TUMOR CELL METABOLISM AND AUTOPHAGY AS THERAPEUTIC TARGETS

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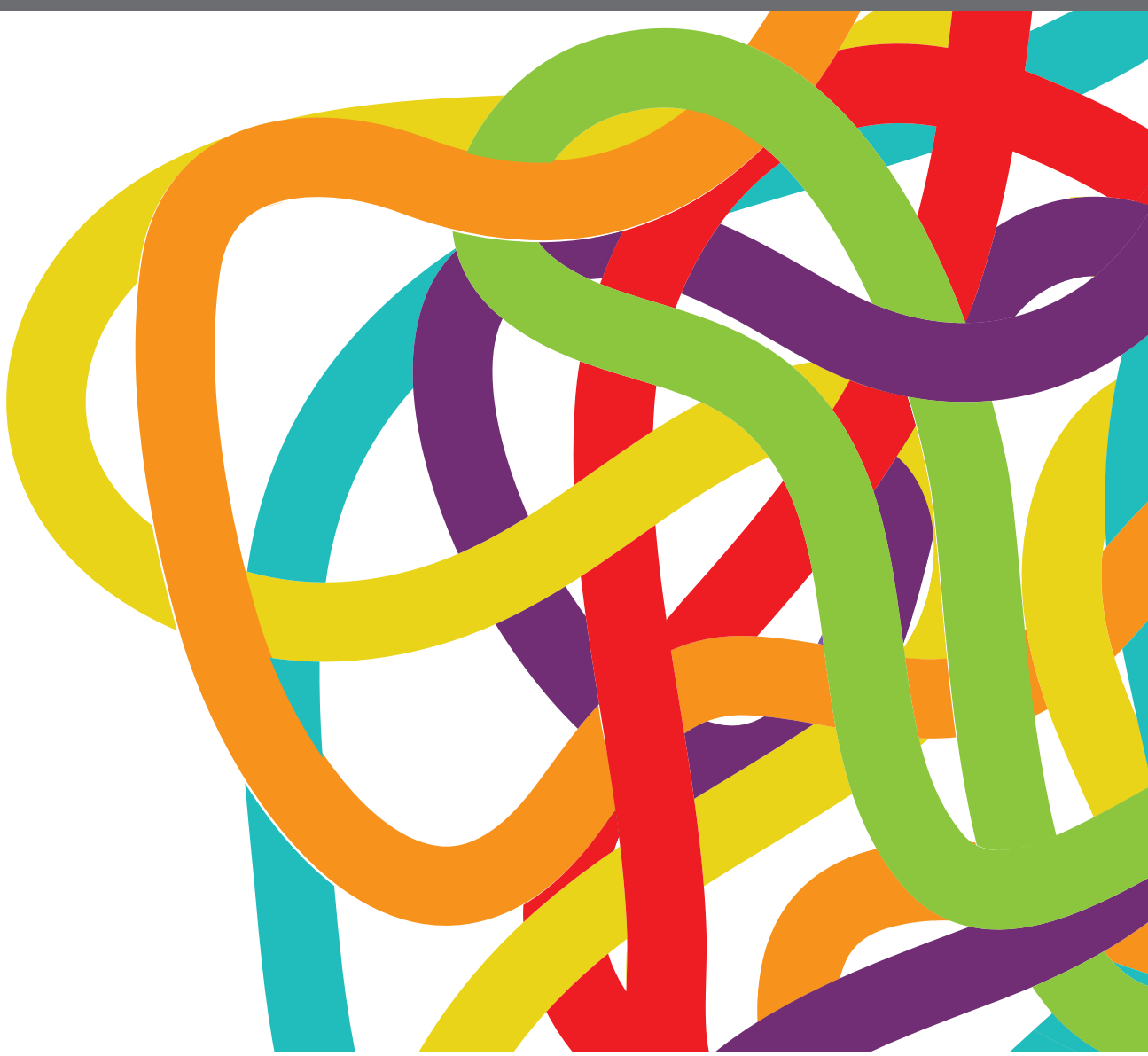

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TUMOR CELL METABOLISM AND AUTOPHAGY AS THERAPEUTIC TARGETS

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EDITORIAL
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Editorial: Tumor Cell Metabolism and Autophagy as Therapeutic Targets

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Editorial on the Research Topic

Tumor Cell Metabolism and Autophagy as Therapeutic Targets

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Cancer is a group of alterations in the normal functioning of a cell. As is known, the tumor cell has adaptations to its environment that allow it to proliferate in conditions unfavorable to a normal cell. Within these changes is the so-called aberrant metabolism, which permits the tumor cell to continue with its replication rate, avoid programmed cell death, or escape from the immune system cells. Although Otto Warburg first described in 1924 that tumor cells employ glycolysis to produce ATP instead of oxidizing glucose in the tricarboxylic acid (TCA) cycle, it was not until recently that tumor metabolism has gained the attention of the scientific community and studied in detail. The seminal work of the Nobel Prize winner Otto Warburg was a pioneer in the realization not only of glycolysis but also in the description of the deregulation of various enzymes involved in the glycolytic cleavage and concluded with the activation of the enzyme lactate dehydrogenase A (LDH-A).

Recent research has shown that the aberrant metabolism of cancer is not only involved in maintaining a high proliferative rate, but it is also necessary to escape from the stress produced by the hypoxic environment to avoid programmed cell death. The high levels of lactate in the tumor microenvironment prevent the cells of the immune system from reacting efficiently against the incipient tumor growth. Novel findings show that there is a delightful regulation of tumor metabolism exerted by oncogenes, as signaling pathways involved in cell growth and proliferation, such as the phosphoinositide 3-kinase/Akt pathway, actively regulates aerobic glycolysis. Transcriptional networks exert control in tumor metabolic activities; thus, c-Myc activates the expression of LDH-A, a metabolic enzyme that converts pyruvate (the final product of glycolysis) to lactate as well as glutamine synthetase. Hypoxia-inducible transcription factor 1α promotes survival under hypoxic conditions by regulating genes relevant in glycolysis, redox homeostasis, among others. Sterol regulatory element-binding protein transcription factor is involved in the synthesis of fatty acids, activates different enzymes needed to convert acetyl-coenzyme A into fatty acids (1).

There is an exquisite regulatory network that sustains tumor metabolism at both the genetic and epigenetic levels. Non-coding RNAs, such as long non-coding RNAs (lncRNAs) and microRNAs (miRNAs), have been described as managers of the tumor microenvironment, involved in the development of cancer and the regulation of the different hallmarks of cancer. These non-coding

molecules are modulators of adaptation to stressful situations such as hypoxia, oxidative stress, and nutrient deprivation.

There are pharmacological strategies designed to inhibit aberrant tumor metabolism. However, the inhibition of enzymes involved in tumor metabolism often causes acute systemic toxicity, injuring enzymes of normal tissues too. Therefore, the possibility of inhibiting metabolic pathways or specific enzymes will depend on whether the systemic blockade is tolerated. Examples of drugs already used in the oncology clinic include inhibitors of DNA synthesis such as antifolates (methotrexate, pemetrexed, and others). Notwithstanding these drugs produce toxicity in normal proliferating tissues like the intestinal epithelium and bone marrow, their use is still valid in therapeutic schemes in different tumors. Therefore, it is an extraordinary opportunity in drug discovery to include novel molecular targets for cancer treatment.

Autophagy is a highly conserved intracellular molecular mechanism in eukaryotes that allows the recycling of proteins and cellular organelles. Several stimuli initiate autophagy; under normal conditions, it starts to degrade misfolded proteins and damaged organelles, depletion of nutrients, embryonic development, or cell death by apoptosis. Although different types of autophagy have been characterized, macroautophagy is the best-studied mechanism and probably the most important in cancer.

The maintenance of cell homeostasis in conditions of nutrient depletion is fundamental to cell survival. At different stages during the development of the tumor mass, conditions of metabolic stress due to starvation are produced, which compromises the viability of the malignant tissue. Moreover, reports mention that hypoxic shock activates autophagy, which plays a central role in chemoresistance (2). On the other hand, activation of autophagy enhances the tumor-associated endothelial cells during the development of new blood vessels supplying the tumor mass (3).

While suppression of glycolysis activates autophagy to recycle nutrients and maintain cell survival, the tumor cell eventually will be able to adapt to its environment and activate other pathways to keep TCA active and thus oxidative phosphorylation. Interestingly, one way to escape the blockage of glycolysis is to raise the metabolism of glutamine and glutamate consumption in different tumors (2). It is essential to recognize that the dual role of autophagy in the promotion of tumor phenotype as a therapeutic target puts in the balance the need for further research in this area to generate therapies that allow the successful treatment of cancer (4).

Cancer metabolism and autophagy have attracted the attention of multiple research centers since the possibility of intervening in their different signaling pathways opens the way for more efficient and specific treatments to diminish toxicity towards normal cells and consequently lessens the repercussions for the patient. The topic is extensive, but thanks to the acceptance of the scientific community, in this Research Topic, we have collected 15 articles, five originals research, and 10 reviews. All of them of high academic quality and with future perspectives that allow a clearer vision about the participation of these metabolic processes in cancer. Following, a brief description of the research works.

In their original research, Sarmiento-Salinas et al. showed that there is a molecular signature based on mitochondrial genes that differentiate basal-like tumors from other molecular subtypes. This gene signature is enriched with genes that participate in reactive oxygen species (ROS) metabolism. Finally, the authors demonstrate that the production of mitochondrial ROS supports the proliferative signaling in triple negative breast cancer whereas its inhibition, using an anti-oxidant agent, induces cells to a decreased proliferation.

Regarding breast cancer treatment, Serrano-Carbajal et al. investigated the metabolic deregulation landscapes in breast cancer (BC) molecular subtypes. They designed through a computation tool, developed by their group, a treatment scheme to regulate purine metabolism independently of the BC subtype with Food and Drug Administration approved drugs in public pharmacological databases.

As above mentioned, tumor cell requires low concentrations of glucose and oxygen, as well as a lactic acidosis medium to survive. Romero-Garcia et al. observed that cell growth is affected depending on the lactic acidosis and the presence or absence of oxygen in the medium. For example, in the cell lines A-427 and MCF-7 in hypoxia conditions, cells do not survive in neutral lactosis but lactic acidosis. On the other hand, they noted that in a lactic acidosis medium, either in normoxia or hypoxia, the mitochondrial mass and mitochondria DNA levels were increased in comparison to neutral lactosis in tumor cells but not in fibroblasts. Therefore, they concluded that lung adenocarcinoma cells induce mitochondrial biogenesis to continue survival and proliferation in lactic acidosis and the absence of glucose.

The discovery of new pharmacological therapies in oncology is a tireless area of research. Natural products have been an inexhaustible source of new molecules that provide chemical and molecular bases for the development of new drugs. In the Couder-Garcia et al. article, the results obtained from the antiproliferative effect of peniocerol in an *in vivo* model of colon cancer are displayed. The mechanism of action of this compound is related to the inhibition of poly (ADP-ribose) polymerase-1 and the decrease in the expression of proliferation cell nuclear antigen (cell proliferation marker) both *in vitro* and in xenografts.

Liu et al. performed a liquid chromatography-mass spectrometry-based methodology to discover potential biomarkers for differential diagnosis of patients with bladder cancer and renal cell carcinoma. The authors found that plasma metabolomics and lipidomics could be useful for the discrimination of the global plasma profiles of the two cancer types evaluated from healthy controls.

The reviews published in this special issue cover several interesting topics. Bermúdez et al. reviewed the lncRNAs related to chemoresistance led by autophagy and the clinical implications of the lncRNAs in colorectal cancer. Autophagy was evaluated by Núñez-Olvera et al. as a therapeutic target in endometrial cancer. Besides, the Acevo-Rodríguez et al.'s group discussed the crosstalk between translation and autophagy and the implications of this process in tumorigenesis. The review of de la Cruz López et al. covered the importance of the mammalian target of rapamycin complex 1 (mTORC1) in the regulation of mitochondrial metabolism in cancer and also discussed the therapeutic efficacy of mTORC1 inhibitors.

Disturbed glucose metabolism is a characteristic of the cancer cell. The review by Vanhove et al. addressed the importance of glucose pathway, glycolysis, gluconeogenesis, and mitochondrial metabolism in lung cancer, and the importance of understanding all these processes to implement new treatments in the future. Moreover, de la Cruz-López et al. also examined the meaning of the lactate in carcinogenesis and tumor immune evasion, as well as a target for cancer therapy.

Regarding dysregulated metabolism, Pedroza-Torres et al. analyzed the miRNAs and their participation in the tumor cell metabolism and as therapeutic target opportunities in cancer.

The hepatocellular carcinoma (HCC) deserved two reviews. In Che et al.'s article, the authors focused on the role of the fatty acid synthase (FASN) and the molecular mechanisms involved in the activation of this fatty acid in liver carcinogenesis. Also, put on the table that the inhibition of FASN and related lipogenesis as targets for HCC treatment. The second review by Marquardt and Edlich covers the chronic inflammation and cell death resistance in HCC as two of the main hallmarks of this disease.

Finally, the upcoming field of the role of growth differentiation factor 11 (GDF11) in cancer was analyzed by Simoni-Nieves et al. The growth factor GDF11 has attracted attention in cancer due to its age-related role, targeting mainly the stem cells. This particularity becomes imperative in the tumor cell as they acquire the stemness ability which is reflected in tumor aggressiveness and poor clinical prognosis. The effect of GDF11 is controversial, some authors report tumor suppression active, while others blame the opposite. In this study, the authors discuss the role of GDF11 and its functions known so far in cancer biology and metabolism, including examples in liver, breast, pancreatic, and colorectal cancers, as well as in oral squamous cell and melanoma (7).

In summary, GDF11 is a fascinating component of the transforming growth factor-beta superfamily. Its functions are known to depend on cell progeny, tissue type, degree of differentiation, and even age, which is why its activities are diverse and why its involvement in cancer has become a new area of research.

Ample evidence of the importance of autophagy in the development and maintenance of tumor phenotype is documented. However, controversy arises when autophagy is evaluated from a pro- or antitumor perspective. Along with the development of the tumor mass, autophagy protects the tumor cell from the different attacks it suffers on its progress, hypoxic shock, lack of nutrients, chemotherapeutic agents, radiation, production of reactive oxygen species, to only mention some sources of cellular stress (5). On the other hand, different drugs that promote autophagy and lead to the inhibition of tumor growth have been tested *in vitro* and *in vivo* (6). In both scenarios, it is necessary to deepen on the role played by autophagy either as a tumor growth promoter or as a pharmacological target. Concerning aberrant tumor metabolism, the challenge is to identify enzymes that are expressed exclusively in the tumor cell, contrarily their inhibition may compromise the viability of normal cells and tissues. The identification of lncRNAs and miRNAs that regulate both fundamental processes in cancer biology will have an impact not only on the treatment of this disease but also on the knowledge of the cellular machinery and its involvement in the development of new drugs and personalized medicine.

AUTHOR CONTRIBUTIONS

NJ-H and CP-P contributed equally to the conception and writing of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Sun Q, Yu X, Peng C, Liu N, Chen W, Xu H, et al. Activation of SREBP-1c alters lipogenesis and promotes tumor growth and metastasis in gastric cancer. *BioMed Pharmacother* (2020) 128:110274. doi: 10.1016/j.biopha.2020.110274
2. Shiratori R,, Furuichi K, Yamaguchi M, Miyazaki N, Aoki H, Chibana H, et al. Glycolytic suppression dramatically changes the intracellular metabolic profile of multiple cancer cell lines in a mitochondrial metabolism-dependent manner. *Sci Rep* (2019) 10;9(1):18699. doi: 10.1038/s41598-019-55296-3
3. Kardideh B, Samimi Z, Norooznezhad F, Kiani S, Mansouri K. Autophagy, cancer and angiogenesis: where is the link? *Cell Biosci* (2019) 9:65. doi: 10.1186/s13578-019-0327-6
4. García-Castillo V, López-Urrutia E, Villanueva-Sánchez O, Ávila-Rodríguez MÁ., Zentella-Dehesa A, Cortés-González C, et al. Targeting Metabolic Remodeling in Triple Negative Breast Cancer in a Murine Model. *J Cancer* (2017) 8(2):178–89. doi: 10.7150/jca.16387
5. Poillet-Perez L, Despouy G, Delage-Mourrous R, Boyer-Guittaut M. Interplay between ROS and autophagy in cancer cells, from tumor initiation to cancer therapy. *Redox Biol* (2015) 184:192. doi: 10.1016/j.redox.2014.12.003
6. Cuomo F, Altucci L, Cobellis G. Autophagy function and dysfunction: potential drugs as anti-cancer therapy. *Cancers* (2019) 11(10):1464. doi: 10.3390/cancers11101465
7. Sinha M, Jang YC, Oh J, Khong D, Wu EY, Manohar R, et al. Restoring systemic GDF11 levels reverses age-related dysfunction in mouse skeletal muscle. *Science* (2014) 344:649–52. doi: 10.1126/science.1251152

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Breast Cancer Subtypes Present a Differential Production of Reactive Oxygen Species (ROS) and Susceptibility to Antioxidant Treatment

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Due to their crucial role in cell metabolism and homeostasis, alterations in mitochondrial biology and function have been related to the progression of diverse diseases including cancer. One of the consequences associated to mitochondrial dysfunction is the production of reactive oxygen species (ROS). ROS are known to have a controversial role during cancer initiation and progression and although several studies have tried to manipulate intracellular ROS levels using antioxidants or pro-oxidation conditions, it is not yet clear how to target oxidation for cancer therapy. In this study, we found differences in mitochondrial morphology in breast cancer cells when compared to a non-tumorigenic cell line and differences in mitochondrial function among breast cancer subtypes when exploring gene-expression data from the TCGA tumor dataset. Interestingly, we found increased ROS levels in triple negative breast cancer (TNBC) cell lines and a dependency on ROS for survival since antioxidant treatment induced cell death in TNBC cells but not in an estrogen receptor positive (ER+) cell line. Moreover, we identified the mitochondria as the main source of ROS in TNBC cell lines. Our results indicate a potential use for ROS as a target for therapy in the TNBC subtype which currently has the worst prognosis among all breast cancers and remains as the only breast cancer subtype which lacks a targeted therapy.

Keywords: breast cancer, ROS, mitochondria, mitochondrial morphology, mitochondrial ROS

INTRODUCTION

Breast cancer is a highly heterogeneous disease whose classification has proven to be central for proper patient management, follow-up, clinical trial selection and focus on translational research (1). Breast cancer classification has gradually shifted from a classification based on morphological findings into a more integrative approach which incorporates tumor biomarkers and molecular

information. About 75% of breast tumors express the estrogen (ER) and/or progesterone (PgR) receptors and can be targeted with endocrine therapy. Hierarchical clustering of gene expression data of several tumors has led to the definition of molecular intrinsic tumor subgroups, classifying most ER+ tumors in the luminal subtype due to the expression of genes characteristic of luminal epithelial cells (2, 3). Patients with a low risk of relapse are found in the Luminal A subtype while patients in the Luminal B subgroup have a higher risk of relapse and their tumors express increased proliferation-related markers (1, 4, 5). More recently, integrative cluster classification based on DNA rearrangement patterns from whole genome sequencing data have further characterized ER+ tumors into 9 different subtypes with differences in clinical outcomes (1). About 10–15% of breast cancers over-express the HER2/erb2/neu receptor protein, a receptor tyrosine kinase that signals cellular proliferation and patients with HER2+ tumors used to have one of the worst prognoses until the advent of anti-HER2 targeted therapies (4). Molecularly, most of the HER2 enriched tumors are HER2+ by immunohistochemistry (4); and HER2+ tumors have been shown to have different combinations of mutations, supporting the existence of subclasses of HER2+ tumors and also indicating a high heterogeneity within this subgroup (1, 5). Finally, triple-negative breast cancer (TNBC), also classified as basal-like breast cancer, is defined by the absence of ER, PgR, and HER2 receptors and thus lacks a targeted therapy. Only chemotherapy options are available for this breast cancer subtype which has the worst prognosis in all cancer stages and also shows a great intrinsic diversity (6–8). Gene expression patterns have led to the identification of 6 different TNBC subgroups (6) and TNBC tumors show multiple copy number alterations affecting most of the chromosomes (1). Since basal-like breast cancers are identified by gene expression profiling and TNBC are characterized by analyzing the absence of receptors by immunohistochemistry, both terms are not strictly synonyms. It is known that approximately 25% of TNBC are not basal-like on gene expression, but it has also been shown that the TNBC phenotype enriches for basal-like cancer (9). Since TNBC cell lines used in this study have been defined as basal (10), both terms (TNBC and basal) are used for TNBC cell lines in this work.

So, despite recent advances in the classification of breast cancer that have led to effective targeted therapies for most patients, evidence suggests that there is a high heterogeneity in breast tumors even among the ones belonging to the same subtype and that patients would benefit the most from a precise classification and a targeted therapy for each individual tumor. On the other hand, finding targetable biological features for each breast cancer subtype has proven to be successful for ER(+)/luminal and HER2(+) patients (8), underscoring the need to find an effective, targeted therapy for TNBC patients.

One of the hallmarks of cancer cells is the de-regulation of cellular energetics in order to fuel cell growth and division (11). Otto Warburg first observed this anomaly in cancer cells which had a high glycolytic activity even in the presence of oxygen and proposed this metabolic shift to be a cancer driver. However, although multiple oncogenes commonly activated in cancer are known to activate glycolysis, they have been shown to also activate mitochondrial metabolism (12). These metabolic changes have brought attention to the role of mitochondria in tumorigenesis and tumor progression but there seems to be no simple explanation for the role of mitochondria in cancer. Instead, mitochondrial functions have been found to vary depending on genetic, environmental and tissue-of-origin differences between tumors (13). One of the characteristics associated with mitochondrial dysfunction is the production of ROS and sensitivity to ROS-induced apoptosis. In this regard, increased ROS have been found in diverse types of cancer and it has been suggested that increased ROS levels in non-transformed cells or in cancer cells could have pro-tumorigenic effects by damaging nucleic acids and promoting genomic instability. However, there is controversy in the literature regarding the role of ROS in tumor progression. While some studies indicate that ROS in cancer cells can activate pro-tumorigenic signaling pathways (14–16), other studies have shown that treatment with anti-oxidants accelerated tumor growth, metastasis and decreased survival in mouse models of cancer (17, 18). In this work, we studied differences in mitochondrial dynamics as well as in the production of ROS in breast cancer cell lines belonging to different subtypes of the disease with the purpose of identifying differences in mitochondrial-dynamics or ROS-related biomarkers which could work as molecular targets for therapy or lead to a better classification of the disease.

MATERIALS AND METHODS

Hierarchical Clustering and Principal Component Analysis

Mitochondria-related genes were obtained from GSEA (19, 20) (mitochondria, OXPHOS signatures) and genes related to mitophagy and mitochondrial dynamics were added for a total of 167 different probes (**Supplementary Table 1**). ROS-related genes were selected from GSEA (GO_OXIDATION_REDUCTION_PROCESS; ANTIOXIDANT_ACTIVITY; and REACTOME_BIOLOGICAL_OXIDATIONS) as well as from a previously published ROS-signature and complemented with NOX-related genes for a total of 370 different probes (21) (**Supplementary Table 1**). Gene expression data was obtained from cbioportal.org using mRNA Expression Z scores from The Cancer Genome Atlas (TCGA); Nature, 2012 study (7). Molecular subtype classification in this sample set was performed according to the PAM50 gene signature assay. Samples with mutations were excluded from the mRNA expression analysis and after elimination of non-classified samples or non-available values, 518 samples were analyzed and Pearson hierarchical clustering as well as principal component analysis (PCA) was performed using Expander7 software (22).

Cell Culture

Breast cancer cell lines were cultured in the following media: MCF10A (DMEM/F12, Caisson DFP18-1LT, 5% horse serum, 0.5 μg/mL hydrocortisone, 20 ng/mL EGF, 100 ng/mL cholera toxin, 10 μg/mL insulin); MCF7 (Eagle's MEM, Caisson MEP-10X1LT, 10 μg/mL insulin, 10% fetal bovine serum, FBS);

T47D (RPMI-1640, Caisson, RPP10-10XLT 7.5 μg/mL insulin, 10% FBS); MDAMB231 (DMEM/F12, 10% FBS); MDAMB468 (DMEM/F12, 10% FBS); BT549 (RPMI-1640, Caisson RPP10-10XLT, 7.5 μg/mL insulin, 10% FBS).

Mitotracker Labeling and Mitochondrial Classification

Mitochondria were labeled with Mitotracker Red CMXRos (ThermoFisher Scientific, M7512). Since fixation is known to disrupt the mitochondrial network (23), we used live cells for mitotracker labeling. Briefly, 100,000 cells were plated on coverslips and after 24 h stained with 250 nM Mitotracker Red in culture medium at 37°C, protected from light. After incubation, cells were washed twice, first with pre-warmed, serum free medium and then with complete medium. Live cells were mounted with 10 μl complete medium and immediately observed on a Zeiss Observer.Z1 microscope equipped with an Axiocam MRm camera and an Apotome illumination system with a 63X oil immersion objective. Cells were classified as completely tubular (I), tubular with some fragments (II), fragmented with some tubules (III), or completely fragmented (IV) as shown in **Figure 2B** by two independent observers.

For mitochondrial ROS labeling, cells grown in coverslips were incubated with 2 μM Mitosox Red (ThermoFisher Scientific, M36008) and 200 nM Mitotracker green (ThermoFisher Scientific, M7514) for 15 min in complete medium, washed with 1X PBS, mounted with 10 μl complete medium and immediately observed on a Zeiss Observer.Z1 microscope equipped with an Axiocam MRm camera and an Apotome illumination system with a 63X oil immersion objective.

ROS Measurement

ROS were evaluated by fluorescence microscopy and flow cytometry. For microscopy, 30,000 cells were plated in 24-well-plates and, after 24 h, stained with 10 μM dihydroethidium (Sigma Aldrich, D7008-10MG) in culture medium for 30 min at room temperature, protected from light. After incubation, cells were washed three times, first with pre-heated complete medium and then with PBS, fixed and stained with Hoechst. Stained cells were observed with 1 ml of PBS in a Zeiss Observer.Z1 microscope. ROS quantification was performed by flow cytometry. Briefly, 100,000 cells were plated on 6 well-plates and after 24 h stained with 10 μM DHE as previously described. After incubation, cells were washed tree times, first with pre-warmed complete medium and then with PBS, trypsinized and centrifuged at 2,500 rpm. The pellet was resuspended in PBS with 3% FBS for immediate analysis in a BD FACS Canto II flow cytometer. Graphs show mean fluorescence intensity minus autofluorescence control. For ROS^{high} and ^{low} populations, cells were analyzed according to the flow cytometry pipeline shown in **Supplementary Figure 3** using Flow Jo V 10.0 software. For mitochondrial ROS evaluation cells were plated as for DHE staining but stained with 5 μM MitoSox Red in pre-warmed medium for 15 min at 37°C. After incubation, cells were washed twice, first with pre-warmed complete medium and then with PBS, trypsinized and centrifuged at 2,500 rpm, the pellet was resuspended in PBS with 3% FBS for immediate analysis in a BD FACS Canto II flow cytometer. Graphs show mean fluorescence intensity minus autofluorescence control.

Proliferation and Cell Death Assays

Cell proliferation was assessed in a live-cell Incucyte ZOOM System. Cells were plated at a density of 3,000–5,000 cells per well and after 24 h, treated with hydrogen peroxide (H_2O_2) or N- acetylcysteine at the indicated concentrations and imaged every 4 h for 24 h. Proliferation was evaluated using the Incucyte software and expressed as % confluency. Cell death was evaluated after 24 h with 10 μM propidium iodide (PI) staining for 10 min. Fluorescence images were taken in the Incucyte ZOOM system and cell death was expressed as % red (PI+) confluency/% total confluency.

Reagents

All reagents were purchased from Sigma Aldrich unless otherwise specified.

Statistical Analysis

Graphs show three or more independent experiments and every figure shows the mean ± standard error. *T*-tests, ANOVA and *post-hoc* tests were performed using GraphPad Prism 5 software. Tukey was performed when means were compared to every other mean and Dunnett's *post-hoc* was used for multiple-to one comparison in **Figure 4**. For two group mean comparison performed in **Figure 4A**, a student *t*-test was used.

RESULTS

Mitochondrial Functional Status May Reveal Association With Breast Cancer Intrinsic Subtypes

Expression analysis of mitochondria-related genes in tumor samples from the TCGA dataset revealed clusters of samples related to molecular breast cancer subtypes, both when analyzed by unsupervised hierarchical clustering (**Figure 1A**) or principal component analysis (PCA, **Figure 1B**). Hierarchical clustering analysis revealed two major clusters (I and II). Cluster I was enriched in luminal samples while cluster II was more heterogeneous and two big sub-groups were observed. The first subgroup in cluster II was enriched in Basal-like tumors, which clustered together with HER2-enriched and some luminal samples. The other sub-group in cluster II had a small basal-like cluster, a HER-2 enriched cluster, a luminal B-enriched one and a big luminal cluster containing both Luminal A and B samples. Importantly, luminal tumors in cluster II, were more similar to basal and HER2-enriched tumors than to the other luminal samples in cluster I, evidencing Luminal tumors with potential differences in mitochondrial biology and function.

Mitochondrial differences in breast cancer subtypes were more evident in a PCA analysis (**Figure 1B**) where a cluster of luminal A and B tumors was found to the left of the graph, followed by an intermediate group with HER2-enriched

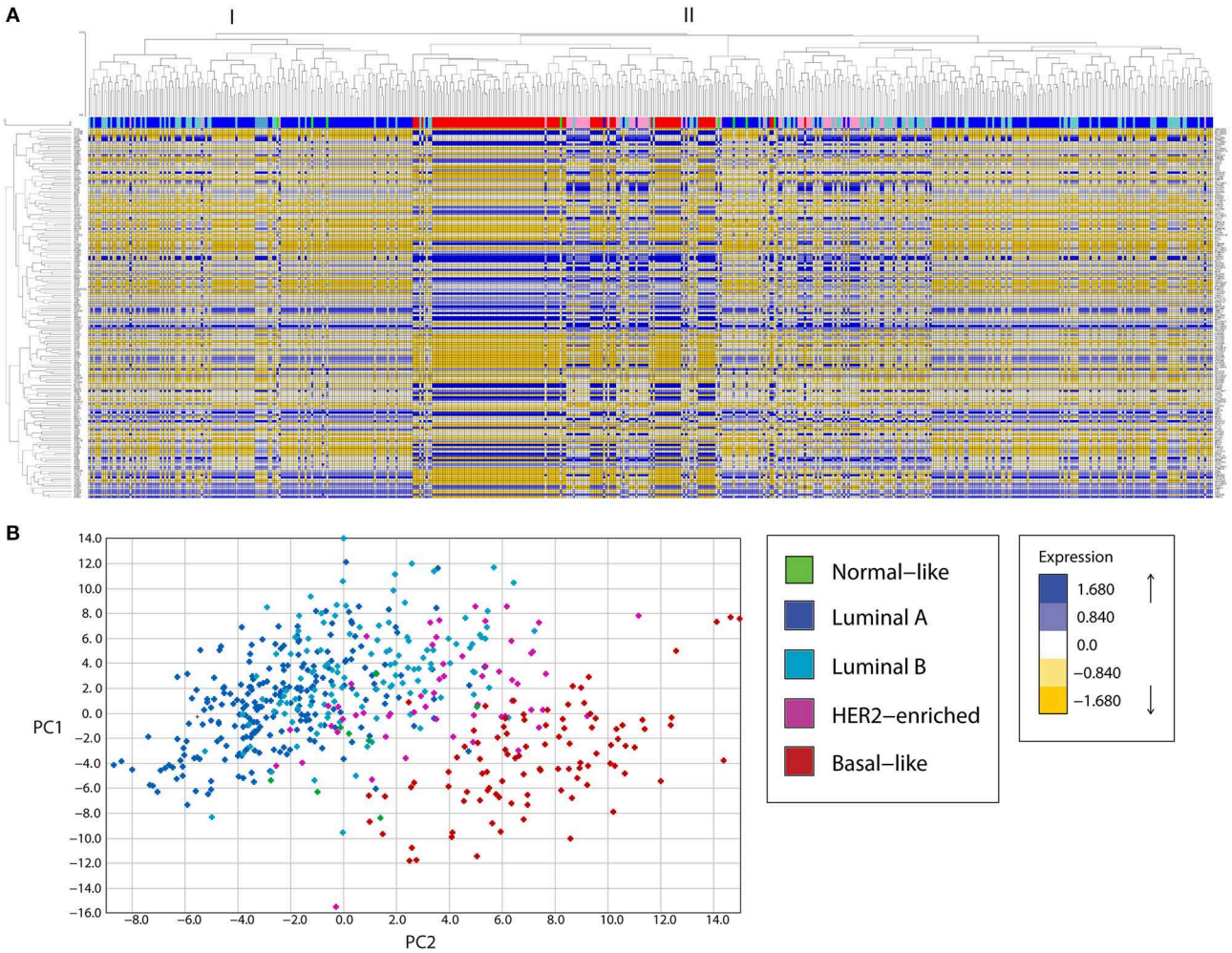

FIGURE 1 | A mitochondria-related gene signature separates breast cancer tumor samples in clusters according to the tumor subtype, in a bioinformatic analysis of gene expression. **(A)** A mitochondria-related gene expression signature was analyzed in TCGA breast tumor samples using unsupervised hierarchical clustering analysis, revealing clusters of samples enriched in molecular subtypes. Two major clusters were found (I, II). Cluster I was enriched in luminal samples while cluster II showed two major sub-groups. The first subgroup was enriched in basal and HER-2 enriched samples while the other was heterogeneous with one sub-group enriched in luminal samples and another containing luminal B and HER2-enriched tumors. **(B)** Principal component analysis (PCA) revealed three main clusters, a cluster of Luminal A and B tumors (left), an intermediate group composed of HER2-enriched and some Luminal B samples and a Basal-like enriched cluster (right). PC, principal component.

and luminal B samples and a well-defined Basal-like enriched cluster to the right of the graph which also included some HER2-enriched samples. Thus, PCA analysis of the expression of mitochondria-related genes clustered tumor samples not only according to breast cancer subtypes but also according to malignancy, with the most malignant triple negative or basal-like subtype to the right and the least malignant luminal A samples to the left of the graph (**Figure 1B**). These findings suggest important differences in mitochondrial function among tumors from different breast cancer subtypes. Importantly, luminal B samples were the most heterogeneous, with some samples clustering with Luminal A tumors (**Figure 1B** and also **Figure 1A**, cluster I) and others clustering with HER2-enriched tumors (**Figure 1B** and also **Figure 1A**, cluster II, sub-cluster vi). This likely reflects differences in the mitochondrial biology of Luminal B samples which are HER2+ and those which are HER2- and suggests a possible role for the HER2 receptor in the regulation of mitochondrial gene expression and function.

Mitochondrial shape has been extensively linked to mitochondrial function and although it is determined by a highly dynamic and regulated process, diverse cellular functions and alterations have been associated to changes in mitochondrial morphology (12, 24). Fluorescent mitochondrial labeling has been used to assess mitochondrial shape and changes in function in breast cancer cells and in cancer cells from other tissues (23, 25–27). Hence, we evaluated mitochondrial morphology in mitotracker-stained breast cancer cell lines representative of different subtypes (**Figure 2A**). We used MCF10A cells as a non-tumorigenic (NT) control and, according to the classification by Neve et al. (10), we used MCF7 and T47D

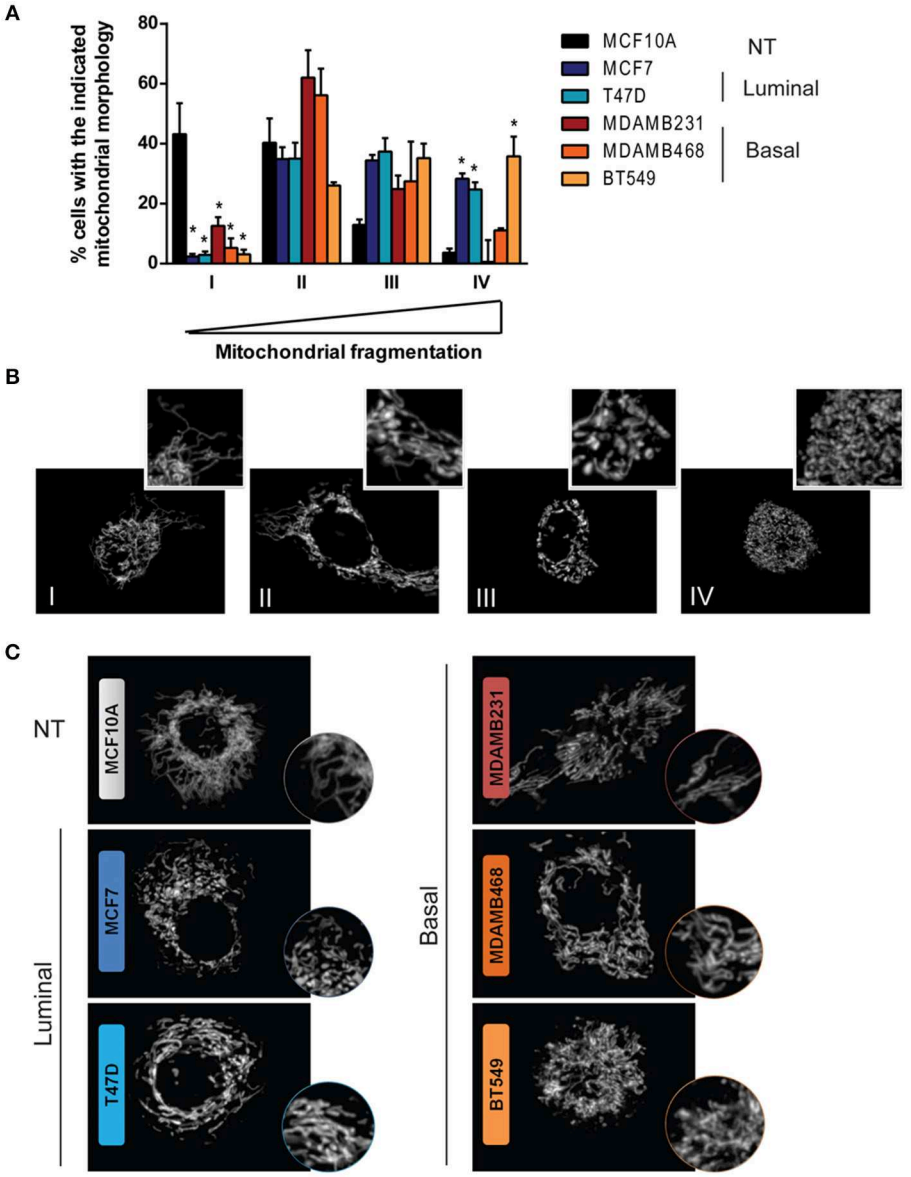

FIGURE 2 | Breast cancer cell lines show differences in mitochondrial morphology and increased mitochondrial fragmentation than a non-tumorigenic cell line. **(A)** Mitotracker staining revealed differences in mitochondrial morphology among breast cancer cell lines. **(B)** Cells were classified as completely tubular (I), tubular with some fragments (II), fragmented with some tubules (III), or completely fragmented (IV). The graph in **(A)** shows the percentage of cells with the corresponding mitochondrial morphology as shown in **(B)**. In **(C)** representative images of the characteristic morphology per cell line is shown. The graph in **(A)** shows mean ± SEM of 3–5 independent experiments. Sixty to one hundred individual cells were classified per experiment by two independent observers. *Different to MCF10A with $p < 0.05$.

cell lines as luminal cells and MDAMB231, MDAMB468, and BT549 as triple negative, basal-like cancer cell lines. Cells were classified as completely tubular (I), tubular with few fragments (II), fragmented with few tubules (III) or completely fragmented (IV) according to representative images in **Figure 2B**. We found an important difference in the number of cells classified as having mostly tubular mitochondria (I) between NT and breast cancer cell lines. The MCF10A cell line showed the highest tubular mitochondria when compared to cancer cells, indicating an important role for mitochondrial fission in breast cancer. Importantly, we found cells with fragmented mitochondria (IV) as being the most heterogeneous population among the cell lines, with a low percentage of fragmented mitochondria in MCF10A and a great diversity among the cancer cell lines studied. An image of the most representative mitochondrial morphology found in each cell line is shown in **Figure 2C**.

We selected genes in mitochondrial gene signatures related to mitophagy, mitochondrial dynamics and biogenesis to evaluate differences in mitochondrial function among breast cancer subtypes. We found differences in the expression level of genes

related to mitochondrial biology in tumors from the TCGA sample set according to the molecular classification of tumors (**Table 1**, **Supplementary Figure 1**). Significant changes in the expression level of genes related to mitochondrial dynamics were observed among breast cancer subtypes. Fusion-related (OPA1, MFN1) as well as fission related (DNM1L) genes were found to be increased in the basal-like subtype when compared to Luminal A tumor samples. However, adaptor proteins for Drp-1 on the outer mitochondrial membrane like MID49/MIEF2 or FIS1 (**Table 1**, **Supplementary Figure 1**), were found to be decreased in the basal-like subtype when compared to the other breast cancer subtypes, and no changes among the different subtypes were observed in MFF, another Drp-1 adaptor protein (**Table 1**). Importantly, decreased levels of mitophagy-related BNIP3L and PINK1, as well as increased levels of mitochondrial biogenesis related genes PPARGC1A and PPARGC1B were found in the basal-like tumor samples when compared to the Luminal A subtype (**Table 1**, **Supplementary Figure 1**). This evidence suggests changes in mitochondrial quality control mechanisms, turnover and important differences in mitochondrial biology among breast cancer subtypes.

TNBC Was Characterized by an Increased Oxidation State

Mitochondria have a crucial role in triggering redox signaling through ROS release from the electron transport chain (ETC) and ROS production is one of the aspects that has been involved in the promotion of malignancy by mitochondrial dysfunction (17). In this regard, ROS generation from mitochondria or from other cellular sources can contribute to the initiation of cancer in normal or non-malignant cells. Moreover, once a cell is transformed, redox signaling can amplify the malignant phenotype in terms of proliferation, survival, and migration through the activation of pro-tumorigenic signaling pathways (17, 28). Since we were particularly interested in TNBC, we analyzed ROS levels in the different TNBC cell lines under basal conditions using dihydroethidium (DHE) staining and compared to an ER+ and a non-tumorigenic control (**Figures 3A–C**). DHE is widely used as a small-molecule fluorescent ROS probe which is oxidized to 2-hydroxyethidium in the presence of $O_2^{\cdot-}$ and to ethidium enzymatically or in the presence of 1-electron oxidants, which reflect total oxidant generation (29, 30). Since both oxidation products are fluorescent in red and are not distinguishable in intact cells with the methods that we used (fluorescence microscopy and flow cytometry), we used DHE staining as a measurement of total ROS levels.

We found increased DHE(+) nuclei in basal-like, TNBC cell lines with more than 45% DHE positive nuclei in the three TNBC cell lines studied (MDAMB231, MDAMB468 and BT549) when compared to the luminal (MCF7) or non-tumorigenic (MCF10A) cell lines (**Figures 3A,B**). Interestingly, H_2O_2 treatment increased DHE positive nuclei only in the non-tumorigenic, MCF10A cell line, probably indicating a better redox balance in cancer cells or a high oxidation status in TNBC cells that cannot be further increased by oxidants. Also, flow cytometry analysis of DHE mean fluorescence intensity in each cell line showed increased DHE staining in basal-like, TNBC cell lines when compared to the non-tumorigenic MCF10A or

TABLE 1 | Breast cancer subtypes show differences in the expression level of mitochondria-related genes.

		Normal-like		Luminal A		Luminal B		HER2-enriched		Basal-like	
	Gene	**Mean ± SD**	**ANOVA**	**Mean ± SD**	**ANOVA**	**Mean ± SD**	**ANOVA**	**Mean ± SD**	**ANOVA**	**Mean ± SD**	**ANOVA**
Fusion	OPA1	−0.25 ± 0.9		−0.244 ± 0.96		0.34 ± 1.3	1	0.978 ± 1.3	0,1,2	1.303 ± 1.5	0,1,2
	MFN1	−0.74 ± 1.18		−0.015 ± 1.15		0.516 ± 1.2	1	0.617 ± 1.4	0,1	1.117 ± 1.5	0,1,2
	MFN2	−0.17 ± 0.82		−0.124 ± 1.08		−0.54 ± 1.3	1	−0.57 ± 1.4		−0.56 ± 1.4	
Fission	DNM1L	−0.62 ± 1.04		−0.302 ± 1.03		0.276 ± 1.1	1	0.293 ± 1.2	1	1.023 ± 1.5	0,1,2,3
	MTP18/MTFP1	−0.28 ± 0.86		−0.585 ± 1.05		−0.01 ± 1.1	1	0.201 ± 1	1	0.235 ± 1.1	1
	YME1L1	−0.79 ± 1.77		−0.204 ± 1.1		0.378 ± 1.1	1	0.2 ± 1.4		0.843 ± 1.9	0,1
	MID51/MIEF1	−0.38 ± 0.75		−0.681 ± 1.05		−0.56 ± 1.3		0.297 ± 1.4	1,2	0.629 ± 1.2	1,2
	MARCH5	−1.47 ± 1.25		−0.14 ± 0.87	0	−0.09 ± 1.1	0	0.142 ± 1.3	0	−0.43 ± 1.3	3
	MFF	−0.29 ± 1.26		−0.119 ± 1.01		−0.23 ± 1		−0.17 ± 1.2		0.071 ± 1.3	
	FIS1	−0.61 ± 1.13		0.25 ± 0.97		0.151 ± 1.1		−0.44 ± 1	1,2	−0.82 ± 1	1,2
	OMA1	−0.29 ± 0.87		0.241 ± 0.95		0.115 ± 1.1		−0.66 ± 1.3	1,2	−0.56 ± 1	1,2
	MID49/MIEF2	−0.49 ± 0.79		0.11 ± 0.95		−0.28 ± 1.1		−0.39 ± 0.8	1,2	−1.24 ± 1	1,2,3
Mitophagy	BNIP3L	−0.5 ± 1.09		−0.084 ± 1.06		−0.76 ± 1.1	1	−0.75 ± 1.2	1	−1.33 ± 1.1	1, 2, 3
	PINK1	0.153 ± 0.93		0.146 ± 0.92		−0.44 ± 1	1	−0.51 ± 1	1	−0.84 ± 1.1	1
	PARK2	−0.5 ± 1.09		−0.084 ± 1.06		−0.76 ± 1.1	1	−0.75 ± 1.2	1	−1.33 ± 1.1	
Biogenesis	PPARGC1A	0.388 ± 0.39		−0.066 ± 0.7		−0.51 ± 0.7	1	0.278 ± 1	2	0.961 ± 1.6	1,2,3
	PPARGC1B	0.395 ± 0.87		−0.285 ± 0.84		−0.16 ± 1		0.531 ± 1	1,2	1.001 ± 0.9	1,2,3
Other	SIRT3	−1.04 ± 0.7		0.382 ± 0.93	0	−0.06 ± 1.1	0, 1	−0.82 ± 0.9	1,2	−1.4 ± 0.9	1,2,3

mRNA expression z-scores (microarray) analyses from TGCA samples according to their breast cancer sybtype (PAM50 classification). Gray cell means differences with $p < 0.05$ to Normal-like (0), Luminal A (1), Luminal B (2), or HER2 (3).

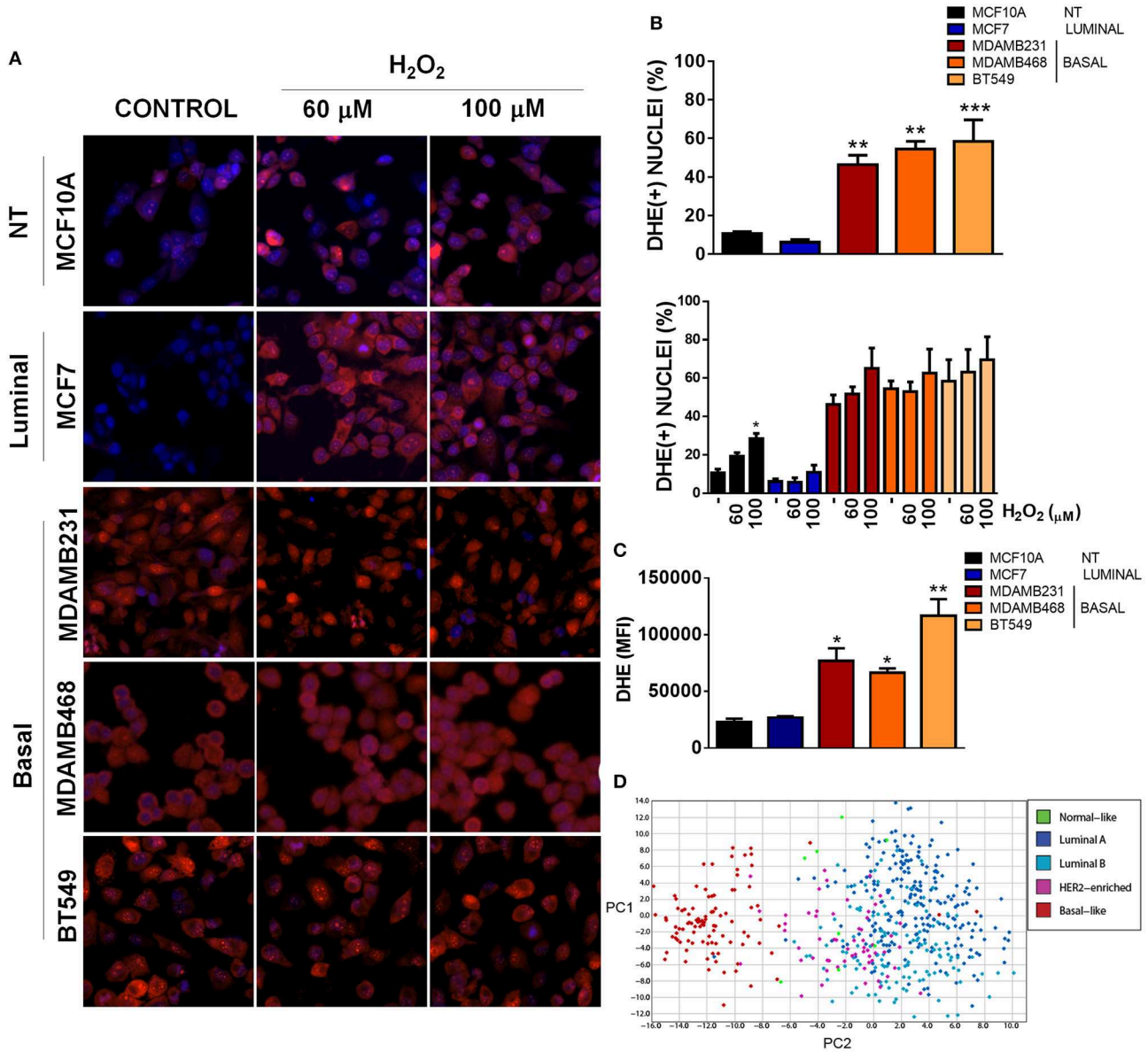

FIGURE 3 | Basal, TNBC cell lines have elevated ROS levels when compared to non-tumorigenic or luminal breast cancer cell lines. **(A)** ROS levels were evaluated by fluorescence microscopy using dihydroethidium (DHE) staining (red) and total nuclei were stained with Hoechst (blue). Cells were treated with the indicated concentrations of H_2O_2. **(B)** DHE positive nuclei were counted and graphed as a percentage of total nuclei. **(C)** ROS levels were quantitatively evaluated by flow cytometry in the different cell lines. **(D)** A ROS-related gene signature PCA analysis clearly clustered basal-like tumors separate from the other breast cancer subtypes. Graphs show mean ± SEM of 3–5 independent experiments, *Different to MCF10A and MCF7 or to their respective control with *$p < 0.05$; **$p < 0.01$, and ***$p < 0.001$. PC, principal component.

the ER+/luminal MCF7 cells (**Figure 3C**). Finally, expression analysis of a ROS gene signature in tumor samples from the TCGA dataset clearly separated basal-like tumors from the other breast cancer subtypes, both when analyzed by unsupervised hierarchical clustering (**Supplementary Figure 2**) or principal component analysis (PCA, **Figure 3D**), indicating that oxidant and anti-oxidant gene expression is similar among basal-like tumor samples and different to other breast cancer subtypes. Thus, TNBC cells had a high level of oxidation when compared to cell lines from other subtypes, which could not be further increased with H_2O_2 treatment.

Mitochondrial ROS Sustain Oncogenic Signaling and Survival in TNBC

To test if elevated ROS levels sustained oncogenic signaling and survival of TNBC cells, we used H_2O_2 to induce oxidation or the antioxidant N-acetyl cysteine (NAC) to decrease basal ROS levels in TNBC cell lines and compared to an ER+ cell line

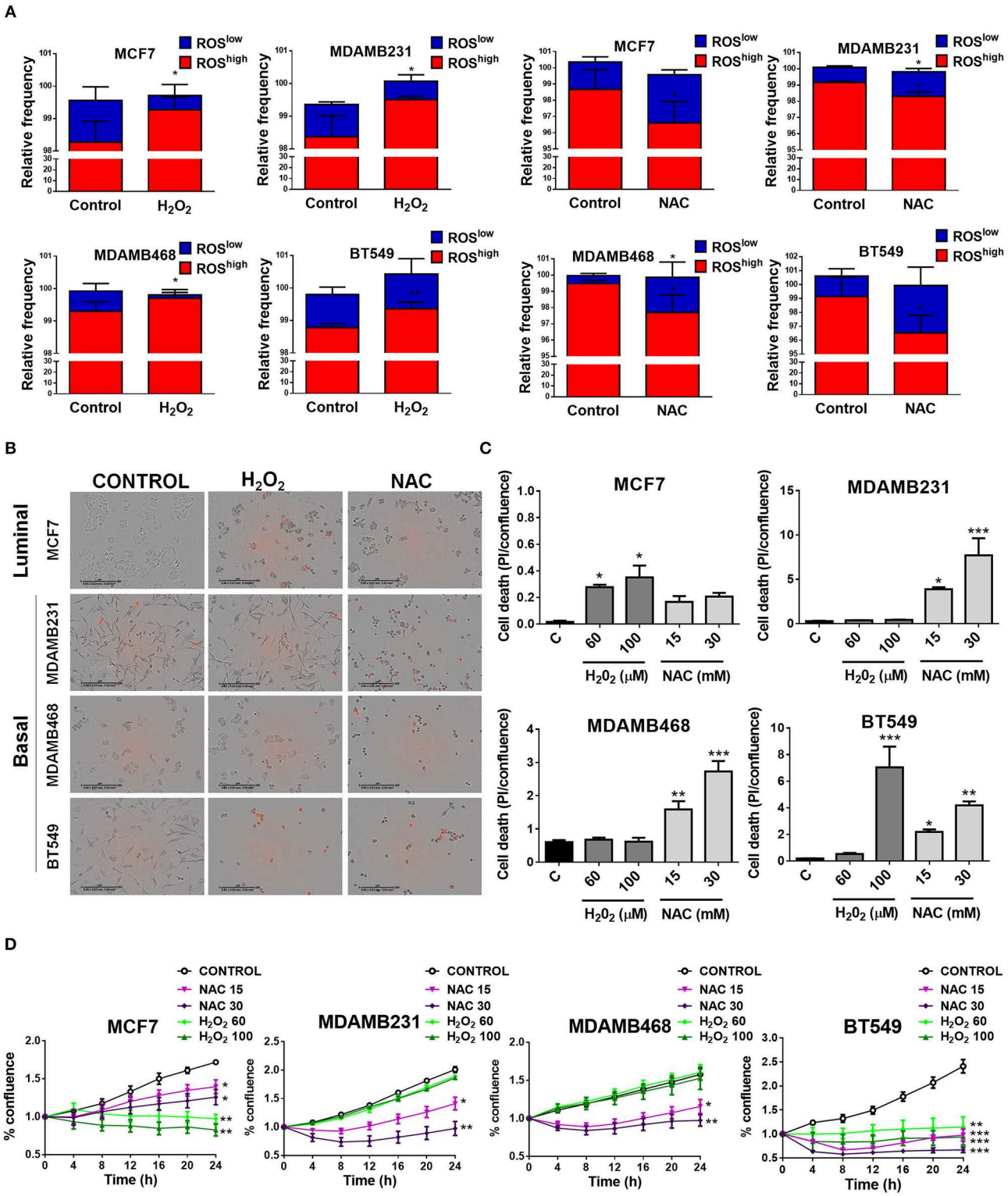

FIGURE 4 | Basal, TNBC cell lines are dependent on ROS for survival. **(A)** Cells were treated with oxidating conditions (H_2O_2) or with an antioxidant (NAC, N-acetyl cysteine) at the indicated concentrations. One hundred micromolar H_2O_2 treatment increased the ROS^{high} or decreased the ROS^{low} population and 30 mM NAC had the opposite effect in all breast cancer cell lines studied. **(B,C)** Cell death was evaluated as propidium iodide staining [% confluency of PI(+) cells] and normalized to total % confluency. **(D)** Cell proliferation was evaluated as changes in cell confluency in an Incucyte real time cell imaging system. Graphs show mean ± SEM of more than 3 independent experiments. *different to control, $p < 0.05$; **different to control, $p < 0.01$; and ***different to control, $p < 0.001$.

(MCF7) with low oxidation levels (**Figure 4A**). H_2O_2 treatment increased the ROS^{high} population and/or decreased the relative frequency in the ROS^{low} population in the cancer cell lines studied. NAC treatment decreased the relative frequency of the ROS^{high} or increased the ROS^{low} population (**Figure 4A**). We measured cell confluency as a measure of cellular viability and cell death using the cell impermeable dye propidium iodide (PI). Dead cells with compromised plasma membrane are permeable to the dye and red nuclei represent dead cells (**Figures 4B,C**). The MCF7 luminal breast cancer cell line was sensitive to H_2O_2 treatment since increased PI staining as well as decreased proliferation was observed (**Figures 4B–D**), whereas with NAC treatment, only proliferation was affected (decreased, **Figure 4D**) and no induction of cell death was observed (**Figures 4B,C**). Interestingly, TNBC cell lines (MDAMB231, MDAMB468, and BT549) showed the most striking changes in cell morphology, increased cell death, and decreased proliferation after antioxidant treatment (**Figures 4B–D**). Surprisingly, H_2O_2 had no effect on cell death (**Figures 4B,C**) or cell proliferation (**Figure 4D**) on MDAMB231 and MDAMB468 TNBC cell lines and both NAC and H_2O_2 induced cell death in the BT549 TNBC cell line. These results suggest that high ROS levels are responsible for maintaining oncogenic signaling in TNBC cells.

In order to evaluate the source of ROS production, we explored if elevated ROS in TNBC cell lines were derived from mitochondria by co-staining cell lines with MitoSox (red) to evaluate mitochondrial ROS production and MitoTracker (green) to stain mitochondria and compared to ER+ MCF7 or non-tumorigenic MCF10A cell lines (**Figure 5A**). MitoSox is a DHE derivative with an additional cationic triphenylphosphonium group (TPP+). Due to its positive charge, MitoSox preferentially accumulates within the mitochondrial matrix and its red fluorescent oxidation products have been used to measure mitochondrial ROS production (31).

We found increased MitoSox staining in all the TNBC cell lines studied as well as increased co-localization of both stains indicating higher mitochondrial ROS production in the TNBC cell lines (**Figure 5A**). In addition, MitoSox levels were higher in the TNBC cell lines when compared to the luminal MCF7 cell line when MitoSox fluorescence intensity was quantified by flow cytometry (**Figure 5B**). Importantly, antimycin A (AA), a mitochondrial complex III inhibitor, only increased MitoSox fluorescence in the MCF10A, MCF7, and MDAMB231 cell

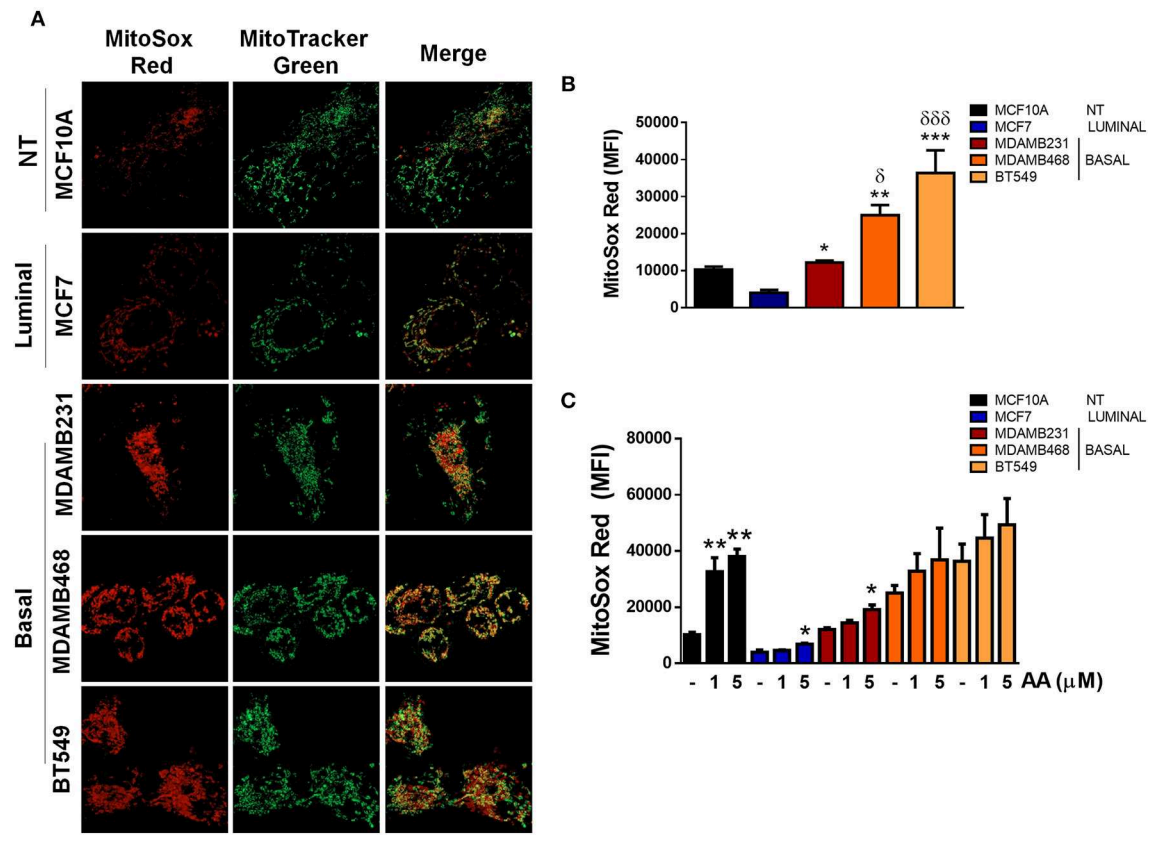

FIGURE 5 | ROS in basal, TNBC cell lines are derived from the mitochondria. **(A)** Mitochondria were stained with Mitotracker (green) and MitoSox (red) to evaluate production of mitochondrial ROS. **(B)** Quantification of MitoSox fluorescence was performed by flow cytometry in basal conditions or **(C)** in cells treated with Antimycin A (AA) at the indicated concentrations. Graphs show mean ± SEM of 3 independent experiments. In **(B)** *different to MCF7 and $^{\delta}$different to MCF10A. In **(C)** *different to control. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; *δ <0.05; and ***δδδ <0.001.

lines, probably indicating that MDAMB468 and BT549 cell line mitochondria were producing high mitochondrial ROS in basal conditions that cannot be further increased by AA treatment (**Figure 5C**). Although the MDAMB231 TNBC cell line had increased MitoSox levels when compared to the MCF7 cell line, it showed high DHE fluorescence (**Figure 3C**) and its DHE fluorescence intensity was similar to MDAMB468 cells. Furthermore, frequency of $MitoSox^{high}$ cells was lower than the DHE^{high} population in both the MDAMB231 and MDAMB468 but not in the BT549 cell line (**Supplementary Figure 4**). The previous data indicate that the MDAMB231 and the MDAMB468 cell line, to a lesser extent, have active ROS sources additional to the mitochondria that contribute to the maintenance of their high oxidation state.

DISCUSSION

Alterations in mitochondrial dynamics and function have been related to malignancy in different types of cancer. In breast cancer, increased mitochondrial fission-related Drp1 protein levels have been found in breast carcinomas and lymph node metastases (25), increased mitochondrial fission has been observed in TNBC cell lines and it has been shown that mitochondrial fission is necessary for cell migration and invasion (25). It has also been shown that invasive breast cancer cells have increased oxidative phosphorylation (OXPHOS), mitochondrial biogenesis, and oxygen consumption rates when compared to their non-invasive counterparts (32). Differences in mitochondrial mass have been observed in primary human breast tumors (33), and mitochondria from breast cancer cell lines with different metastatic capacities have been shown to have different functional characteristics (34). In agreement with the previously published reports, a mitochondria-related gene signature clustered breast cancer tumor samples according to their intrinsic subtype (**Figure 1**) and we found important differences in mitochondrial morphology when comparing the non-tumorigenic MCF10A with all the breast cancer cell lines tested (**Figures 2A,C**). Breast cancer cell lines had more fragmented mitochondria than the non-tumorigenic cell line, which had almost 40% of cells with a tubular morphology (I) while all the cancer cell lines had <15% indicating an important role for mitochondrial fragmentation in transformation. However, despite significant differences in the expression levels of genes related to mitochondrial dynamics among breast tumor subtypes (**Table 1**, **Supplementary Figure 1**), we did not find a clear relationship between mitochondrial morphology and breast cancer subtype in the cell lines studied. Although luminal cell lines MCF7 and T47D showed a very similar mitochondrial morphology, mitochondrial shape from basal, TNBC cell lines was highly heterogeneous (**Figure 2A**). Nevertheless, the basal-like subtype was the most different to other breast cancer subtypes with regards to changes in mitochondria-related gene expression (**Table 1**, **Figure 1**), suggesting mitochondrial alterations different to mitochondrial shape. So, although protein levels of individual mitochondrial-dynamics related proteins (e.g., Drp1) have been related to increased levels of malignancy (25), the global changes in mitochondria-related gene expression that we studied (**Figure 1**), clustered breast cancer samples according to tumor subtypes and seem to be related to changes in mitochondrial function rather to a specific mitochondrial morphology. Indeed, we found increased DNM1L/Drp1 mRNA levels in Basal-like breast cancer samples (**Table 1**, **Supplementary Figure 1**) but we also found increased mitochondrial fusion and biogenesis-related gene expression, as well as decreased mitophagy-related genes, suggesting global changes in mitochondrial function beyond mitochondrial morphology. Importantly, changes in mitochondrial gene expression, particularly of genes related to mitochondrial dynamics could also reflect a distinct association of mitochondria with other organelles, like the endoplasmic reticulum, which has been shown to regulate cellular processes like endoplasmic reticulum stress, autophagy and inflammasome signaling (35), but this was not evaluated in this study.

One of the consequences of mitochondrial disfunction that has been involved in several aspects of carcinogenesis is the production of ROS and elevated ROS levels have been found in tumor cells from different tissues (14, 36). In this regard, it has been proposed that cancer cells are able to modulate their antioxidant capacity to achieve a different redox balance than normal cells. In this setting, overproduction of ROS due to oncogenic signaling or metabolic alterations has been shown to result in increased antioxidant capacity that is able to maintain oncogenic ROS signaling, allowing disease progression and avoiding cell death (28). Oncogenic signaling pathways that are known to be activated by ROS include the NF-kB (37), NRF2, Wnt (38), and EGFR signaling pathways (39, 40). Also, ROS have been shown to activate the tumor suppressor p53 and mediate apoptosis (41). Importantly, basal-like tumors are known to present TP53 loss of function in most, if not all, tumors and show amplification of the RAS-RAF-MEK pathway including amplifications in EGFR (7). We found increased ROS levels in TNBC cell lines when compared to a non-tumorigenic or an ER+/luminal breast cancer cell line. The increased oxidation state was necessary for cell survival since antioxidant treatment induced cell death in TNBC cell lines and not in non-tumorigenic or an ER+/luminal breast cancer cell line. Our results demonstrate that increased ROS production in TNBC cell lines have a pro-tumorigenic role by sustaining the oncogenic signaling necessary for their proliferation and survival and suggest that the loss of function of p53 characteristic of this type of breast cancer might be necessary to survive this strongly oxidizing conditions. Mitochondrial ROS were the main source of ROS in TNBC cell lines (**Figure 5**) and mitochondrial ROS levels were related to mitochondrial shape (**Figure 2**), since MitoSox fluorescence correlated with the percentage of cells in the fragmented classification (IV, **Figure 2A**) for the TNBC subtype. Our results relate differences in mitochondrial shape among TNBC cell lines with mitochondrial ROS production and suggest that those TNBC cell lines with low levels of fragmented mitochondria (e.g., MDAMB231 cell line) could have additional ROS sources to maintain oxidative conditions and signaling, while those TNBC cells with high levels of fragmented mitochondria (BT549)

would rely only on mitochondrial ROS production to sustain oncogenic signaling.

In the literature, there is conflicting evidence regarding the use of antioxidants during cancer progression and treatment. In normal cells or pre-cancerous lesions, ROS have been proposed to induce DNA damage and increase oncogenic mutations, raising the possibility that dietary supplementation with antioxidants could suppress the initiation or progression of some types of cancer. However, antioxidant treatment is known to suppress cancer initiation in some contexts and increase cancer progression in others (16). Moreover, the use of dietary antioxidants has not been shown to reduce cancer incidence and in fact, antioxidant supplementation has actually increased incidence and death from some types of cancer including lung cancer (42) or increase the risk of developing another type of unrelated diseases (43, 44). In cancer progression models there is also contradictory evidence regarding the use of antioxidants for the treatment of cancer. For instance, in a melanoma mouse model, metastatic cells had higher cytoplasmic and mitochondrial ROS levels and lower mitochondrial mass. The authors proposed that the high levels of oxidation in metastasis are limiting for the establishment of metastasis, since the treatment with antioxidants increased the number of circulating tumor cells and metastatic burden without affecting the growth of the tumor (45). On the other hand, in another study, highly metastatic cells were found to have increased mitochondrial activity and superoxide production. In this case, antioxidant treatment decreased migration and invasiveness which was proposed to be due to ROS-mediated Src activation in tumor cells (46). In breast cancer, in the PyMT mouse cancer model, mice with decreased glutathione content due to deficiency in GCLM (glutamate cysteine ligase modifier), a subunit of glutamate cysteine ligase, necessary for glutathione synthesis or mice treated with BSO (buthionine-[S,R]-sulfoximine) to chemically inhibit glutathione synthesis, had decreased mammary tumor burden. When formed, tumors in PyMT-Gclm-/- mice, showed reduced proliferation and progression (47). So, at least in this model, pre-cancerous lesions need the antioxidant effect of glutathione to progress to breast cancer, which would argue against the use of antioxidants for breast cancer treatment. On the other hand, also in breast cancer, antioxidant treatment decreased DNA lesions and tumorigenesis in a murine model of BRCA1-deficient, $p53^{+/-}$ breast cancer, where excessive estrogen metabolism increased cancer cell ROS production and DNA damage (48). Also, ROS scavenging by overexpression of exogenous EcSOD (extracellular superoxide dismutase), decreased invasion of breast cancer cells *in vitro* (49), and decreased metastasis *in vivo* in TNBC mouse models (50). Moreover, increased ROS levels have been associated with BRCA1 mutations in this type of breast cancer (40).

In agreement with the previous studies, where BRCA1 mutations, which predispose to TNBC have been found to have increased ROS levels, our data shows increased ROS levels in all the TNBC cell lines studied in comparison to an ER+ breast cancer cell line or the non-tumorigenic cells. Moreover, high ROS levels seem to be necessary for the maintenance and survival of this type of cancer since antioxidant treatment greatly decreased proliferation and induced cell death. Our results suggest a potential use for ROS or oxidation products in cancer cells as biomarkers of malignancy in TNBC which is currently diagnosed by the absence of immunohistochemistry biomarkers. Furthermore, mitochondrial ROS could function as a therapeutic target against this cancer subtype, which currently lacks a targeted therapy. Our data explains controversies in the literature regarding the use of antioxidants for cancer therapy and we propose that antioxidant treatments should only be used in those cancer cells with high basal ROS levels which are likely to use this pro-oxidant conditions to sustain oncogenic signaling. Our results also suggest that a similar approach could be used in those types of cancer which have been shown to have increased oxidation levels or similar mechanisms of transformation as TNBC (e.g., *BRCA1* inactivation, *RB1* loss, *TP53* inactivation or amplifications in the *MAPK* pathway) (7). In this regard, serous ovarian carcinomas have been shown to have a similar mutation and expression profile as TNBC (7) and has also been shown to manifest a pro-oxidant state (51), indicating a potential use for antioxidant treatment in this type of cancer. Other cancer types that have also been characterized by increased oxidation levels and in which a role of ROS has been proposed in the promotion of malignancy include prostate (52), gastric (53, 54), and pancreatic cancer (55). Importantly, our data also suggests that antioxidant treatment should not be suggested as a general therapy for cancer in these tissues since heterogeneity in tumors from those tissues has also been reported (56–58) and a careful analyses of those cancer subtypes which utilize pro-oxidant signaling for survival should be performed.

Our data also suggests a possible explanation for the anti-cancer effect of drugs with yet unclear mechanisms of action like metformin. This drug has been shown to have anti-cancer effects on breast (59) and other types of cancer (60, 61), has been proposed to have an antioxidant effect (62), and is known to be particularly effective on the TNBC subtype (63). So, our results indicate a potential use for this drug or for antioxidant nutraceuticals (64) for the treatment of this type of cancer and for cancers with similar mechanisms of oncogenicity involving increased oxidation states.

Finally, our results underscore the role of mitochondrial ROS production in sustaining oncogenic signaling in the basal, TNBC subtype as the main, but likely not only, source of ROS and highlights their potential use as a therapeutic target in this breast cancer subtype with current limited therapeutic options.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

FS-S, PC-H, RP, LM-P-P, and PM contributed conception and design of the study. FS-S, AD-M, JM-A, DR-R, and JF-A performed the experiments. FS-S, JR-L, IH-C, MA-R,

RP, and PM wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00480/full#supplementary-material

REFERENCES

1. Russnes HG, Lingjaerde OC, Borresen-Dale AL, Caldas C. Breast cancer molecular stratification: from intrinsic subtypes to integrative clusters. *Am J Pathol*. (2017) 187:2152–62. doi: 10.1016/j.ajpath.2017.04.022
2. Sorlie T. Molecular portraits of breast cancer: tumour subtypes as distinct disease entities. *Eur J Cancer*. (2004) 40:2667–75. doi: 10.1016/j.ejca.2004.08.021
3. Sorlie T. The impact of gene expression patterns in breast cancer. *Clin Chem*. (2016) 62:1150–1. doi: 10.1373/clinchem.2015.253229
4. Prat A, Perou CM. Deconstructing the molecular portraits of breast cancer. *Mol Oncol*. (2011) 5:5–23. doi: 10.1016/j.molonc.2010.11.003
5. Coates AS, Winer EP, Goldhirsch A, Gelber RD, Gnant M, Piccart-Gebhart M, et al. Tailoring therapies–improving the management of early breast cancer: St. Gallen international expert consensus on the primary therapy of early breast cancer 2015. *Ann Oncol*. (2015) 26:1533–46. doi: 10.1093/annonc/mdv221
6. Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Investigat*. (2011) 121:2750–67. doi: 10.1172/JCI45014
7. The Cancer Genome Atlas T. Comprehensive molecular portraits of human breast tumours. *Nature*. (2012) 490:61–70. doi: 10.1038/nature11412
8. Howlader N, Cronin KA, Kurian AW, Andridge R. Differences in breast cancer survival by molecular subtypes in the United States. *Cancer Epidemiol Biomarkers Prev*. (2018) 27:619–26. doi: 10.1158/1055-9965.EPI-17-0627
9. Kumar P, Aggarwal R. An overview of triple-negative breast cancer. *Arch Gynecol Obstet*. (2016) 293:247–69. doi: 10.1007/s00404-015-3859-y
10. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell*. (2006) 10:515–27. doi: 10.1016/j.ccr.2006.10.008
11. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
12. Maycotte P, Marin-Hernandez A, Goyri-Aguirre M, Anaya-Ruiz M, Reyes-Leyva J, Cortes-Hernandez P. Mitochondrial dynamics and cancer. *Tumour Biol*. (2017) 39:1-16. doi: 10.1177/1010428317698391
13. Vyas S, Zaganjor E, Haigis MC. Mitochondria and cancer. *Cell*. (2016) 166:555–66. doi: 10.1016/j.cell.2016.07.002
14. Barrera G. Oxidative stress and lipid peroxidation products in cancer progression and therapy. *ISRN Oncol*. (2012) 2012:137289. doi: 10.5402/2012/137289
15. Huang Q, Zhan L, Cao H, Li J, Lyu Y, Guo X, et al. Increased mitochondrial fission promotes autophagy and hepatocellular carcinoma cell survival through the ROS-modulated coordinated regulation of the NFKB and TP53 pathways. *Autophagy*. (2016) 12:999–1014. doi: 10.1080/15548627.2016.1166318
16. Chio IIC, Tuveson DA. ROS in cancer: the burning question. *Trends Mol Med*. (2017) 23:411–29. doi: 10.1016/j.molmed.2017.03.004
17. Sabharwal SS, Schumacker PT. Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles' heel? *Nat Rev Cancer*. (2014) 14:709–21. doi: 10.1038/nrc3803
18. Liou GY, Storz P. Detecting reactive oxygen species by immunohistochemistry. *Methods Mol Biol*. (2015) 1292:97–104. doi: 10.1007/978-1-4939-2522-3_7
19. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet*. (2003) 34:267–73. doi: 10.1038/ng1180
20. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA*. (2005) 102:15545–50. doi: 10.1073/pnas.0506580102
21. Bime C, Zhou T, Wang T, Slepian MJ, Garcia JG, Hecker L. Reactive oxygen species-associated molecular signature predicts survival in patients with sepsis. *Pulm Circ*. (2016) 6:196–201. doi: 10.1086/685547
22. Ulitsky I, Maron-Katz A, Shavit S, Sagir D, Linhart C, Elkon R, et al. Expander: from expression microarrays to networks and functions. *Nat Protoc*. (2010) 5:303–22. doi: 10.1038/nprot.2009.230
23. Giedt RJ, Fumene Feruglio P, Pathania D, Yang KS, Kilcoyne A, Vinegoni C, et al. Computational imaging reveals mitochondrial morphology as a biomarker of cancer phenotype and drug response. *Sci Rep*. (2016) 6:32985. doi: 10.1038/srep32985
24. Wai T, Langer T. Mitochondrial dynamics and metabolic regulation. *Trends Endocrinol Metabol TEM*. (2016) 27:105–17. doi: 10.1016/j.tem.2015.12.001
25. Zhao J, Zhang J, Yu M, Xie Y, Huang Y, Wolff DW, et al. Mitochondrial dynamics regulates migration and invasion of breast cancer cells. *Oncogene*. (2013) 32:4814–24. doi: 10.1038/onc.2012.494
26. Kashatus JA, Nascimento A, Myers LJ, Sher A, Byrne FL, Hoehn KL, et al. Erk2 phosphorylation of Drp1 promotes mitochondrial fission and MAPK-driven tumor growth. *Mol Cell*. (2015) 57:537–51. doi: 10.1016/j.molcel.2015.01.002
27. Serasinghe MN, Wieder SY, Renault TT, Elkholi R, Asciolla JJ, Yao JL, et al. Mitochondrial division is requisite to RAS-induced transformation and targeted by oncogenic MAPK pathway inhibitors. *Mol Cell*. (2015) 57:521–36. doi: 10.1016/j.molcel.2015.01.003
28. Moloney JN, Cotter TG. ROS signalling in the biology of cancer. *Semin Cell Dev Biol*. (2018) 80:50–64. doi: 10.1016/j.semcdb.2017.05.023
29. Wang X, Fang H, Huang Z, Shang W, Hou T, Cheng A, et al. Imaging ROS signaling in cells and animals. *J Mol Med*. (2013) 91:917–27. doi: 10.1007/s00109-013-1067-4
30. Fernandes DC, Goncalves RC, Laurindo FR. Measurement of superoxide production and NADPH oxidase activity by HPLC analysis of dihydroethidium oxidation. *Methods Mol Biol*. (2017) 1527:233–49. doi: 10.1007/978-1-4939-6625-7_19
31. Forkink M, Smeitink JA, Brock R, Willems PH, Koopman WJ. Detection and manipulation of mitochondrial reactive oxygen species in mammalian cells. *Biochim Biophys Acta*. (2010) 1797:1034–44. doi: 10.1016/j.bbabio.2010.01.022
32. Lebleu VS, O'connell JT, Gonzalez Herrera KN, Wikman H, Pantel K, Haigis MC, et al. PGC-1alpha mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis. *Nat Cell Biol*. (2014) 16:992–1003. doi: 10.1038/ncb3039
33. Boland ML, Chourasia AH, Macleod KF. Mitochondrial dysfunction in cancer. *Front Oncol*. (2013) 3:292. doi: 10.3389/fonc.2013.00292
34. Tu YF, Kaipparettu BA, Ma Y, Wong LJ. Mitochondria of highly metastatic breast cancer cell line MDA-MB-231 exhibits increased autophagic properties. *Biochim Biophys Acta*. (2011) 1807:1125–32. doi: 10.1016/j.bbabio.2011.04.015

35. Van Vliet AR, Verfaillie T, Agostinis P. New functions of mitochondria associated membranes in cellular signaling. *Biochim Biophys Acta*. (2014) 1843:2253–62. doi: 10.1016/j.bbamcr.2014.03.009
36. Gu H, Huang T, Shen Y, Liu Y, Zhou F, Jin Y, et al. Reactive oxygen species-mediated tumor microenvironment transformation: the mechanism of radioresistant gastric cancer. *Oxid Med Cell Longev*. (2018) 2018:8. doi: 10.1155/2018/5801209
37. Morgan MJ, Liu ZG. Crosstalk of reactive oxygen species and NF-kappaB signaling. *Cell Res*. (2011) 21:103–15. doi: 10.1038/cr.2010.178
38. de Sá Junior PL, Câmara DAD, Porcacchia AS, Fonseca PMM, Jorge SD, Araldi RP, et al. The roles of ROS in cancer heterogeneity and therapy. *Oxid Med Cell Longev*. (2017):2467940. doi: 10.1155/2017/2467940
39. Liu LZ, Hu XW, Xia C, He J, Zhou Q, Shi X, et al. Reactive oxygen species regulate epidermal growth factor-induced vascular endothelial growth factor and hypoxia-inducible factor-1alpha expression through activation of AKT and P70S6K1 in human ovarian cancer cells. *Free Radic Biol Med*. (2006) 41:1521–33. doi: 10.1016/j.freeradbiomed.2006.08.003
40. Kubli SP, Bassi C, Roux C, Wakeham A, Gobl C, Zhou W, et al. *AhR controls redox homeostasis and shapes the tumor microenvironment in BRCA1-associated breast cancer. Proc Natl Acad Sci USA*. (2019) 116:3604–13. doi: 10.1073/pnas.1815126116
41. Liu B, Chen Y, St. Clair DK. ROS and p53: a versatile partnership. *Free Radic Biol Med*. (2008) 44:1529–35. doi: 10.1016/j.freeradbiomed.2008.01.011
42. Fortmann SP, Burda BU, Senger CA, Lin JS, Whitlock EP. Vitamin and mineral supplements in the primary prevention of cardiovascular disease and cancer: an updated systematic evidence review for the U.S. Preventive Services Task Force. *Ann Intern Med*. (2013) 159:824–34. doi: 10.7326/0003-4819-159-12-201312170-00729
43. Bjelakovic G, Gluud LL, Nikolova D, Whitfield K, Krstic G, Wetterslev J, et al. Vitamin D supplementation for prevention of cancer in adults. *Cochrane Database Syst Rev*. (2014) 6:CD007469. doi: 10.1002/14651858.CD007469.pub2
44. Bjelakovic G, Nikolova D, Gluud C. Antioxidant supplements and mortality. *Curr Opin Clin Nutr Metab Care*. (2014) 17:40–4. doi: 10.1097/MCO.0000000000000009
45. Piskounova E, Agathocleous M, Murphy MM, Hu Z, Huddlestun SE, Zhao Z, Oxidative stress inhibits distant metastasis by human melanoma cells. *Nature*. (2015) 527:186–91. doi: 10.1038/nature15726
46. Porporato PE, Payen VL, Perez-Escuredo J, De Saedeleer CJ, Danhier P, Copetti T, et al. A mitochondrial switch promotes tumor metastasis. *Cell Rep*. (2014) 8:754–66. doi: 10.1016/j.celrep.2014.06.043
47. Harris IS, Treloar AE, Inoue S, Sasaki M, Gorrini C, Lee KC, et al. Glutathione and thioredoxin antioxidant pathways synergize to drive cancer initiation and progression. *Cancer Cell*. (2015) 27:211–22. doi: 10.1016/j.ccell.2015.01.009
48. Li M, Chen Q, Yu X. Chemopreventive effects of ROS targeting in a murine model of BRCA1-deficient breast cancer. *Cancer Research*. (2017) 77:448–58. doi: 10.1158/0008-5472.CAN-16-2350
49. Teoh ML, Fitzgerald MP, Oberley LW, Domann FE. Overexpression of extracellular superoxide dismutase attenuates heparanase expression and inhibits breast carcinoma cell growth and invasion. *Cancer Res*. (2009) 69:6355–63. doi: 10.1158/0008-5472.CAN-09-1195
50. Teoh-Fitzgerald ML, Fitzgerald MP, Zhong W, Askeland RW, Domann FE. Epigenetic reprogramming governs EcSOD expression during human mammary epithelial cell differentiation, tumorigenesis and metastasis. *Oncogene*. (2014) 33:358–68. doi: 10.1038/onc.2012.582
51. Saed GM, Diamond MP, Fletcher NM. Updates of the role of oxidative stress in the pathogenesis of ovarian cancer. *Gynecol Oncol*. (2017) 145:595–602. doi: 10.1016/j.ygyno.2017.02.033
52. Lim SD, Sun C, Lambeth JD, Marshall F, Amin M, Chung L, et al. Increased Nox1 and hydrogen peroxide in prostate cancer. *Prostate*. (2005) 62:200–7. doi: 10.1002/pros.20137
53. Tamura M, Matsui H, Tomita T, Sadakata H, Indo HP, Majima HJ, et al. Mitochondrial reactive oxygen species accelerate gastric cancer cell invasion. *J Clin Biochem Nutr*. (2014) 54:12–7. doi: 10.3164/jcbn.13-36
54. Du S, Miao J, Zhu Z, Xu E, Shi L, Ai S, et al. NADPH oxidase 4 regulates anoikis resistance of gastric cancer cells through the generation of reactive oxygen species and the induction of EGFR. *Cell Death Dis*. (2018) 9:948. doi: 10.1038/s41419-018-0953-7
55. Storz P. KRas, ROS and the initiation of pancreatic cancer. *Small GTPases*. (2017) 8:38–42. doi: 10.1080/21541248.2016.1192714
56. Cancer Genome Atlas Research N. The molecular taxonomy of primary prostate cancer. *Cell*. (2015) 163:1011–25. doi: 10.1016/j.cell.2015.10.025
57. Li X, Wu WK, Xing R, Wong SH, Liu Y, Fang X, et al. Distinct subtypes of gastric cancer defined by molecular characterization include novel mutational signatures with prognostic capability. *Cancer Res*. (2016) 76:1724–32. doi: 10.1158/0008-5472.CAN-15-2443
58. Sidaway P. Pancreatic cancer: TCGA data reveal a highly heterogeneous disease. *Nat Rev Clin Oncol*. (2017) 14:648. doi: 10.1038/nrclinonc.2017.146
59. Faria J, Negalha G, Azevedo A, Martel F. Metformin and breast cancer: molecular targets. *J Mammary Gland Biol Neoplasia*. (2019). doi: 10.1007/s10911-019-09429-z. [Epub ahead of print].
60. Coyle C, Cafferty FH, Vale C, Langley RE. Metformin as an adjuvant treatment for cancer: a systematic review and meta-analysis. *Ann Oncol*. (2016) 27:2184–95. doi: 10.1093/annonc/mdw410
61. Antognelli C, Cecchetti R, Riuzzi F, Peirce MJ, Talesa VN. Glyoxalase 1 sustains the metastatic phenotype of prostate cancer cells via EMT control. *J Cell Mol Med*. (2018) 22:2865–83. doi: 10.1111/jcmm.13581
62. Kurhaluk N, Bojkova B, Winklewski PJ. Liver antioxidant and aerobic status improves after metformin and melatonin administration in a rat model of high-fat diet and mammary carcinogenesis. *Can J Physiol Pharmacol*. (2018) 96:790–7. doi: 10.1139/cjpp-2018-0001
63. Liu B, Fan Z, Edgerton SM, Deng XS, Alimova IN, Lind SE, et al. Metformin induces unique biological and molecular responses in triple negative breast cancer cells. *Cell Cycle*. (2009) 8:2031–40. doi: 10.4161/cc.8.13.8814
64. Prasad S, Gupta SC, Tyagi AK. Reactive oxygen species (ROS) and cancer: role of antioxidative nutraceuticals. *Cancer Lett*. (2017) 387:95–105. doi: 10.1016/j.canlet.2016.03.042

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REVIEW
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LncRNAs as Regulators of Autophagy and Drug Resistance in Colorectal Cancer

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Colorectal cancer (CRC) is a common malignancy with 1. 8 million cases in 2018. Autophagy helps to maintain an adequate cancer microenvironment in order to provide nutritional supplement under adverse conditions such as starvation and hypoxia. Additionally, most of the cases of CRC are unresponsive to chemotherapy, representing a significant challenge for cancer therapy. Recently, autophagy induced by therapy has been shown as a unique mechanism of resistance to anticancer drugs. In this regard, long non-coding RNAs (lncRNAs) analysis are important for cancer detection, progression, diagnosis, therapy response, and prognostic values. With increasing development of quantitative detection techniques, lncRNAs derived from patients' non-invasive samples (i.e., blood, stools, and urine) has become into a novel approach in precision oncology. Tumorspecific GAS5, HOTAIR, H19, and MALAT are novels CRC related lncRNAs detected in patients. Nonetheless, the effect and mechanism of lncRNAs in cancer autophagy and chemoresistance have not been extensively characterized. Chemoresistance and autophagy are relevant for cancer treatment and lncRNAs play a pivotal role in resistance acquisition for several drugs. LncRNAs such as HAGLROS, KCNQ1OT1, and H19 are examples of lncRNAs related to chemoresistance leaded by autophagy. Finally, clinical implications of lncRNAs in CRC are relevant, since they have been associated with tumor differentiation, tumor size, histological grade, histological types, Dukes staging, degree of differentiation, lymph node metastasis, distant metastasis, recurrent free survival, and overall survival (OS).

Keywords: lncRNA, colorectal cancer, autophagy, chemoresistance, drug resistance, macroautophagy

INTRODUCTION

Cancer is one of the most deathly illness worldwide with an estimated 9.6 million deaths in 2018 (1). One of the most common is colorectal cancer (CRC) with 1.8 million cases and 862,000 deaths only during the last year (1). Development of CRC involves different genetic and epigenetic changes. Most cases are sporadic and show a slow development through the time, advancing from adenoma to carcinoma (2). Even though there are important progress in treatment and molecular mechanisms involved in CRC, the OS rate still remains relatively low (3, 4).

Chemotherapy has been widely used for cancer treatment, for instance, the fluoropyrimidine 5-fluorouracil (5-FU) is a first selection anticancer drugs for CRC treatment (5). Besides, new drugs such as cetuximab and panitumumab have been incorporated into clinical practice (6). Nevertheless, drug resistance acquisition is one of the main issues in effective chemotherapy (7). This due to different factors as Pharmacokinetic Resistance, that includes since absorption until, distribution, metabolism, and the excretion of drugs. In addition, the evolutionary resistance, a process that occurs in the tumor where the cells acquire the ability to survive chemotherapy, this through expression of different proteins, such as P-glycoprotein 1 (P-gp) also known as, multidrug resistance protein 1 (MDR1). Besides the physics of the tumor site is involved in chemotherapy resistance such as, number and morphology of vessels and blood viscosity, are important factors involved (8). Drug ineffectiveness could be the result from tumor-host interactions and a clear understanding of such an interaction will open new opportunities not only for the discovery of new drugs but also for new therapeutic strategies to overcome the development and evolution of resistance to cancer chemotherapy.

Autophagy is an important cellular response to stress or starvation and starts when organelles and proteins are sequestered in vesicles and delivered to lysosomes for degradation (9). New research revealed that autophagy has different functions in the development, maintenance, and tumor progression (10) and recently, autophagy induced by therapy has been shown as a new mechanism of resistance to chemotherapeutic drugs (11). Through carcinogenic process of CRC, autophagy could promote tumor survival or cancer cell death, and it depends on the tumor type, stage, and the metabolic setting (12).

Non-coding RNAs (ncRNAs) represent 99% of total transcribed RNAs in the human genome, being the principal components of the human transcriptome (13). Recently, ncRNAs have shown to play key roles in important biological processes by interfering with gene expression in several cancer types (14, 15).

The best characterized of the "expanding universe" of ncRNAs are the ∼22 nucleotide microRNAs (miRNAs) and the long non-coding RNAs (lncRNAs). The lncRNAs are classified as >200 nucleotides in length and are involved in a wide variety of molecular genetics and cellular processes in many aspects of gene regulation, including imprinting, epigenetic modulation, transcription, mRNA splicing, and tracking between the nucleus and cytoplasm (15–18). Moreover, lncRNAs are involved in variety biological processes such as, proliferation, differentiation, apoptosis, invasion, and metastasis.

Recently, lncRNAs have been implicated in tumor-drug resistance and autophagy in different types of cancer including CRC (16, 19–22). Therefore, the aim of this review is to compile the current knowledge about lncRNAs and their implication on chemoresistance and autophagy in CRC. To this end, we searched on PubMed, PMC, Web of Science, Google scholar, and EMBASE up to July 2019 for pertinent articles using the keywords as follows: (lncRNA or long non-coding RNA) and (CRC or colorectal cancer) and (autophagy or autophagia) and (chemoresistance or drug resistance). The titles and abstracts were screened, and we acquired the relevant full-text manuscripts for perusal.

LONG NON-CODING RNAS

Biogenesis, Classification, and Function

LncRNAs include different types of RNA polymerase II (Pol II)-transcribed molecules with sizes over 200 nt in length. It has been reported an estimated abundance of 5,400 to more than 10,000 lncRNAs transcripts in humans (23, 24). All mammalian lncRNAs share a few structural, functional, or mechanistic characteristics among them. They often harbor a poly-A tail and can be spliced, similar to mRNAs (25). Besides, they regulate gene expression at transcriptional and post-transcriptional levels in multiple biological processes and cellular contexts (26–28).

Spurlock et al., classified LncRNAs based on their structural origin context (**Figure 1**). Overlapping when a protein-coding genes is included in the intron of a lncRNA (29, 30), divergent when the lncRNA and neighboring protein coding gene are transcribed on opposite strands (31), intronic when the whole sequence of the lncRNA belongs to the intron of a protein-coding gene (32), intergenic when a lncRNA sequence belongs to two genes as a distinct unit (33), and sense (34) or antisense (35) when the lncRNA is located between one or more exons of another transcript on the same sense or antisense strand (36–38). Lastly, enhancer RNAs can be transcribed in one or two senses, 1D-eRNAs and 2D-eRNAs, respectively, at genomic transcriptional enhancers, frequently very close to protein-coding genes (39).

It has been shown that lncRNAs functions depend on their subcellular location (26). There is evidence in human cell lines using single molecule RNA fluorescence *in situ*-hybridization that revealed a wide range of subcellular localization patterns, including nucleus, cytoplasm and both (40). Nevertheless, it is most common to catalog lncRNAs based on similar action mechanisms (25) (**Figure 2**).

Some lncRNAs have a very important role in nuclear structure, since they help to the structure of nuclear speckles, paraspeckles, and interchromatin granules (41). Another nuclear lncRNAs are able to regulate gene expression by epigenetic mechanisms and recruiting chromatin-modification factors in order to switch-on or switch-off different loci (42). Besides, there are other types of stable lncRNAs, such as competing endogenous RNAs (ceRNAs) and circular RNAs, which are accumulated in the cell acting as decoys or sponges for miRNAs modulating gene expression (43).

LncRNAs also has an important role in transcription since they help in assembling transcriptional activators and repressors for modulating the activation of transcription (44). Besides, lncRNAs are able to modulate gene expression post-transcriptionally by interfering with RNA-binding proteins to impact splicing and translation and by modulating the translation and stability of partially complementary mRNAs (45, 46). In addition, some lncRNAs function post-transductionally in order to regulate protein turnover to enhance ubiquitination (47).

Detection Methods

The lncRNAs importance in cancer characteristics such as progression, autophagy, and chemotherapy resistance has been

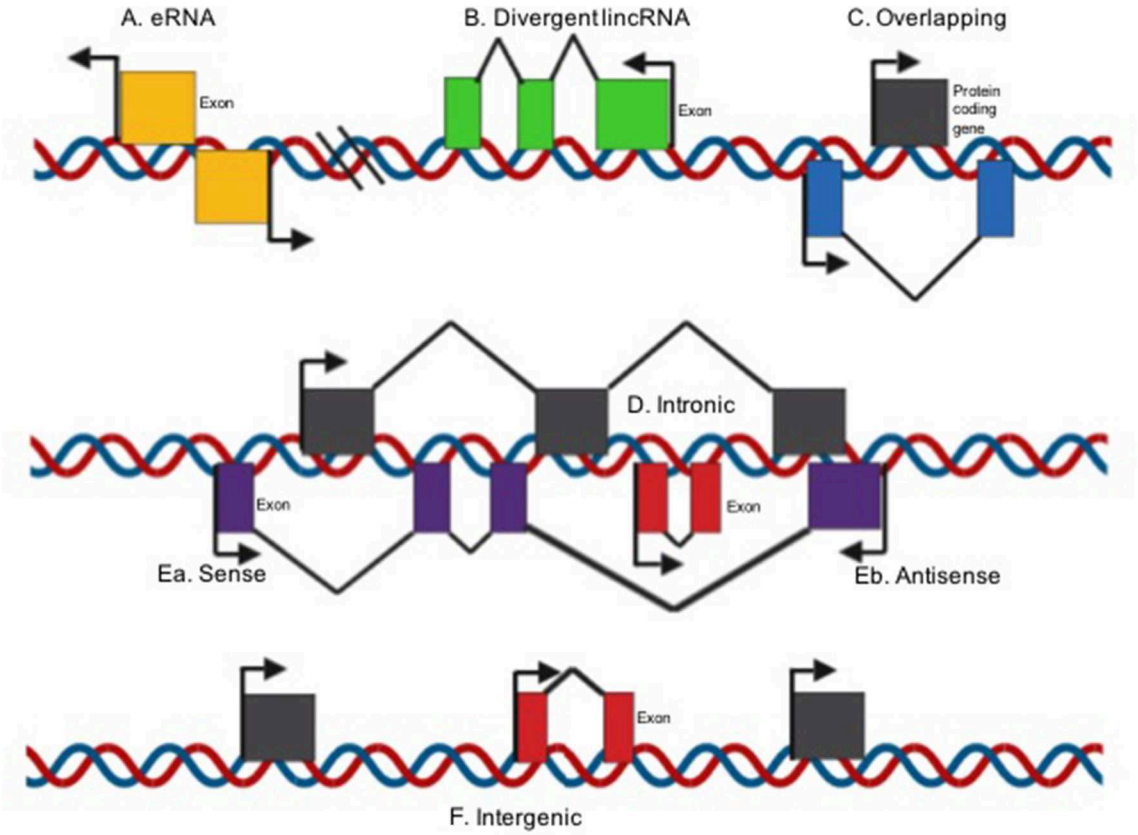

FIGURE 1 | LncRNA classification on their structural origin context [modified image from Spurlock et al. (29)]. **(A)** Enhancer RNAs can be transcribed in one or two senses 1D-eRNAs and 2D-eRNAs, respectively, at genomic transcriptional enhancers, frequently in close proximity to protein-coding genes; **(B)** Divergent when the lincRNA and nearby protein coding gene are transcribed on opposite strands; **(C)** overlapping when a protein-coding genes is included in the intron of a lncRNA; **(D)** Intronic when the whole sequence of the lncRNA belongs to the intron of a protein-coding gene; Ea. Sense or **(E)** antisense if the lncRNA is located between one or more exons of another transcript on the same sense or antisense strand; **(F)** intergenic when a lncRNA sequence belongs to two genes as a distinct unit.

established thanks to more advanced detection technologies. The main two methods for lncRNAs detection are microarrays and RNA sequencing (RNA-seq), Microarrays contain probe sequences that match with lncRNAs (48). Whereas, RNA-seq provides comprehensive coverage of whole transcriptomes compared to microarrays. Due to unbiased genome-wide screening, it is possible to exclude ribosomal RNAs (rRNAs) from total RNA to enhance it, including protein-coding genes and lncRNAs. Besides, it is possible to enrich mRNAs using oligo-dT beads with poly A tails, giving as a result the detection of protein-coding genes and lncRNAs with poly A tails that are nearly 60% of total lncRNAs (49, 50).

Since lncRNAs has been described as biomarkers in several types of cancer, non-invasive detection methods have been developed (**Table 1**) for early diagnostic, evolution, and poor prognosis of cancer (62). Thus, there are several carcinomas that can be detected by specific serum circulating lncRNAs (**Table 1**) (63). Moreover, lncRNAs are detectable in urine and may serve as biomarker predictor in T-cell mediated kidney transplant rejection as well as bladder cancer tumor-stage (64, 65). In addition, US Food and Drug administration (FDA) has recently approved PCA3 lncRNA as a biomarker for prostate cancer in urine (66, 67) showing better sensitivity and specificity than Prostate-specific antigen (PSA) blood test (68). Whole saliva also represents a source for cancer biomarkers by lncRNAs detection, given this, saliva contains certain lncRNAs that can be used as biomarkers for oral squamous cell carcinoma diagnosis such as HOTAIR, which presence in saliva samples is correlated with high levels in metastatic tissues (69).

LNCRNAS IN COLORECTAL CANCER

LncRNAs play key roles regulating gene expression during cell development and differentiation, regulating or maintaining cellular homeostasis (70, 71). Abnormal expression of lncRNAs has been reported in numerous cancer types such as; hematopoietic, urologic, lung, liver, breast, ovarian, and colorectal (72–79). Alterations of these molecules are studied in CRC in order to obtain clinical biomarkers for diagnostic, prognostic, and therapeutic applications (80, 81). Multiple lncRNAs have been related with CRC as important clinical and

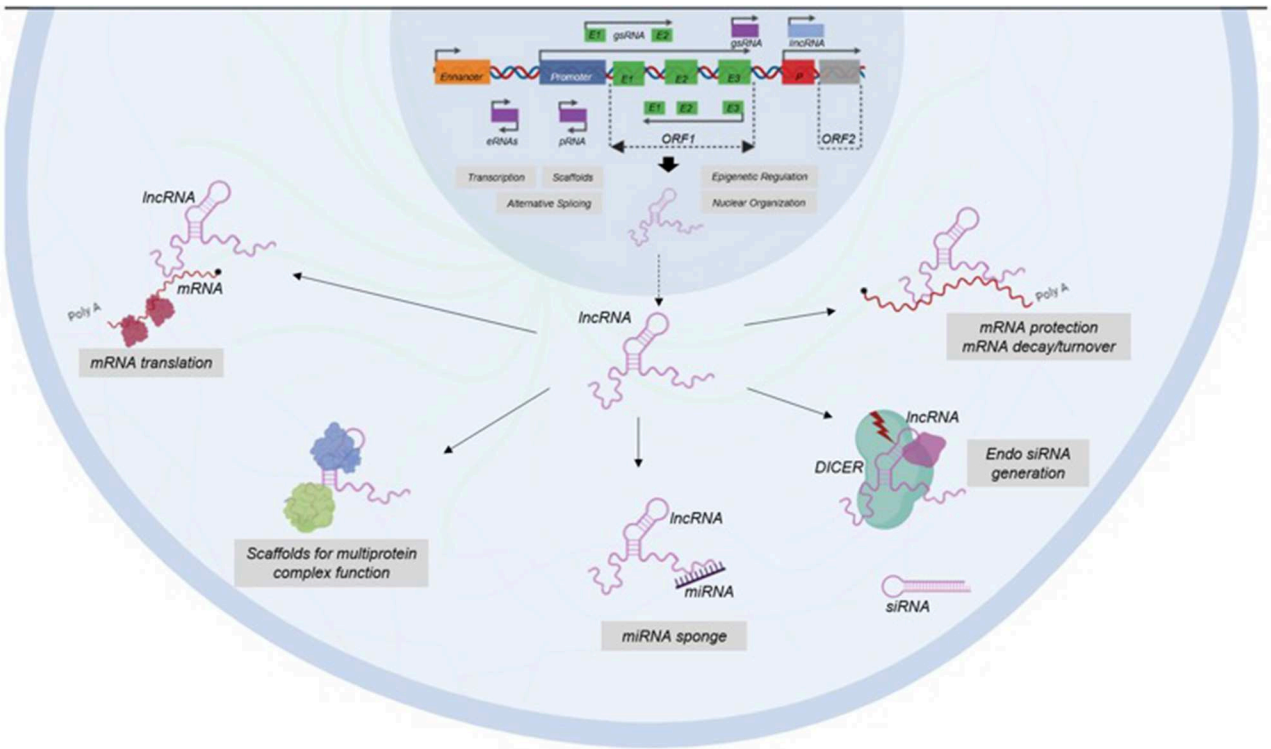

FIGURE 2 | Classification of LncRNAs based on their functions. LncRNAs participate in transcription, epigenetic regulation, nuclear organization, and alternative splicing at nuclear level. In cytoplasm, LncRNAs have functions as enhancers of mRNA translation, scaffolds of protein complex, miRNA sponges, generators of endo siRNA, and protectors of mRNA.

TABLE 1 | Circulating lncRNAs detected in serum in different types of cancer.

LncRNA	Associated cancer	References
RP11-04K16.1, LOC_012542, PVT1	Cervical cancer	(51, 52)
SNHG1, RMRP	Lung	(53)
H19	Multiple myeloma	(54)
PCA3, BCAR4, CRNDE-h, LNCV6_116109, LNCV6_98390, LNCV6_38772, LNCV_108266, LNCV6_84003, LNCV6_98602, u50535	Colorectal	(55–58)
H19, lncUEGC1	Gastric	(59, 60)
LINC00161	Hepatocellular carcinoma	(61)

mechanistic molecules (**Table 2**) and there are some lncRNA that are strongly associated to CRC and presented below.

In this regard, the growth arrest-specific transcript 5 (GAS5), is located at 1q25, with a length of 630 nt (95). GAS5 is upregulated during growth arrest induced by the absence of growth factors or serum starvation. It has been shown that GAS5 binds to the DNA-binding domain of the glucocorticoid receptor (GR) and acts as a decoy glucocorticoid response element (GRE), therefore it can compete with DNA GREs for binding to the GR (95). This lncRNA is able to inhibit cell proliferation and promote apoptosis, by acting as tumor suppressor (96). Nowadays, researches demonstrate that GAS5 is downregulated in several cancer cells such as, breast cancer, prostate cancer, and renal carcinoma (97–99).

In human CRC tumor tissues, Gas5 has been found downregulated and it is correlated with tumor size, TNM staging, lymph node metastasis, low histological grade and less OS (100–104). Besides, overexpression of GAS5 shows that could inhibit cell proliferation *in vitro* and *in vivo* (102), prevent migration and invasion (100, 105), and promotes apoptosis (100, 101, 103) through inhibition of mRNA expression of Akt and Erk and protein expression of p-Akt and p-Erk, giving as a result A pho-Casp9 protein expression and inhibition of pho-Casp3 protein expression (100). Another mechanism of GAS5 to inhibit the apoptosis could be through the GAS5/miR-182-5p/FOXO3a axis, since GAS5 acts as ceRNA of miR-18-5p, which regulates a pro-apoptotic transcription factor named FOXO3a, and target directly the PI3K-AKT signaling pathway (101).

In the case of HOTAIR (Homeobox Transcript Antisense Intergenic RNA), a 2.2 kb lncRNA, is transcribed from the mammalian HOXC gene cluster located in 12q13.13 (106). It participates in epigenetic regulation of gene transcription and interacts on its 5′ end with Polycomb repressive complex 2 in order to remodel chromatin and guarantee silencing of HOX

TABLE 2 | Important lncRNAs involved in CRC.

lncRNA	Status of expression	Participation in CRC	References
XLOC_010588	Upregulated	Associated with metastasis, poor prognosis, invasion, migration, and the progression of CRC via EMT pathway	(82)
FTX	Upregulated	Tumor diameter, TNM stage, the lymph node, and distant metastasis and poor prognosis of patients with CRC. *In vitro*, promotes CRC cell proliferation, migration, invasion, and interacts with miR-215 and vimentin	(83)
BLACAT1	Upregulated	Proliferation, both *in vitro* and *in vivo*, and have a role in G1/G0 arrest by binding to EZH2	(84)
lnc-CRCMSL	Downregulated	Overexpression restricts tumor growth and metastasis *in vivo* and *in vitro* and the silencing accelerates CRC cell proliferation and migration. Also, mediates suppression EMT process by HMGB2	(85)
DANCR	Upregulated	Promotes proliferation and metastasis in CRC. DANCR promotes HSP27 expression and its mediation of proliferation/metastasis via miR-577 sponging. *In vivo*, DANCR promotes CRC tumor growth and liver metastasis	(86)
lnc-DILC	Upregulated	Inhibits the growth and metastasis of CRC cells. Knockdown, facilitates the proliferation and metastasis of CRC cells. Lnc-DILC is a CRC suppressor by inactivating IL-6/STAT3 signaling	(87)
kcna3	Downregulated	Higher TNM grade and the higher occurrence rate of lymphatic metastasis and distant metastasis, and shorter OS. Overexpression, inhibits proliferation, migration and invasion and induces cell apoptosis *in vitro*, and represses CRC tumor growth *in vivo*. Also, exerts a tumor-inhibit role in CRC progression through down-regulating YAP1 expression	(88)
Loc554202	Downregulated	Associated with advanced TNM and a larger tumor size. The overexpression decreases the cell proliferation and induces apoptosis *in vitro* and delay tumorigenesis *in vivo*. Regulates cell apoptosis through the activation of specific caspase cleavage cascades	(89)
MAPKAPK5- AS1	Upregulated	Greater tumor size and advanced TNM in CRC patients. Knockdown, inhibits proliferation and causes apoptosis in CRC cells. Also, p21 is a target of MAPKAPK5- AS1	(90)
ZNFX1-AS1	Upregulated	Associated with aggressive tumor phenotype and poor prognosis in CRC. Knockdown inhibits cell proliferation and invasion *in vitro*, and tumorigenesis and metastasis *in vivo*. ZNFX1-AS1 works as a ceRNA for miR-144, inhibiting to EZH2	(91)
u50535	Upregulated	Activates CCL20 signaling to promote cell proliferation and migration in CRC	(58)
DUXAP10	Upregulated	Positively correlated with advanced pathological stages, larger tumor sizes, and lymph node metastasis. Knockdown inhibits cell proliferation, induces cell apoptosis and increase G0/G1 cells. DUXAP10 silencing inhibits tumor growth *in vivo*, also promotes CRC cell growth and reduces cell apoptosis through silencing the expression of p21 and PTEN by binding LSD1	(92)
NNT-AS1	Upregulated	Correlated with lymph node metastasis, TNM stage, vessel invasion and differentiation, Also, is an independent predictor of OS and progression free survival. Knockdown, inhibits CRC cell proliferation, migration and invasion *in vitro* and suppress tumor growth and metastasis in nude mice by NNT-AS1-mediated activating of MAPK/Erk signaling pathway and EMT	(93)
91H	Upregulated	Associated with distant metastasis and poor prognosis in patients with CRC. Also, is an independent prognostic indicator and of distant metastasis. *In vitro*, knockdown of 91H inhibits the proliferation, migration, and invasiveness of CRC cells	(94)

genes during embryonic development. On 3′ end HOTAIR interacts with histone demethylase (107). Evidence shows that HOTAIR exhibits an oncogenic role in renal, breast, gastric, lung, and ovarian cancer (108–112).

HOTAIR is overexpressed at high levels in CRC (113–116) and some studies show that HOTAIR is only overexpressed in right (proximal) CRCs samples (117). This overregulation has been associated to lymph node and tumor node metastasis, distant metastases, Duke's staging, histological types, the degree of differentiation (113), and unfavorable prognosis (114, 118). *In vitro*, the inhibition of its expression shows decreased proliferation, invasion, and migration, as well as low cyclin E and CDK2 expression, increased apoptosis and p21 expression (113). Besides, HOTAIR promotes tumorigenesis and aggressiveness (114). This lncRNA directly harbors miR-326 binding sites and regulates FUT6 expression, a specific fucosyl transferase. The HOTAIR/miR-326/FUT6 axis modifies α1, 3-fucosylation of CD44, which triggers PI3K/AKT/mTOR pathway mediating CRC malignancy (114). In addition, HOTAIR knockdown and miR-203a-3p upregulation in CRC cell lines produces inhibited Wnt/β-catenin signaling, cell proliferation, and reduced chemoresistance (116).

The H19 gene is located on 11p15 and plays pivotal roles in embryonal development and growth regulation (119, 120). The H19 gene encodes for a processed 2.7 kb RNA (121). H19 is highly expressed from the onset of embryogenesis to fetal life in vital organs such as the fetal adrenal, liver, and placenta but is downregulated postnatal stages (122). Recent evidence shows that H19 is upregulated in several cancers as, esophageal cancer, hepatocellular carcinoma, ovarian cancer, bladder cancer, and breast cancer (123–127).

It has been demonstrated that H19 is upregulated in CRC tissues compared with adjacent noncancerous tissues (9, 128, 129). Data from The Cancer Genome Atlas (TCGA)

shows that H19 is the lncRNA with the most substantial correlation to CRC patient survival (130), serving as an independent predictor for OS and disease-free survival (DFS) (9, 131). Besides, this lncRNA has been related with poor prognosis (132).

Besides, miR-200a binds H19 and inhibits its expression, thus decreasing proliferation of CRC cells, also H19 regulates the expression and activity of β-catenin by competitive binding to miR-200a (128). In addition, depletion of H19 inhibits cell viability and induces growth arrest whereas overexpression of H19 upregulates a series of cell-cycle genes. Moreover, H19 binds to eIF4A3 resulting in an abnormal cell-cycle-regulatory genes expression (131).

H19 promotes invasion and metastasis in CRC through activation of RAS-MAPK signaling pathway (133) and its overexpression in MTX-resistant colorectal cell line HT-29 prove that is involved in Metrotexate (MTX) resistance via activating Wnt/β-catenin signaling (134). The overexpression of H19 and miR-675 in CRC implies that both are important factors in the tumorigenesis of CRC since H19-derived miR-675, targets tumor suppressor RB (129).

Interestingly, mesenchymal-like cancer cells and primary CRC tissues show high expression of H19, whereas its stable expression accelerates tumor growth and enhances epithelial–mesenchymal transition (EMT) progression. Finally, H19 can function as ceRNA by antagonizing the functions of miR-138 and miR-200a, giving as a result the de-repression of Vimentin, ZEB1, and ZEB2 (135).

Finally, metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1), is on 11q13 and transcribed from the nuclear-enriched transcript 2 (NEAT2), which has been identified as a prognostic factor in patients with stage I lung cancer (136, 137). It has been reported that this lncRNA is expressed in mouse and normal human tissues (137, 138) and its overexpression have been demonstrated in many cancer types including lung, cervical, liver, bladder and sarcomas of uterus (139–144), and correlated to metastasis (137).

The MALAT1 levels are up-regulated in human primary CRC tissues (136), being 2.26 times higher than noncancerous tissues (145), serving as a negative prognostic marker in stage II/III CRC patients, since, these patients show a high hazard ratio (HR) for OS and DFS (145). Moreover, upregulation of MALAT1 has been found in CRC tissues with lymph node metastasis (136). *In vitro*, MALAT1 could promote CRC cell proliferation, invasion, and migration through up-regulating SOX9 and down-regulating miR-145. On the other hand, cell cycle and apoptosis can be suppressed by MALAT1/miR-145/SOX9 axis (146). Furthermore, MALAT1 regulates proliferation, migration, and promotes tumor growth and metastasis in nude mice (136), this regulation could be through SFPQ and AKAP-9 as MALAT1 interact with SFPQ, hence releasing PTBP2 from the SFPQ/PTBP2 complex, facilitating cell proliferation and migration (147). AKAP-9 is overexpressed in CRC cells with metastatic potential and human primary CRC tissues with lymph node metastasis, and its knockdown blocks CRC cell proliferation, migration, and invasion mediated by MALAT1 (136).

Angiogenesis and the EMT to promote metastasis in CRC are enhanced by YAP1-induced MALAT1-miR126-5p axis since YAP1 forms a complex with β-catenin/TCF4 bound to the MALAT1 promoter, which can act as a sponge of miR-126-5p to induce SLUG, VEGFA, and TWIST expression (148). miR-20b-5p-mimic and si-MALAT1 give as a result attenuated microsphere formation and self-renewal capability, reduces the proportion of CSCs, downregulating the expression of stemness markers as Oct4, Nanog, Sox2, and Notch1, and cellular metabolism such as GLUT1, LDHB, HK2, and PKM2 in HCT-116 cells *in vitro*. Additionally, the administration of either si-MALAT1 or miR-20b-5p-mimic in a xenograft model based on BALB/c mice demonstrated that they can suppress tumorigenicity of HCT-116 cells *in vivo* (149).

As we reviewed above, HOTAIR, H19, and MALAT are overexpressed in CRC samples. Interestingly, HOTAIR and MALAT level expression are related to lymph node and tumor node metastasis (113, 136). In addition, H19 is considered as an important independent predictor for OS and DFS (9, 131), besides, H19 is the most significant lncRNA associated to CRC (130). Moreover, MALAT1 is one important negative prognostic marker in II/III CRC patients (145). Conversely, down regulation of Gas5 has been found in CRC and is associated with poor prognosis (100–104).

Interestingly, LncRNAs regulate multiples pathways in CRC as PI3K-AKT signaling pathway, that is regulated by GAS5, promoting apoptosis via GAS5/miR-182-5p/FOXO3a axis (101), as well as, PI3K/AKT/mTOR that is managed through HOTAIR/miR-326/FUT6 axis stimulating CRC (114). In addition, H19 regulates RAS-MAPK and Wnt/β-catenin signaling pathways, activating invasion, metastasis, and chemoresistance mechanism (133, 134). Another important axis is MALAT1/miR-145/SOX9 that mediates cell cycle and apoptosis (146).

LNCRNA AS REGULATORS OF AUTOPHAGY IN CRC

Autophagy is a basal physiological mechanism in normal cells that assure cellular homeostasis. Besides, autophagy is a very well-conserved catabolic process where the cell is self-digested through the removal of proteins or dysfunctional organelles (150). This process can also be, under specific circumstances (hypoxia, stress, and nutrient deprivation), a survival mechanism in which the cell recycles nutrients and energy (151).

There are three forms of autophagy based on its morphology, macroautophagy in which autophagosomes engulf cytoplasmic components and interact whit lysosomes for degradation, microautophagy in which there is a direct lysosomal membrane invagination to engulf damaged proteins, and chaperone-mediated autophagy which involves the translocation of soluble cytosolic proteins by chaperone-dependent selection across the lysosomal membrane (152–154).

LncRNAs generally modulate autophagy by regulating the expression of ATG genes which are important effectors in autophagy process (155, 156). Frequently, LncRNAs behaves

as competing endogenous RNAs (ceRNAs) for modulating autophagy-related microRNAs (miRNAs). LncRNAs have a very important implication in autophagy regulation (155). For instance, activation of autophagy can be given by NBR2 via AMPK activation (157) or by repression of PI3K/AKT/mTOR pathway leaded by Ad5-AlncRNA, and PTENP1, whereas MEG3 and H19 enhances the opposite effect. Another LncRNAs involved in activation of autophagy are HOTAIRM1, PTENP1, and MALAT1, which increase the expression of ULK (158–162). Conversely, RISA suppress autophagy initiation through ULK1 inhibition (163). Additionally, key genes in autophagy such as ATG and adaptor proteins involved in later steps of autophagy regulation are affected by H19, MEG3, AK156230, PTENP1, and MALAT1(141, 158, 161, 164, 165).

It is clear that LncRNAs are non-canonical regulators and participates in keeping homeostasis in a variety of pathophysiological processes, but also they can be illness effectors, since they can interact directly with DNA, RNA, and proteins. In this regard, it has been demonstrated that autophagiaparticipates in cancer progression and drug resistance mechanisms (166). Besides, autophagy may suppress tumors (167), but also, their induction promotes tumorigenesis since it provide survival capacity of tumor under adverse microenvironment (168, 169).

In CRC, little is known about lncRNAs involved in autophagy, for instance, POU3F3, a lincRNA, is overexpressed in CRC tissue samples and when is silenced, autophagy is enhanced, suggesting the involvement of autophagy in the induction of apoptosis (170). Another lncRNA highly expressed in CRC is HAGLROS, which is correlated with shorter survival time of CRC patients and its decreased expression can produce apoptosis and suppress autophagy in CRC HCT116 cells by regulation of miR-100/ATG5 axis and PI3K/AKT/mTOR pathway (171).

UCA1 is also abnormally overexpressed in SW620 and HT29 CRC cell lines when compared to CCD-18Co. There is evidence that UCA1 downregulation inhibits the growth, apoptosis, and autophagy of CRC cell lines *in vitro*. Besides, UCA1 directly interacts with miR-185-5p downregulates its expression. Additionally, UCA1 could reverse this effect of miR-185-5p on the growth and autophagy, suggesting its involvement in the derepression of WISP2 expression and the stimulation of the WISP2/β-catenin signaling pathway (172).

Another lncRNA involved in CRC is KCNQ1OT1 (173), which is also upregulated. It has been demonstrated that expression patterns of Atg4B, which cleavages LC3 (thus promotes the formation of autophagosome) (174) is downregulated in CRC HCT116 and SW480 cells in KCNQ1OT1 knockdown cells. Besides, these cells treated with oxaliplatin, decrease cell viability, meaning that KCNQ1OT1 induce protective autophagy and chemoresistance. Finally, overexpression of KCNQ1OT1 is correlated with poor OS of CRC patients, suggesting that higher levels in patients make them resistant to chemotherapy treatments (173).

H19 is another upregulated lncRNA in CRC samples and has been correlated with patient OS suggesting that can predicts 5-FU chemoresistance. These findings reveal that SIRT1 (which is modulated by H19/miR-194-5p axis) dependent autophagy pathway can affect 5-FU resistance in CRC cells (9).

There is no doubt that LncRNAs are key molecules involved in regulation of autophagy in CRC. Nevertheless, more research in this field is needed to clarify interactions on regulation axis in order to understand complex processes in which autophagy is implicated, such as apoptosis and chemoresistance.

LNCRNA AS REGULATOR OF DRUG RESISTANCE IN CRC

Malignant CRC tumors develop pharmacological resistance, which is a complex phenomenon that triggers increase in DNA repair and loss of apoptosis induction, resulting from several factors that include individual variation in patients such as genetic and/or epigenetic differences within the tumors (7, 175, 176). Drug resistance is influenced by abnormal expression or mutation on efflux proteins, which reduce uptakes of drugs (177).

Chemotherapy for CRC depends on the stage of cancer; however, other factors are important as well. For stage 0 to II, surgical treatment alone might be successful, nonetheless, for stage II some oncologists opt for including 5-FU and leucovorin, oxaliplatin, or capecitabine if chemotherapy is needed (178–180). Treatment for stages III and IV includes chemo and/or targeted drugs, commonly include CAPEOX (capecitabine plus oxaliplatin), FOLFOX (oxaliplatin, 5-FU, and leucovorin), 5-FU and leucovorin, or capecitabine for stage III and FOLFIRI (leucovorin, 5-FU, and irinotecan), FOLFOXIRI (leucovorin, 5-FU, oxaliplatin, and irinotecan) plus some target drugs such as bevacizumab, ramucirimab, cetuximab, or panitumumab added for stage IV (181–186).

Regulation of gene expression by different types of non-coding RNAs such as miRNAs and lncRNAs are involved in acquisition of drug resistance characteristics after treatment (187). Most important dysregulated lncRNAs are summarized in **Table 3**. For instance, the characteristic acquisition of 5-FU resistance in CRC has been related with a plethora of lncRNAs miss-expression. In the case of UCA-1, it plays an important role in 5-FU chemoresponse by exerting a sponge activity to miR-204-5p, thus, indirectly increases CREB1 which have been related with poor OS (172). Another LncRNA implicated in the development of 5-FU resistance is GIHCG, since its overexpression is found in both CRC tissues and cell lines and is related to invasion, migration, and chemoresistant properties (188). There is also evidence that downregulation of PVT1, MALAT1, and PCAT-1 sensitizes CRC cells to 5-FU treatment, inducing early and late apoptosis by regulation of MDR genes (193, 194, 196). On the other hand, downregulation of snaR and SLC25A25-AS1 promotes chemoresistance in CRC (198, 199).

Certain aspects of chemoresistance have been related with lncRNAs regulated by miRNAs, for instance, ENST00000547547 promotes sensitivity to 5-FU in CRC cells by competitive arresting miR-31/ABCB9 (200) and LINC00152/miR-139-5p/NOTCH1 axis increases chemoresistance by suppressing apoptosis (191).

TABLE 3 | Long non-coding RNAs and their physiological function in colorectal cancer drug resistance.

LncRNA	Function	References
GIHCG	Potential target in 5-FU and Oxaliplatin resistance mechanisms.	(188)
MIR100HG	Coordinately MIR100HG, miR-100 and miR-125b overexpression drives Cetuximab resistance by targeting five negative regulators of Wnt signaling which have a potential clinical relevant interaction with EGFR.	(189)
UCA1	UCA1 can decrease the sensitivity of CRC cells to 5-FU by sponging miR-204-5p resulting in attenuating apoptosis. Moreover, UCA1 expression levels are increased in Cetuximab resistant cells and can be transferred to sensitive cells through exosomes increasing resistant cells number.	(172, 190)
LINC00152	LIN00152 confers Oxa and 5-FU chemoresistance by sponging miR-193a-3p by ERBB4 modulation and then inducing the activation of AKT signaling pathway that mediates cell survival and chemoresistance. miR-193a-3p also targets NOTCH1 regulating CRC growth, metastasis, stemness, and chemoresistance.	(191, 192)
HOTAIR	HOTAIR could regulate the progression and Cisplatin and Paclitaxel chemoresistance enhancements in CRC by targeting miR-203a-3p and the activity of Wnt/β-catenin signaling pathway.	(116)
PCAT-1	PCAT-1 regulates the invasiveness and 5-FU resistance in CRC cells and that PCAT-1 may promote CRC cell invasion by modulating the expression of c-Myc.	(193)
PVT1	PVT1 is associated with 5-FU resistance in human CRC tissues and cells by inhibiting apoptosis and upregulating the expression of MRP1, P-gp, mTOR, and Bcl-2	(194)
XIST	XIST promotes Doxorubicin resistance through sponging miR-124 which targets SGK1 increasing cell survival, loss of control in cell cycle, inhibiting apoptosis, and increasing chemoresistance.	(195)
MALAT1	Overexpression of MALAT1 enhances chemoresistance in 5-FU resistant cells through potentiation of multidrug resistant genes such as MDR1, MRP1, BCRP, and ABC. Moreover, modulates EZH2 pathway in Oxa resistance	(196, 197)
H19	H19 mediated Methotrexate resistance via activating Wnt/β-catenin signaling, which help to develop H19 as a promising therapeutic target for MTX resistant CRC. Besides, CAFs promote stemness and Oxa chemoresistance in CRC by transferring exosomal H19 to CRC sensitive cells through sponging miR-141.	(20, 134)
SLC25A25-AS1	SLC25A25-AS1 has a pivotal role in CRC cells promoting chemo sensitivity to 5-FU and DOX via Erk and p38 pathway modulation. Hence, SLC25A25-AS1 was determined to play a tumor suppressive role in CRC.	(198)
snaR	snaR has a negative regulator role in responsible of the development of 5-FU resistance through cell growth of CRC cells. Nonetheless, snaR detailed roles have not yet been established.	(199)
ENST00000547547	ENST00000547547 reduced the chemoresistance of 5-FU via competitive sponging to miR-31 which targets ABCB9 involved in chemotherapy induced apoptosis. This suggests that lncRNA ENST00000547547 may be a positive prognostic factor for 5-FU-based chemotherapy.	(200)
TUG1	TUG1 mediates MTX resistance in colorectal cancer via sponging miR-186 that targets CPEB2 increasing its protein levels that play an important role in tumorigenesis and chemoresistance.	(201)
PVT1	PVT1 is a significant regulator in tumorigenesis and cisplatin resistance of CRC by inhibiting apoptotic pathways in CRC and may serve as a promising target for CRC therapy.	(202)
MEG3	MEG3 promotes chemosensitivity to Oxa by inducing cytotoxicity in CRC cells promoting apoptosis. In addition, MEG3 sponges miR-141 that targets PDCD4.	(203, 204)

5-FU, 5-fluorouracil, Oxa, oxaliplatin. CAFs, cancer associated fibroblasts, DOX, doxorubicin.

In the case of oxaliplatin CRC treatment, several lncRNAs such as GIHCG (172), LIN00152 (192), MALAT1 (197), H19 (20), and MEG3 (203, 204) promote apoptosis by inducing cytotoxicity by different mechanisms, mainly by axis with miRNAs targeting important genes in cell death behavior. Nevertheless, cisplatin CRC resistance is mainly mediated by HOTAIR and PVT1 through inhibition of apoptotic pathways, modulation of expression levels of miR-203a-3p and the activity of Wnt/β-catenin signaling pathway, respectively (116, 202).

Interestingly, H19 also exert drug resistance modulation in Methotrexate treatment via Wnt/β-catenin signaling pathway (134). Regarding to TUG1, the resistance is given by CPBE2 gene modulation after arresting of miR-186 (201). Finally, Doxorubicin resistance is manly influenced by the XIST/miR-124/SGK1 axis which promotes chemoresistance in CRC cells (195).

Evaluating lncRNAs expression profiles is very important since it can be used to identify novel biomarkers for CRC resistance and use them as a therapeutically potential targets based on their biological behavior, improving in this way, the efficacy of chemotherapy in CRC patients.

CLINICAL RELEVANCE ON LNCRNA IN AUTOPHAGY AND DRUG RESISTANCE IN COLORECTAL CANCER

Clinical implications of lncRNAs in CRC are relevant as there is evidence of its participation and correlation with staging and survival. In this regard, GAS5 down-regulation is common in CRC tissues being associated with distant metastasis, tumor differentiation, tumor size and advanced TNM staging (100), low histological grade (102), later tumor-node-metastasis stage and less OS (103).

Clinical relevance of H19 has been related with poor recurrent free survival (RFS) (9) tumor differentiation and advanced

TNM stage, and is an independent predictor for OS and DFS. Moreover, previous studies using HOTAIR have determined that its overexpression is related to lymph node and, tumor node metastasis, distant metastases, Duke's staging, histological types, degree of differentiation (113) and poor clinical prognosis (114). Some studies show that it is upregulated in right CRCs biopsies (117). In addition, high levels of HOTAIR in tumors and blood are associated with higher mortality of patients (118).

MALAT1, patients have shown worse prognosis in tumors that appearance overexpression of this lncRNA in human primary CRC (145). In addition, MALAT1 have being related with lymph node metastasis in CRC patients (136).

Regarding to autophagy and chemoresistance in CRC, HAGLROS, a lncRNA related to autophagy, is correlated with shorter survival time (153). KCNQ1OT1, has also prove that induce protective autophagy and chemoresistance and its high expression is associated with poor OS of colon cancer patients, suggesting that patients with overexpression of KCNQ1OT1 might be resistant to chemotherapy treatments (173). Finally, H19 has been correlated with patient OS suggesting being a potential biomarker for predicting 5-FU resistance that could be modulated by H19/miR-194-5p axis (157).

CONCLUDING REMARKS

Recently lncRNAs analysis is important for cancer detection, progression, diagnosis, therapy response, and prognostic values. With increasing development of quantitative detection techniques, lncRNAs derived from patients' non-invasive samples (i.e., blood, stools, and urine) has become into a novel approach in precision oncology.

Tumorspecific GAS5, HOTAIR, H19, and MALAT are novels CRC related lncRNAs detected in patients. Nonetheless, the effect and mechanism of lncRNAs in cancer autophagy and chemoresistance have not been extensively characterized.

Chemoresistance and autophagy are top issues for cancer treatment and lncRNAs play a pivotal role in resistance acquisition for several drugs. LncRNAs such as HAGLROS, KCNQ1OT1, and H19 are examples of lncRNA related to chemoresistance leaded by autophagy. Nevertheless, identifying the network interactions of lncRNAs can provide an insight in their mechanisms of action, adding clinical significance and hence, improve detection, diagnosis, and treatment.

AUTHOR CONTRIBUTIONS

MB, MA-M, EL-V, MA-F, and RR-P conceived and designed the content of this review and wrote the paper. ES-B and CL-C contributed to the final version of the manuscript.

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REFERENCES

1. WHO. *Cancer Fact Sheet.* (2018). Available online at: http://www.who.int/mediacentre/factsheets/fs297/en/ (accessed August 15, 2019).
2. Mishra DK, Veena U, Kaliki S, Kethiri AR, Sangwan VS, Ali MH, et al. Differential expression of stem cell markers in ocular surface squamous neoplasia. *PLoS ONE.* (2016) 11:e0161800. doi: 10.1371/journal.pone.0161800
3. Brenner H, Kloor M, Pox CP. Colorectal cancer. *Lancet.* (2014) 383:1490–502. doi: 10.1016/S0140-6736(13)61649-9
4. Li W, Zhang G, Wang HL, Wang L. Analysis of expression of cyclin E, p27kip1 and Ki67 protein in colorectal cancer tissues and its value for diagnosis, treatment and prognosis of disease. *Eur Rev Med Pharmacol Sci.* (2016) 20:4874–9.
5. Kurkjian C, Kummar S. Advances in the treatment of metastatic colorectal cancer. *Am J Ther.* (2009) 16:412–20. doi: 10.1097/MJT.0b013e3181907ed9
6. Holubec L, Polivka J, Safanda M, Karas M, Liska V. The role of cetuximab in the induction of anticancer immune response in colorectal cancer treatment. *Anticancer Res.* (2016) 36:4421–6. doi: 10.21873/anticanres.10985
7. Kang H, Kim C, Lee H, Kim W, Lee EK. Post-transcriptional controls by ribonucleoprotein complexes in the acquisition of drug resistance. *Int J Mol Sci.* (2013) 14:17204–20. doi: 10.3390/ijms140817204
8. Alfarouk KO, Stock C-M, Taylor S, Walsh M, Muddathir AK, Verduzco D, et al. Resistance to cancer chemotherapy: failure in drug response from ADME to P-gp. *Cancer Cell Int.* (2015) 15:71. doi: 10.1186/s12935-015-0221-1
9. Wang M, Han D, Yuan Z, Hu H, Zhao Z, Yang R, et al. Long non-coding RNA H19 confers 5-Fu resistance in colorectal cancer by promoting SIRT1-mediated autophagy. *Cell Death Dis.* (2018) 9:1149. doi: 10.1038/s41419-018-1187-4
10. Mah LY, Ryan KM. Autophagy and cancer. *Cold Spring Harb Perspect Biol.* (2012) 4:a008821. doi: 10.1101/cshperspect.a008821
11. Galluzzi L, Bravo-San Pedro JM, Levine B, Green DR, Kroemer G. Pharmacological modulation of autophagy: therapeutic potential and persisting obstacles. *Nat Rev Drug Discov.* (2017) 16:487–511. doi: 10.1038/nrd.2017.22
12. Mokarram P, Albokashy M, Zarghooni M, Moosavi MA, Sepehri Z, Chen QM, et al. New frontiers in the treatment of colorectal cancer: autophagy and the unfolded protein response as promising targets. *Autophagy.* (2017) 13:781–819. doi: 10.1080/15548627.2017.1290751
13. Patrushev LI, Kovalenko TF. Functions of noncoding sequences in mammalian genomes. *Biochemistry.* (2014) 79:1442–69. doi: 10.1134/S0006297914130021
14. Lanzafame M, Bianco G, Terracciano LM, Ng CKY. The role of long non-coding RNAs in hepatocarcinogenesis. *Int J Mol Sci.* (2018) 19:E682. doi: 10.3390/ijms19030682
15. Klinge CM. Non-coding RNAs: long non-coding RNAs and microRNAs in endocrine-related cancers. *Endocr Relat Cancer.* (2018) 25:R259-r82. doi: 10.1530/ERC-17-0548
16. Clark MB, Mattick JS. Long noncoding RNAs in cell biology. *Semin Cell Dev Biol.* (2011) 22:366–76. doi: 10.1016/j.semcdb.2011.01.001

17. Guarino M, rubino B, ballabio g. The role of epithelial-mesenchymal transition in cancer pathology. *Pathology.* (2007) 39:305–18. doi: 10.1080/00313020701329914
18. Fang J, Sun C-C, Gong C. Long noncoding RNA XIST acts as an oncogene in non-small cell lung cancer by epigenetically repressing KLF2 expression. *Biochem Biophys Res Commun.* (2016) 478:811–7. doi: 10.1016/j.bbrc.2016.08.030
19. Zinovieva OL, Grineva EN, Prokofjeva MM, Karpov DS, Zheltukhin AO, Krasnov GS, et al. Expression of long non-coding RNA LINC00973 is consistently increased upon treatment of colon cancer cells with different chemotherapeutic drugs. *Biochimie.* (2018) 151:67–72. doi: 10.1016/j.biochi.2018.05.021
20. Ren J, Ding L, Zhang D, Shi G, Xu Q, Shen S, et al. Carcinoma-associated fibroblasts promote the stemness and chemoresistance of colorectal cancer by transferring exosomal lncRNA H19. *Theranostics.* (2018) 8:3932–48. doi: 10.7150/thno.25541
21. Yu ZY, Wang Z, Lee KY, Yuan P, Ding J. Effect of silencing colon cancer-associated transcript 2 on the proliferation, apoptosis and autophagy of gastric cancer BGC-823 cells. *Oncol Lett.* (2018) 15:3127–32. doi: 10.3892/ol.2017.7677
22. Kim ED, Sung S. Long noncoding RNA: unveiling hidden layer of gene regulatory networks. *Trends Plant Sci.* (2012) 17:16–21. doi: 10.1016/j.tplants.2011.10.008
23. Jia H, Osak M, Bogu GK, Stanton LW, Johnson R, Lipovich L. Genome-wide computational identification and manual annotation of human long noncoding RNA genes. *RNA.* (2010) 16:1478–87. doi: 10.1261/rna.1951310
24. Cabili MN, Trapnell C, Goff L, Koziol M, Tazon-Vega B, Regev A, et al. Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. *Genes Dev.* (2011) 25:1915–27. doi: 10.1101/gad.17446611
25. Hombach S, Kretz M. Non-coding RNAs: classification, biology and functioning. *Adv Exp Med Biol.* (2016) 937:3–17. doi: 10.1007/978-3-319-42059-2_1
26. Chen LL. Linking long noncoding RNA localization and function. *Trends Biochem Sci.* (2016) 41:761–72. doi: 10.1016/j.tibs.2016.07.003
27. Jarroux J, Morillon A, Pinskaya M. History, discovery, and classification of lncRNAs. *Adv Exp Med Biol.* (2017) 1008:1–46. doi: 10.1007/978-981-10-5203-3_1
28. Kashi K, Henderson L, Bonetti A, Carninci P. Discovery and functional analysis of lncRNAs: methodologies to investigate an uncharacterized transcriptome. *Biochim Biophys Acta.* (2016) 1859:3–15. doi: 10.1016/j.bbagrm.2015.10.010
29. Spurlock CF III, Crooke PS III, Aune TM. Biogenesis and transcriptional regulation of long noncoding RNAs in the human immune system. *J Immunol.* (2016) 197:4509–17. doi: 10.4049/jimmunol.1600970
30. Clark MB, Amaral PP, Schlesinger FJ, Dinger ME, Taft RJ, Rinn JL, et al. The reality of pervasive transcription. *PLoS Biol.* (2011) 9:e1000625-e1102. doi: 10.1371/journal.pbio.1000625
31. Hangauer MJ, Vaughn IW, McManus MT. Pervasive transcription of the human genome produces thousands of previously unidentified long intergenic noncoding RNAs. *PLoS Genet.* (2013) 9:e1003569. doi: 10.1371/journal.pgen.1003569
32. Yu AD, Wang Z, Morris KV. Long noncoding RNAs: a potent source of regulation in immunity and disease. *Immunol Cell Biol.* (2015) 93:277–83. doi: 10.1038/icb.2015.2
33. Khachane AN, Harrison PM. Mining mammalian transcript data for functional long non-coding RNAs. *PLoS ONE.* (2010) 5:e10316. doi: 10.1371/journal.pone.0010316
34. Moran VA, Perera RJ, Khalil AM. Emerging functional and mechanistic paradigms of mammalian long non-coding RNAs. *Nucleic Acids Res.* (2012) 40:6391–400. doi: 10.1093/nar/gks296
35. Aune TM, Crooke PS, Spurlock CF. Long noncoding RNAs in T lymphocytes. *J Leukoc Biol.* (2016) 99:31–44. doi: 10.1189/jlb.1RI0815-389R
36. Mattick JS, Rinn JL. Discovery and annotation of long noncoding RNAs. *Nat Struct Mol Biol.* (2015) 22:5–7. doi: 10.1038/nsmb.2942
37. Guttman M, Rinn JL. Modular regulatory principles of large non-coding RNAs. *Nature.* (2012) 482:339–46. doi: 10.1038/nature10887
38. Rinn JL, Chang HY. Genome regulation by long noncoding RNAs. *Annu Rev Biochem.* (2012) 81:145–66. doi: 10.1146/annurev-biochem-051410-092902
39. Lam MT, Li W, Rosenfeld MG, Glass CK. Enhancer RNAs and regulated transcriptional programs. *Trends Biochem Sci.* (2014) 39:170–82. doi: 10.1016/j.tibs.2014.02.007
40. Cabili MN, Dunagin MC, McClanahan PD, Biaesch A, Padovan-Merhar O, Regev A, et al. Localization and abundance analysis of human lncRNAs at single-cell and single-molecule resolution. *Genome Biol.* (2015) 16:20. doi: 10.1186/s13059-015-0586-4
41. Naganuma T, Hirose T. Paraspeckle formation during the biogenesis of long non-coding RNAs. *RNA Biol.* (2013) 10:456–61. doi: 10.4161/rna.23547
42. Quinodoz S, Guttman M. Long noncoding RNAs: an emerging link between gene regulation and nuclear organization. *Trends Cell Biol.* (2014) 24:651–63. doi: 10.1016/j.tcb.2014.08.009
43. Tay Y, Rinn J, Pandolfi PP. The multilayered complexity of ceRNA crosstalk and competition. *Nature.* (2014) 505:344–52. doi: 10.1038/nature12986
44. Bonasio R, Shiekhattar R. Regulation of transcription by long noncoding RNAs. *Annu Rev Genet.* (2014) 48:433–55. doi: 10.1146/annurev-genet-120213-092323
45. Ferre F, Colantoni A, Helmer-Citterich M. Revealing protein-lncRNA interaction. *Brief Bioinform.* (2016) 17:106–16. doi: 10.1093/bib/bbv031
46. Xu Y, Wu W, Han Q, Wang Y, Li C, Zhang P, et al. New insights into the interplay between non-coding RNAs and RNA-binding protein HnRNPK in regulating cellular functions. *Cells.* (2019) 8:62. doi: 10.3390/cells8010062
47. Yoon J-H, Abdelmohsen K, Kim J, Yang X, Martindale JL, Tominaga-Yamanaka K, et al. Scaffold function of long non-coding RNA HOTAIR in protein ubiquitination. *Nat Commun.* (2013) 4:2939. doi: 10.1038/ncomms3939
48. Uchida S. High-throughput methods to detect long non-coding RNAs. *High Throughput.* (2017) 6:12. doi: 10.3390/6030012
49. Lodish HF. *Molecular Cell Biology*. 6th ed. Basingstoke: W.H. Freeman (2008).
50. Shi Y, Shang J. Long noncoding RNA expression profiling using arraystar LncRNA microarrays. *Methods Mol Biol.* (2016) 1402:43–61. doi: 10.1007/978-1-4939-3378-5_6
51. Iempridee T, Wiwithaphon S, Piboonprai K, Pratedrat P, Khumkhrong P, Japrung D, et al. Identification of reference genes for circulating long noncoding RNA analysis in serum of cervical cancer patients. *FEBS Open Bio.* (2018) 8:1844–54. doi: 10.1002/2211-5463.12523
52. Yang JP, Yang XJ, Xiao L, Wang Y. Long noncoding RNA PVT1 as a novel serum biomarker for detection of cervical cancer. *Eur Rev Med Pharmacol Sci.* (2016) 20:3980–6.
53. Lin Y, Leng Q, Zhan M, Jiang F. A plasma long noncoding RNA signature for early detection of lung cancer. *Transl Oncol.* (2018) 11:1225–31. doi: 10.1016/j.tranon.2018.07.016
54. Pan Y, Chen H, Shen X, Wang X, Ju S, Lu M, et al. Serum level of long noncoding RNA H19 as a diagnostic biomarker of multiple myeloma. *Clin Chim Acta.* (2018) 480:199–205. doi: 10.1016/j.cca.2018.02.019
55. Liu T, Zhang X, Gao S, Jing F, Yang Y, Du L, et al. Exosomal long noncoding RNA CRNDE-h as a novel serum-based biomarker for diagnosis and prognosis of colorectal cancer. *Oncotarget.* (2016) 7:85551–63. doi: 10.18632/oncotarget.13465
56. Qi P, Dong L, Lin W, Zhou X, Du X. Abstract B42: a two-lncRNA signature in serous exosomes serves as a new biomarker for colorectal cancer diagnosis. *Cancer Res.* (2016) 76 (Suppl. 6):B42.
57. Hu D, Zhan Y, Zhu K, Bai M, Han J, Si Y, et al. Plasma exosomal long non-coding RNAs serve as biomarkers for early detection of colorectal cancer. *Cell Physiol Biochem.* (2018) 51:2704–15. doi: 10.1159/000495961
58. Yu X, Yuan Z, Yang Z, Chen D, Kim T, Cui Y, et al. The novel long noncoding RNA u50535 promotes colorectal cancer growth and metastasis by regulating CCL20. *Cell Death Dis.* (2018) 9:751. doi: 10.1038/s41419-018-0771-y
59. Lin LY, Yang L, Zeng Q, Wang L, Chen ML, Zhao ZH, et al. Tumor-originated exosomal lncUEGC1 as a circulating biomarker for early-stage gastric cancer. *Mol Cancer.* (2018) 17:1–6. doi: 10.1186/s12943-018-0834-9
60. Arita T, Ichikawa D, Konishi H, Komatsu S, Shiozaki A, Shoda K, et al. Circulating long non-coding RNAs in plasma of patients with gastric cancer. *Anticancer Res.* (2013) 33:3185–94.

61. Sun L, Su Y, Liu X, Xu M, Chen X, Zhu Y, et al. Serum and exosome long non coding RNAs as potential biomarkers for hepatocellular carcinoma. *J Cancer.* (2018) 9:2631–9. doi: 10.7150/jca.24978
62. Bolha L, Ravnik-Glavač M, Glavač D. Long noncoding RNAs as biomarkers in cancer. *Disease Markers.* (2017) 2017:1–14. doi: 10.1155/2017/7243968
63. Qi P, Zhou Xy, Du X. Circulating long non-coding RNAs in cancer: current status and future perspectives. *Mol Cancer.* (2016) 15:1–11. doi: 10.1186/s12943-016-0524-4
64. Du L, Duan W, Jiang X, Zhao L, Li J, Wang R, et al. Cell-free lncRNA expression signatures in urine serve as novel non-invasive biomarkers for diagnosis and recurrence prediction of bladder cancer. *J Cell Mol Med.* (2018) 22:2838–45. doi: 10.1111/jcmm.13578
65. Lorenzen JM, Schauerte C, Kölling M, Hu A, Knapp M. Long noncoding RNAs in urine are detectable and may enable early detection of acute T cell—mediated rejection of renal allografts. *Clin Chem.* (2015) 61:1505–14. doi: 10.1373/clinchem.2015.243600
66. Hu B, Yang H, Yang H. Diagnostic value of urine prostate cancer antigen 3 test using a cutoff value of 35 mug/L in patients with prostate cancer. *Tumour Biol.* (2014) 35:8573–80. doi: 10.1007/s13277-014-2109-4
67. Xue W-J, Ying X-L, Jiang J-H, Xu Y-H. Prostate cancer antigen 3 as a biomarker in the urine for prostate cancer diagnosis: a meta-analysis. *J Cancer Res Ther.* (2014) 10 (Suppl. 1):C218–21. doi: 10.4103/0973-1482.145881
68. Sartori DA, Chan DW. Biomarkers in prostate cancer: what's new? *Curr Opin Oncol.* (2014) 26:259–64. doi: 10.1097/CCO.0000000000000065
69. Tang H, Wu Z, Zhang J, Su B. Salivary lncRNA as a potential marker for oral squamous cell carcinoma diagnosis. *Mol Med Rep.* (2013) 7:761–6. doi: 10.3892/mmr.2012.1254
70. Huarte M, Rinn JL. Large non-coding RNAs: missing links in cancer? *Human Mol Genet.* (2010) 19:R152–61. doi: 10.1093/hmg/ddq353
71. Taft RJ, Pang KC, Mercer TR, Dinger M, Mattick JS. Non-coding RNAs: regulators of disease. *J Pathol.* (2010) 220:126–39. doi: 10.1002/path.2638
72. Parasramka MA, Maji S, Matsuda A, Yan IK, Patel T. Long non-coding RNAs as novel targets for therapy in hepatocellular carcinoma. *Pharmacol Ther.* (2016) 161:67–78. doi: 10.1016/j.pharmthera.2016.03.004
73. Zeng S, Xiao YF, Tang B, Hu CJ, Xie R, Yang SM, et al. Long noncoding RNA in digestive tract cancers: function, mechanism, and potential biomarker. *Oncologist.* (2015) 20:898–906. doi: 10.1634/theoncologist.2014-0475
74. Sun J, Li W, Sun Y, Yu D, Wen X, Wang H, et al. A novel antisense long noncoding RNA within the IGF1R gene locus is imprinted in hematopoietic malignancies. *Nucleic Acids Res.* (2014) 42:9588–601. doi: 10.1093/nar/gku549
75. Martens-Uzunova ES, Bottcher R, Croce CM, Jenster G, Visakorpi T, Calin GA. Long noncoding RNA in prostate, bladder, and kidney cancer. *Eur Urol.* (2014) 65:1140–51. doi: 10.1016/j.eururo.2013.12.003
76. Ricciuti B, Mencaroni C, Paglialunga L, Paciullo F, Crino L, Chiari R, et al. Long noncoding RNAs: new insights into non-small cell lung cancer biology, diagnosis and therapy. *Med Oncol.* (2016) 33:18. doi: 10.1007/s12032-016-0731-2
77. Shi Y, Li J, Liu Y, Ding J, Fan Y, Tian Y, et al. The long noncoding RNA SPRY4-IT1 increases the proliferation of human breast cancer cells by upregulating ZNF703 expression. *Mol Cancer.* (2015) 14:51. doi: 10.1186/s12943-015-0318-0
78. Shi Y, Liu Y, Wang J, Jie D, Yun T, Li W, et al. Downregulated long noncoding RNA BANCR promotes the proliferation of colorectal cancer cells via downregualtion of p21 expression. *PLoS ONE.* (2015) 10:e0122679. doi: 10.1371/journal.pone.0122679
79. Jin Y, Feng SJ, Qiu S, Shao N, Zheng JH. LncRNA MALAT1 promotes proliferation and metastasis in epithelial ovarian cancer via the PI3K-AKT pathway. *Eur Rev Med Pharmacol Sci.* (2017) 21:3176–84.
80. Ge X, Chen Y, Liao X, Liu D, Li F, Ruan H, et al. Overexpression of long noncoding RNA PCAT-1 is a novel biomarker of poor prognosis in patients with colorectal cancer. *Med Oncol.* (2013) 30:588. doi: 10.1007/s12032-013-0588-6
81. Fu J, Cui Y. Long noncoding RNA ZEB1-AS1 expression predicts progression and poor prognosis of colorectal cancer. *Int J Biol Markers.* (2017) 32:e428-e33. doi: 10.5301/ijbm.5000303
82. Li Y, Zhao L, Zhang Y, Guan L, Zhang H, Zhou H, et al. Downregulation of the long non-coding RNA XLOC_010588 inhibits the invasion and migration of colorectal cancer. *Oncol Rep.* (2018) 39:1619–30. doi: 10.3892/or.2018.6260
83. Yang Y, Zhang J, Chen X, Xu X, Cao G, Li H, et al. LncRNA FTX sponges miR-215 and inhibits phosphorylation of vimentin for promoting colorectal cancer progression. *Gene Ther.* (2018) 25:321–30. doi: 10.1038/s41434-018-0026-7
84. Su J, Zhang E, Han L, Yin D, Liu Z, He X, et al. Long noncoding RNA BLACAT1 indicates a poor prognosis of colorectal cancer and affects cell proliferation by epigenetically silencing of p15. *Cell Death Dis.* (2017) 8:e2665. doi: 10.1038/cddis.2017.83
85. Han Q, Xu L, Lin W, Yao X, Jiang M, Zhou R, et al. Long noncoding RNA CRCMSL suppresses tumor invasive and metastasis in colorectal carcinoma through nucleocytoplasmic shuttling of HMGB2. *Oncogene.* (2019) 38:3019–32. doi: 10.1038/s41388-018-0614-4
86. Wang Y, Lu Z, Wang N, Feng J, Zhang J, Luan L, et al. Long noncoding RNA DANCR promotes colorectal cancer proliferation and metastasis via miR-577 sponging. *Exp Mol Med.* (2018) 50:57. doi: 10.1038/s12276-018-0082-5
87. Gu LQ, Xing XL, Cai H, Si AF, Hu XR, Ma QY, et al. Long non-coding RNA DILC suppresses cell proliferation and metastasis in colorectal cancer. *Gene.* (2018) 666:18–26. doi: 10.1016/j.gene.2018.03.100
88. Zhong X, Lu M, Wan J, Zhou T, Qin B. Long noncoding RNA kcna3 inhibits the progression of colorectal carcinoma through down-regulating YAP1 expression. *Biomed Pharmacother.* (2018) 107:382–9. doi: 10.1016/j.biopha.2018.07.118
89. Ding J, Lu B, Wang J, Wang J, Shi Y, Lian Y, et al. Long non-coding RNA Loc554202 induces apoptosis in colorectal cancer cells via the caspase cleavage cascades. *J Exp Clin Cancer Res.* (2015) 34:100. doi: 10.1186/s13046-015-0217-7
90. Ji H, Hui B, Wang J, Zhu Y, Tang L, Peng P, et al. Long noncoding RNA MAPKAPK5-AS1 promotes colorectal cancer proliferation by partly silencing p21 expression. *Cancer Sci.* (2019) 110:72–85. doi: 10.1111/cas.13838
91. Shi L, Hong X, Ba L, He X, Xiong Y, Ding Q, et al. Long non-coding RNA ZNFX1-AS1 promotes the tumor progression and metastasis of colorectal cancer by acting as a competing endogenous RNA of miR-144 to regulate EZH2 expression. *Cell Death Dis.* (2019) 10:150. doi: 10.1038/s41419-019-1332-8
92. Lian Y, Xu Y, Xiao C, Xia R, Gong H, Yang P, et al. The pseudogene derived from long non-coding RNA DUXAP10 promotes colorectal cancer cell growth through epigenetically silencing of p21 and PTEN. *Sci Rep.* (2017) 7:7312. doi: 10.1038/s41598-017-07954-7
93. Wang Q, Yang L, Hu X, Jiang Y, Hu Y, Liu Z, et al. Upregulated NNT-AS1, a long noncoding RNA, contributes to proliferation and migration of colorectal cancer cells *in vitro* and *in vivo*. *Oncotarget.* (2017) 8:3441–53. doi: 10.18632/oncotarget.13840
94. Deng Q, He B, Gao T, Pan Y, Sun H, Xu Y, et al. Up-regulation of 91H promotes tumor metastasis and predicts poor prognosis for patients with colorectal cancer. *PLoS ONE.* (2014) 9:e103022. doi: 10.1371/journal.pone.0103022
95. Kino T, Hurt DE, Ichijo T, Nader N, Chrousos GP. Noncoding RNA gas5 is a growth arrest- and starvation-associated repressor of the glucocorticoid receptor. *Sci Signal.* (2010) 3:ra8-ra. doi: 10.1126/scisignal.2000568
96. Pickard MR, Williams GT. Molecular and cellular mechanisms of action of tumour suppressor GAS5 LncRNA. *Genes.* (2015) 6:484–99. doi: 10.3390/genes6030484
97. Pickard MR, Mourtada-Maarabouni M, Williams GT. Long non-coding RNA GAS5 regulates apoptosis in prostate cancer cell lines. *Biochim Biophys Acta.* (2013) 1832:1613–23. doi: 10.1016/j.bbadis.2013.05.005
98. Qiao HP, Gao WS, Huo JX, Yang ZS. Long non-coding RNA GAS5 functions as a tumor suppressor in renal cell carcinoma. *Asian Pacific J Cancer Prevent.* (2013) 14:1077–82. doi: 10.7314/APJCP.2013.14.2.1077
99. Mourtada-Maarabouni M, Pickard MR, Hedge VL, Farzaneh F, Williams GT. GAS5, a non-protein-coding RNA, controls apoptosis and is downregulated in breast cancer. *Oncogene.* (2009) 28:195–208. doi: 10.1038/onc.2008.373
100. Li J, Wang Y, Zhang C-G, Xiao H-J, Hou J-M, He J-D. Effect of long non-coding RNA Gas5 on proliferation, migration, invasion and

apoptosis of colorectal cancer HT-29 cell line. *Cancer Cell Int.* (2018) 18:4. doi: 10.1186/s12935-017-0478-7
101. Cheng K, Zhao Z, Wang G, Wang J, Zhu W. lncRNA GAS5 inhibits colorectal cancer cell proliferation via the miR1825p/FOXO3a axis. *Oncol Rep.* (2018) 40:2371–80. doi: 10.3892/or.2018.6584
102. Yin D, He X, Zhang E, Kong R, De W, Zhang Z. Long noncoding RNA GAS5 affects cell proliferation and predicts a poor prognosis in patients with colorectal cancer. *Med Oncol.* (2014) 31:253. doi: 10.1007/s12032-014-0253-8
103. Yang Y, Shen Z, Yan Y, Wang B, Zhang J, Shen C, et al. Long non-coding RNA GAS5 inhibits cell proliferation, induces G0/G1 arrest and apoptosis, and functions as a prognostic marker in colorectal cancer. *Oncol Lett.* (2017) 13:3151–8. doi: 10.3892/ol.2017.5841
104. Liu L, Meng T, Yang XH, Sayim P, Lei C, Jin B, et al. Prognostic and predictive value of long non-coding RNA GAS5 and mircoRNA-221 in colorectal cancer and their effects on colorectal cancer cell proliferation, migration and invasion. *Cancer Biomarkers.* (2018) 22:283–99. doi: 10.3233/CBM-171011
105. Song J, Shu H, Zhang L, Xiong J. Long noncoding RNA GAS5 inhibits angiogenesis and metastasis of colorectal cancer through the Wnt/beta-catenin signaling pathway. *J Cell Biochem.* (2019) 2019:27743. doi: 10.1002/jcb.27743
106. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, et al. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell.* (2007) 129:1311–23. doi: 10.1016/j.cell.2007.05.022
107. Zeng X, Chen S, Huang H. Phosphorylation of EZH2 by CDK1 and CDK2: a possible regulatory mechanism of transmission of the H3K27me3 epigenetic mark through cell divisions. *Cell Cycle.* (2011) 10:579–83. doi: 10.4161/cc.10.4.14722
108. Ding J, Yeh C-R, Sun Y, Lin C, Chou J, Ou Z, et al. Estrogen receptor β promotes renal cell carcinoma progression via regulating LncRNA HOTAIR-miR-138/200c/204/217 associated CeRNA network. *Oncogene.* (2018) 37:5037–53. doi: 10.1038/s41388-018-0175-6
109. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. *Nature.* (2010) 464:1071–6. doi: 10.1038/nature08975
110. Liu XH, Sun M, Nie FQ, Ge YB, Zhang EB, Yin DD, et al. Lnc RNA HOTAIR functions as a competing endogenous RNA to regulate HER2 expression by sponging miR-331–3p in gastric cancer. *Mol Cancer.* (2014) 13:92. doi: 10.1186/1476-4598-13-92
111. Liu XH, Liu ZL, Sun M, Liu J, Wang ZX, De W. The long non-coding RNA HOTAIR indicates a poor prognosis and promotes metastasis in non-small cell lung cancer. *BMC Cancer.* (2013) 13:464. doi: 10.1186/1471-2407-13-464
112. Zhang Z, Cheng J, Wu Y, Qiu J, Sun Y, Tong X. LncRNA HOTAIR controls the expression of Rab22a by sponging miR-373 in ovarian cancer. *Mol Med Rep.* (2016) 14:2465–72. doi: 10.3892/mmr.2016.5572
113. Lin K, Jiang H, Zhang LL, Jiang Y, Yang YX, Qiu GD, et al. Down-regulated LncRNA-HOTAIR suppressed colorectal cancer cell proliferation, invasion, and migration by mediating p21. *Digest Dis Sci.* (2018) 63:2320–31. doi: 10.1007/s10620-018-5127-z
114. Pan S, Liu Y, Liu Q, Xiao Y, Liu B, Ren X, et al. HOTAIR/miR-326/FUT6 axis facilitates colorectal cancer progression through regulating fucosylation of CD44 via PI3K/AKT/mTOR pathway. *Biochim Biophys Acta Mol Cell Res.* (2019) 1866:750–60. doi: 10.1016/j.bbamcr.2019.02.004
115. Yang XD, Xu HT, Xu XH, Ru G, Liu W, Zhu JJ, et al. Knockdown of long non-coding RNA HOTAIR inhibits proliferation and invasiveness and improves radiosensitivity in colorectal cancer. *Oncol Rep.* (2016) 35:479–87. doi: 10.3892/or.2015.4397
116. Xiao Z, Qu Z, Chen Z, Fang Z, Zhou K, Huang Z, et al. LncRNA HOTAIR is a prognostic biomarker for the proliferation and chemoresistance of colorectal cancer via MiR-203a-3p-mediated Wnt/ss-catenin signaling pathway. *Cell Physiol Biochem.* (2018) 46:1275–85. doi: 10.1159/000489110
117. Tatangelo F, Di Mauro A, Scognamiglio G, Aquino G, Lettiero A, Delrio P, et al. Posterior HOX genes and HOTAIR expression in the proximal and distal colon cancer pathogenesis. *J Transl Med.* (2018) 16:350. doi: 10.1186/s12967-018-1725-y
118. Svoboda M, Slyskova J, Schneiderova M, Makovicky P, Bielik L, Levy M, et al. HOTAIR long non-coding RNA is a negative prognostic factor not only in primary tumors, but also in the blood of colorectal cancer patients. *Carcinogenesis.* (2014) 35:1510–5. doi: 10.1093/carcin/bgu055
119. Gabory A, Ripoche MA, Yoshimizu T, Dandolo L. The H19 gene: regulation and function of a non-coding RNA. *Cytogenet Genome Res.* (2006) 113:188–93. doi: 10.1159/000090831
120. Gabory A, Jammes H, Dandolo L. The H19 locus: role of an imprinted non-coding RNA in growth and development. *Bioessays.* (2010) 32:473–80. doi: 10.1002/bies.200900170
121. Brannan CI, Dees EC, Ingram RS, Tilghman SM. The product of the H19 gene may function as an RNA. *Mol Cell Biol.* (1990) 10:28–36. doi: 10.1128/MCB.10.1.28
122. Lustig O, Ariel I, Ilan J, Lev-Lehman E, De-Groot N, Hochberg A. Expression of the imprinted gene H19 in the human fetus. *Mol Reprod Dev.* (1994) 38:239–46. doi: 10.1002/mrd.1080380302
123. Ariel I, Miao HQ, Ji XR, Schneider T, Roll D, de Groot N, et al. Imprinted H19 oncofetal RNA is a candidate tumour marker for hepatocellular carcinoma. *Mol Pathol.* (1998) 51:21–5. doi: 10.1136/mp.51.1.21
124. Hibi K, Nakamura H, Hirai A, Fujikake Y, Kasai Y, Akiyama S, et al. Loss of H19 imprinting in esophageal cancer. *Cancer Res.* (1996) 56:480–2.
125. Tanos V, Prus D, Ayesh S, Weinstein D, Tykocinski ML, De-Groot N, et al. Expression of the imprinted H19 oncofetal RNA in epithelial ovarian cancer. *Eur J Obstetr Gynecol Reprod Biol.* (1999) 85:7–11. doi: 10.1016/S0301-2115(98)00275-9
126. Berteaux N, Lottin S, Monte D, Pinte S, Quatannens B, Coll J, et al. H19 mRNA-like noncoding RNA promotes breast cancer cell proliferation through positive control by E2F1. *J Biol Chem.* (2005) 280:29625–36. doi: 10.1074/jbc.M504033200
127. Byun HM, Wong HL, Birnstein EA, Wolff EM, Liang G, Yang AS. Examination of IGF2 and H19 loss of imprinting in bladder cancer. *Cancer Res.* (2007) 67:10753–8. doi: 10.1158/0008-5472.CAN-07-0329
128. Yang W, Ning N. The lncRNA H19 promotes cell proliferation by competitively binding to miR-200a and derepressing beta-catenin expression in colorectal cancer. *Biomed Res Int.* (2017) 2017:2767484. doi: 10.1155/2017/2767484
129. Tsang WP, Ng EK, Ng SS, Jin H, Yu J, Sung JJ, et al. Oncofetal H19-derived miR-675 regulates tumor suppressor RB in human colorectal cancer. *Carcinogenesis.* (2010) 31:350–8. doi: 10.1093/carcin/bgp181
130. Ohtsuka M, Ling H, Ivan C, Pichler M, Matsushita D, Goblirsch M, et al. H19 noncoding RNA, an independent prognostic factor, regulates essential Rb-E2F and CDK8-beta-catenin signaling in colorectal cancer. *EBio Med.* (2016) 13:113–24. doi: 10.1016/j.ebiom.2016.10.026
131. Han D, Gao X, Wang M, Qiao Y, Xu Y, Yang J, et al. Long noncoding RNA H19 indicates a poor prognosis of colorectal cancer and promotes tumor growth by recruiting and binding to eIF4A3. *Oncotarget.* (2016) 7:22159–73. doi: 10.18632/oncotarget.8063
132. Ding D, Li C, Zhao T, Li D, Yang L, Zhang B. LncRNA H19/miR-29b-3p/PGRN axis promoted epithelial-mesenchymal transition of colorectal cancer cells by acting on Wnt signaling. *Mol Cells.* (2018) 41:423–35. doi: 10.14348/molcells.2018.2258
133. Yang W, Redpath RE, Zhang C, Ning N. Long non-coding RNA H19 promotes the migration and invasion of colon cancer cells via MAPK signaling pathway. *Oncol Lett.* (2018) 16:3365–72. doi: 10.3892/ol.2018.9052
134. Wu KF, Liang WC, Feng L, Pang JX, Waye MM, Zhang JF, et al. H19 mediates methotrexate resistance in colorectal cancer through activating Wnt/beta-catenin pathway. *Exp Cell Res.* (2017) 350:312–7. doi: 10.1016/j.yexcr.2016.12.003
135. Liang WC, Fu WM, Wong CW, Wang Y, Wang WM, Hu GX, et al. The lncRNA H19 promotes epithelial to mesenchymal transition by functioning as miRNA sponges in colorectal cancer. *Oncotarget.* (2015) 6:22513–25. doi: 10.18632/oncotarget.4154
136. Yang MH, Hu ZY, Xu C, Xie LY, Wang XY, Chen SY, et al. MALAT1 promotes colorectal cancer cell proliferation/migration/invasion via PRKA kinase anchor protein 9. *Biochim Biophys Acta.* (2015) 1852:166–74. doi: 10.1016/j.bbadis.2014.11.013
137. Ji P, Diederichs S, Wang W, Boing S, Metzger R, Schneider PM, et al. MALAT-1, a novel noncoding RNA, and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer. *Oncogene.* (2003) 22:8031–41. doi: 10.1038/sj.onc.1206928

138. Tseng JJ, Hsieh YT, Hsu SL, Chou MM. Metastasis associated lung adenocarcinoma transcript 1 is up-regulated in placenta previa increta/percreta and strongly associated with trophoblast-like cell invasion *in vitro*. *Mol Hum Reprod.* (2009) 15:725–31. doi: 10.1093/molehr/gap071
139. Lin R, Maeda S, Liu C, Karin M, Edgington TS. A large noncoding RNA is a marker for murine hepatocellular carcinomas and a spectrum of human carcinomas. *Oncogene.* (2007) 26:851–8. doi: 10.1038/sj.onc.1209846
140. Guo F, Li Y, Liu Y, Wang J, Li Y, Li G. Inhibition of metastasis-associated lung adenocarcinoma transcript 1 in CaSki human cervical cancer cells suppresses cell proliferation and invasion. *Acta Biochim Biophys Sin.* (2010) 42:224–9. doi: 10.1093/abbs/gmq008
141. Schmidt LH, Spieker T, Koschmieder S, Schaffers S, Humberg J, Jungen D, et al. The long noncoding MALAT-1 RNA indicates a poor prognosis in non-small cell lung cancer and induces migration and tumor growth. *J Thoracic Oncol.* (2011) 6:1984–92. doi: 10.1097/JTO.0b013e3182307eac
142. Ying L, Chen Q, Wang Y, Zhou Z, Huang Y, Qiu F. Upregulated MALAT-1 contributes to bladder cancer cell migration by inducing epithelial-to-mesenchymal transition. *Mol Biosystems.* (2012) 8:2289–94. doi: 10.1039/c2mb25070e
143. Gutschner T, Hammerle M, Eissmann M, Hsu J, Kim Y, Hung G, et al. The noncoding RNA MALAT1 is a critical regulator of the metastasis phenotype of lung cancer cells. *Cancer Res.* (2013) 73:1180–9. doi: 10.1158/0008-5472.CAN-12-2850
144. Yamada K, Kano J, Tsunoda H, Yoshikawa H, Okubo C, Ishiyama T, et al. Phenotypic characterization of endometrial stromal sarcoma of the uterus. *Cancer Sci.* (2006) 97:106–12. doi: 10.1111/j.1349-7006.2006.00147.x
145. Zheng HT, Shi DB, Wang YW, Li XX, Xu Y, Tripathi P, et al. High expression of lncRNA MALAT1 suggests a biomarker of poor prognosis in colorectal cancer. *Int J Clin Exp Pathol.* (2014) 7:3174–81.
146. Xu Y, Zhang X, Hu X, Zhou W, Zhang P, Zhang J, et al. The effects of lncRNA MALAT1 on proliferation, invasion and migration in colorectal cancer through regulating SOX9. *Mol Med.* (2018) 24:52. doi: 10.1186/s10020-018-0050-5
147. Ji Q, Zhang L, Liu X, Zhou L, Wang W, Han Z, et al. Long non-coding RNA MALAT1 promotes tumour growth and metastasis in colorectal cancer through binding to SFPQ and releasing oncogene PTBP2 from SFPQ/PTBP2 complex. *Br J Cancer.* (2014) 111:736–48. doi: 10.1038/bjc.2014.383
148. Sun Z, Ou C, Liu J, Chen C, Zhou Q, Yang S, et al. YAP1-induced MALAT1 promotes epithelial-mesenchymal transition and angiogenesis by sponging miR-126–5p in colorectal cancer. *Oncogene.* (2019) 38:2627–44. doi: 10.1038/s41388-018-0628-y
149. Tang D, Yang Z, Long F, Luo L, Yang B, Zhu R, et al. Long noncoding RNA MALAT1 mediates stem cell-like properties in human colorectal cancer cells by regulating miR-20b-5p/Oct4 axis. *J Cell Physiol.* (2019) 234:20816–28. doi: 10.1002/jcp.28687
150. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell.* (2008) 132:27–42. doi: 10.1016/j.cell.2007.12.018
151. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature.* (2008) 451:1069–75. doi: 10.1038/nature06639
152. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol.* (2007) 8:741–52. doi: 10.1038/nrm2239
153. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. *Cell.* (2011) 147:728–41. doi: 10.1016/j.cell.2011.10.026
154. Klionsky DJ. The molecular machinery of autophagy: unanswered questions. *J Cell Sci.* (2005) 118 (Pt 1):7–18. doi: 10.1242/jcs.01620
155. Yang L, Wang H, Shen Q, Feng L, Jin H. Long non-coding RNAs involved in autophagy regulation. *Cell Death Dis.* (2017) 8:e3073. doi: 10.1038/cddis.2017.464
156. Devenish RJ, Prescott M. Autophagy: starvation relieves transcriptional repression of ATG genes. *Curr Biol.* (2015) 25:R238–R40. doi: 10.1016/j.cub.2015.01.045
157. Liu X, Xiao ZD, Han L, Zhang J, Lee SW, Wang W, et al. LncRNA NBR2 engages a metabolic checkpoint by regulating AMPK under energy stress. *Nat Cell Biol.* (2016) 18:431–42. doi: 10.1038/ncb3328
158. Zhuo C, Jiang R, Lin X, Shao M. LncRNA H19 inhibits autophagy by epigenetically silencing of DIRAS3 in diabetic cardiomyopathy. *Oncotarget.* (2017) 8:1429–37. doi: 10.18632/oncotarget.13637
159. Tang S, Tan G, Jiang X, Han P, Zhai B, Dong X, et al. An artificial lncRNA targeting multiple miRNAs overcomes sorafenib resistance in hepatocellular carcinoma cells. *Oncotarget.* (2016) 7:73257–69. doi: 10.18632/oncotarget.12304
160. Pawar K, Hanisch C, Palma Vera SE, Einspanier R, Sharbati S. Down regulated lncRNA MEG3 eliminates mycobacteria in macrophages via autophagy. *Sci Rep.* (2016) 6:19416. doi: 10.1038/srep19416
161. Chen CL, Tseng YW, Wu JC, Chen GY, Lin KC, Hwang SM, et al. Suppression of hepatocellular carcinoma by baculovirus-mediated expression of long non-coding RNA PTENP1 and MicroRNA regulation. *Biomaterials.* (2015) 44:71–81. doi: 10.1016/j.biomaterials.2014.12.023
162. Chen ZH, Wang WT, Huang W, Fang K, Sun YM, Liu SR, et al. The lncRNA HOTAIRM1 regulates the degradation of PML-RARA oncoprotein and myeloid cell differentiation by enhancing the autophagy pathway. *Cell Death Differ.* (2017) 24:212–24. doi: 10.1038/cdd.2016.111
163. Wang Y, Hu Y, Sun C, Zhuo S, He Z, Wang H, et al. Down-regulation of Risa improves insulin sensitivity by enhancing autophagy. *FASEB J.* (2016) 30:3133–45. doi: 10.1096/fj.201500058R
164. Xiu Y-L, Sun K-X, Chen X, Chen S, Zhao Y, Guo Q-G, et al. Upregulation of the lncRNA Meg3 induces autophagy to inhibit tumorigenesis and progression of epithelial ovarian carcinoma by regulating activity of ATG3. *Oncotarget.* (2017) 8:31714–25. doi: 10.18632/oncotarget.15955
165. Chen Y-N, Cai M-Y, Xu S, Meng M, Ren X, Yang JW, et al. Identification of the lncRNA, AK156230, as a novel regulator of cellular senescence in mouse embryonic fibroblasts. *Oncotarget.* (2016) 7:52673–84. doi: 10.18632/oncotarget.10170
166. Santana-Codina N, Mancias JD, Kimmelman AC. The role of autophagy in cancer. *Ann Rev Cancer Biol.* (2017) 1:19–39. doi: 10.1146/annurev-cancerbio-041816-122338
167. Kung CP, Budina A, Balaburski G, Bergenstock MK, Murphy M. Autophagy in tumor suppression and cancer therapy. *Crit Rev Eukaryot Gene Expr.* (2011) 21:71–100. doi: 10.1615/CritRevEukarGeneExpr.v21.i1.50
168. Avalos Y, Canales J, Bravo-Sagua R, Criollo A, Lavandero S, Quest AF. Tumor suppression and promotion by autophagy. *Biomed Res Int.* (2014) 2014:603980. doi: 10.1155/2014/603980
169. White E. The role for autophagy in cancer. *J Clin Invest.* (2015) 125:42–6. doi: 10.1172/JCI73941
170. Shan TD, Xu JH, Yu T, Li JY, Zhao LN, Ouyang H, et al. Knockdown of linc-POU3F3 suppresses the proliferation, apoptosis, and migration resistance of colorectal cancer. *Oncotarget.* (2016) 7:961–75. doi: 10.18632/oncotarget.5830
171. Zheng Y, Tan K, Huang H. Long noncoding RNA HAGLROS regulates apoptosis and autophagy in colorectal cancer cells via sponging miR-100 to target ATG5 expression. *J Cell Biochem.* (2019) 120:3922–33. doi: 10.1002/jcb.27676
172. Bian Z, Jin L, Zhang J, Yin Y, Quan C, Hu Y, et al. LncRNA—UCA1 enhances cell proliferation and 5-fluorouracil resistance in colorectal cancer by inhibiting miR-204–5p. *Sci Rep.* (2016) 6:23892. doi: 10.1038/srep23892
173. Li Y, Li C, Li D, Yang L, Jin J, Zhang B. lncRNA KCNQ1OT1 enhances the chemoresistance of oxaliplatin in colon cancer by targeting the miR-34a/ATG4B pathway. *Oncotargets Ther.* (2019) 12:2649–60. doi: 10.2147/OTT.S188054
174. Hemelaar J, Lelyveld VS, Kessler BM, Ploegh HL. A single protease, Apg4B, is specific for the autophagy-related ubiquitin-like proteins GATE-16, MAP1-LC3, GABARAP, and Apg8L. *J Biol Chem.* (2003) 278:51841–50. doi: 10.1074/jbc.M308762200
175. Raguz S, Yague E. Resistance to chemotherapy: new treatments and novel insights into an old problem. *Br J Cancer.* (2008) 99:387–91. doi: 10.1038/sj.bjc.6604510
176. Tan DSW, Gerlinger M, Teh B-T, Swanton C. Anti-cancer drug resistance: understanding the mechanisms through the use of integrative genomics and functional RNA interference. *Eur J Cancer.* (2010) 46:2166–77. doi: 10.1016/j.ejca.2010.03.019
177. Senthebane DA, Rowe A, Thomford NE, Shipanga H, Munro D, Mazeedi MAMA, et al. The role of tumor microenvironment in chemoresistance:

to survive, keep your enemies closer. *Int J Mol Sci.* (2017) 18:1586. doi: 10.3390/ijms18071586
178. Marshall JL, Haller DG, de Gramont A, Hochster HS, Lenz H-J, Ajani JA, et al. Adjuvant therapy for stage II and III colon cancer: consensus report of the International Society of Gastrointestinal Oncology. *Gastrointest Cancer Res.* (2007) 1:146–54.
179. Moertel CG, Fleming TR, Macdonald JS, Haller DG, Laurie JA, Tangen CM, et al. Intergroup study of fluorouracil plus levamisole as adjuvant therapy for stage II/Dukes' B2 colon cancer. *J Clin Oncol.* (1995) 13:2936–43. doi: 10.1200/JCO.1995.13.12.2936
180. Gill S, Loprinzi CL, Sargent DJ, Thome SD, Alberts SR, Haller DG, et al. Pooled analysis of fluorouracil-based adjuvant therapy for stage II and III colon cancer: who benefits and by how much? *J Clin Oncol.* (2004) 22:1797–806. doi: 10.1200/JCO.2004.09.059
181. Martini G, Troiani T, Cardone C, Vitiello P, Sforza V, Ciardiello D, et al. Present and future of metastatic colorectal cancer treatment: a review of new candidate targets. *World J Gastroenterol.* (2017) 23:4675–88. doi: 10.3748/wjg.v23.i26.4675
182. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med.* (2004) 350:2335–42. doi: 10.1056/NEJMoa032691
183. Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med.* (2004) 351:337–45. doi: 10.1056/NEJMoa033025
184. Van Cutsem E, Peeters M, Siena S, Humblet Y, Hendlisz A, Neyns B, et al. Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. *J Clin Oncol.* (2007) 25:1658–64. doi: 10.1200/JCO.2006.08.1620
185. Tabernero J, Yoshino T, Cohn AL, Obermannova R, Bodoky G, Garcia-Carbonero R, et al. Ramucirumab versus placebo in combination with second-line FOLFIRI in patients with metastatic colorectal carcinoma that progressed during or after first-line therapy with bevacizumab, oxaliplatin, and a fluoropyrimidine (RAISE): a randomised, double-blind, multicentre, phase 3 study. *Lancet Oncol.* (2015) 16:499–508. doi: 10.1016/S1470-2045(15)70127-0
186. Peeters M, Price TJ, Cervantes A, Sobrero AF, Ducreux M, Hotko Y, et al. Randomized phase III study of panitumumab with fluorouracil, leucovorin, and irinotecan (FOLFIRI) compared with FOLFIRI alone as second-line treatment in patients with metastatic colorectal cancer. *J Clin Oncol.* (2010) 28:4706–13. doi: 10.1200/JCO.2009.27.6055
187. Corrà F, Agnoletto C, Minotti L, Baldassari F, Volinia S. The network of non-coding RNAs in cancer drug resistance. *Front Oncol.* (2018) 8:1–25. doi: 10.3389/fonc.2018.00327
188. Jiang X, Li Q, Zhang S, Song C, Zheng P. Long noncoding RNA GIHCG induces cancer progression and chemoresistance and indicates poor prognosis in colorectal cancer. *Oncotargets Ther.* (2019) 12:1059–70. doi: 10.2147/OTT.S192290
189. Lu Y, Zhao X, Liu Q, Li C, Graves-Deal R, Cao Z, et al. LncRNA MIR100HG-derived miR-100 and miR-125b mediate cetuximab resistance via Wnt/β-catenin signaling. *Nat Med.* (2017) 23:1331–41. doi: 10.1038/nm.4424
190. Yang Y-n, Zhang R, Du J-w, Yuan H-h, Li Y-j, Wei X-l, et al. Predictive role of UCA1-containing exosomes in cetuximab-resistant colorectal cancer. *Cancer Cell Int.* (2018) 18:1–11. doi: 10.1186/s12935-018-0660-6
191. Bian Z, Zhang J, Li M, Feng Y, Yao S, Song M, et al. Long non-coding RNA LINC00152 promotes cell proliferation, metastasis, and confers 5-FU resistance in colorectal cancer by inhibiting miR-139–5p. *Oncogenesis.* (2017) 6:395. doi: 10.1038/s41389-017-0008-4
192. Yue B, Cai D, Liu C, Fang C, Yan D. Linc00152 functions as a competing endogenous RNA to confer oxaliplatin resistance and holds prognostic values in colon cancer. *Am Soc Gene Cell Ther.* (2016) 24:2064–77. doi: 10.1038/mt.2016.180
193. Qiao L, Liu X, Tang Y, Zhao Z, Zhang J. Knockdown of long non-coding RNA prostate cancer-associated ncRNA transcript 1 inhibits multidrug resistance and c-Myc- dependent aggressiveness in colorectal cancer Caco-2 and HT-29 cells. *Mol Cell Biochem.* (2018) 441:99–108. doi: 10.1007/s11010-017-3177-8
194. Fan H, Zhu JHUA, Yao XUEQ. Knockdown of long non-coding RNA PVT1 reverses multidrug resistance in colorectal cancer cells. *Mol Med Rep.* (2018) 17:8309–15. doi: 10.3892/mmr.2018.8907
195. Zhu J, Zhang R, Yang D, Li J, Yan X, Jin K, et al. Knockdown of long non-coding RNA XIST inhibited doxorubicin resistance in colorectal cancer by upregulation of miR-124 and downregulation of SGK1. *Cell Physiol Biochem.* (2018) 51:113–28. doi: 10.1159/000495168
196. Tang D, Yang Z, Long F, Luo L, Yang B, Zhu R, et al. Inhibition of MALAT1 reduces tumor growth and metastasis and promotes drug sensitivity in colorectal cancer. *Cell Signal.* (2019) 57:21–8. doi: 10.1016/j.cellsig.2019.01.013
197. Li P, Zhang X, Wang H, Wang L, Liu T, Du L, et al. MALAT1 is associated with poor response to oxaliplatin-based chemotherapy in colorectal cancer patients and promotes chemoresistance through EZH2. *Mol Cancer Ther.* (2017) 16:739–52. doi: 10.1158/1535-7163.MCT-16-0591
198. Li Y, Huang S, Li Y, Zhang W, He K, Zhao M, et al. Decreased expression of LncRNA SLC25A25-AS1 promotes proliferation, chemoresistance, and EMT in colorectal cancer cells. *Tumor Biol.* (2016) 37:14205–15. doi: 10.1007/s13277-016-5254-0
199. Lee H, Kim C, Ku J-l, Kim W, Yoon SK, Kuh H-j, et al. A long non-coding RNA snaR contributes to 5-fluorouracil resistance in human colon cancer cells. *Mol Cells.* (2014) 37:540–6. doi: 10.14348/molcells.2014.0151
200. Li J, Li X, Cen C, Ai X, Lin C, Hu G. The long non-coding RNA ENST00000547547 reduces 5-fluorouracil resistance of colorectal cancer cells via competitive binding to microRNA-31. *Oncol Rep.* (2018) 39:217–26. doi: 10.3892/or.2017.6082
201. Li C, Gao Y, Li Y, Ding D. TUG1 mediates methotrexate resistance in colorectal cancer via miR-186/CPEB2 axis. *Biochem Biophys Res Commun.* (2017) 491:552–7. doi: 10.1016/j.bbrc.2017.03.042
202. Ping G, Xiong W, Zhang L, Li Y, Zhang Y, Zhao Y. Silencing long noncoding RNA PVT1 inhibits tumorigenesis and cisplatin resistance of colorectal cancer. *Am J Transl Res.* (2018) 10:138–49.
203. Wang H, Li H, Zhang L, Yang D. Overexpression of MEG3 sensitizes colorectal cancer cells to oxaliplatin through regulation of miR-141/PDCD4 axis. *Biomed Pharmacother.* (2018) 106:1607–15. doi: 10.1016/j.biopha.2018.07.131
204. Li L, Jian S, Yupeng Z, Liu S, Yanan P, Zhou Z, et al. MEG3 is a prognostic factor for CRC and promotes chemosensitivity by enhancing oxaliplatin-induced cell apoptosis. *Oncol Rep.* (2017) 38:1383–92. doi: 10.3892/or.2017.5828

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REVIEW
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GDF11 Implications in Cancer Biology and Metabolism. Facts and Controversies

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Growth Differentiation Factor 11 (GDF11), a member of the super family of the Transforming Growth Factor β, has gained more attention in the last few years due to numerous reports regarding its functions in other systems, which are different to those related to differentiation and embryonic development, such as age-related muscle dysfunction, skin biology, metabolism, and cancer. GDF11 is expressed in many tissues, including skeletal muscle, pancreas, kidney, nervous system, and retina, among others. GDF11 circulating levels and protein content in tissues are quite variable and are affected by pathological conditions or age. Although, GDF11 biology had a lot of controversies, must of them are only misunderstandings regarding the variability of its responses, which are independent of the tissue, grade of cellular differentiation or pathologies. A blunt fact regarding GDF11 biology is that its target cells have stemness feature, a property that could be found in certain adult cells in health and in disease, such as cancer cells. This review is focused to present and analyze the recent findings in the emerging research field of GDF11 function in cancer and metabolism, and discusses the controversies surrounding the biology of this atypical growth factor.

Keywords: GDF11, PCSK5, cancer, liver, HCC, metabolism

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INTRODUCTION

On May 2013 the research groups, led by doctors Amy J. Wagers and Richard T. Lee, published outstanding work suggesting that the growth differentiation factor 11 (GDF11) could be a good candidate for the age-related heart hypertrophy reversion observed in the model of heterochronic parabiosis (1). One year later, on May 2014, Science journal published a couple of works by the same research team at Harvard University, unveiling that systemic injection of the GDF11 reverses age-related dysfunction in skeletal muscle (2) and vascular and neurogenic function in the brain (3). Both reports were astonishing, particularly because myostatin, also known as GDF8, shares high structural homology with GDF11, but GDF8 induces exactly the contrary effect, muscle growth inhibition (4). At that moment, GDF11 was called "the rejuvenation factor," a term taken by a commentary note published by Jocelyn Kaiser in the same number of the Science journal (5), and Karoline E. Brun published another similar commentary in Cell journal entitle "GDF11 and the Mythical Fountain of Youth" (6).

The findings, beyond this unfortunate motto, revealed that GDF11 could exert functions in adult systems, in addition of those characterized in embryonic and fetal tissues. The works by the groups of doctors Wagers and Lee provided evidence that the main target cells are those with certain stemness phenotype, such as the satellite cells in the muscle, which are the progenitor ones for new functional muscle cells.

If GDF11 targets cells with stemness capacity, then many cancer cells should be targeted by this growth factor.

Many cancer cells gain stemness capacity and this correlates with aggressiveness and poor prognosis. The findings raised by the group of doctors, Wagers and Lee, position cancer cells as a target of GDF11 since they proved that stemness is a key condition for GDF11 effect. However, the results could be opposite depending of the cancer cell origin, metabolic status, or the stage of the cancer. We must wait for incoming works in the next few years, perhaps months, revealing a more precise mechanism regarding these apparent controversies in cancer and metabolism.

This work is focused to review the general knowledge of GDF11, and its functions in cancer biology and metabolism, taking into consideration recent findings in the specialized literature and in the public databases and scientific on-line resources.

GDF11 AN ATYPICAL TGF-β FAMILY MEMBER

GDF11 (also known as Bone Morphogenetic Protein, BMP11), is a member of the super family of the Transforming Growth Factor beta (TGF-β) and a subfamily of the BMP which is widely secreted in many species, including mouse, rat and human, and it is accepted as a key factor in embryo development, particularly in the anterior/posterior patterning (7–9).

GDF11 was identified by McPherron et al. in 1999, who cloned the human and mouse GDF11 and characterized its function in pattering the axial skeleton (9). Two years prior, the same group also discovered and characterized the GDF8 (10).

In humans, GDF11 gene is located in chromosome 12 (12q13.2, forward strand, Ensembl accession number: ENSG00000135414). Two splice variants products have been identified, according to Ensembl (**Figure 1**), the first one, GDF11-201 is a 8657 bp RNA, formed by three coding exons, generating a 407 amino acids protein, and the second one, GDF11-202, is a 1,258 bp, formed by three exons generating a 380 amino acids protein (11). Jeanplong (12) reported another RNA splice variant determined as GDF-11ΔEx11, characterized by the absence of exon 1, and composed for exon 2 and 3 with transcriptional initiation in intron 1 (4,701 bp). It is predicted this variant could be regulated by transcription factors, such as some myogenic factors (MRF, Myf5, MyoD, Myogenin, and MRF4), Pax3, NF1, AP1, among others (12), suggesting that it could be involved in muscle development and/or repair as reported in other work (2). Interestingly, the promoter of GDF11 could also be activated by trichostatin A (13), an inhibitor of histone deacetylases (HDAC), suggesting a clear epigenetic regulation of the GDF11 gene expression; HDAC3 regulates zebrafish liver development by modulating GDF11. The overexpression of HDAC3 increases liver size, while the increase of GDF11 expression induces a small size liver; interestingly, the knockdown of GDF11 did not induce any relevant change in liver morphology. The role of HDAC3 in GDF11 function in liver development is likely a direct control over the hepatocyte precursor (hepatoblast) proliferation, as observed in HCC-derived cells (14), but this must be deeply addressed.

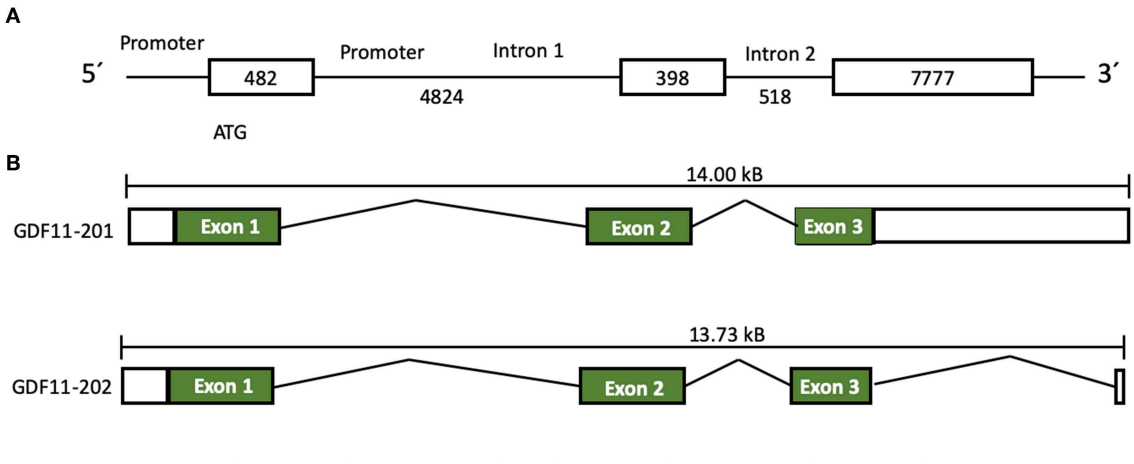
GDF11 mRNA is translated in a precursor protein (**Figure 2**), which is processed by specific proteases generating the mature GDF11 (C-terminal, 12.5 kDa) and the pro-domain (N-terminal, 30.1 kDa). GDF11 shares 89% amino acid sequence homology with GDF8, however GDF8 expression in human tissues is restricted to cardiac and skeletal muscle (1), while GDF11 is practically expressed in all tissues (15). Although there is high homology between mature GDF8 and GDF11, the pro-domains of both proteins share only 54% homology. The pro-domain is fundamental for proper protein folding, disulfide bond formation and exportation of the homodimers (16), suggesting differences in post-translational process.

The protein convertase subtilisin/kexin 5 (PCSK5) is one of the main acting proteins on GDF11, activating the mature GDF11 by proteolytic process at basic sites of the pro-domain (17). The elimination of PCSK5 in the mouse embryo was associated with abnormal expression of Hlxb9 and Hox genes, two well-known GDF11 target genes, generating defects in the anteroposterior patterning and strongly proposing a relationship with GDF11 functions (7, 8).

In humans, GDF11 is expressed in practically all tissues, but is particularly relevant in the brain (hippocampus), the kidneys, the endometrium, and the heart muscle; while the liver is the organ with the lowest expression (1, 15, 18).

THE SIGNAL TRANSDUCTION

As a member of the BMP family, GDF11 uses the canonical receptors and the SMAD proteins for signaling. The GDF11 dimer (a disulfide-linked homodimer of carboxy-terminal fragments) binds the activin receptors type II A or B (ActRIIA, ActRIIB), proteins with serine/threonine kinase activity; leading to the recruitment and transphosphorylation of two type I serine/threonine kinase receptors, also known as activin-like kinase receptors (ALK), particularly the 4, 5, or 7 (19, 20). The activated ALK receptor phosphorylates and activates the receptor-regulated SMAD (R-SAMD). GDF11 particularly transduces by using SMAD2 and 3 (14, 21), and some reports also propose the participation of SMAD1, 5 and 8 (22). The R-SMAD dimer recruits the co-SMAD, SMAD4, to form a trimeric complex, which eventually translocates to the nucleus for gene expression regulation (23). Although the signal transduction of the TGF-β family might seem simple, it is highly regulated by extracellular and intracellular mechanisms. Inside the cell, the regulation can occur at the membrane or in the cytosol, during nuclear translocation and DNA biding, at this level, is a



Name Splice Variant	Ensambl trasncript number	bp	Protein	Ensambl protein number	Biotype
GDF11-201	ENST00000257868.9	8657	407aa 45.1 KDa	ENSP00000257868.5	Protein coding
GDF11-202	ENST00000546799.1	1258	380aa 42.2 KDa	ENSP00000448390.1	Protein coding

FIGURE 1 | Schematic representation of GDF11 gene and mRNA. **(A)** Gdf11 gene and **(B)** Gdf11 transcripts and table with the two transcripts specifications according to Ensembl (www.ensembl.org, ENSG00000135414.9) and Jeanplog 2014.

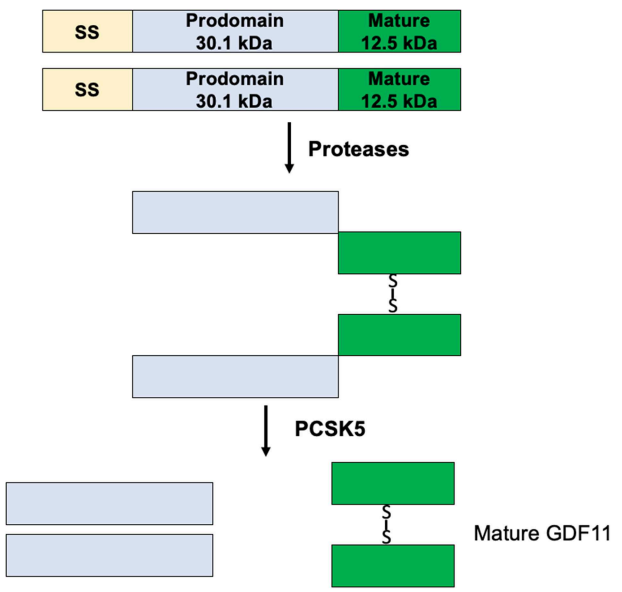


FIGURE 2 | Maturation process of GDF11.

tetrameric complex because the interaction with a fourth protein component or partner (24) (**Figure 3**).

GDF11 can also transduce by non-canonical pathways. Mitogen activated protein kinase (MAPK) is perhaps the main non-SMAD pathway controlled by the growth factor, activating routes such as p38, AKT, and JNK (25, 26), however, in some cases, inhibiting the activation of JNK or NF-κB (27) depending of the cell lineage. Further, it has been described that the family can also transduce by MAPKKK7 [also known as TGF-β activated kinase 1 (TAK1)] via MEK6 (28–30). TAK1 is part of a signaling complex formed by TAK1 binding protein 1 (TAB1) and with either TAB2 or TAB3 (31). TAK1 complex follows an intricate mechanism of activation involving the tumor necrosis factor receptor-associated factor (TRAF) 2 or 6, adaptor proteins with non-conventional activity of E3 ubiquitin ligase. TRAF proteins exert regulation over TAB2 or 3. Finally, the autophosphorylation of TAK1 leads to the activation of its downstream targets, particularly members of the MAPK and NF-κB signaling pathways (32).

Negative regulation of the GDF11-mediated signaling can also occur at different levels. Extracellularly, GDF11 can be negatively regulated by the interaction with many proteins such as follistatin (33, 34), GDF-associated serum protein-1 (GASP-1), GASP-2 (35), decorin and follistatin-like 3, among others (4). Follistatin, a secreted glycoprotein, binds GDF11 and inhibits its interaction with ActRIIB. Follistatin is the main extracellular inhibitor of GDF11, and is transcriptionally regulated by the same GDF11 signaling, indicating that the signal transduction is restricted by a negative feedback mechanism (36).

The BMP and activin membrane-bound inhibitor (BAMBI), a co-receptor that is not functional due to it lacks cytosolic domain, has been suggested to be another negative regulator in plasma membrane, but that still remains to be confirmed (19).

In the cytosol, GDF11 follows the canonical negative regulation of the family. It has been reported that GDF11 is regulated by SMAD7 (37) and SMAD6 (19). The SMAD specific E3 ubiquitin protein ligase 2 (SMURF2) also displays negative regulation of the signaling pathway (28). Negative-regulation

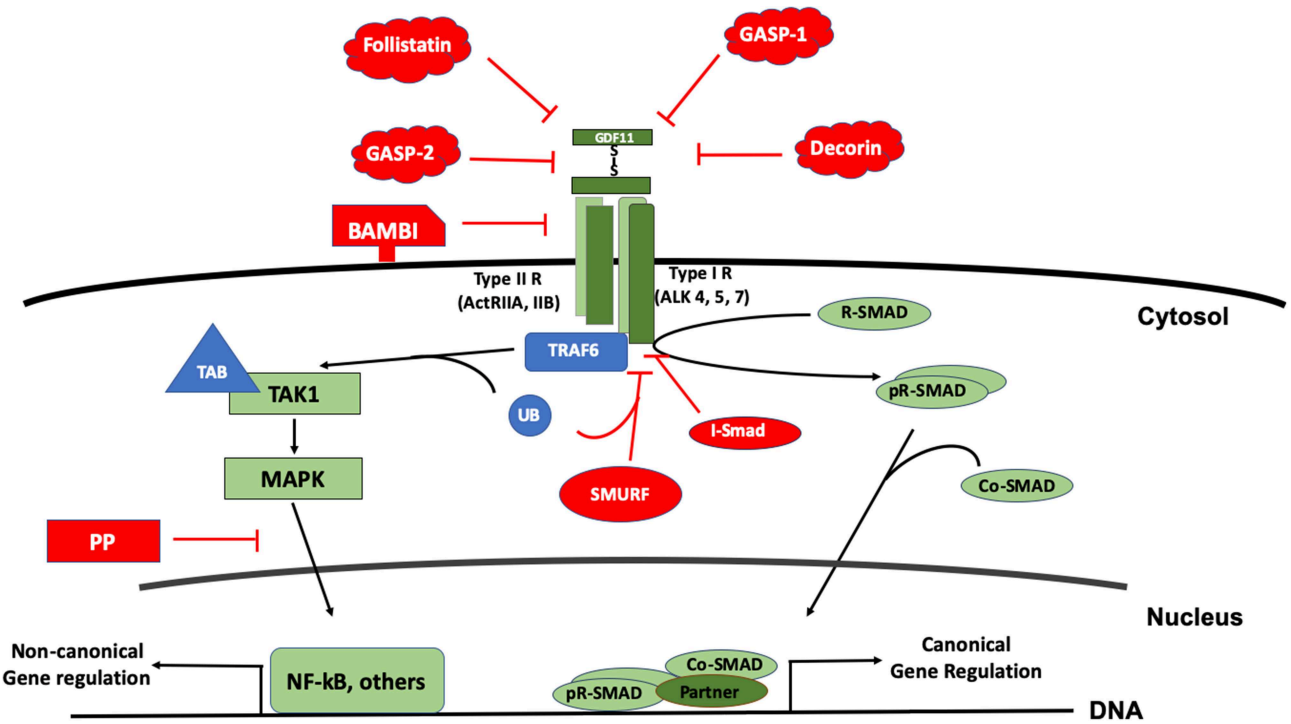


FIGURE 3 | The signal transduction of GDF11. The figure displays the canonical signal transduction mediated by R-SMAD (SMAD 2/3, SMAD 1,5,8), assisted by the Co-SMAD (SMAD4). The signal could inhibited by inhibitory SMAD (I-SMAD) 6 or 7 or by the SMAD specific E3 ubiquitin protein ligase (SMURF). Extracellularly, GDF11 could be inhibited by the BMP and activin membrane-bound inhibitor (BAMBI), or the action of proteins such as Follistatin, Decorin, and GDF-associated serum protein-1 (GASP-1) and GASP-2. The non-canonical pathway is driven particularly by the Mitogen Activated Proteins Kinases (MAPK), signaling continues to the tumor necrosis factor receptor-associated factor (TRAF) 2 or 6; and TGF-β activated kinase 1 (TAK1), which in addition uses the TAK1 binding protein (TAB) 1 and one of both 2 or 3. Non-canonical regulation could influence the nuclear factor kappa B (NF-kB) among others, and the inhibition of this pathway could be blocked by protein phosphatases (PP).

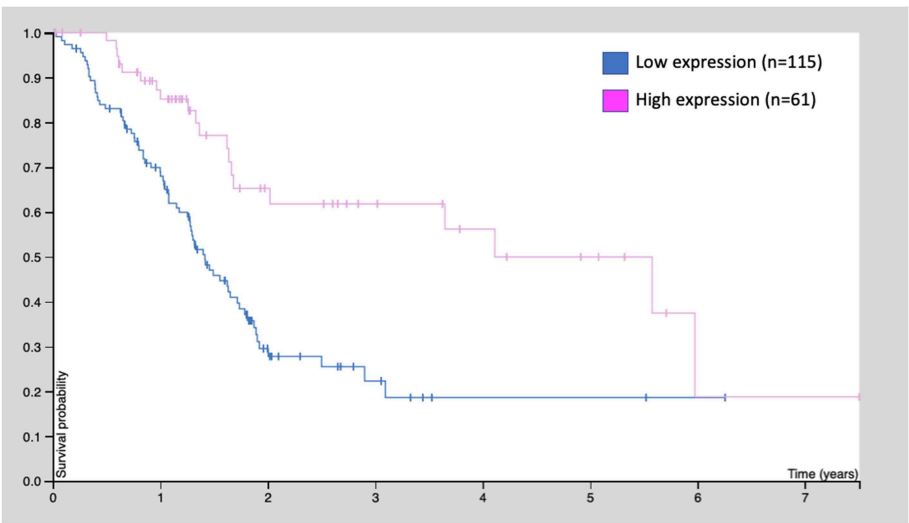


FIGURE 4 | Kaplan-Meier survival curve of 176 patients with pancreatic cancer. Patients were classified as low GDF11 expression and high GDF11 expression, $p < 0.001$, according to the human protein atlas (www.proteinatlas.org/ENSG00000135414-GDF11/pathology).

of the non-canonical pathway is driven by specific protein phosphatases (PP), such as PPC1, among others.

DEVELOPMENT AND AGED-RELATED FUNCTION

Although GDF11 was identified in 1999 (9), as previously mentioned, in 2014 the growth factor was transiently located in the "*Sancta sanctorum*" of the "miraculous" molecule, when the laboratory of Amy Wagers (2) reported that GDF11 was responsible for the skeletal muscle regeneration in mice heterochronic parabiosis. A profound controversy arose regarding the rejuvenating property of GDF11; some groups stated that this property is displayed by the growth factor (1–3, 38), while others reported the opposite effect (39–42), as previously mentioned. To have a good point of view regarding this debate, we suggest a deep view of cited works and commentaries regarding the controversy (3, 5, 6, 19, 42, 43).

Regardless of this disagreement, there is no doubt about the GDF11 function in differentiation and embryonic development, particularly in anterior/posterior axial skeleton (9) and brain function (44), which are nicely reviewed elsewhere (19, 45, 46).

GDF11 VS. GDF8 AND THE RACE FOR THE DISCOVERY OF THE REJUVENATION PROPERTIES

GDF11 and GDF8 are close related members of the activins subclass in the TGF-β superfamily. Sharing 90% of their amino acid sequence (38, 47), these two proteins have been a technical challenge for antibody manufacturers and, therefore, protagonists of one of the most controversial studies in recent years (29, 39, 48–50), regarding to the issue of GDF11 being the protein responsible for "rejuvenation" of aged organisms (1, 2), as previously mentioned.

The race from the discovery of the rejuvenation properties of GDF11 to the following debate of the antibody specificity led to a deeper structural analysis of these proteins and the interaction with their receptor. Due to the similarities of ∼90% of sequence identity of the C-terminal signaling domain between GDF11 and GDF8, their mature form is nearly identical, which causes these proteins to share the same activin type II receptor (38).

Although they are indeed similar in their monomeric form, in fact these proteins are thought to have opposite functions, where GDF11 works as a muscle generator in embryogenesis (9) but GDF8 acts as a muscle mass inhibitor (10, 51), which may be the result of the final homodimer structure. Thus, it is important to understand that the GDF11 and GDF8 homodimer formation leads to a different conformation that allows them to interact with the same receptor in a unique and specific way. It is reported that both homodimers are linked by a single disulfide bond in an antiparallel conformation, but the flexibility in the relative orientations generated by the differences in their structure are determinant for the quaternary structure variations that lead to a distinctive biological response (47, 52).

It has also been reported that GDF11 has a stronger affinity for the receptor than GDF8 (38) and that it is more dependent on direct receptor contacts (53), but there is also an issue with crystal structures of both proteins. Human myostatin alone has not been reported and the available structures are bound to extracellular antagonists (follistatin and follistatin-like 3) (54, 55), which have been compared to a small-angle X-ray scattering (SAXS) analysis to determine the mechanism of activation (52). On the other hand, human GDF11 structure has been resolved in recent years (47), thus, it is possible to discover the real impact of the structure of both proteins in future, at which point we can begin to uncover exactly what makes the responses so different.

GDF11 EFFECTS IN CANCER BIOLOGY

An emerging field of research is the impact of GDF11 in cancer biology. Most of the cancer cells, particularly those with high aggressiveness, retain or recover stemness capacity, placing them as a potential target of GDF11 (14, 23).

There exist some controversies in cancer biology as well; in some cases GDF11 induces clear tumor suppressive properties (14, 23), and in others it is the opposite (56, 57). Once again, the versatility displayed by this growth factor depends of cell progeny, grade of differentiation or transformation.

LIVER CANCER

We recently published work describing how GDF11 induces tumor suppressive properties in human hepatocellular carcinoma-derived cells, Huh7 and Hep3B cell lines, restricting spheroid formation and clonogenic capacity, an effect that is also observed in other liver cancer cell lines (SNU-182, Hepa1-6, and HepG2), decreasing proliferation, motogenesis, and invasion. These characteristics were associated with transcriptional repression of cyclin D1 and A, and the overexpression of p27 (14). GDF11 effects, on hepatic cell proliferation, have been found in liver development, where GDF11 targets the hepatoblast, the hepatocyte precursor (13, 58).

Remarkably, the invasion experiments using the chick embryo chorioallantoic membrane (CAM) model (14, 59) revealed a static phenotype in Huh7 cells treated for 72 h with GDF11 (50 ng/ml), an outcome well-correlated with a decrease in cell migration and proliferation. Furthermore, GDF11 treated cells were incapable of sustaining colony and sphere capacity in the absence of GDF11, up to 5 days, indicating that the effect of GDF11 on self-renewal capacity is not transient, suggesting a reprogramming effect.

Similar results were obtained in the hepatoblastoma cell lines, HepG2 and SMMC-7721: the treatment with GDF11 up to 72 h reduced cell viability. Although SMMC-7721 cells are probably a HELA-derivative cell line, the effect was also present (60). This report also provides preliminary evidence that the expression of GDF11 was significantly lower in cancerous tissue rather than in normal liver.

Outstandingly, GDF11 was capable of decreasing aggressiveness-associated markers in Huh7 and Hep3B cells,

producing a deregulation in the expression of *Epcam, promo1* (CD133), *cd24, and ck19*, that was associated with the repression of Snail and N-cadherin, and the overexpression of occluding and E-cadherin, strongly indicating a mesenchymal to epithelial transition (14).

It is interesting that, under normal conditions, liver cells, which are the poorest in GDF11 production, are highly responsive to GDF11 in the context of cancer could be relevant in terms of a possible use of GDF11 for treatment. The work by Gerardo-Ramírez clearly showed that all HCC cells used in the study responded to the exogenous GDF11 treatment, decreasing all aggressiveness-associated markers. Interestingly, the effects in HCC cells were differentiated, and it was dependent of the stemness capacity, being more responsive to Hep3B cells, which express fewer stemness markers compared to Huh7 cells. Supporting this statement, in liver development GDF11 has been related to inhibition of liver growth, mainly targeting proliferation of hepatoblast, the cell precursor or mature hepatocytes by a mechanism involving HDAC3, which inhibits the expression of GDF11 as proven by Farooq and collaborators (58). This work clearly demonstrates that GDF11 targets hepatic cells with stemness features, not necessarily those observed in cancer, but in the normal liver, particularly in development.

BREAST CANCER

Similarly, Bajikar et al. (23) identified a tumor-suppressive role of GDF11 in a triple-negative breast cancer (TNBC). These cells, under 3D culture, heterogeneously express GDF11 and very low levels of GDF8, as well as the main canonical receptors, such as ALK4, and ALK5, among other protein machinery required for a proper signal transduction. This clearly indicates that breast epithelial cells express the required components to recognize GDF11 as an autocrine or paracrine stimulus (23). GDF11 also induced a decrease in number and size of the spheroids and generated more-compacted structures by the increment in E-cadherin, as observed in liver cancer cell lines, and GDF11 treatment induces a cell-cell adhesion preventing metastasis phenomena (14, 23).

Authors also found a defective GDF11 maturation and secretion in seven of nine studied TNBC cell lines. The linker was the convertase PCSK5, in which a deficiency was found in the TNBC cells, inducing the extracellular accumulation of the immature proGDF11 and, for instance, loss in the bioactivity of GDF11. This mechanism was also observed in mice; the lack of Pcsk5 in $Apc^{min/+}$ animals (61) increases adenocarcinoma formation in the small intestine, decreasing the survival (23, 62), which demonstrates a clear function in tandem of GDF11 and PCSK5 to induce the tumor suppressive properties. In fact, the restoration of the PCSK5 activity in the TNBC cells suppresses lung metastasis (23).

Another work by Wallner et al. (63) revealed that super-physiological levels of GDF11 (2 μg/ml) could provide advantages in chemotherapy in breast adenocarcinoma, inducing a decrement in the migrative capacity of MCF-7 cells in a scratch assay. Similar findings were observed in the presence of follistatin (2 μg/ml), while GDF8 (2 μg/ml) induced cell death at the same time. This study also showed that GDF11 is expressed in low grade adenocarcinoma tissue (G1), but lower levels in G3 tissue were found, and it was correlated with high expression of follistatin in G1, suggesting an inhibitory effect of GDF11 at higher levels of differentiation, which is consistent with the idea that high aggressiveness in cancer associates with less GDF11 function, confirming the tumor suppressive capacity of GDF11.

PANCREATIC CANCER

Pancreatic cancer (PC) represents one of the most lethal cancers worldwide (64). It has been reported that GDF11 is down-regulated in PC tissue, compared with surrounding tissue, and pancreatic cell lines exhibit a low expression of the growth factor (65). This group also reported that, in a cohort of 63 PC patients, those with high GDF11 expression had significantly better survival rates in comparison with those with low GDF11 expression. These effects were related to decreased proliferation, migration and invasion, and these observations are in agreement with those reported in HCC and TNBC. GDF11 is also capable of inducing apoptosis in PC cell lines (65).

Similarly, the human protein atlas (https://www.proteinatlas.org) provides evidence from 176 patients: those with high GDF11 expression ($n = 61$) exhibited better survival rates, compared with those with low expression ($n = 115$, $p < 0.001$) (**Figure 4**). These observations strongly suggest that GDF11 could also exert tumor suppressive properties that should be deeply addressed to gain confidence, particularly the effect of exogenous active GDF11 (18).

Interestingly, another member of the family GDF15 is directly correlated with poor survival in PC patients, and it is proposed as a better marker than CA-125 (66), again raising the atypical functions of this growth factor.

As observed in HCC, in PC, the targets of GDF11 are poorly differentiated cells. In the mouse embryo, GDF11 is expressed in the pancreatic epithelium, at embryonic day E12-E14 (67), as it happens in the liver, but in $GDF11^{-/-}$ animals the pancreas size are 2-fold smaller than wild type.

In the context of the educated guess that cells with some stemness phenotype respond to GDF11, even in cancer, it has been proven that GDF11 negatively regulates $NGN3^{+}$ progenitor cells and GDF11 induces β-cell differentiation (68), supporting the role of GDF11 in metabolism. Under this context, GDF11 exerts its functions in pancreatic cells with stemness phenotype.

COLORECTAL CANCER

In 130 patients with colorectal cancer (CRC), the expression of GDF11 was significantly higher compared with normal tissue (56). The classification of the patient cohort in low and high GDF11 expression revealed that those patients with high levels of GDF11 showed a higher frequency of lymph node metastasis, more deaths and lower survival. The study suggests that GDF11 could be a prognostic biomarker in patients with this disease.

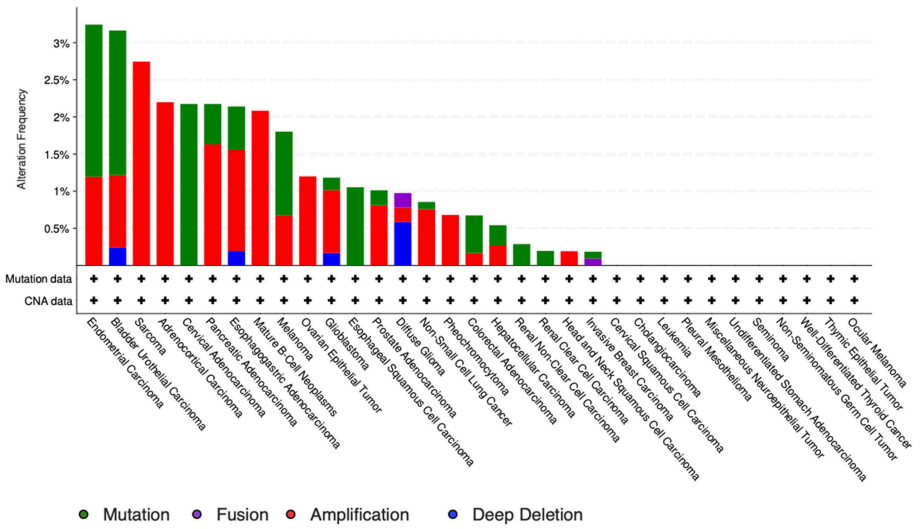


FIGURE 5 | Genomic alterations in Gdf11 gene. Alteration frequency by type of cancer according to cBioportal for cancer genomics (https://www.cbioportal.org).

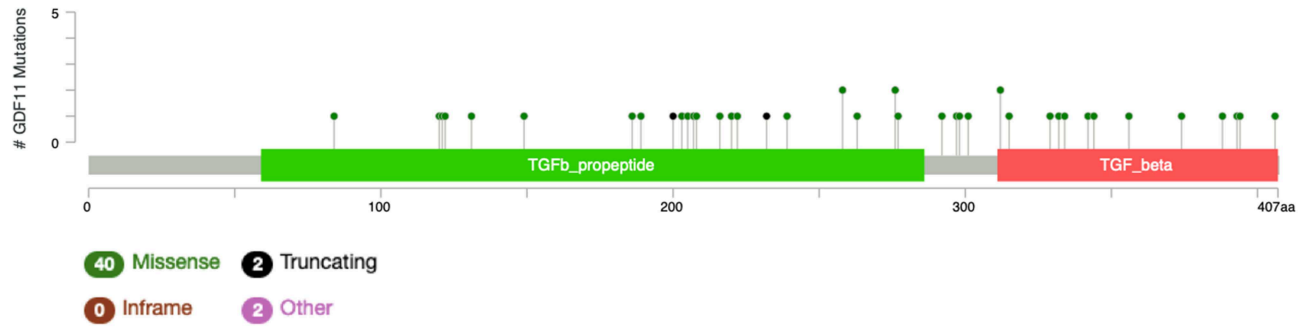


FIGURE 6 | Number of mutations in Gdf11 gene. According to cBioportal for cancer genomics (https://www.cbioportal.org). RefSeq: NM_005811. Ensembl ENST00000257868. CCDS: CCDS8891. UniProt:GDF11_HUMAN. Somatic Mutations Frequency: 0.4%. Forty missense mutations. Two truncating, 0 inframe, 2 other.

It is known that lymphangiogenesis is a fundamental phenomenon for colorectal cancer dissemination (69). Recently, Ungaro and collaborators reported that the microenvironment in the lymphatic vessels provides support to the tumor-derived cells by manipulating the production of extracellular matrix proteins and soluble factors, such as cytokines and growth factors (70). Whole transcriptomic analysis addressed by RNA-seq of isolated human intestinal lymphatic endothelial cells (HILEC) from surgically resected CRC and healthy corresponding controls, revealed that among those genes differentially expressed, GDF11 was observed as a significant increment with high statistical confidence. CACO-2 cells demonstrated high proliferation in co-culture with CRC-HILEC, but the GDF11 silencing by siRNA abrogated this effect indicating a tumor promotion role of GDF11 in CRC. Interestingly, GDF11 was expressed not only in lymphatic vessels in CRC, but also in normal tissue (69). The study also provides evidence of a direct correlation of GDF11 expression and tumor stage, confirming in this particular cancer that GDF11 expression could be a marker of tumor progression (70), and also raises mechanistic evidence that microenvironment in the lymphatic vessel could play a pivotal role in metastasis by local production of GDF11.

OTHER TYPES OF CANCER

Some reports have pointed to the pro-tumorigenic properties of GDF11, with major or minor confidence of rigorous scientific approach.

In oral squamous cell carcinoma, Qin and coauthors (57) showed that in a small patient cohort GDF11 expression is positively correlated with aggressiveness, finding a higher expression in metastatic oral cancer ($n = 19$) in comparison with non-metastatic oral cancer ($n = 15$). Authors also sustain that GDF11 induced epithelial to mesenchymal transition by downregulating epithelial markers such as E-cadherin, and the overexpression of vimentin or metalloproteinase 9.

In uveal melanoma, GDF11 expression was significantly upregulated compared with surrounding tissue, the expression

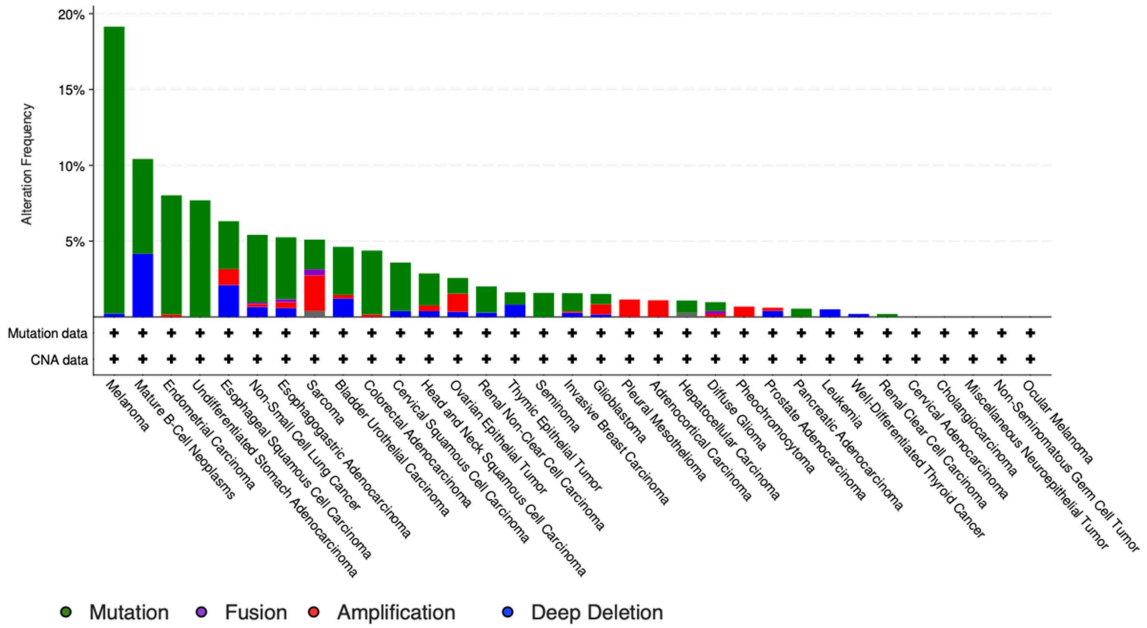


FIGURE 7 | Genomic alterations in Pcsk5 gene. Alteration frequency by type of cancer according to cBioportal for cancer genomics (https://www.cbioportal.org).

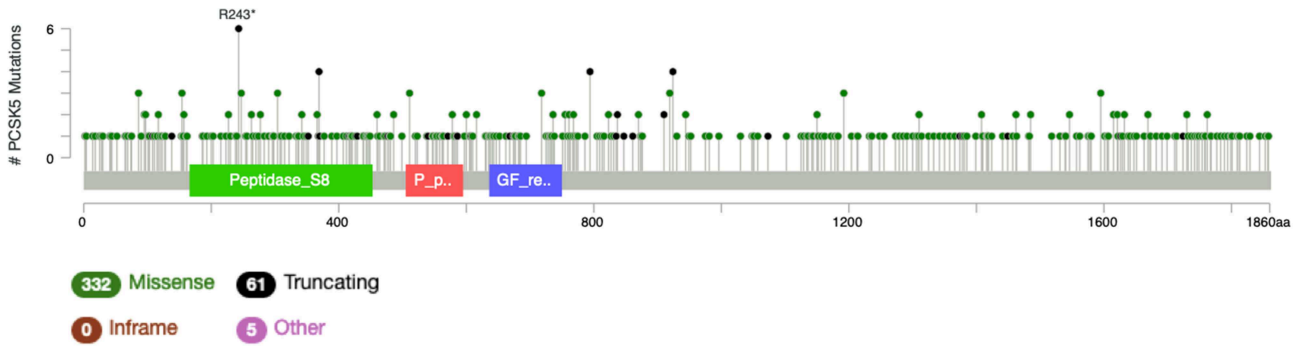


FIGURE 8 | Number of mutations in Pcsk5 gene. According to cBioportal for cancer genomics (https://www.cbioportal.org). RefSeq: NM_001190482. Ensembl ENST00000545128. CCDS: CCDS55320. UniProt: PCSK5_HUMAN. Somatic Mutations Frequency: 2.8%. Three hundred thirty-two missense mutations, 61 truncating, 0 inframe, 5 others.

was higher in stage IV and substantially greater in the deceased cases regarding living cases (71). The multivariate analysis confirmed that GDF11 is an independent prognostic indicator of unfavorable overall survival.

GDF11 AND PCSK MUTATIONS

The study by Liu et al. (71) also showed that no relevant mutations were observed in the GDF11 gene in fact. The cBioportal for cancer genomics web site (https://www.cbioportal.org) indicates that GDF11 is altered in 1% of database patients. **Figure 4** shows the alteration frequency in *Gdf11* gene in some cancers, and **Figure 5** depicts the number of somatic mutations, most of which are missense (72). It seems that mutations in the Gdf11 gene are not the main consequence in those cancers where GDF11 is a prognostic factor, which increases research interest in transcriptional and post-translational regulation.

It is particularly relevant to consider the convertase PCSK5, a key regulator of GDF11 activity. Pcsk5 gene presents a high frequency of genomic alterations in 3% of the patients, according to cBioportal, being particularly relevant in melanoma, endometrial carcinoma, and stomach adenocarcinoma, among others (**Figure 6**). Missense mutations are particularly observed in the peptidase transcript (**Figure 7**) (72). As proven remarkably by the team of doctor Kevin A. Janes (23), maturation of bioactive GDF11 is defective in TNBC due to insufficient PCSK5 activity but, as shown, the frequency of mutations appear not to be related with the flaw (**Figure 8**). Once again, transcriptional and post-translational regulation should be considered in future research.

TABLE 1 | Overview of cancer cell lines or tissue from patients with differential effect of GDF11, as tumor suppressive or tumor promotion protein.

Cancer	Cell/Tissue	Tumor suppressive	Tumor promotion	References
Liver	Huh7 Hep3B SNU-182 Hepa1-6 HepG2	X		(14)
Liver	HepG2 SMMC-7721 and tissue	X		(60)
Breast	MDA-MB-231 MDA-MB-468 and tissue	X		(23)
Breast	MCF-7	X		(63)
Pancreas	PANC-1 CFPAC-1 Tissue	X		(65)
Colorectal	Tissue		X	(56)
Colorectal	Tissue		X	(70)
Colorectal	CACO-2		X	(69)
Oral squamous cell carcinoma	Tissue		X	(57)
Uveal melanoma	Tissue		X	(71)

EFFECTS OF GDF11 AS METABOLISM REGULATOR

The impact of GDF11 in the development of pancreas implies that the growth factor could exert some metabolic regulation on this organ in the adult, particularly in the endocrine pancreas (67). Dichmann and coauthors found that in the gdf11$^{-/-}$ mouse, the maturation and number of β- and α-cells are normal, however, another group led by Harmon reported that the gdf11$^{-/-}$ mouse exhibited impairment maturation of β-cells and an increment in α-cells, which could produce glucagon in comparison with the wild type mouse (68). This controversy, which is not unusual, must be addressed, but makes it clear that GDF11 could be inducing effects in the metabolism mediated by the pancreas.

Recently, a work by Anon-Hidalgo et al. (73) reported a convincing study associating the circulating levels of GDF11 with thyroid-stimulating hormone (TSH) in humans. The study showed subjects with high or normal levels of TSH present high level contents of GDF11, compared with patients with low levels of TSH. This finding could be due to the fact that other members of the family, such as GDF8 and GDF15, are regulators of the energy homeostasis (74, 75). Anon-Hidalgo team states that it could be related to a regulation of TSH by GDF11, or GDF11 could be positively regulated by TSH or any other thyroid hormones (73).

Luo et al. published that GDF11 decreased lipid content in human mesenchymal stem cells and the mouse 3T3-L1 cell line. This was associated with the repression of adipogenic genes, such as the transcription factors *Pparg, Cebpa*, and the executer proteins *Plp, Cd36, Plin1, Adipoq*, among others, in a mechanism associated to the canonical signal transduction mediated by SMAD2/3 (76). The report provides evidence that GDF11 could exert control over lipid content in unclear fashion. The role of GDF11 in lipid homeostasis could be directed to lipid uptake or efflux, intervening in lipogenic or lipolysis pathways, or lipid removal by autophagy, but data provided by Luo et al. suggest an intervention in lipogenesis. Interestingly, obese mice fed with a high lipid diet present significantly decreased circulating GDF11 levels, compared with mice under low fat diet (77). The mRNA and protein content of GDF11 in skeletal muscle from mice under the high fat diet correlated with the serum content of the growth factor, exhibiting lower expression and protein content, compared with animals under low fat diet. Furthermore, palmitate treatment in the mouse-derived myoblast cell line, C2C12, decreases GDF11 expression. However, the GDF11 did not ameliorate the palmitate-induced insulin resistance and GDF11 treatment did not change expression of Glut4 or Irs-1.

The evidence sustains the metabolic intervention by GDF11, at least in terms of lipid homeostasis, and again in cells with stemness features. This could be relevant in cancer, since lipid overload is one of the main characteristics required for a proper cancer cell proliferation (78, 79). In fact, it is reported that GDF11 impairs mitochondrial function in cancer cell lines, particularly in HCC-derived cells (14). The impact of GDF11 in the central metabolic organelle could explain the tumor suppressive properties exerted by the growth factor. Mitochondria provide essential intermediaries required for cell proliferation: driving redox and calcium homeostasis, coordinating energy supply and mediating cell survival; all of which are fundamental for all cells, and particularly for transformed ones (80). A report by Hernandez-Rizo and collaborators states that GDF11 restricts cell proliferation in hepatic tumor cells through glycolysis and lipid metabolism impairment (81). In agreement with these findings, Garrido-Moreno et al. (82) recently reported that GDF11 prevents cardiomyocyte hypertrophy by preserving the communication between the mitochondria and sarcoplasmic reticulum and calcium mobility, preserving oxidative mitochondria metabolism by a mechanism mediated by the maintenance of mitochondrial cytosolic calcium buffering capacity.

Although the evidence of GDF11 regulation of the energetic and lipid metabolism is limited, it clearly indicates an effect tending to maintain the cellular energetic homeostasis. More research is required to characterize the mechanism underlying metabolic regulation by the growth factor, particularly in cancer cells.

CONCLUDING REMARKS AND FUTURE PROSPECTIVE

GDF11 is an intriguing non-conventional growth factor, perhaps the most fascinating new member of the TGF-β superfamily. It transduces, as practically all members, by the canonical

SMAD and non-canonical MAPK pathways, but its functions can be quite variable, even contradictory, depending of the cell lineage, tissue (**Table 1**), or even age. This raises a complex body of physiological control, which could also differ in health or disease. GDF11 displays a versatile response that must be fully characterized, due to it representing an interesting point of intervention in many diseases or physiological conditions, particularly in cancer. It is remarkable that one of the main characteristics in GDF11 target cells, in normal or pathological conditions, is the stemness capacity. The effects exerted by the growth factor in cancer have begun to be characterized with greater scientific rigor and mechanistic approaches.

Perhaps it is time that GDF11, due to its diverse functionality, constitutes its own subfamily as an atypical and versatile member of the TGF-β family.

We must be cautious to oversimplify its functions. The controversies found clearly indicate that GDF11 displays particular activities depending of cell type, grade of differentiation, and pathological or normal conditions. This remarkable atypical member of the TGF-β family must be carefully studied in clear and well-controlled biological systems. The knowledge, regarding GDF11, will surely be increased in the next few years. The mechanism of action in each particular cancer or cell type must be elucidated to clarify these controversies, and perhaps they will stop being such, thanks to the mechanistic enlightenments obtained in the incoming research in the field.

AUTHOR CONTRIBUTIONS

AS-N, MG-R, GP-V, and LC-R conception and preparation of the manuscript. LB, VS, and RM-L reviewed and corrected the manuscript. MCG-R and LG-Q final review of the manuscript and financial support.

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REFERENCES

1. Loffredo FS, Steinhauser ML, Jay SM, Gannon J, Pancoast JR, Yalamanchi P, et al. Growth differentiation factor 11 is a circulating factor that reverses age-related cardiac hypertrophy. *Cell*. (2013) 153:828–39. doi: 10.1016/j.cell.2013.04.015
2. Sinha M, Jang YC, Oh J, Khong D, Wu EY, Manohar R, et al. Restoring systemic GDF11 levels reverses age-related dysfunction in mouse skeletal muscle. *Science*. (2014) 344:649–52. doi: 10.1126/science.1251152
3. Katsimpardi L, Litterman NK, Schein PA, Miller CM, Loffredo FS, Wojtkiewicz GR, et al. Vascular and neurogenic rejuvenation of the aging mouse brain by young systemic factors. *Science*. (2014) 344:630–4. doi: 10.1126/science.1251141
4. Walker RG, Poggioli T, Katsimpardi L, Buchanan SM, Oh J, Wattrus S, et al. Biochemistry and biology of GDF11 and myostatin: similarities, differences, and questions for future investigation. *Circ Res*. (2016) 118:1125–41; discussion 1142. doi: 10.1161/CIRCRESAHA.116.308391
5. Kaiser J. Aging. 'Rejuvenation factor' in blood turns back the clock in old mice. *Science*. (2014) 344:570–1. doi: 10.1126/science.344.6184.570
6. Brun CE, Rudnicki MA. GDF11 and the mythical fountain of youth. *Cell Metab*. (2015) 22:54–6. doi: 10.1016/j.cmet.2015.05.009
7. Lee YJ, McPherron A, Choe S, Sakai Y, Chandraratna RA, Lee SJ, et al. Growth differentiation factor 11 signaling controls retinoic acid activity for axial vertebral development. *Dev Biol*. (2010) 347:195–203. doi: 10.1016/j.ydbio.2010.08.022
8. Tsuda T, Iwai N, Deguchi E, Kimura O, Ono S, Furukawa T, et al. PCSK5 and GDF11 expression in the hindgut region of mouse embryos with anorectal malformations. *Eur J Pediatr Surg*. (2011) 21:238–41. doi: 10.1055/s-0031-1273691
9. McPherron AC, Lawler AM, Lee SJ. Regulation of anterior/posterior patterning of the axial skeleton by growth/differentiation factor 11. *Nat Genet*. (1999) 22:260–4. doi: 10.1038/10320
10. McPherron AC, Lawler AM, Lee SJ. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature*. (1997) 387:83–90. doi: 10.1038/387083a0
11. Hunt SE, McLaren W, Gil L, Thormann A, Schuilenburg H, Sheppard D, et al. Ensembl variation resources. *Database*. (2018) 2018:bay119. doi: 10.1093/database/bay119
12. Jeanplong F, Falconer SJ, Oldham JM, Maqbool NJ, Thomas M, Hennebry A, et al. Identification and expression of a novel transcript of the growth and differentiation factor-11 gene. *Mol Cell Biochem*. (2014) 390:9–18. doi: 10.1007/s11010-013-1949-3
13. Zhang X, Wharton W, Yuan Z, Tsai SC, Olashaw N, Seto E. Activation of the growth-differentiation factor 11 gene by the histone deacetylase (HDAC) inhibitor trichostatin A and repression by HDAC3. *Mol Cell Biol*. (2004) 24:5106–18. doi: 10.1128/MCB.24.12.5106-5118.2004
14. Gerardo-Ramirez M, Lazzarini-Lechuga R, Hernandez-Rizo S, Jimenez-Salazar JE, Simoni-Nieves A, Garcia-Ruiz C, et al. GDF11 exhibits tumor suppressive properties in hepatocellular carcinoma cells by restricting clonal expansion and invasion. *Biochim Biophys Acta Mol Basis Dis*. (2019) 1865:1540–54. doi: 10.1016/j.bbadis.2019.03.003
15. Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Proteomics. Tissue-based map of the human proteome. *Science*. (2015) 347:1260419. doi: 10.1126/science.1260419
16. Gray AM, Mason AJ. Requirement for activin A and transforming growth factor–beta 1 pro-regions in homodimer assembly. *Science*. (1990) 247:1328–30. doi: 10.1126/science.2315700
17. Essalmani R, Zaid A, Marcinkiewicz J, Chamberland A, Pasquato A, Seidah NG, et al. *In vivo* functions of the proprotein convertase PC5/6 during mouse development: Gdf11 is a likely substrate. *Proc Natl Acad Sci USA*. (2008) 105:5750–5. doi: 10.1073/pnas.0709428105
18. Uhlen M, Zhang C, Lee S, Sjostedt E, Fagerberg L, Bidkhori G, et al. A pathology atlas of the human cancer transcriptome. *Science*. (2017) 357:eaan2507. doi: 10.1126/science.aan2507
19. Jamaiyar A, Wan W, Janota DM, Enrick MK, Chilian WM, Yin L. The versatility and paradox of GDF 11. *Pharmacol Ther*. (2017) 175:28–34. doi: 10.1016/j.pharmthera.2017.02.032
20. Andersson O, Reissmann E, Ibanez CF. Growth differentiation factor 11 signals through the transforming growth factor-beta receptor ALK5 to regionalize the anterior-posterior axis. *EMBO Rep*. (2006) 7:831–7. doi: 10.1038/sj.embor.7400752
21. Gaunt SJ, George M, Paul YL. Direct activation of a mouse Hoxd11 axial expression enhancer by Gdf11/Smad signalling. *Dev Biol*. (2013) 383:52–60. doi: 10.1016/j.ydbio.2013.08.025
22. Zhang YH, Cheng F, Du XT, Gao JL, Xiao XL, Li N, et al. GDF11/BMP11 activates both smad1/5/8 and smad2/3 signals but shows no significant effect on proliferation and migration of human umbilical vein endothelial cells. *Oncotarget*. (2016) 7:12063–74. doi: 10.18632/oncotarget.10664

23. Bajikar SS, Wang CC, Borten MA, Pereira EJ, Atkins KA, Janes KA. Tumor-suppressor inactivation of GDF11 occurs by precursor sequestration in triple-negative breast cancer. *Dev Cell*. (2017) 43:418–35 e13. doi: 10.1016/j.devcel.2017.10.027
24. Hata A, Chen YG. TGF-beta signaling from receptors to smads. *Cold Spring Harb Perspect Biol*. (2016) 8:a022061. doi: 10.1101/cshperspect.a022061
25. Wang Z, Dou M, Liu F, Jiang P, Ye S, Ma L, et al. GDF11 induces differentiation and apoptosis and inhibits migration of C17.2 neural stem cells via modulating MAPK signaling pathway. *PeerJ*. (2018) 6:e5524. doi: 10.7717/peerj.5524
26. Demontis F, Patel VK, Swindell WR, Perrimon N. Intertissue control of the nucleolus via a myokine-dependent longevity pathway. *Cell Rep*. (2014) 7:1481–94. doi: 10.1016/j.celrep.2014.05.001
27. Mei W, Xiang G, Li Y, Li H, Xiang L, Lu J, et al. GDF11 protects against endothelial injury and reduces atherosclerotic lesion formation in apolipoprotein e-null mice. *Mol Ther*. (2016) 24:1926–38. doi: 10.1038/mt.2016.160
28. van Wijk B, Moorman AF, van den Hoff MJ. Role of bone morphogenetic proteins in cardiac differentiation. *Cardiovasc Res*. (2007) 74:244–55. doi: 10.1016/j.cardiores.2006.11.022
29. Hammers DW, Merscham-Banda M, Hsiao JY, Engst S, Hartman JJ, Sweeney HL. Supraphysiological levels of GDF11 induce striated muscle atrophy. *EMBO Mol Med*. (2017) 9:531–44. doi: 10.15252/emmm.201607231
30. Hindi SM, Sato S, Xiong G, Bohnert KR, Gibb AA, Gallot YS, et al. TAK1 regulates skeletal muscle mass and mitochondrial function. *JCI Insight*. (2018) 3:98441. doi: 10.1172/jci.insight.98441
31. Mihaly SR, Ninomiya-Tsuji J, Morioka S. TAK1 control of cell death. *Cell Death Differ*. (2014) 21:1667–76. doi: 10.1038/cdd.2014.123
32. Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature*. (2001) 412:346–51. doi: 10.1038/35085597
33. Fife E, Kostka J, Kroc L, Guligowska A, Piglowska M, Soltysik B, et al. Relationship of muscle function to circulating myostatin, follistatin and GDF11 in older women and men. *BMC Geriatr*. (2018) 18:200. doi: 10.1186/s12877-018-0888-y
34. Schneyer AL, Sidis Y, Gulati A, Sun JL, Keutmann H, Krasney PA. Differential antagonism of activin, myostatin and growth and differentiation factor 11 by wild-type and mutant follistatin. *Endocrinology*. (2008) 149:4589–95. doi: 10.1210/en.2008-0259
35. Lee YS, Lee SJ. Regulation of GDF-11 and myostatin activity by GASP-1 and GASP-2. *Proc Natl Acad Sci USA*. (2013) 110:E3713–22. doi: 10.1073/pnas.1309907110
36. Gamer LW, Cox KA, Small C, Rosen V. Gdf11 is a negative regulator of chondrogenesis and myogenesis in the developing chick limb. *Dev Biol*. (2001) 229:407–20. doi: 10.1006/dbio.2000.9981
37. Smart NG, Apelqvist AA, Gu X, Harmon EB, Topper JN, MacDonald RJ, et al. Conditional expression of Smad7 in pancreatic beta cells disrupts TGF-beta signaling and induces reversible diabetes mellitus. *PLoS Biol*. (2006) 4:e39. doi: 10.1371/journal.pbio.0040039
38. Walker RG, Czepnik M, Goebel EJ, McCoy JC, Vujic A, Cho M, et al. Structural basis for potency differences between GDF8 and GDF11. *BMC Biol*. (2017) 15:19. doi: 10.1186/s12915-017-0350-1
39. Egerman MA, Cadena SM, Gilbert JA, Meyer A, Nelson HN, Swalley SE, et al. GDF11 increases with age and inhibits skeletal muscle regeneration. *Cell Metab*. (2015) 22:164–74. doi: 10.1016/j.cmet.2015.05.010
40. Glass DJ. Elevated GDF11 is a risk factor for age-related frailty and disease in humans. *Cell Metab*. (2016) 24:7–8. doi: 10.1016/j.cmet.2016.06.017
41. Zhao L, Zhang S, Cui J, Huang W, Wang J, Su F, et al. TERT assists GDF11 to rejuvenate senescent VEGFR2(+)/CD133(+) cells in elderly patients with myocardial infarction. *Lab Invest*. (2019). doi: 10.1038/s41374-019-0290-1. [Epub ahead of print].
42. Egerman MA, Glass DJ. The role of GDF11 in aging and skeletal muscle, cardiac and bone homeostasis. *Crit Rev Biochem Mol Biol*. (2019) 54:174–83. doi: 10.1080/10409238.2019.1610722
43. Fan X, Gaur U, Sun L, Yang D, Yang M. The growth differentiation factor 11 (GDF11) and myostatin (MSTN) in tissue specific aging. *Mech Ageing Dev*. (2017) 164:108–12. doi: 10.1016/j.mad.2017.04.009
44. Gokoffski KK, Wu HH, Beites CL, Kim J, Kim EJ, Matzuk MM, et al. Activin and GDF11 collaborate in feedback control of neuroepithelial stem cell proliferation and fate. *Development*. (2011) 138:4131–42. doi: 10.1242/dev.065870
45. Zhang Y, Wei Y, Liu D, Liu F, Li X, Pan L, et al. Role of growth differentiation factor 11 in development, physiology and disease. *Oncotarget*. (2017) 8:81604–16. doi: 10.18632/oncotarget.20258
46. Gad JM, Tam PP. Axis development: the mouse becomes a dachshund. *Curr Biol*. (1999) 9:R783–6. doi: 10.1016/S0960-9822(00)80013-5
47. Padyana AK, Vaidialingam B, Hayes DB, Gupta P, Franti M, Farrow NA. Crystal structure of human GDF11. *Acta Crystallogr F Struct Biol Commun*. (2016) 72:160–4. doi: 10.1107/S2053230X16001588
48. Jones JE, Cadena SM, Gong C, Wang X, Chen Z, Wang SX, et al. Supraphysiologic administration of GDF11 induces cachexia in part by upregulating GDF15. *Cell Rep*. (2018) 22:1522–30. doi: 10.1016/j.celrep.2018.01.044
49. Zimmers TA, Jiang Y, Wang M, Liang TW, Rupert JE, Au ED, et al. Exogenous GDF11 induces cardiac and skeletal muscle dysfunction and wasting. *Basic Res Cardiol*. (2017) 112:48. doi: 10.1007/s00395-017-0642-1
50. Hinken AC, Powers JM, Luo G, Holt JA, Billin AN, Russell AJ. Lack of evidence for GDF11 as a rejuvenator of aged skeletal muscle satellite cells. *Aging Cell*. (2016) 15:582–4. doi: 10.1111/acel.12475
51. McPherron AC, Lee SJ. Double muscling in cattle due to mutations in the myostatin gene. *Proc Natl Acad Sci USA*. (1997) 94:12457–61. doi: 10.1073/pnas.94.23.12457
52. Walker RG, McCoy JC, Czepnik M, Mills MJ, Hagg A, Walton KL, et al. Molecular characterization of latent GDF8 reveals mechanisms of activation. *Proc Natl Acad Sci USA*. (2018) 115:E866–75. doi: 10.1073/pnas.1714622115
53. Goebel EJ, Corpina RA, Hinck CS, Czepnik M, Castonguay R, Grenha R, et al. Structural characterization of an activin class ternary receptor complex reveals a third paradigm for receptor specificity. *Proc Natl Acad Sci USA*. (2019) 116:15505–13. doi: 10.1073/pnas.1906253116
54. Cash JN, Angerman EB, Kattamuri C, Nolan K, Zhao H, Sidis Y, et al. Structure of myostatin.follistatin-like 3: N-terminal domains of follistatin-type molecules exhibit alternate modes of binding. *J Biol Chem*. (2012) 287:1043–53. doi: 10.1074/jbc.M111.270801
55. Cash JN, Rejon CA, McPherron AC, Bernard DJ, Thompson TB. The structure of myostatin:follistatin 288: insights into receptor utilization and heparin binding. *EMBO J*. (2009) 28:2662–76. doi: 10.1038/emboj.2009.205
56. Yokoe T, Ohmachi T, Inoue H, Mimori K, Tanaka F, Kusunoki M, et al. Clinical significance of growth differentiation factor 11 in colorectal cancer. *Int J Oncol*. (2007) 31:1097–101. doi: 10.3892/ijo.31.5.1097
57. Qin X, Kuang H, Chen L, Wei S, Yu D, Liang F. Coexpression of growth differentiation factor 11 and reactive oxygen species in metastatic oral cancer and its role in inducing the epithelial to mesenchymal transition. *Oral Surg Oral Med Oral Pathol Oral Radiol*. (2017) 123:697–706. doi: 10.1016/j.oooo.2017.03.010
58. Farooq M, Sulochana KN, Pan X, To J, Sheng D, Gong Z, et al. Histone deacetylase 3 (hdac3) is specifically required for liver development in zebrafish. *Dev Biol*. (2008) 317:336–53. doi: 10.1016/j.ydbio.2008.02.034
59. Lazzarini-Lechuga R, Alcantar-Ramirez O, Jaime-Cruz R, Gómez-Quiroz L. Efecto teratogénico de nanopartículas de oro de 20 nm durante la septación cardiaca. *Mundo Nano*. (2014) 7:69–77. doi: 10.22201/ceiich.24485691e.2014.13.48710
60. Zhang YH, Pan LH, Pang Y, Yang JX, Lv MJ, Liu F, et al. GDF11/BMP11 as a novel tumor marker for liver cancer. *Exp Ther Med*. (2018) 15:3495–500. doi: 10.3892/etm.2018.5861
61. Su LK, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, et al. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science*. (1992) 256:668–70. doi: 10.1126/science.1350108
62. Sun X, Essalmani R, Seidah NG, Prat A. The proprotein convertase PC5/6 is protective against intestinal tumorigenesis: *in vivo* mouse model. *Mol Cancer*. (2009) 8:73. doi: 10.1186/1476-4598-8-73
63. Wallner C, Drysch M, Becerikli M, Jaurich H, Wagner JM, Dittfeld S, et al. Interaction with the GDF8/11 pathway reveals treatment options for adenocarcinoma of the breast. *Breast*. (2018) 37:134–41. doi: 10.1016/j.breast.2017.11.010

64. Jemal A, Ward EM, Johnson CJ, Cronin KA, Ma J, Ryerson B, et al. Annual report to the nation on the status of cancer, 1975-2014, featuring survival. *J Natl Cancer Inst.* (2017) 109:djx030. doi: 10.1093/jnci/djx030
65. Liu Y, Shao L, Chen K, Wang Z, Wang J, Jing W, et al. GDF11 restrains tumor growth by promoting apoptosis in pancreatic cancer. *Onco Targets Ther*. (2018) 11:8371–9. doi: 10.2147/OTT.S181792
66. Hogendorf P, Durczynski A, Skulimowski A, Kumor A, Poznanska G, Strzelczyk J. Growth differentiation factor (GDF-15) concentration combined with Ca125 levels in serum is superior to commonly used cancer biomarkers in differentiation of pancreatic mass. *Cancer Biomark*. (2018) 21:505–11. doi: 10.3233/CBM-170203
67. Dichmann DS, Yassin H, Serup P. Analysis of pancreatic endocrine development in GDF11-deficient mice. *Dev Dyn*. (2006) 235:3016–25. doi: 10.1002/dvdy.20953
68. Harmon EB, Apelqvist AA, Smart NG, Gu X, Osborne DH, Kim SK. GDF11 modulates NGN3+ islet progenitor cell number and promotes beta-cell differentiation in pancreas development. *Development*. (2004) 131:6163–74. doi: 10.1242/dev.01535
69. Barresi V, Reggiani-Bonetti L, Di Gregorio C, De Leon MP, Barresi G. Lymphatic vessel density and its prognostic value in stage I colorectal carcinoma. *J Clin Pathol.* (2011) 64:6–12. doi: 10.1136/jcp.2010.083550
70. Ungaro F, Colombo P, Massimino L, Ugolini GS, Correale C, Rasponi M, et al. Lymphatic endothelium contributes to colorectal cancer growth via the soluble matrisome component GDF11. *Int J Cancer.* (2019) 145:1913–20. doi: 10.1002/ijc.32286
71. Liu X, Zhang Q, Fan C, Tian J, Liu X, Li G. GDF11 upregulation independently predicts shorter overall-survival of uveal melanoma. *PLoS ONE.* (2019) 14:e0214073. doi: 10.1371/journal.pone.0214073
72. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal.* (2013) 6:pl1. doi: 10.1126/scisignal.2004088
73. Anon-Hidalgo J, Catalan V, Rodriguez A, Ramirez B, Idoate-Bayon A, Silva C, et al. Circulating concentrations of GDF11 are positively associated with TSH levels in humans. *J Clin Med.* (2019) 8:E878. doi: 10.3390/jcm8060878
74. Zamani N, Brown CW. Emerging roles for the transforming growth factor-{beta} superfamily in regulating adiposity and energy expenditure. *Endocr Rev*. (2011) 32:387–403. doi: 10.1210/er.2010-0018
75. Hsu JY, Crawley S, Chen M, Ayupova DA, Lindhout DA, Higbee J, et al. Non-homeostatic body weight regulation through a brainstem-restricted receptor for GDF15. *Nature.* (2017) 550:255–9. doi: 10.1038/nature24042
76. Luo H, Guo Y, Liu Y, Wang Y, Zheng R, Ban Y, et al. Growth differentiation factor 11 inhibits adipogenic differentiation by activating TGF-beta/Smad signalling pathway. *Cell Prolif.* (2019) 52:e12631. doi: 10.1111/cpr.12631
77. Jing YY, Li D, Wu F, Gong LL, Li R. GDF11 does not improve the palmitate induced insulin resistance in C2C12. *Eur Rev Med Pharmacol Sci.* (2017) 21:1795–802.
78. Dominguez-Perez M, Simoni-Nieves A, Rosales P, Nuno-Lambarri N, Rosas-Lemus M, Souza V, et al. Cholesterol burden in the liver induces mitochondrial dynamic changes and resistance to apoptosis. *J Cell Physiol*. (2019) 234:7213–23. doi: 10.1002/jcp.27474
79. Calvisi DF, Wang C, Ho C, Ladu S, Lee SA, Mattu S, et al. Increased lipogenesis, induced by AKT-mTORC1-RPS6 signaling, promotes development of human hepatocellular carcinoma. *Gastroenterology*. (2011) 140:1071–83. doi: 10.1053/j.gastro.2010.12.006
80. Porporato PE, Filigheddu N, Pedro JMB, Kroemer G, Galluzzi L. Mitochondrial metabolism and cancer. *Cell Res*. (2018) 28:265–80. doi: 10.1038/cr.2017.155
81. Hernandez-Rizo S, Gerardo-Ramirez M, Fucho R, Perez-Aguilar B, Gomez-Quiroz LE, Fernandez-Checa JC, et al. Growth differentiation factor 11 (GDF11) restricts cell proliferation in hepatic tumor cells through glycolysis and lipid metabolism. *Ann Hepatol*. (2018) 17:683. doi: 10.5604/01.3001.0012.0962
82. Garrido-Moreno V, Diaz-Vegas A, Lopez-Crisosto C, Troncoso MF, Navarro-Marquez M, Garcia L, et al. GDF-11 prevents cardiomyocyte hypertrophy by maintaining the sarcoplasmic reticulum-mitochondria communication. *Pharmacol Res*. (2019) 146:104273. doi: 10.1016/j.phrs.2019.104273

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Lactic Acidosis Promotes Mitochondrial Biogenesis in Lung Adenocarcinoma Cells, Supporting Proliferation Under Normoxia or Survival Under Hypoxia

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Lactic acidosis, glucose deprivation and hypoxia are conditions frequently found in solid tumors because, among other reasons, tumors switch to Warburg effect and secrete high levels of lactate, which decreases the pH (<6. 9) in the microenvironment. We hypothesized that lung cancer cells consume lactate and induce mitochondrial biogenesis to support survival and proliferation in lactic acidosis with glucose deprivation even under hypoxia. We examined lung adenocarcinoma cell lines (A-427 and A-549), a breast cancer cell line (MCF-7) and non-transformed fibroblasts (MRC-5). Cells were cultured using RPMI-1640 medium with 28 mM lactate varying pH (6.2 or 7.2) under normoxia (atmospheric O_2) or hypoxia (2% O_2). Cellular growth was followed during 96 h, as well as lactate, glutamine and glutamate levels, which were measured using a biochemical analyzer. The expression levels of monocarboxylate transporters (MCT1 and MCT4) were evaluated by flow cytometry. To evaluate mitochondrial biogenesis, mitochondrial mass was analyzed by flow cytometry and epifluorescence microscopy. Also, mitochondrial DNA (mtDNA) was measured by qPCR. Transcript levels of Nuclear Respiratory Factors (NRF-1 and NRF-2) and Transcription Factor A Mitochondrial (TFAM) were determined using RT-qPCR. The specific growth rate of A-549 and A-427 cells increased in lactic acidosis compared with neutral lactosis, either under normoxia or hypoxia, a phenomenon that was not observed in MRC-5 fibroblasts. Under hypoxia, A-427 and MCF-7 cells did not survive in neutral lactosis but survived in lactic acidosis. Under lactic acidosis, A-427 and MCF-7 cells increased MCT1 levels, reduced MCT4 levels and consumed higher lactate amounts, while A-549 cells consumed glutamine and decreased MCT1 and MCT4 levels with respect to neutral lactosis condition. Lactic acidosis, either under normoxia or hypoxia, increased mitochondrial mass and mtDNA levels compared with neutral lactosis in all tumor cells but not in fibroblasts. A-549 and MCF-7 cells increased levels of NRF-1, NRF-2, and TFAM with respect to MRC-5 cells, whereas A-427 cells

upregulated these transcripts under lactic acidosis compared with neutral lactosis. Thus, lung adenocarcinoma cells induce mitochondrial biogenesis to support survival and proliferation in lactic acidosis with glucose deprivation.

Keywords: mitochondrial mass, mitochondrial DNA (mtDNA), nuclear respiratory factor (NRF), monocarboxylate transporter (MCT), glutamine, tumor growth rate, glucose deprivation

INTRODUCTION

Lactic acidosis is a common condition found in solid tumors (1–3); for instance, in breast cancer patients, intratumoral lactate levels range from 0.6 to 8.0 μmol/g (4). Lactate can be employed as a carbon source; accordingly, some non-small cell lung cancer (NSCLC) tumors were recently shown to use lactate as a respiratory substrate to survive and proliferate (5). There are different sources that promote lactic acidosis in tumors: first, the altered metabolism of tumor cells augment both glucose consumption and glycolysis with a concomitant increase in lactate production in the presence of oxygen (Warburg effect) (6) or in the absence of oxygen (Pasteur effect) (7, 8). Second, the inefficient formation of microvasculature favors the development of concentration gradients of lactate, glucose, oxygen and pH (9, 10). Third, cancer-associated fibroblasts (CAFs) can also promote high levels of lactate and an acidic microenvironment ($pH < 6.9$) (6).

Because the tumor microenvironment is variable, cells that initially had access to high glucose levels and normal oxygen concentrations may eventually encounter an environment with lactic acidosis (1, 7), absence of glucose (1) and hypoxia (8). For example, in solid tumors such as colon and stomach cancer, glucose concentrations are much lower within the tumor (0.15 mmol/g) than in normal tissues (1.2 mmol/g) (2). In breast cancer patients, the tumor mass has an estimated median PO_2 value of 10 mmHg (1.5% O_2), whereas in normal breast tissue, it is 65 mmHg (8). These changes in the microenvironment might favor tumor survival and invasion; in this regard, hypoxia is known to favor angiogenesis, metastasis, and resistance to radiation and chemotherapies (8).

The acidic microenvironment promotes extracellular matrix degradation and angiogenesis, inhibits the immune response and is toxic for non-transformed cells (7). For instance, human melanoma cells maintained under acidic conditions reduce their capacity to form flank tumors, but they exhibit a greater range of motility and invasive phenotypes (7, 11). Also, lactic acidosis in the presence of glucose (3 mM) promotes autophagy, increases resistance to glucose-deprivation-induced apoptosis and arrests cells in the G0/G1 cell cycle phase as survival mechanisms in murine breast cancer 4T1 cells (10). As a proof of concept, bicarbonate infusions into tumors revert lactic acidosis into lactosis, where tumor cells switch back to the Warburg effect, which induces glucose-deprivation-associated death (3).

Lactate transport requires of monocarboxylate transporters (MCTs). In particular, MCT1 and MCT4 are plasma membrane transporter proteins of lactate and pyruvate. MCT1 is a symporter that co-introduces equimolar lactate and protons. MCT4 may import or export lactate, but it frequently transports lactate out of the cell (5). MCT1 and MCT4 expression are deregulated in several types of cancers. For instance, high MCT4 expression in CAFs accompanied by strong MCT1 expression in tumor cells has been shown to be associated with poor prognosis in prostate cancer (6, 10, 12). A previous report also showed that some NSCLC tissues can incorporate lactate by increasing the expression of MCT1, MCT4, and lactate dehydrogenase A and B (LDHA and LDHB) (5). This study also indicated that other NSCLC tissues neither introduce lactate nor express MCT1, MCT4, LDHA, and LDHB, highlighting the heterogeneity in human NSCLC tumor cells (5). Nevertheless, to our knowledge, regulation of MCTs expression on lung cancer cells by lactate itself has not been studied.

Among the many pathways involved in tumor development, mitochondrial biogenesis has been shown to be important for sustaining cell proliferation. Cells generate more mitochondria from pre-existing organelles in a process called mitochondrial biogenesis to increase their mitochondrial mass to divide them up and inherit sufficient mitochondria to daughter cells (13). Increases in mitochondrial mass and mtDNA are considered markers of mitochondrial biogenesis (14, 15). The main transcriptional regulators of mitochondrial biogenesis include peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) and mitochondrial transcription factor (TFAM) (14, 16). A significant increase in the PGC-1α, NRF-1 and TFAM proteins with a concomitant increase in mitochondrial biogenesis has been reported in arsenic-induced carcinogenesis of human keratinocytes (14) and endometrial cancer tissue (16). Increases in mtDNA content have been reported in prostatic cancer (12) and in the transformation from hyperplastic to cancer endometrium (15). In contrast, mtDNA depletion has been associated with ovarian cancer progression (17) or with the acquisition of a more invasive phenotype in early prostate carcinoma (18). However, none of these studies describe whether mitochondrial biogenesis is modulated by lactate, acidosis or hypoxia in cancer cells.

Mitochondrial biogenesis appears to be critical for several process of the malignant transformation (19). Mitochondrial biogenesis is increased to favor migratory and invasive tumor phenotypes in breast cancer (8, 20), propagation and survival of stem-like cancer cells (19), and adaptation to hypoxia in a neutral pH microenvironment of human hepatocellular carcinoma (HCC) cells (13). In contrast, a reduction of mitochondrial biogenesis and the subsequent alteration of respiratory capacity has been observed in lung tumors, these features were associated with a lower expression level of Bcl-2 (21). Thus, mitochondrial biogenesis has been thought to be dysfunctional in lung cancer cells.

Since lactic acidosis with extremely limited glucose levels and hypoxia are conditions frequently found in solid tumors, we hypothesized that if lung adenocarcinoma cells survive under glucose-deprivation with lactic acidosis, then they will consume lactate and induce mitochondrial biogenesis independently of the oxygen tension. Thus, we tested *in vitro* the influence of each variable (carbon source, pH and oxygen) on tumor survival and proliferation, we also analyzed the expression of MCT1 and MCT4 and evaluated whether mitochondrial biogenesis is modified in response to lactic acidosis. The results of this study may contribute to develop novel strategies for cancer treatment.

MATERIALS AND METHODS

Cell Lines

Two human lung adenocarcinoma cell lines were used in this study, A-549 and A-427. Additionally, one breast carcinoma cell line (MCF-7) and human fetal lung fibroblast cells (MRC-5) were included. All cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA).

Growth Kinetics of Tumor Cells

The tumor cell lines and fibroblasts were maintained in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, HyClone, Logan, Utah, USA) with 100 μg/mL of streptomycin and 100 U/mL of penicillin at 37°C, atmospheric O_2 and 5% CO_2. The cell lines grew in monolayers and were harvested by trypsinization.

The growth of all carcinoma cell lines and the fibroblasts was tested using RPMI-1640 glucose-free medium (Sigma-Aldrich) supplemented with sodium L-lactate (28 mM) (Sigma-Aldrich), 10% heat-inactivated fetal calf serum, 100 μg/mL of streptomycin and 100 U/mL of penicillin. Because FCS contained a small amount of glucose, the initial glucose concentration was 350 μM. Additionally, RPMI-1640 medium contained L-glutamine and after FCS addition, the initial concentration of L-glutamine was 1.4 mM. The medium was adjusted at pH 7.2 or pH 6.2 using HCl. Normoxic cells were incubated in a humidified chamber at 37°C with filtered atmospheric air (21% O_2) and 5% CO_2. Hypoxic cells were incubated in a humidified Billups-Rothenberg chamber (Del Mar, CA, USA) with 2% O_2, 93% N_2 and 5% CO_2 at 37°C.

A-427, A-549 and MCF-7 cells were seeded at a density of 1×10^5 cells/mL, and MRC-5 cells were seeded at a density of 5×10^4 cells/mL. Cellular suspensions prepared in lactate-supplemented medium at pH 7.2 or pH 6.2 were seeded in sextuplicate in a 24-well plate. Two 24-well plates were seeded in an equivalent fashion. One plate was incubated under normoxia, while the other was incubated under hypoxia for 96 h. Depending on the cell line, the supernatant from each well was removed and measured every 8, 12, or 24 h for analysis of metabolites considering evaporation. Cell-free supernatants were stored at −20°C for later analysis. The cells were counted, and cell viability was determined by trypan blue dye exclusion using a TC20 Automated Cell Counter (Bio-Rad Laboratories, Inc., USA). All cultures were repeated at least twice. The specific growth rate was determined during exponential growth according to the following formula:

$\mu = \ln 2/(\text{duplication time})$.

Determination of Metabolites

The levels of glucose, L-lactate, L-glutamine and glutamate were determined using a YSI 2900 biochemistry analyzer (Yellow Springs Instruments, Ohio, USA) and membranes containing the immobilized enzymes d-glucose oxidase, L-lactate oxidase, L-glutamine oxidase or L-glutamic acid oxidase (YSI, Ohio, USA). For each metabolite, specific standards were prepared and used according to the manufacturer's instructions. The evaporation volume was determined to correct the quantity of each metabolite.

Analysis of MCT1, MCT4, and CD98 Cell Surface Expression

MCT1 and MCT4 expression was evaluated in cancer and fibroblast cells by flow cytometry, whereas CD98 was evaluated in A-549 and A-427 adenocarcinoma cell lines. Briefly, cells were cultured in 24-well plates under the four above-described conditions over a 48 h time period. The initial cellular concentrations per well were adjusted to 1×10^5 cells/mL for the A-549, A-427, and MCF-7 cell lines and 1.5×10^5 cells/mL for MRC-5. All cultures were repeated at least twice. After incubation, the cells were harvested by non-enzymatic treatment (EDTA-MOPS). The cells were then washed with PBS. Dead cells were excluded by using the Zombie NIR fixable viability kit following manufacturer's instructions (BioLegend, CA, USA). For immunostaining, cells were resuspended with bovine serum albumin (BSA, 1% w/v) and sodium azide (0.1% w/v) and incubated at room temperature for 30 min with rabbit polyclonal antibodies against MCT1 (MCT1-Alexa 647) and MCT4 (MCT4-Alexa 488) from Bioss (Massachusetts, USA), or with monoclonal antibody for CD98 (clone5E5, FITC) from eBioscience (San Diego, CA, USA). After incubation, the cells were washed and fixed with paraformaldehyde (1% w/v) for further cytometric analysis. At least 10,000 events were acquired from the region of viable cells. The median fluorescence intensity (MFI) of MCT1-Alexa 647, MCT4-Alexa 488, and CD98-FITC was determined. The results were analyzed with FlowJo V10 software (TreeStar, Inc., Ashland, Or, USA).

Mitochondrial Mass Determination

We used MitoTracker Green (Thermo Fisher Scientific Eugene, OR, USA) to determine mitochondrial content in the cells. The initial cellular concentration per well was the same for the different culture conditions and was adjusted to 1×10^5 cells/mL for A-549, A-427, and MCF-7 and 5×10^4 cells/mL for MRC-5. After 48 h of incubation, cells were harvested and washed with phosphate-buffered saline (PBS). After counting, the cells were resuspended in MitoTracker Green 250 nM (200 μL per 2×10^5 cells) and incubated at 37°C for 30 min in the dark. Cells were washed with PBS and resuspended in 200 μL of 7-aminoactinomycin D (7-AAD, BioLegend, CA, USA). Then, stained cells were analyzed by flow cytometry, and at least 2×10^4 events from the viable cellular region (7AAD-negative

cells) were acquired in a FACS Canto II Flow Cytometer (Becton Dickinson, San Jose, CA). The median fluorescence intensity (MFI) of MitoTracker Green, which is directly proportional to the mitochondrial content, was determined only in the viable cell population.

Mitochondrial Mass Using Epifluorescence Microscopy

The cellular preparation for epifluorescence was made as follows. After 48 h of incubation in 48-well plates, the supernatant was removed. Then, each well was washed with 500 μL PBS, and 100 μL of MitoTracker Green 250 nM (Invitrogen, USA) was added. The plates were incubated at 37°C for 30 min. After washing with 500 μL PBS, 100 μL of CellMask Orange 1X (Molecular Probes) was added. The preparations were incubated at 37°C for 5 min. After washing with 500 μL PBS, 100 μL of 0.1 μg/mL Hoechst 33342 solution (Invitrogen) was added. The plates were incubated at 37°C for 10 min; later, digital images were taken using an EVOS FL Imaging System (Life Technologies, USA). Micrographs were analyzed with ImageJ Software v 1.50i (Wayne Rasband, National Institutes of Health, USA) to obtain fluorescence intensity due to MitoTracker Green per cell line.

DNA and RNA Extraction

One milliliter of 1×10^5 cells/mL for A-549, A-427, and MCF-7 and 5×10^4 cells/mL for MRC-5 was seeded per well in 24-well plates under the four conditions mentioned above. After 48 h of incubation, total DNA and RNA were extracted from cell lines using a ZR-Duet DNA/RNA Miniprep system according to the manufacturer's instructions (Zymo Research, Irvine, CA, USA). Total isolated DNA was stored at −20°C for further analysis. The quality and quantity of RNA were determined by absorbance at 260 and 280 nm using a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). The RNA was treated with RNase-free DNase I (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The RNA was reverse-transcribed to produce cDNA using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Waltham, MA, USA). The cDNA obtained was stored at −20°C until further analysis.

Quantification of mtDNA Using qPCR (Real-Time PCR)

Mitochondrial mass determination and mtDNA copy number are indicators of mitochondrial content (15). We used qPCR to quantify mtDNA molecules. Briefly, the concentration of purified total DNA was measured using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA). The mtDNA (12S) copy number was determined by qPCR and compared to genomic DNA (RNase P), as previously described (22). Briefly, mtDNA was quantified using 125 nM specific primers for the gene 12S (12S-F: 5-CCA CGG GAA ACA GCA GTG ATT-3 and 12S-R: 5-TAT TGA CTT GGG TTA ATC GTG TG-3) and 140 nM of the TaqMan MGB Probe (6'FAM GTG CCA GCC ACC GCG MGENFQ). gDNA copy number was quantified using 1X of the PDARS RNase P Kit (VIC, Thermo Scientific, P/N 4316944) and 1X Universal master mix (Thermo Scientific, USA). mtDNA and gDNA were amplified in a single tube using four 1:5 serial dilutions, beginning with 5 ng/μL of total DNA. All samples, including serial dilutions, were analyzed in triplicate. The amount of mtDNA relative to RNase P was calculated using the following formula: mtDNA/RNase P $= 2^{-(\mathrm{CtmtDNA}-\mathrm{CtRNAseP})}$, where Ct is the threshold cycle.

Transcriptional Analysis of NRF-1, NRF-2, and TFAM Using RT-qPCR

To determine the transcript levels of the main transcriptional regulators of mitochondrial biogenesis, we used semiquantitative RT-qPCR in an ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA). NRF-1, NRF-2, and TFAM mRNA levels were quantified using specific primers (NRF-1-F: 5′-ATG AAG ACT CGC CTT CTT CTC-3′ and NRF-1-R: 5′-TTG TTG CCT CTT CCG GAT AGA-3′; NRF-2-F: 5′-AGT GCA ATC TGC TAC ACC TAC-3′ and NRF-2-R: 5′-ATG CAG TCT CGA GCG TCC TT-3′; TFAM-F: 5′-TGT GCA CCG GCT GTG GAA GT-3′ and TFAM-R: 5′-TCC CTC CAA CGC TGG GCA AT-3′), SYBR Select Master Mix (Thermo Scientific, Waltham, MA, USA) and cDNA as a template.

The PCR reactions were performed in 96-well reaction plates using the recommended parameters (10 min at 95°C, 40 cycles of 95°C for 15 s and 60°C for 1 min). Validation curves were run using 18S rRNA (18S-F: 5′-TAC CGC AGC TAG GAA TAA TGG-3′ and 18S-R: 5′-CGT CTT CGA ACC TCC GAC TT-3′) and HPRT1 (HPRT1-F: 5′-CCT GCT GGA TTA CAT CAA AGC-3′ and HPRT1-R: 5′-CTG CAT TGT TTT GCC AGT GTC-3′) to determine the suitable endogenous control for all the analyzed genes. The 18S rRNA was selected as the endogenous control for all transcripts. Each PCR reaction was performed in triplicate, and two non-template controls were included. Data were analyzed with Sequence Detection Software v 1.3.1 (Thermo Scientific, Waltham, MA, USA) to establish the PCR cycle at which the fluorescence exceeded a set of cycle thresholds (Ct) for each sample. Target gene expression analysis was performed according to the comparative $2^{-\Delta\Delta Ct}$ method (23).

Analysis of NRF-1 and NRF-2 Protein Levels

NRF-1 and NRF-2 protein levels were determined in A-549 and A-427 adenocarcinoma cell lines by flow cytometry. After 72 h of incubation under the four tested conditions described above, cells were harvested by trypsinization, washed with PBS, and stained with Zombie NIR to exclude dead cells. Then cells were fixed and permeabilized with Transcription Factor Staining Buffer Set (Invitrogen) according to manufacturer's instructions. After permeabilization, cells were resuspended in 100 μL of rabbit anti-NRF-1 polyclonal antibody (dilution 1:1000, cat. no. bs-1342R, Bioss Antibodies) or rabbit anti-GABPA/NRF2A polyclonal antibody (dilution 1:1000, cat. no. bs-13261R, Bioss Antibodies). After 45 min of incubation, cells were washed and incubated with Alexa 488 mouse anti-rabbit monoclonal antibody (Molecular Probes, Eugene Oregon) for 30 min. Cells were washed with Perm Buffer (provided by the manufacturer) and resuspended in 200 μL PBS/BSA to proceed to the flow

cytometry analysis. At least 10,000 events were acquired from the region of viable cells. The MFI values for NRF-1 and NRF-2 were determined. The results were analyzed with FlowJo V10 software.

Statistical Analysis

All values are expressed as the mean ± standard error of at least two independent experiments. Changes between groups were analyzed using unpaired Student's *T*-test and *post-hoc* tests were performed using GraphPad Prism 7 software. Significant differences between groups were defined at $p < 0.05$.

RESULTS

Lactic Acidosis Increased the Proliferation of Lung Adenocarcinoma Cells Compared With Neutral Lactosis

Lactic acidosis is a condition frequently found in solid tumors. Thus, we wanted to evaluate whether lung adenocarcinoma cells could proliferate in the presence of lactate with or without acidosis under normoxia (21% O_2) or hypoxia (2% O_2). We included the non-transformed cells (MRC-5) as a negative control and the breast tumor cell line (MCF-7) as a positive control because these cells can survive consuming lactate (4). We found that the specific growth rate (μ) of A-549 and A-427 cells significantly increased under lactic acidosis compared with neutral lactosis either under normoxia or hypoxia. Although A-549 cells only showed a tendency to increase growth rate under lactic acidosis and normoxia (**Table 1**), cell number up to 96 h of culture was greater under lactic acidosis (**Figure 1**). Of note, from the start of the experiment to the beginning of the growth phase, there was an adaptation phase (lag phase) that was variable for each cell line and culture condition (**Figure 1**). A-427 and MCF-7 tumor cells did not survive in neutral lactosis under hypoxia and they showed a decline in proliferation associated with cell death, because the cell number at 48 h was smaller than time zero (**Figure 1**). In contrast, after an adaptation phase of 8 h under lactic acidosis and hypoxia, the A-549 tumor cells exhibited a low proliferation rate; nonetheless, this rate increased after 48 h of incubation (**Figure 1**).

In the case of the non-transformed MRC-5 cells cultured under lactic acidosis, the μ diminished under normoxia but

TABLE 1 | Specific growth rate of tumor cell lines and fibroblast cells.

	Normoxia		Hypoxia	
Cell line	pH 7.2 ($\times 10^{-2}$ h^{-1})	pH 6.2 ($\times 10^{-2}$ h^{-1})	pH 7.2 ($\times 10^{-2}$ h^{-1})	pH 6.2 ($\times 10^{-2}$ h^{-1})
MRC-5	1.9 (0.2)	1.4 (2.2)	[−1.9 (1.1)]**	1.4 (1.1)[a]
A-549	1.5 (0.7)	2.0 (0.5)	[0.3 (0.4)]*	1.1 (0.6)[a]
A-427	1.2 (0.6)	2.4 (0.4)*	[−0.7 (0.4)]**	0.8 (1.0)[a]
MCF-7	2.6 (0.3)	1.5 (0.6)	[−0.4 (0.4)]**	0.5 (0.8)*

*Specific rate of growth was determined on exponential phase. All cultures were made by triplicate in tissue-culture plate using RPMI-1640 supplemented with lactate (28 mM), glucose (0.35 mM), and pH 7.2 or pH 6.2 under normoxia or hypoxia. Values are expressed as mean (std dev). *p < 0.05 and **p < 0.01 respect to normoxia pH 7.2 condition. [a]p < 0.05 respect to hypoxia pH 7.2 condition.*

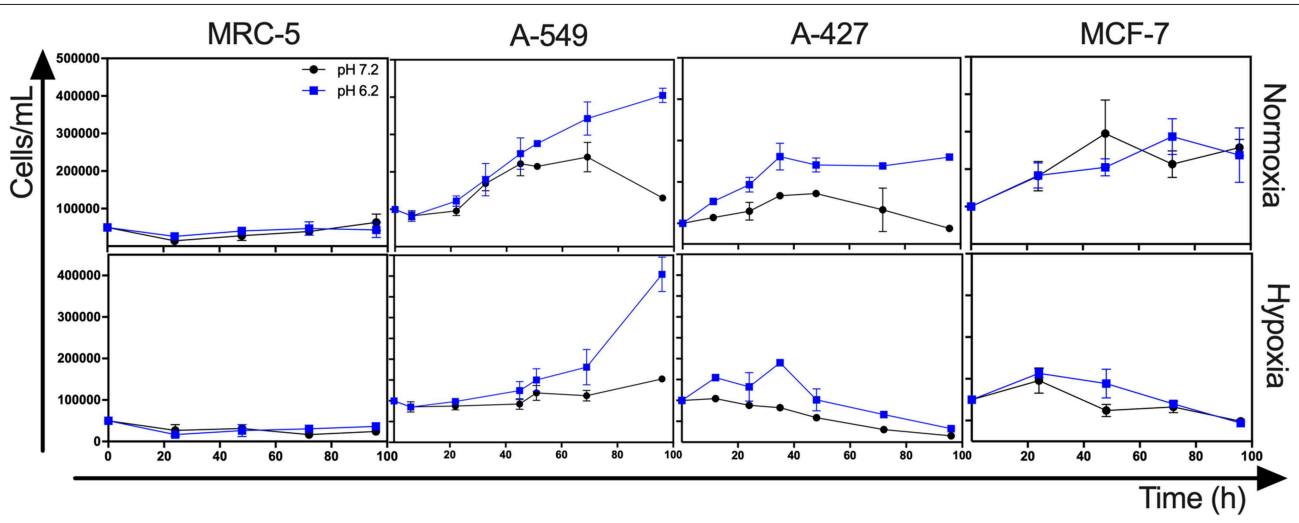

FIGURE 1 | Growth curves of MRC-5, A-549, A-427, and MCF-7 followed for 96 h, using RPMI-1640 low glucose (350 μM) supplemented lactate (28 mM) adjusted at pH 7.2 or pH 6.2 under normoxia (21% O_2) or hypoxia (2% O_2).

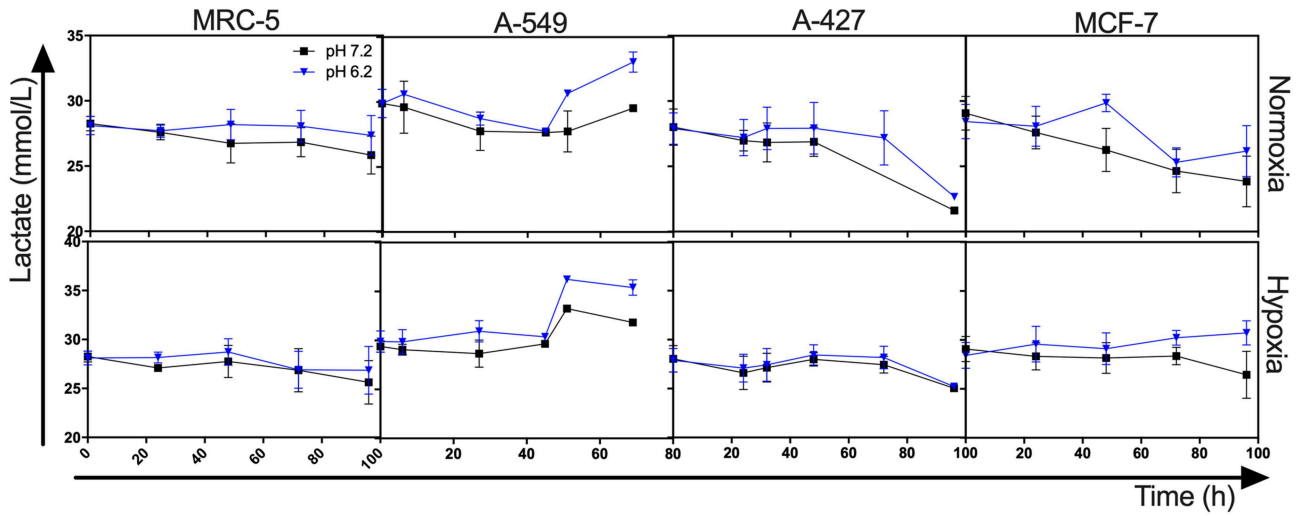

FIGURE 2 | Lactate levels from cultures of MRC-5, A-549, A-427, and MCF-7 followed for 96 h, using RPMI-1640 low glucose (350 μM) supplemented lactate (28 mM) adjusted at pH 7.2 or pH 6.2 under normoxia (21% O_2) or hypoxia (2% O_2).

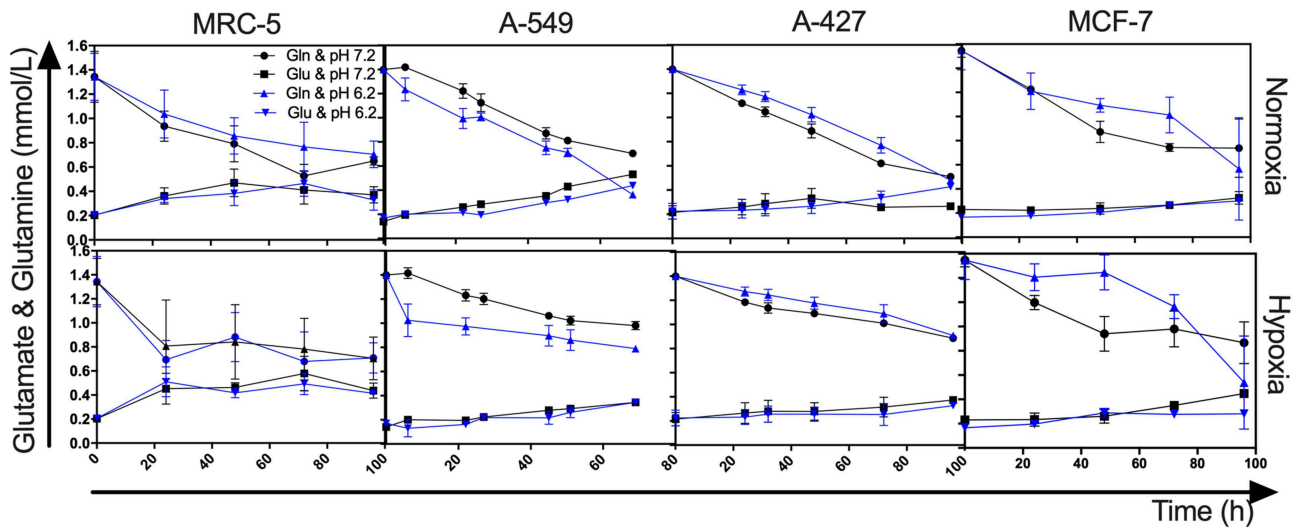

FIGURE 3 | Glutamine and glutamate levels from cultures of MRC-5, A-549, A-427, and MCF-7 followed for 96 h, using RPMI-1640 low glucose (350 μM) supplemented lactate (28 mM) adjusted at pH 7.2 or pH 6.2 under normoxia (21% O_2) or hypoxia (2% O_2). Gln, Glutamine; Glu, Glutamate.

increased under hypoxia compared with the neutral condition (**Table 1**, **Figure 1**). Nonetheless, MRC-5 cells presented a long adaptation phase and entered a survival stage, with the exception of neutral lactosis and hypoxia.

These results indicate that lactic acidosis allows lung adenocarcinoma cells to survive and even proliferate in lactic acidosis and glucose deprivation.

A-427 and MCF-7 but Not A-549 Cells Cultured Under Normoxia Consumed Lactate Independently of the pH

After finding that adenocarcinoma cells in lactic acidosis proliferated under normoxia or survived longer under hypoxia, we hypothesized that adenocarcinoma cells would consume lactate because all tumor cells and fibroblasts completely consumed the initial small amount of glucose (350 μM) during the first 8 h of incubation. Then, the cultures remained glucose-free to the end of the incubation period of 96 h (data not shown).

Interestingly, we found that when A-427 and MCF-7 cells were cultured under normoxia independently of pH, they consumed lactate during both the growth and stationary phases (**Figures 1**, **2**). In contrast, A-549 and MRC-5 consumed low lactate quantities when they were cultured under neutral lactosis and normoxia (**Figure 2**). Surprisingly, none of the cell lines consumed lactate under hypoxia regardless of the pH (**Figure 2**).

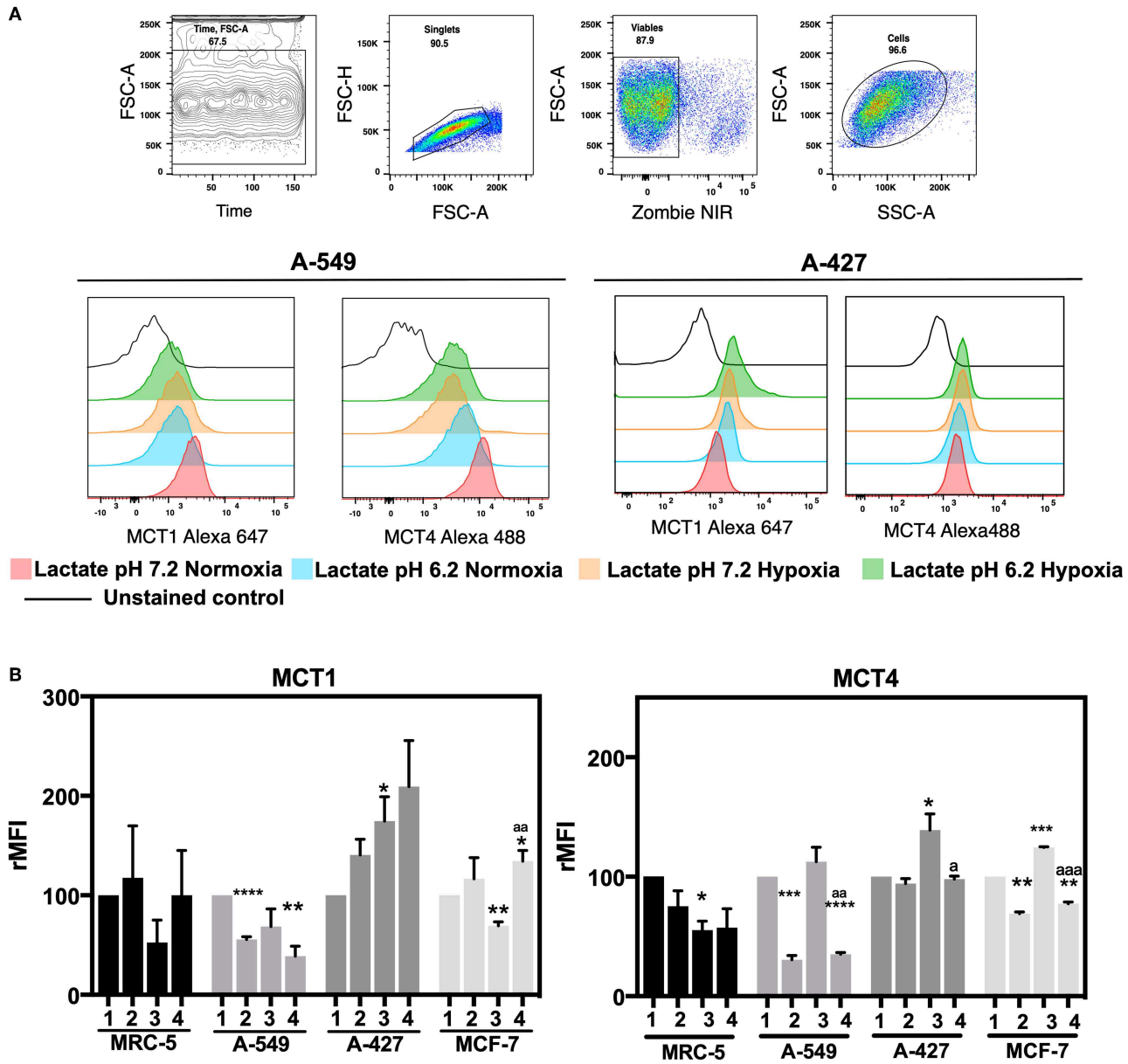

FIGURE 4 | Determination of monocarboxilate transport levels in cancer cell lines and fibroblasts, cultured for 48 h. **(A)** Representative flow cytometric analysis and representative histograms of MCT1 and MCT4 expression on lung adenocarcinoma cell lines. **(B)** Relative median fluorescence intensity (rMFI) values for MCT1 and MCT4 on tumor and fibroblast cells. (1) Normoxia, pH 7.2; (2) Normoxia, pH 6.2; (3) Hypoxia, pH 7.2 and (4) Hypoxia, pH 6.2. Bars represent mean ± SEM of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ respect to normoxia pH 7.2. $^{a}p < 0.05$, $^{aa}p < 0.01$, $^{aaa}p < 0.01$ respect to hypoxia pH 7.2.

Under Lactic Acidosis, A-549 Cells Consumed Higher Amounts of Glutamine Than A-427, MCF-7, and MRC-5 Cells

Because we observed that under lactic acidosis, A-549 cells proliferated but did not consume lactate, we investigated whether A-549 cells consumed glutamine to support proliferation and survival under lactic acidosis with glucose deprivation. We found that A-549 cells cultured under lactic acidosis consumed more glutamine (1.1 mM at 72 h) and produced more glutamate (0.4 mM at 72 h) than A-427, MCF-7 and MRC-5 cells (0.4–0.6 mM of consumed glutamine and 0–0.1 mM of produced glutamine at 72 h) (**Figure 3**), indicating that glutaminolysis supports the proliferation of A-549 cells. Additionally, A-549 cells consumed glutamine faster under lactic acidosis than neutral conditions independent of oxygen tension (**Figure 3**). In contrast, A-427, MCF-7 and MRC-5 cells consumed glutamine faster under neutral lactosis than in lactic acidosis (**Figure 3**).

TABLE 2 | rMFI of CD98 protein on the cell surface of lung adenocarcinoma cells.

	Normoxia		Hypoxia	
Cell line	pH 7.2	pH 6.2	pH 7.2	pH 6.2
A-549	100	61.9 (10.8)*	79.0 (2.7)**	55.1 (5.3)**[a]
A-427	100	35.3 (7.2)**	82.8 (12.3)	76.0 (4.5)*

*All cultures were made by triplicate in tissue-culture plate using RPMI-1640 supplemented with lactate (28 mM), glucose (0.35 mM), and pH 7.2 or pH 6.2 under normoxia or hypoxia during 48 h. Values are reported as percentages with respect to lactate pH 7.2 and expressed as mean (std dev). *$p < 0.05$ and **$p < 0.01$ respect to normoxia pH 7.2 condition. [a]$p < 0.05$ respect to hypoxia pH 7.2 condition.*

MCT1 Expression Was Differentially Modulated, While CD98 Expression Diminished on Both Adenocarcinoma Cells Under Lactic Acidosis

After finding that A-427 and MCF-7 cells consumed lactate under normoxia, we analyzed the expression of MCT1 and MCT4 on the cell surface. MFI values for the expression of MCT1 and MCT4 were normalized with respect to neutral lactosis condition and reported as relative MFI values (rMFI). A representative flow cytometric analysis is shown in **Figure 4A**.

We found that A-427 and MCF-7 cells tended to increase MCT1 levels under acidosis compared with neutral conditions independent of oxygen tension (**Figure 4B**). Additionally, A-549 cells significantly diminished MCT1 expression under acidosis regardless of oxygen tension, whereas MRC-5 cells did not change the MCT1 expression levels (**Figure 4B**). Hypoxia tended to increase MCT4 levels compared with normoxia in all tumor cell lines when they were cultured under neutral pH. Interestingly, A-549, A-427 and MCF-7 cells significantly diminished MCT4 levels under acidosis compared with neutral conditions (**Figure 4B**).

CD98 alongside with LAT1 form an antiporter that introduces long amino acids at the expense of intracellular glutamine (24). Because CD98 has an important role in glutamine metabolism, we analyzed CD98 protein levels on the surface of A-549 and A-427 adenocarcinoma cells (**Table 2**). We found that CD98 expression on both A-549 and A-427 adenocarcinoma cells significantly decreased under lactic acidosis independent of oxygen tension (**Table 2**). These results suggest that lung adenocarcinoma cells inhibit the intracellular glutamine release. Thus, under lactic acidosis and glucose withdrawal, A-427 and MCF-7 cells consumed lactate by increasing MCT1 expression, whereas A-549 cells did not consume lactate neither increased MCT1 expression. However, A-549 cells inhibited glutamine release and increased glutamine consumption which may indicate that glutaminolysis was favored under lactic acidosis.

Tumor Cells Increased Mitochondrial Mass Under Lactic Acidosis

We next wanted to determine whether mitochondrial biogenesis correlated with tumor growth or tumor survival. Thus, we evaluated mitochondrial mass by staining tumor cell lines and fibroblast cells with MitoTracker Green dye, which is a fluorescent compound that can accumulate in the lipid environment of the mitochondria and emit fluorescence independently of the mitochondrial membrane potential. We included 7-AAD staining to guarantee the analysis of exclusively viable cells. A representative flow cytometric analysis is shown in **Figure 5A**. MFI values for MitoTracker Green were normalized with respect to neutral lactosis and normoxia condition.

We found that A-549, A-427, and MCF-7 cell lines cultured under lactic acidosis significantly increased mitochondrial mass compared with neutral lactosis condition, either under normoxia or hypoxia; by contrast, MRC-5 fibroblasts did not increase their mitochondrial mass under lactic acidosis (**Figure 5B**). We corroborated these data using epifluorescence microscopy. Representative epifluorescence images of A-427, A-549, MCF-7 cells and MRC-5 fibroblasts cultured under normoxia are shown in **Figure 6A**, MitoTracker Green fluorescence intensity tended to increase in tumor cells but not in fibroblasts when cells were cultured in lactic acidosis with glucose deprivation under normoxia (**Figure 6B**).

These data indicated that mitochondrial mass content increased when tumor cells proliferated under normoxia or presented a survival stage under hypoxia when cells were cultured in lactic acidosis.

Adenocarcinoma Cells Increased mtDNA Levels Under Lactic Acidosis

To corroborate the mitochondrial mass findings made by flow cytometry and epifluorescence microscopy, we also measured the mtDNA levels in the tumor cell lines and fibroblast cells by qPCR and used the RNase P gene for normalization.

Under lactic acidosis, A-549 and A-427 cells significantly increased mtDNA levels compared with neutral lactosis under normoxia (**Figure 5C**). When these adenocarcinoma cells were cultured under lactic acidosis and hypoxia, mtDNA levels tended to increase compared with neutral lactosis (**Figure 5C**). These findings correlated with the findings of increased mitochondrial mass of adenocarcinoma cells cultured in lactic acidosis under normoxia and hypoxia. In contrast, the mtDNA levels of MCR-5 cells did not increase when these cells were cultured under lactic acidosis, either under normoxia or hypoxia (**Figure 5C**). In the case of the MCF-7 cell line, the mtDNA levels showed a tendency to increase under lactic acidosis.

A-549 and MCF-7 Cell Lines Overexpressed NRF-1 NRF-2 and TFAM Compared With MRC-5

We next evaluated the transcript levels of the main biogenesis regulators, such as nuclear respiratory factor (NRF) 1 and 2 and mitochondrial transcription factor A (TFAM). After normalization using 18S rRNA, we determined the relative expression of the abovementioned genes in the tumor cells compared with their counterparts expressed in MRC-5 fibroblasts when all cells were cultured under neutral lactosis and normoxia. We found that the NRF-1 and NRF-2 transcript levels were significantly upregulated in A-549 and MCF-7 cell lines compared with their counterparts in MRC-5 fibroblasts (**Figure 7A**). Remarkably, all tumor cell lines significantly

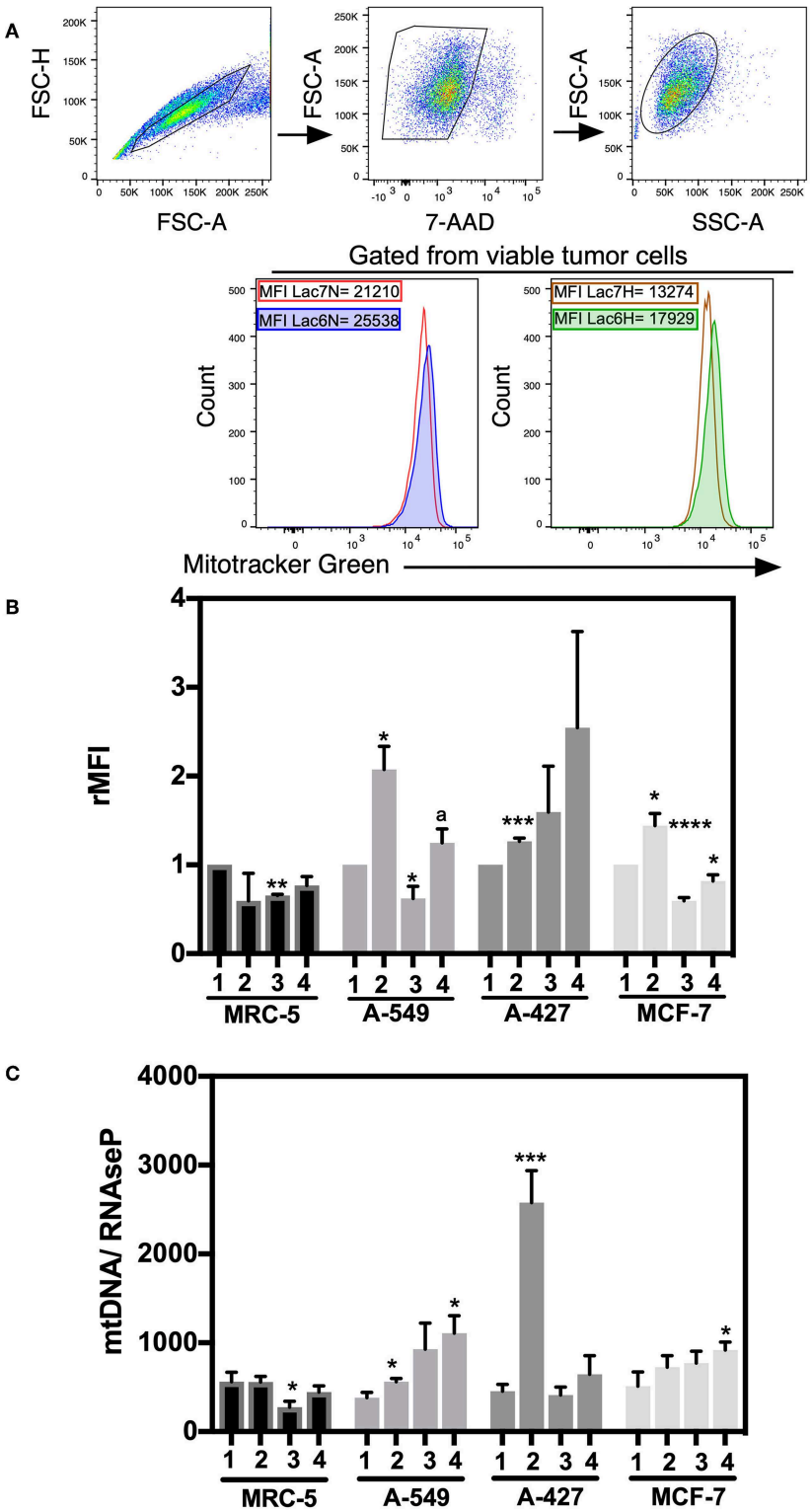

FIGURE 5 | Analysis of mitochondrial mass and mtDNA levels in cancer cell lines and fibroblasts cultured in RPMI-1640 low glucose (350 μM) supplemented with lactate (28 mM) for 48 h. **(A)** Histograms of a representative experiment of the A-427 tumor cell line. Analysis was performed using 7AAD-negative (viable) cells. **(B)** Relative median fluorescence intensity (rMFI) values for MitoTracker Green (MTG) in cancer and fibroblast cells. **(C)** Amount of mtDNA relative to the amount of the nuclear RNase P gene in cancer cell lines and fibroblasts. (1) Normoxia, pH 7.2; (2) Normoxia, pH 6.2; (3) Hypoxia, pH 7.2 and (4) Hypoxia, pH 6.2. Bars represent the mean with SEM of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ respect to normoxia pH 7.2. $^{a}p < 0.05$ respect to hypoxia pH 7.2.

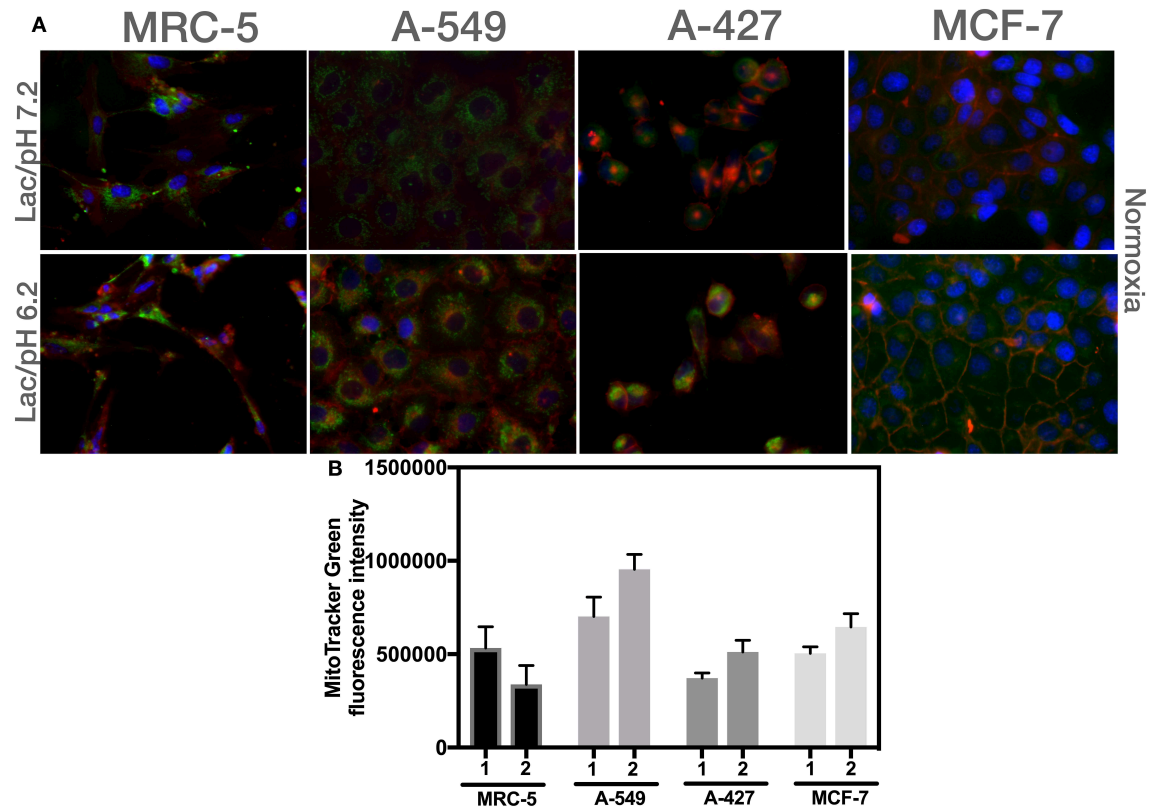

FIGURE 6 | Analysis of mitochondrial mass using epifluorescence microscopy of different cells cultured in RPMI-1640 with lactate and glucose deprivation at (1) pH 7.2 or (2) pH 6.2 under normoxia for 48 h. **(A)** Representative epifluorescence images of A-427, A-549, MCF-7 cells (60X), and MRC-5 fibroblast (40X). **(B)** Analysis of MitoTracker Green fluorescence intensity per cell line.

increased TFAM transcript levels compared with MRC-5 cells (**Figure 7A**), possibly to protect and stabilize mtDNA molecules and consequently avoid their degradation. Previous reports showed that this is an mtDNA replication-independent pathway that increases mitochondrial biogenesis, enhancing proliferation (14, 25).

Next, we evaluated how the expression of the different genes in tumor and fibroblast cells varied in acidosis compared with neutral conditions. We found that A-427 cells significantly increased NRF-1 and NRF-2 mRNA levels in lactic acidosis under normoxia and hypoxia (**Figures 7B,C**). At protein level, only NRF-2 intracellular protein significantly increased in A-427 cell line cultured under lactic acidosis with glucose deprivation independent of oxygen tension (**Table 3**). MCF-7 cells significantly increased TFAM transcript levels in lactic acidosis under normoxia or hypoxia (**Figure 7D**). In contrast, MRC-5 cells cultured under lactic acidosis did not increase the NRF-1, NRF-2, and TFAM transcript levels.

These findings indicate that tumor cells differentially express the NRF-1, NRF-2, and TFAM genes. Although some tumor cell lines (A-549 and MCF-7) maintained increased levels of NRF-1, NRF-2 and TFAM, lactic acidosis promoted the increase in NRF-1, NRF-2, and TFAM transcript levels, only when the tumor cells expressed low levels of the transcription factors. This was the case for the A-427 cell line, where intracellular NRF-2 protein levels, but not NRF-1, correlated with increased mRNA levels.

DISCUSSION

Lactic acidosis with very low glucose quantities is a condition found in solid tumors (2). Some reports have shown the importance of lactic acidosis in the transformation to a more aggressive tumor phenotype, favoring invasion and metastasis (7, 9). Although some groups have tried to find the mechanisms by which tumor cells proliferate in media containing lactic acidosis with high glucose levels from 3 to 10 mM (1, 3, 5); there are no reported data concerning the means by which tumor cells survive and proliferate under lactic acidosis with extremely low concentrations of glucose (350 μM), under normoxia (O_2, 21%) and hypoxia (O_2, 2%), though these are common conditions found in solid tumors. Lactic acidosis with an appreciable supply of glucose (3 mM) promotes a significantly longer sustainable proliferation of a murine breast cell line (4T1) than cells cultured only with glucose, suggesting that lactic acidosis but not acidosis alone favors cellular survival (3, 10). Accordingly, we found that lung adenocarcinoma cell lines (A-549 and A-427) cultured

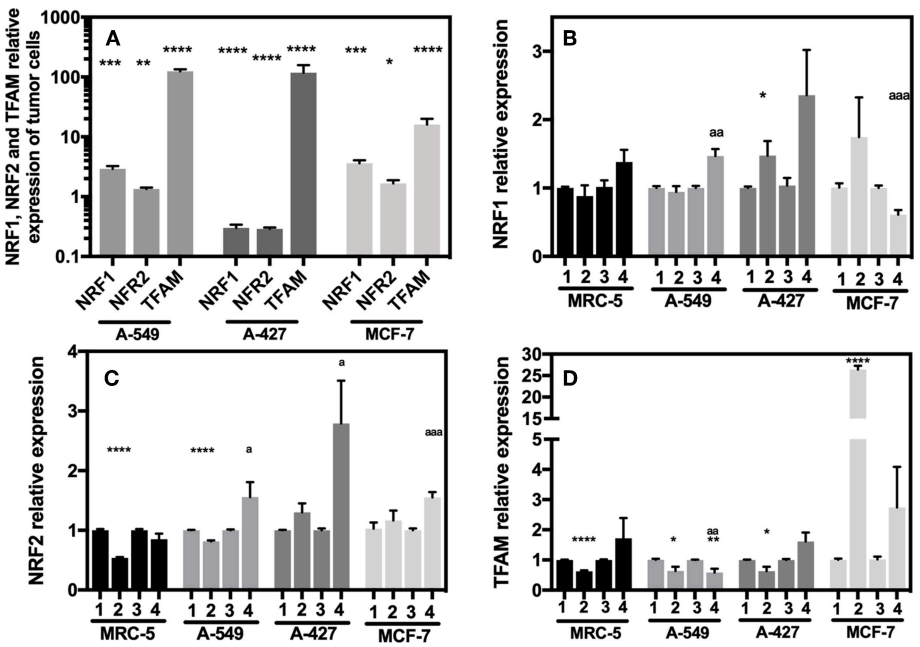

FIGURE 7 | Relative quantification of NRF-1, NRF-2, TFAM in cancer cell lines and fibroblasts cultured for 48 h. **(A)** Relative expression of NRF-1, NRF-2, TFAM in tumor cells compared with their expression in MRC-5, all cultured under neutral lactosis and normoxia. Relative expression of NRF-1 **(B)**, NRF-2 **(C)**, and TFAM **(D)** in the tumor cells and fibroblast cultured under lactic acidosis with respect to same cells cultured in neutral lactosis under normoxia or hipoxia. (1) Normoxia, pH 7.2; (2) Normoxia, pH 6.2; (3) Hypoxia, pH 7.2 and (4) Hypoxia, pH 6.2. Bars represent transcriptional data of two independent culture experiments expressed in mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ respect to the transcript levels in MRC-5 or normoxia pH7.2. $^{a}p < 0.05$, $^{aa}p < 0.01$, $^{aaa}p < 0.01$ respect to hypoxia pH7.2.

in lactic acidosis with extremely low glucose concentrations (350 μM) showed an increased growth rate under normoxia or survived for a longer period under hypoxia than cells cultured under glucose deprivation with neutral lactosis. In particular, lactic acidosis was especially beneficial for MCF-7 cells when they were cultured under hypoxia. In contrast, MRC-5 non-transformed cells cultured under lactic acidosis did not survive. Furthermore, each separate variable, lactosis, or acidosis alone, does not support tumor survival under glucose deprivation. These data indicated that lactic acidosis under normoxia or hypoxia, but not lactosis alone, activates an adaptive response that supports tumor survival, which is not present in normal cells. Although this study has the limitation of being *in vitro*, our results may help to explain why the experimental treatment of tumors with bicarbonate infusion has presented some benefits (3, 7). Taken together, our results indicate that lactic acidosis is a potent survival factor that allows lung adenocarcinoma and breast tumor cells to develop resistance to glucose deprivation even under hypoxia. It will be important to extend this study in animal models and to correlate our results with human tumor samples.

MCT1 expression has been associated with increased mitochondrial OXPHOS, whereas MCT4 is abundantly expressed on glycolytic cells to allow lactate expulsion and is also upregulated by hypoxia (12, 26, 27). This last phenomenon was also observed in our data. Accordingly, we found that A-427 and MCF-7 cells cultured under lactic acidosis and normoxia consumed lactate, which was associated to an increase in MCT1 expression and diminished MCT4 expression. Thus, our results suggest that A-427 and MCF-7 cells have an oxidative phenotype that allows proliferation and survival. Interestingly, A-549 cells did not consume lactate and diminished MCT1 and MCT4 expression levels. These results complement the findings reported by Faubert et al., who showed that some biopsies from NSCLC can use lactate by increasing the expression of MCT1, MCT4, LDHA, and LDHB, but due to the heterogeneity in human NSCLC cells, there are NSCLC tumors that neither introduce lactate nor express MCT1, MCT4, LDHA, and LDHB (5). Nevertheless, we found that A-549 lung adenocarcinoma cells did not introduce lactate nor upregulated MCT1 or MCT4; instead these cells consumed glutamine under lactic

TABLE 3 | rMFI of NRF-1 and NRF-2 intracellular protein levels in A-427 adenocarcinoma cell line.

	Normoxia		Hypoxia	
Protein	pH 7.2	pH 6.2	pH 7.2	pH 6.2
NRF-1	1.0	0.97 (0.09)	1.0	1.05 (0.03)
NRF-2	1.0	1.3 (0.04)*	1.0	1.23 (0.04)*[a]

All cultures were made by triplicate in tissue-culture plate using RPMI-1640 supplemented with lactate (28 mM), glucose (0.35 mM), and pH 7.2 or pH 6.2 under normoxia or hypoxia during 72 h. Values are expressed as mean (std dev). $^{*}p < 0.05$ respect to normoxia pH 7.2 condition. $^{a}p < 0.05$ respect to hypoxia pH 7.2 condition.

acidosis and glucose deprivation, allowing proliferation under normoxia or hypoxia. We corroborated the important role of glutamine in tumor survival under lactic acidosis with glucose deprivation, analyzing CD98 expression, which has an important participation in glutamine release. CD98 (4F2hc), covalently associated to glutamine transporter LAT1 (SLC7A5), contributes to glutamine efflux and large neutral amino acids influx, whereas ASCT2 (SLC1A5) allows Na^+-glutamine influx and neutral amino acids efflux (24, 28). Although increased LAT1 and ASCT2 expressions have also been reported in human melanoma samples, prostate cancer and breast cancer (29–31), the role of glutamine in cellular homeostasis is complex. We found that under lactic acidosis and glucose deprivation, lung adenocarcinoma cells inhibited glutamine release by diminishing CD98 expression, alongside with the increased intake of glutamine, both results indicate that lung adenocarcinoma cells increase glutaminolysis under lactic acidosis. Nevertheless, it will be important to evaluate ASCT2 expression on lung cancer cells cultured under lactic acidosis. Hence, our study shows that intracellular glutamine supports tumor survival and proliferation under lactic acidosis with glucose deprivation.

Mitochondrial biogenesis has been evaluated through different parameters, such as mitochondria and mtDNA count (13–15), mitochondrial mass determination (13), and the analysis of transcript or protein levels for NRF-1, NRF-2, TFAM, PGC-1α, and CS (13, 14, 16). Here, we found that lactic acidosis induced mitochondrial biogenesis in tumor cell lines (A-549, A-427, MCF-7), as evidenced by an increase in both mitochondrial mass and mtDNA, accompanied by high transcript levels for NRF-1, NRF-2, and TFAM in A-549 and MCF-7 cells or upregulation of these transcripts by lactic acidosis in A-427 cells. Tumor cells stimulate mitochondrial biogenesis not only for proliferation but also for promoting malignant transformation (15), in migration and invasiveness (8, 20) and during tumor adaptation to hypoxia (13). Here, we showed that lung adenocarcinoma cells (A-549, A-427) and breast cancer cells (MCF-7) stimulate mitochondrial biogenesis to survive under hypoxia; thus, lung and breast cancer cells share this survival response with other very aggressive types of cancer, such as HCC (13). Of note, mitochondrial biogenesis of fibroblast cells (MRC-5) was not increased under lactic acidosis; consequently, these cells were not able to proliferate under normoxia or survive under hypoxia. On the other hand, MCF-7 cells cultured with lactic acidosis in the presence of glucose increase their mitochondrial mass compared with medium with glucose alone (6). Our results complement these findings because we found that, even under glucose deprivation and hypoxia, lactic acidosis increased mitochondrial biogenesis in MCF-7 cells.

Bellance et al. suggested that mitochondrial biogenesis was diminished or damaged in lung cancer because they found diminished levels of mRNA for PGC-1α, reduced expression of PGC-1α and TFAM proteins, as well as, lower quantities of total mitochondrial area/cell area in lung cancer biopsies with respect to non-cancer tissue (21). Conversely, our study proposes that lung cancer cells need to face stressful conditions, such as lactic acidosis with glucose deprivation, to induce mitochondrial biogenesis.

CONCLUSIONS

Cancer cells need to survive when both glucose level and oxygen concentration are low. Under these last conditions, lactic acidosis becomes a key factor in tumor survival promoting mitochondrial biogenesis, although some tumor cells prefer to consume other alternative carbon sources, such as glutamine, rather than lactate. In contrast, non-transformed cells such as MRC-5 failed to induce mitochondrial biogenesis under these stressful and common tumor conditions. Thus, we report a tumor behavior that supports tumor survival.

Understanding metabolic adaptive mechanisms for recovering lung tumor cell proliferation under conditions that mimic the tumor microenvironment may provide promising opportunities to improve traditional cancer therapies or find new therapeutic targets to develop specific treatments.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

SR-G designed the study. SR-G, HP-G, AV-C, and AA-P performed the experiments. SR-G and HP-G wrote and critically reviewed the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

1. Duan K, Liu ZJ, Hu SQ, Huo HY, Xu ZR, Ruan JF, et al. Lactic acid induces lactate transport and glycolysis/OXPHOS interconversion in glioblastoma. *Biochem Biophys Res Commun.* (2018) 503:888–94. doi: 10.1016/j.bbrc.2018.06.092
2. Hirayama A, Kami K, Sugimoto M, Sugawara M, Toki N, Onozuka H, et al. Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry. *Cancer Res.* (2009) 69:4918–25. doi: 10.1158/0008-5472.CAN-08-4806
3. Xie J, Wu H, Dai C, Pan Q, Ding Z, Hu D, et al. Beyond Warburg effect–dual metabolic nature of cancer cells. *Sci Rep.* (2014) 4:4927. doi: 10.1038/srep04927
4. Kennedy KM, Scarbrough PM, Ribeiro A, Richardson R, Yuan H, Sonveaux P, et al. Catabolism of exogenous lactate reveals it as a

legitimate metabolic substrate in breast cancer. *PLoS ONE.* (2013) 8:e75154. doi: 10.1371/journal.pone.0075154
5. Faubert B, Li KY, Cai L, Hensley CT, Kim J, Zacharias LG, et al. Lactate metabolism in human lung tumors. *Cell.* (2017) 171:358–71.e9. doi: 10.1016/j.cell.2017.09.019
6. Martinez-Outschoorn UE, Balliet RM, Rivadeneira DB, Chiavarina B, Pavlides S, Wang C, et al. Oxidative stress in cancer associated fibroblasts drives tumor-stroma co-evolution: a new paradigm for understanding tumor metabolism, the field effect and genomic instability in cancer cells. *Cell Cycle.* (2010) 9:3256–76. doi: 10.4161/cc.9.16.12553
7. Estrella V, Chen T, Lloyd M, Wojtkowiak J, Cornnell HH, Ibrahim-Hashim A, et al. Acidity generated by the tumor microenvironment drives local invasion. *Cancer Res.* (2013) 73:1524–35. doi: 10.1158/0008-5472.CAN-12-2796
8. Cai FF, Xu C, Pan X, Cai L, Lin XY, Chen S, et al. Prognostic value of plasma levels of HIF-1a and PGC-1a in breast cancer. *Oncotarget.* (2016) 7:77793–806. doi: 10.18632/oncotarget.12796
9. Romero-Garcia S, Lopez-Gonzalez JS, Baez-Viveros JL, Aguilar-Cazares D, Prado-Garcia H. Tumor cell metabolism: an integral view. *Cancer Biol Ther.* (2011) 12:939–48. doi: 10.4161/cbt.12.11.18140
10. Wu H, Ding Z, Hu D, Sun F, Dai C, Xie J, et al. Central role of lactic acidosis in cancer cell resistance to glucose deprivation-induced cell death. *J Pathol.* (2012) 227:189–99. doi: 10.1002/path.3978
11. Moellering RE, Black KC, Krishnamurty C, Baggett BK, Stafford P, Rain M, et al. Acid treatment of melanoma cells selects for invasive phenotypes. *Clin Exp Metastasis.* (2008) 25:411–25. doi: 10.1007/s10585-008-9145-7
12. Pertega-Gomes N, Vizcaino JR, Attig J, Jurmeister S, Lopes C, Baltazar F. A lactate shuttle system between tumour and stromal cells is associated with poor prognosis in prostate cancer. *BMC Cancer.* (2014) 14:352. doi: 10.1186/1471-2407-14-352
13. Tohme S, Yazdani HO, Liu Y, Loughran P, van der Windt DJ, Huang H, et al. Hypoxia mediates mitochondrial biogenesis in hepatocellular carcinoma to promote tumor growth through HMGB1 and TLR9 interaction. *Hepatology.* (2017) 66:182–97. doi: 10.1002/hep.29184
14. Lee CH, Wu SB, Hong CH, Liao WT, Wu CY, Chen GS, et al. Aberrant cell proliferation by enhanced mitochondrial biogenesis via mtTFA in arsenical skin cancers. *Am J Pathol.* (2011) 178:2066–76. doi: 10.1016/j.ajpath.2011.01.056
15. Cormio A, Guerra F, Cormio G, Pesce V, Fracasso F, Loizzi V, et al. Mitochondrial DNA content and mass increase in progression from normal to hyperplastic to cancer endometrium. *BMC Res Notes.* (2012) 5:279. doi: 10.1186/1756-0500-5-279
16. Cormio A, Guerra F, Cormio G, Pesce V, Fracasso F, Loizzi V, et al. The PGC-1alpha-dependent pathway of mitochondrial biogenesis is upregulated in type I endometrial cancer. *Biochem Biophys Res Commun.* (2009) 390:1182–5. doi: 10.1016/j.bbrc.2009.10.114
17. Wang Y, Liu VW, Xue WC, Cheung AN, Ngan HY. Association of decreased mitochondrial DNA content with ovarian cancer progression. *Br J Cancer.* (2006) 95:1087–91. doi: 10.1038/sj.bjc.6603377
18. Moro L, Arbini AA, Marra E, Greco M. Mitochondrial DNA depletion reduces PARP-1 levels and promotes progression of the neoplastic phenotype in prostate carcinoma. *Cell Oncol.* (2008) 30:307–22. doi: 10.3233/CLO-2008-0427
19. De Luca A, Fiorillo M, Peiris-Pages M, Ozsvari B, Smith DL, Sanchez-Alvarez R, et al. Mitochondrial biogenesis is required for the anchorage-independent survival and propagation of stem-like cancer cells. *Oncotarget.* (2015) 6:14777–95. doi: 10.18632/oncotarget.4401
20. LeBleu VS, O'Connell JT, Gonzalez Herrera KN, Wikman H, Pantel K, Haigis MC, et al. PGC-1alpha mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis. *Nat Cell Biol.* (2014) 16:992–1003. doi: 10.1038/ncb3039
21. Bellance N, Benard G, Furt F, Begueret H, Smolkova K, Passerieux E, et al. Bioenergetics of lung tumors: alteration of mitochondrial biogenesis and respiratory capacity. *Int J Biochem Cell Biol.* (2009) 41:2566–77. doi: 10.1016/j.biocel.2009.08.012
22. Naini A, Shanske, S. Detection of mutations in mtDNA. In: Pon LA, Shon, EA, editors. *Methods in Cell Biology.* 2nd ed. San Diego, CA: Elsevier (2007). p. 438–64. doi: 10.1016/S0091-679X(06)80022-1
23. Romero-Garcia S, Prado-Garcia H, Lopez-Gonzalez JS. Transcriptional analysis of hnRNPA0, A1, A2, B1, and A3 in lung cancer cell lines in response to acidosis, hypoxia, and serum deprivation conditions. *Exp Lung Res.* (2014) 40:12–21. doi: 10.3109/01902148.2013.856049
24. Poffenberger MC, Jones RG. Amino acids fuel T cell-mediated inflammation. *Immunity.* (2014) 40:635–7. doi: 10.1016/j.immuni.2014.04.017
25. Kang D, Kim SH, Hamasaki N. Mitochondrial transcription factor A (TFAM): roles in maintenance of mtDNA and cellular functions. *Mitochondrion.* (2007) 7:39–44. doi: 10.1016/j.mito.2006.11.017
26. Curry JM, Tuluc M, Whitaker-Menezes D, Ames JA, Anantharaman A, Butera A, et al. Cancer metabolism, stemness and tumor recurrence: MCT1 and MCT4 are functional biomarkers of metabolic symbiosis in head and neck cancer. *Cell Cycle.* (2013) 12:1371–84. doi: 10.4161/cc.24092
27. Ullah MS, Davies AJ, Halestrap AP. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. *J Biol Chem.* (2006) 281:9030–7. doi: 10.1074/jbc.M511397200
28. Kaira K, Ohde Y, Endo M, Nakagawa K, Okumura T, Takahashi T, et al. Expression of 4F2hc (CD98) in pulmonary neuroendocrine tumors. *Oncol Rep.* (2011) 26:931–7. doi: 10.3892/or.2011.1384
29. Wang Q, Beaumont KA, Otte NJ, Font J, Bailey CG, van Geldermalsen M, et al. Targeting glutamine transport to suppress melanoma cell growth. *Int J Cancer.* (2014) 135:1060–71. doi: 10.1002/ijc.28749
30. Sakata T, Ferdous G, Tsuruta T, Satoh T, Baba S, Muto T, et al. L-type amino-acid transporter 1 as a novel biomarker for high-grade malignancy in prostate cancer. *Pathol Int.* (2009) 59:7–18. doi: 10.1111/j.1440-1827.2008.02319.x
31. Shennan DB, Thomson J. Inhibition of system L (LAT1/CD98hc) reduces the growth of cultured human breast cancer cells. *Oncol Rep.* (2008) 20:885–9. doi: 10.3892/or_00000087

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REVIEW
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Lactate in the Regulation of Tumor Microenvironment and Therapeutic Approaches

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Tumor cells must generate sufficient ATP and biosynthetic precursors in order to maintain cell proliferation requirements. Otto Warburg showed that tumor cells uptake high amounts of glucose producing large volumes of lactate even in the presence of oxygen, this process is known as "Warburg effect or aerobic glycolysis." As a consequence of such amounts of lactate there is an acidification of the extracellular pH in tumor microenvironment, ranging between 6.0 and 6.5. This acidosis favors processes such as metastasis, angiogenesis and more importantly, immunosuppression, which has been associated to a worse clinical prognosis. Thus, lactate should be thought as an important oncometabolite in the metabolic reprogramming of cancer. In this review, we summarized the role of lactate in regulating metabolic microenvironment of cancer and discuss its relevance in the up-regulation of the enzymes lactate dehydrogenase (LDH) and monocarboxilate transporters (MCTs) in tumors. The goal of this review is to expose that lactate is not only a secondary product of cellular metabolic waste of tumor cells, but also a key molecule involved in carcinogenesis as well as in tumor immune evasion. Finally, the possible targeting of lactate production in cancer treatment is discussed.

Keywords: lactate, acidification, tumor microenvironment (TME), therapy, immune response

INTRODUCTION

Cellular transformation involves the deregulated control of cell proliferation, resistance to cell death, immune evasion and circumvention of growth suppressor activities, which finally allow cancer establishment (1). Additionally, it has been observed that tumor cells have the remarkable ability to adjust their energetic metabolism as part of their mechanisms for tumor survival, this feature is now recognized as a hallmark of cancer (2). The increased metabolic rate in several neoplasms, was first studied by Otto Warburg in 1926 demonstrating that tumor cells uptake high amounts of glucose as a primary energy source, producing excessive amounts of lactate, even in the presence of oxygen (3). In 1972, Efraim Racker named such effect as the "Warburg Effect," also

known as "aerobic glycolysis" (4). Initially, it was proposed that the driving event of the enhanced glycolysis in tumor cells was caused by an irreversible damage of the mitochondrial function. Although defects in mitochondria function have been shown in some types of cancer (5), this process alone cannot explain the metabolic preference of tumor cells.

The Warburg phenotype is present in several neoplasms including breast, colon, cervical and liver cancer (6–9). The increased glucose uptake and metabolism by neoplastic cells represents the basis for tumor detection using positron emission tomography (PET); PET imaging uses a radioisotope-labeled glucose tracer, ^{18}F-fluorodeoxyglucose (^{18}F-FDG), to identify areas of high glucose uptake/metabolism in the body. After ^{18}F-FDG distribution, the radionuclide is transported into the cells by glucose transporters, and consequently phosphorylated by the hexokinase to produce ^{18}F-FDG-6-phosphate (^{18}F-FDG-6-p). Once inside the cell, the ^{18}F-FDG-6-p accumulates in the cytoplasm since this molecule cannot be further metabolized through the glycolytic pathway because it lacks the necessary 2'hydroxyl group (10). Additionally, due to its highly polar nature the ^{18}F-FDG-6-p is trapped inside the cell, thus the accumulated amounts of ^{18}F-FDG-6-p are used to identify the presence of solid tumors as well as the effectiveness of treatments (10).

The Warburg effect involves the alteration of metabolic enzymes, including hexokinase 2 (HK2), pyruvate kinase type M2 (PKM2), glucose transporter 1 (GLUT1), lactate dehydrogenase (LDH) and lactate transporters (monocarboxilate transporters [MCTs]) (11–14). Importantly, the Warburg phenotype has been associated, not only with an increased obtention of energy but also with the activation of numerous transcription factors, such as c-Myc, NF-κB, and Hypoxia-Inducible Factor 1-α (HIF 1-α) (15–17). These transcription factors can regulate the expression of metabolic enzymes resulting in the deregulated conversion of glucose to lactate (18) then promoting a "tumor lactagenesis" state (19).

Glycolysis is by far less efficient than oxidative phosphorylation for ATP production, and for this reason cancer cells increase their glucose uptake and glycolytic rate. The high utilization of glucose by cancer cells results in the accumulation of extracellular lactate affecting a number of cell types within the tumor microenvironment (TME), composed by a variety of different cell types such as endothelial cells, cancer-associated fibroblasts (CAFs), immune cells and non-cancer stroma cells (20).

For a long time, lactate was only recognized as a "metabolic waste product" derived of aerobic glycolysis, however, it has now been firmly demonstrated that lactate can be incorporated into the tricarboxylic acid (TCA) cycle and be a source of energy, and even act as an oncometabolite with signaling properties. In this review we describe the role of lactate in tumor progression, highlighting its ability to promote invasion and metastasis. We also show the role of lactate as a metabolic fuel for tumor cells, as well as its participation in drug resistance (**Figure 1**). The importance of the suppressive acidic tumor microenvironment induced by lactate is also presented. Finally, we discuss the possible targeting of lactate production as a novel therapeutic approach.

LACTATE METABOLISM BOTH IN NORMAL PHYSIOLOGY AND CANCER

Lactate (2-hydroxypropanoic acid) is a hydroxycarboxylic acid that may exist in the human body as two stereoisomers, D-lactate and L-lactate; the latter is the predominant physiological enantiomer of lactate (21). D-lactate is also present but generally accounts only for 1–5% of L-lactate concentration (22), in this review we only focus in L-lactate, designed as lactate. The pKa of the lactate/lactic acid pair is 3.8 at physiological pH, the lactic acid dissociates immediately into lactate (base form) and hydrogen (H^+) (22, 23). Under physiological conditions, lactate is used as a fuel source by the heart, brain and skeletal muscle (24); it can also be converted into glucose in the liver by the Cori cycle, serving as an alternate source of energy (25). Also, lactate acts as an inter-organ carbon shuttle, supplying both aerobic metabolism and gluconeogenesis pathways (26), in fact, now this is identified as a "lactate shuttle theory," describing that lactate, under fully aerobic conditions can transcend compartment barriers and shuttle occur within and among cells, tissues and organs (27–29), interestingly, this phenomenon is also observed in cancer, and will be described in this review.

The physiological concentration of lactate, in blood and healthy tissues is about 1.5–3 mM (30), but in cancer tissues it can be present in up to 10–30 mM concentrations (31). **Table 1** summarizes the amounts of lactate in different neoplasms.

Glucose is the major source of lactate production in most solid tumors (39). This molecule is an essential metabolic energy source for all living organisms and the structural precursor for cellular biosynthesis of proteins, lipids, and nucleic acids with ATP generation being the essential metabolic process for energy supply to the cells. Mammalian cells generate their ATP through glycolysis in the cytoplasm (2 ATP per glucose molecule) and oxidative phosphorylation (OXPHOS) in the mitochondria (32–34 ATP per glucose molecule). Normal cells (except erythrocytes and skeletal muscle cells during high intensity exercise) depend on OXPHOS for ATP production from glucose; on the contrary, cancer cells obtain their ATP by glycolysis and the final conversion of glucose to lactate (40).

Additionally, it has been demonstrated that glutamine may contribute to a small amount of lactate formation in tumor cells (41). Glutamine comprise the most abundant amino acid in blood circulation (about 500 μM), representing more than 20% of the free amino acid pool in blood and 40% in muscle (42). It was demonstrated that tumor cells require at least 10 times as much glutamine as any other amino acid in culture (43). In the mitochondrion, glutamine is deaminated to glutamate by glutaminase (GLS), later in other deamination reaction, α-ketoglutarate is generated by the enzyme glutamate dehydrogenase (GDH) finally incorporated into TCA cycle to generate malate by fumarase enzyme since α-ketoglutarate is the major anaplerotic source for TCA cycle (44). Malate is exported to the cytosol where is it converted to pyruvate by the malic enzyme which is finally converted to lactate by LDHA (41).

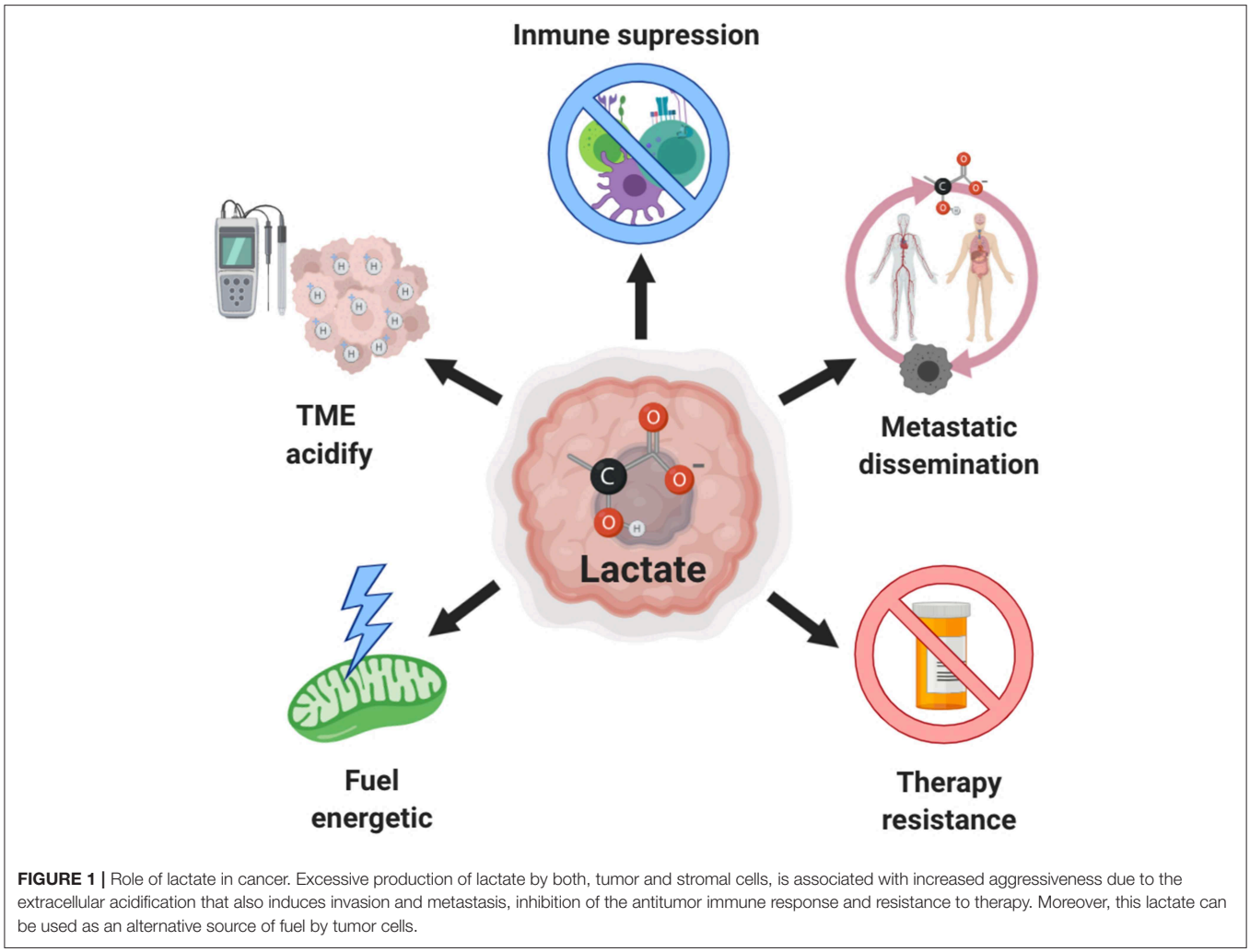

FIGURE 1 | Role of lactate in cancer. Excessive production of lactate by both, tumor and stromal cells, is associated with increased aggressiveness due to the extracellular acidification that also induces invasion and metastasis, inhibition of the antitumor immune response and resistance to therapy. Moreover, this lactate can be used as an alternative source of fuel by tumor cells.

LACTATE AS A FUEL SOURCE FOR CANCER CELLS

Despite lactate was first recognized only as a waste product of anaerobic cell metabolism, it is now known that lactate is used continuously as a fuel by diverse cells under complete aerobic conditions (29). Currently it is known that, certain cancer cells may also actively use OXPHOS or a combination of OXPHOS and glycolysis for ATP production (45, 46). Interestingly, using high-resolution mass spectrometry, it was shown that ^{13}C-lactate resides inside the mitochondria and can be used as a carbon source to synthesize lipids by cervical cancer and human lung cancer cells (47). Furthermore, it was revealed that LDHB is localized to the inner mitochondrial membrane and was associated with the regulation of the mitochondrial respiration using transmission electron microscopy (TEM) with gold-labeled lactate dehydrogenase B (LDHB) (47). Due to these results, it has been suggested that lactate is oxidized to pyruvate in the mitochondria by LDHB. However, it remains unknown how lactate enters inside the mitochondria in this cells, but it has been proposed that mitochondrial lactate import may be mediated by the monocarboxylate transporter (MCT) as in muscle and neuron cells (48, 49). Another study analyzing [3-^{13}C]-lactate metabolism *in vitro* and *in vivo* using nuclear magnetic resonance, indicated that lactate could be transported into and being oxidized by cancer cells (34, 50). Cancer cells are avid consumers of glucose, however, intratumoral levels of glucose are usually exceedingly low (51). Under these circumstances of low glucose, tumor cells uptake and oxidize lactate (52, 53). For instance, breast cancer derived-cells grown in different concentrations of glucose, produce high lactate levels, but switched from net lactate producer to consumers when glucose was limiting (54). Moreover by isotopomer analysis using (U-^{13}C)-labeled lactate, it was determined that under conditions of glucose deprivation, over 50% of the total cellular pool of TCA cycle intermediates were derived from lactate (54). Whereas it was shown that lactate can serve as a fuel source when glucose is limited, a disagreement remains in the field as to whether it enters into the TCA cycle directly or if it must first be converted to glucose through gluconeogenesis (55). Further studies are required to decipher its role in cancer, to specifically elucidate what

TABLE 1 | Lactate quantification in tumors and their association with metastatic spread.

Cancer type	Sample	Lactate concentration	Method	References
Head and neck cancer	Cryobiopsies from head and neck tumors, either with metastatic spread or without	With metastatic spread: 12.3 ± 3.3 μmol/g Without metastatic spread: 4.7 ± 1.5 μmol/g	Quantitative bioluminescence imaging	(32)
Head and neck cancer	Cryobiopsies from tumors from the head and neck	With metastatic spread: 19.9 μmol/g Without metastatic spread: 7.1 μmol/g	Quantitative bioluminescence imaging	(31)
Cervical cancer	Cryobiopsies at first diagnosis	With metastatic spread: 10.0 ± 2.9 μmol/g Without metastatic spread: 6.3 ± 2.8 μmol/g	Quantitative bioluminescence imaging	(30)
Colorectal cancer	Cryobiopsies from primary rectal adenocarcinoma	With metastatic spread: 13.4 ± 3.8 μmol/g Without metastatic spread: 6.9 μmol/g	Quantitative imaging bioluminescence	(33)
Breast cancer	Cryobiopsies from locally advanced breast cancer	Median concentration range of 0.6–8.0 μmol/g	Quantitative imaging bioluminescence	(34)
Metastatic non-small cell lung cancer	Venous and arterial blood sample	Median maximal levels was 1.8 ± 2.2 mmol/L	Enzymatic method	(35)
Human astrocytomas	Cyst content	With metastatic spread: 12.35 mmol/L Without metastatic spread: 8.28 mmol/L	Enzymatic method	(36)
Head and neck squamous carcinoma	Xenograft in nude mice	More radioresistant tumor ranged 7.3–25.9 μmol/g	Quantitative imaging bioluminescence	(37)
Head and neck squamous, melanoma, rectum carcinoma and adenocarcinoma	Xenograft in nude mice	Median concentration in central areas: 7 μmol/g Median concentration in the periphery region: 0.5 μmol/g	Quantitative imaging bioluminescence	(38)

metabolic pathway is preferred and if it is dependent on the tumor metabolism.

Regarding the participation of lactate in the synthesis of TCA cycle intermediaries, Hui et al. (52) used three genetically modified mice cancer models, two for lung cancer and one for pancreas cancer, all under fasting conditions, showing that circulating lactate contributes to the generation of TCA cycle intermediaries. This contribution was higher than of glucose in the two lung cancer mouse models. Using intravenous infusions of ^{13}C-labeled nutrients, Faubert et al. (56) showed that the circulatory turnover flux of lactate is the highest of all metabolites and exceeds that of glucose in human lung tumors. Recently, Bok et al. (57) showed that ^{13}C-pyruvate is mainly directed to lactate production, associated with tumor progression and metastases.

Although it was shown that glutamine generates lactate in human glioma cells (41), it has been also shown that high amounts of lactate promotes glutamine uptake in SiHa and HeLa cells and consequently induces the glutaminolysis pathway. This increase in the intake and metabolism of glutamine was due to the stabilization of HIF 1-α by lactate. HIF 1-α then transactivates c-MYC proto-oncogene in a pathway that mimics a response to hypoxia. c-MYC is one of the main regulators of glutaminolysis and is also overexpressed in the vast majority of tumors (58). Lactate-induced c-MYC activation triggers the expression of the glutamine transporter ASCT2 and glutaminase 1 (GLS1), both resulting in improved glutamine uptake and catabolism (59).

These findings highlight the use of lactate in the generation of TCA cycle intermediaries and its role as a regulatory molecule of glutamine incorporation and metabolism, to finally serve as a source of energy in cancer cells. Also supports the importance of the mitochondrial function in cancer development.

LACTATE SYNTHESIS: ROLE OF LDHA IN CANCER

The inter-conversion between pyruvate and lactate is mediate by the nicotinamide adenine dinucleotide (NAD^+) oxidoreductase LDH enzyme. This is a tetrameric enzyme composed of M and H protein subunits that are encoded by the LDHA and LDHB genes, respectively (60). The two subunits can then combine and form five homo or hetero tetramers in human tissues: LDH-1 (4H), LDH-2 (3H1M), LDH-3 (2H2M), LDH-4 (1H3M), and LDH-5 (4M). LDH5, also known as LDHA, is the predominant isoform found in skeletal muscle. In contrast, LDH1 also known as LDHB, is the predominant isoform found in heart muscle (61). LDHA preferentially reduces pyruvate to lactate, while LDHB supports conversion of lactate to pyruvate in cells that utilize lactate as a nutrient source for oxidative metabolism or gluconeogenesis (62). Pyruvate is reduced to produce lactate while NADH is oxidized to NAD^+ in a thermodynamically favored reaction. In the opposite direction, lactate is oxidized to form pyruvate, while NAD^+ is reduced to NADH (63).

LDHA Expression in Tumors

Several reports indicate that LDHA expression and its activity is increased in numerous types of tumors and is associated with lower event-free survival rate and with resistance to chemotherapy treatment. For instance, high LDHA levels in serum could be a negative prognostic biomarker in osteosarcoma, pancreatic cancer, and lung adenocarcinoma (64–67). On the other hand, knocking down the expression of LDHA in lung adenocarcinoma cells inhibits the proliferation, invasion, migration and colony formation (67). In human lymphoma

and pancreatic cancer, knocking down the expression of LDHA by siRNA reduces ATP levels and induces significant oxidative stress and cell death in human lymphoma and pancreatic cancer xenografts in mice (68).

The ability to monitor when a disease arises, how it progresses and to evaluate the result of treatment through non-invasive techniques is the most desirable goal in clinical setting. Non-invasive sampling is the most useful and valuable alternative because no stress is generated in the oncological patient. LDHA determination in saliva sample has been proposed for detection and monitoring of oral squamous cell carcinoma (OSCC), since the major source of salivary LDHA are the oral epithelium-shedding cells. Any pathological changes in the oral epithelium should be reflected diagnostically in the saliva, and the aggressiveness of different histological grades of OSCC could be assessed (69). Thus, the LDHA levels could be an excellent diagnostic marker. In this regard, a positive correlation between the LDHA expression and the histopathological grading was found in saliva samples from patients with OSCC (70). The expression of salivary LDHA in patients with OSCC was significantly higher than that of healthy individuals. Importantly, the levels of salivary LDHA in patients with squamous cell carcinoma of the tongue and lower oral cavity were significantly higher than other patients affected with squamous cell carcinoma in other parts of the head and neck (71).

Phosphorylation of LDHA and Its Role in Cancer

It was demonstrated that LDHA is phosphorylated at two specific tyrosine sites, tyrosine 10 (Y10), and tyrosine 83 (Y83). Phosphorylation in Y10 increases LDHA activity by enhancing the active tetrameric LDHA conformation, which induces the binding of NADH and promotes Warburg effect in human head and neck squamous cell carcinoma (HNSCC), lung cancer, breast cancer and prostate cancer cells (72). Interestingly, it was demonstrated that high levels of phosphorylated LDHA in human prostate cancer tissues were associated with short recurrence and poor survival times in patients (73). The tyrosine kinases involved in the Y10 phosphorylation of LDHA are HER2, the avian sarcoma viral oncogene v-src homolog (Src) and the Fibroblast growth factor receptor 1 (FGFR1), this phosphorylation promotes the Warburg effect and pro-invasive and pro-metastatic potential of cancer cells (73, 74). Recently, it was identified that cyclin G2 could directly interact with LDHA and negatively regulate the phosphorylation of Y10 in LDHA, although the mechanism by which cyclin G2 reduce the Y10 phosphorylation remains unknown, this interaction inhibits the Warburg effect and tumor progression in glioma (75). Taken together the phosphorylated-induced activation of LDHA provides other mechanism used by tumor cells in order to establish a malignant phenotype. However, there are very few studies on this topic, so it is important to investigate whether post-translational modifications such as phosphorylation in metabolic enzymes such as LDHA are part of the broad mechanism by which tumorigenesis is promoted by associating signaling and metabolism pathways.

NON-CANONICAL FUNCTIONS OF LDH IN CANCER

Metabolic enzymes exhibit "promiscuous" catalytic activities (76). In addition to the above-described canonical functions of LDH, it has recently been demonstrated that LDHA exhibits non-canonical roles, which are also involved in tumor progression.

Based on this, Intlekonfer et al. (77) observed that LDHA produces the oncometabolite L-2-hydroxyglutarate (L-2HG) under hypoxic conditions in glioblastoma, via alternative substrate usage and additional contributions from malate dehydrogenase 1 and 2 (MDH 1/2). The authors also demonstrated that during hypoxia, the resulting increase in L-2HG is necessary and sufficient for the induction of increased methylation of histone repressive marks such as histone 3 lysine 9 (H3K9me3). Later, the same research group also demonstrated that the L-2HG produced by LDHA is favored in an acidic environment and promotes the HIF 1-α stabilization under normoxia conditions (78). HIF 1-α is associated with metabolic regulation, specifically with tumor lactagenesis, because of the induction of the expression of LDHA, MCT4 and the membrane-bound carbonic anhydrase IX (CAIX), and in this way regulates the tumor acid environment and tumor progression (79, 80). Recently, it has been reported that LDHA translocates to the nucleus, induced by reactive oxygen species (ROS) in cervical cancer. Once in the nucleus, LDHA in its dimeric form produces the antioxidant metabolite α-hydroxybutyrate (α-HB). α-HB induces H3K79 hypermethylation through the interaction between methyl-transferase DOT1L and LDHA, demonstrating that LDHA nuclear translocation appears to be essential for maintaining redox balance and sustaining cell proliferation through epigenetic regulation (81).

This promiscuous enzymatic activity of LDHA might represent a metabolic response to multiple environmental stimuli including hypoxia and acidosis, conditions frequently found in tumor microenvironment of aggressive tumors. Future investigations will be directed at elucidating the role as well as how deregulation L-2HG and α-HB by LDHA might contribute to oncogenesis.

LACTATE TRANSPORT: ROLE OF MCTs IN CANCER

Eukaryotic cells require the efflux of lactate and H^+ to the extracellular space to prevent intracellular acidification and sustain continuously high rates of glycolysis, since the accumulation of cytosolic lactate reduces the glycolytic rate via inhibition of the rate-limiting enzyme fosfofructokinase-1 (PFK-1) (82). Lactate itself cannot cross the plasma membrane by free diffusion. Hence, it requires a specific transport mechanism provided by proton-like MCTs (83, 84) It was identified that lactate shuttle is driven by a concentration and pH gradient or by the cellular redox state in rat skeletal muscle (28).

MCTs belong to the family of solute carrier (SLC) transporters, composed by 52 families of the membrane transport proteins; in particular, the SLC16 family encodes 14 MCTs isoforms and

plays a significant role in the absorption, tissue distribution and clearance of both, endogenous and exogenous compounds (83). MCTs 1–4 are known lactate transporters, but they can carry other monocarboxylates such as pyruvate and the ketone bodies such as acetoacetate, β-hydroxybutyrate and acetate (85). Two proteins, basigin (CD147) and embigin (gp70), have been identified as important chaperone proteins implicated in the trafficking of the MCTs 1–4 to the plasma membrane (86–88). Recently, it was discovered that the TMPRSS11B protease also regulates the function of MCT4 mediated by CD147 in cancer cells (89).

Over-expression of lactate transporters is a common feature of some cancers with high metabolic rate (90). For instance, high expression of MCT1, MCT4 and its chaperone CD147 is associated with decreased progression-free survival in clear cell renal cell carcinoma, head and neck cancers and neuroblastoma (91–93). In human bladder cancer, high MCT1 expression was associated to shorter overall survival than those with low-MCT1 expression, and the knockdown of MCT1 inhibits cell proliferation, migration and invasion in a cellular model (94). In cervical cancer, it has been shown that CD147 expression was higher in squamous and adenocarcinoma tissue than in their non-neoplastic counterparts, and both MCT1 and MCT4 were more frequently expressed in CD147 positive cases (90, 95). This over-expression of MCT1 and 4 was associated with lymph node and distant metastases in melanoma and adenocarcinoma (96, 97). Interestingly, disruption of MCT1 or MCT4 in renal cell carcinoma, pancreatic cancer, breast cancer and prostate cancer has been shown to exert significant antitumor effects both *in vivo* and *in vitro* with increased accumulation of intracellular lactate (98, 99). In $MCT4^{-}/^{-}$ and wild type mice with oral cancer, it was observed that mouse tongues from $MCT4^{-}/^{-}$ mice developed significantly fewer and less extended invasive lesions than wild type mice indicating an important role for MCT4 in tumor metastasis (100). Additionally, MCT4 was detected in foci of the basal layer undergoing transformation, in areas of carcinoma *in situ*, and also in invasive carcinomas (100). These findings support the important role of MCTs in tumor metastasis development.

LACTATE IN TUMOR MICROENVIRONMENT: TUMOR-ASSOCIATED ACIDITY

Lactate is largely produced within the TME and is used as an energy-rich substrate, signaling molecule and as an important immune suppressor by tumors (101). TME consist of malignant cancer cells, endothelial cells, cancer associated fibroblasts (CAFs), immune cells and non-cancer cell stroma conformed by numerous peptide factors (growth factors, chemokines, cytokines and antibodies) (20). The glycolytic cancer cells and CAFs are the main producers of lactate, simply because they are the most abundant populations within the neoplasm (101). TME enforce to metabolic adaptability, physical pressure, oxidative stress, nutrient deprivation and competition, immune surveillance as well as adaptability to hypoxic and acidic environment having an enormous impact on tumor malignancy (102). In agreement with this, it was demonstrated that pH 6–6.5 in tumor microenvironment is associated with metastasis, angiogenesis and therapy resistance, a characteristic phenotype of more aggressive tumors (103, 104).This tumor acidification is a consequence of high lactate production in a poorly perfused environment, as well as a high activity of the CAIX (105). CAIX is a transmembrane protein belonging to the α carbonic anhydrase family of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate ions and H^{+} (106) that is overexpressed in tumors and is associated with unfavorable responses to first-line therapy (107). The tumor hypoxia induces the expression of the CA9 gene in a HIF 1-α dependent manner; on the other hand it was shown that lactate promotes normoxic expression of CA9 genes through HIF 1-α stabilization independently of hypoxia (108). CAIX acts as an extracellular pH-stat, maintaining an acidic tumor extracellular pH favoring invasion and metastasis (105).

By cooperating with anion exchanger 2 (AE2) and Na^{+}/bicarbonate co-transporter 1 (NBCe1), CAIX serves as a pH regulatory component that provides acid-base balance. Interestingly, it was shown that CAIX work in support with diverse acid extruders such as MCT1 and MCT4 (109, 110), and as mentioned earlier, MCT-mediated H^{+} efflux exacerbates extracellular acidification and supports the formation of a hostile environment where cancer cells, that have adapted to these conditions, can outcompete normal cells, which further enhances tumor progression.

The proteoglycan like (PG) domain of CAIX could function as a "proton antenna" to facilitate MCT1 and MCT4 transport activity in hypoxic cancer cells (111). Recently, it was found that CAIV also facilitate the activity of MCT1, MCT2, and MCT4 via a non-catalytic mechanism and requires direct biding between CAIV in the amino acid residue His-88 and a charged amino acid in the extracellular domain of the chaperones CD147 and GP70 (112).

LACTATE EXCHANGE BETWEEN CANCER CELLS: METABOLIC SYMBIOSIS

As previously described, the lactate shuttle theory occurs in normal physiology (29). Interestingly, this mechanism is also observed in cancer cells where it is known as "metabolic symbiosis." Sonveaux et al. (53) found that cervical cancer-derived SiHa cells, which expressed higher levels of MCT1 but lower levels of MCT4, consumed significantly more lactate and less glucose than colorectal cancer WiDr cells, conversely, WiDr cells, which expressed higher levels of MCT4 and lower levels of MCT1, consumed less lactate and more glucose than SiHa cells. Consistent with the proposed "tumor metabolic symbiosis," metabolically heterogeneous regions within and between tumors were identified, which are regulated by TME conditions such as hypoxia in the center of the tumor and better oxygenated regions in the periphery (113) (**Figure 2**). Furthermore, it was shown that a high lactate uptake occur only in aerobic tumor regions in breast cancer (34). The oxygenated cancer cells, close

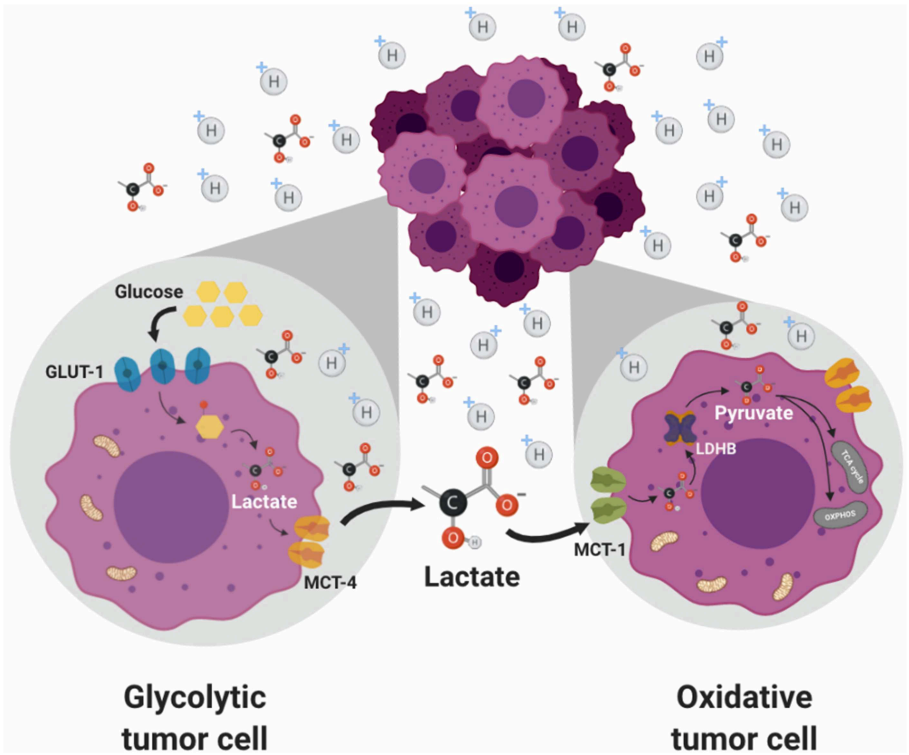

FIGURE 2 | Metabolic symbiosis. Solid tumors are characterized by metabolic heterogeneity. Glycolytic tumor cancers are sustained by a favorable location with high nutritional availability. This phenotype is regulated by a differential expression of MCTs, where glycolytic cells preferentially express MCT4 favoring lactate export. Meanwhile, oxidative cells express MCT1 transporter which preferentially promotes lactate import. Then, lactate is used by these cells as an energetic source due to its conversion to pyruvate which enters the TCA cycle in the mitochondria. The presence of lactate allows a metabolic symbiosis between hypoxic cancer cells (glycolytic) and with normoxic cancer cells (oxidative).

to blood vessels, are sustained by a favorable location with high nutritional availability and can establish a metabolic symbiosis with hypoxic cancer cells, essential for the progression of a fast-growing tumor characterized by hypoxic regions. This tumor metabolic symbiosis is supported by differences in MCT1 and MTC4 expression and activity. MCT1 (SLC16A1) is ubiquitously expressed and has a high affinity for lactate (3–6 mM); this transporter is the main lactate exporter where intracellular lactate levels are low (84). On the other hand, MCT4 (SLC16A3) is expressed strongly in glycolytic tissue (114) and has low affinity for lactate (25–30 mM) and it does not import serum lactate (<2 mM) (115). In tumors, it was demonstrated that both, the glucose transporter 1 (GLUT1) and MCT4 were induced in distal hypoxic cells in a HIF 1-α-dependent fashion (116). Instead, tumor cells proximal to blood vessels, expressed the lactate transporter MCT1. These differences in the regulation of the expression and activity of lactate transporters underpin the metabolic symbiotic model.

Despite the avidity by which tumor cells uptake glucose, glutamine or lactate *in vivo*, encounter conditions of nutrient scarcity are often an issue as a result of the increased rate of nutrients consumption and the inadequacies of the tumor vascular supply, for this reason tumors have develop various nutrient scavenging strategies to bypass these limitations, for instance lactate exchange (117).

LACTATE EXCHANGE BETWEEN CANCER CELLS AND CAFs: REVERSE WARBURG EFFECT

Another way by which it is believed that cancer cells survive under nutrient scarcity is by a cross talk between stroma cells from tumor microenvironment and tumoral cells, process known as "reverse Warburg effect," in which aerobic glycolysis takes place in CAFs, rather than in epithelial cancer cells, fueling cancer cells via metabolite transfer, particularly lactate (118) (**Figure 3**). CAFs constitute the more abundant cell population in tumors and have been associated with tumor progression, invasion and metastasis directly through paracrine pathways (119). Fibroblasts possess a metabolic phenotype characterized by increased glycolysis and decreased OXPHOS. During tumor initiation, neoplastic cells recruit CAFs to the surrounding area through the ROS production inducing oxidative stress. As a consequence, CAFs suffer DNA damage, initiating several catabolic pathways, such as autophagy and more specifically mitophagy (120). Autophagy is a catabolic process of macromolecules (proteins, lipids) and organelles whereby intracellular components are enveloped in double-membrane vesicles, known as autophagosomes, which ultimately fuse with lysosomes where the content is degraded and recycled into the cytosol (121). On the other hand, mitophagy is a specific

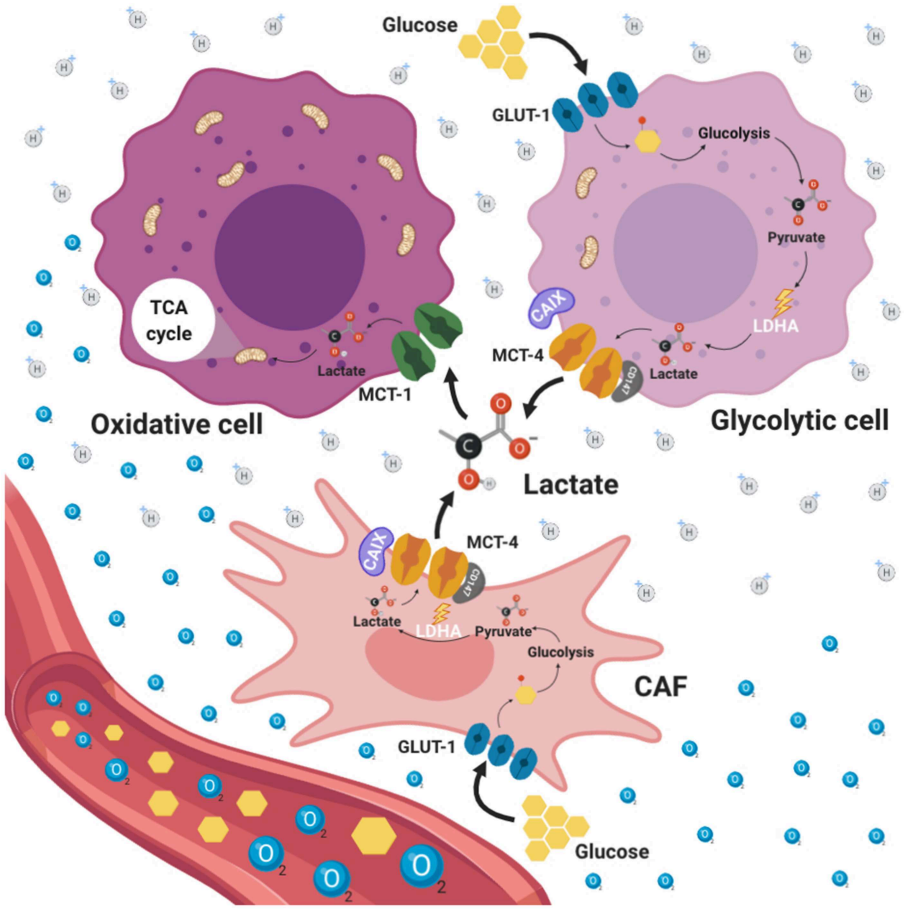

FIGURE 3 | Reverse Warburg effect. Tumor microenvironment (TME) is an ultrastructure consisting of different cell types including tumor cells, stromal cells, immune cells, blood vessels and cellular metabolites such as lactate. TME promotes different processes aimed to enforce metabolic adaptability, oxidative stress, nutrient competition, immune surveillance. This adaptability to hypoxic and acidic environments stimulates tumor malignancy. Tumor cells and cancer associated fibroblasts (CAFs) with a glycolytic phenotype represent the principal source of lactate production within TME which is favored by the presence of GLUT1. Additionally, CAFs exhibit high expression of MCT4 dedicated to lactate export. In this way, CAFs can interchange lactate with oxidative tumor cells which use lactate as a fuel through the TCA cycle. This phenomenon is known as reverse Warburg effect.

process performed in CAFs used for the removal of mitochondria through autophagy. CAFs with dysfunctional mitochondria shift their metabolism toward glycolysis, producing energy-rich molecules, such as lactate, which is exported to the tumor microenvironment and consequently can be used by neighboring cancer cells via oxidative mitochondrial metabolism providing an alternative energy source promoting tumor initiation, progression and metastasis (122). Interestingly, it has been shown that CAFs are able to stimulate cancer cell proliferation and progression through multiple mechanisms. For instance, in a lung cancer model, CAFs underwent increased aerobic glycolysis and promoted the epithelial mesenchymal transition, migration and invasion of non-small-cell lung carcinoma (NSCLC) cells, in contrast, NSCLC cells experienced enhanced oxidative phosphorylation upon CAF stimulation, with an increase in ATP generation, thereby an activation of the PIK3/Akt and MAPK/ERK pathways occurred (123). Furthermore, colorectal cancer cells induce oxidative stress in microenvironment fibroblast, which then undergo metabolic changes, including increased expression of glycolytic enzymes, reduced TCA cycle enzymes and autophagy proteins such as microtubule-associated protein 1A/1B-light chain 3 (LC3), Bcl-2 interacting protein 3(BNIP3), and p62 (124). In this model, the increased autophagy promoted survival of cancer cells by providing nutrients for cell proliferation and protection against oxidative damage. Moreover, hypoxia-induced oxidized ATM promoted the glycolytic activity of CAFs by phosphorylating GLUT1 at S490 and in consequence induced its membrane translocation (125). In addition, the PKM2 expression in CAFs was up regulated by the activation of ATM through PI3K/Akt signaling pathway.

Another study showed that intercellular contact activated stromal fibroblasts, triggering the expression of GLUT1, lactate production, and extrusion of lactate by the *de novo* expressed MCT4 (126). Conversely, prostate cancer cells, upon contact with CAFs, were reprogrammed toward aerobic metabolism, with a

decrease in GLUT1 expression and an increase in lactate upload via MCT1. Metabolic reprogramming of both stromal and cancer cells was under strict control of the HIF 1-α, which drove redox- and SIRT3-dependent stabilization of HIF 1-α in normoxic conditions. Prostate cancer cells gradually became independent of glucose consumption, while developing a dependence on lactate driving anabolic pathways and thereby cell growth (126). Lactate shuttle between CAFs (released by MCT4) and tumor cells (absorbed via MCT1) may accelerate tumor cell invasion by activation of TGF-β1/p38 MAPK/MMP2/9 signaling (125).

This reverse Warburg effect provides tumoral metabolic plasticity that enables tumor cells to adapt to variations in microenvironment and represents a change to the paradigm on the metabolism of neoplastic cells, indicating that not all tumors depend on glycolysis (Warburg effect), since some tumors exhibit high dependence of OXPHOS and consequently of mitochondrial function (127). It has been observed that mitochondrial metabolism is important for cancer development. Interestingly, frozen sections of human breast tumors exhibit have a high expression and activity of cytochrome C oxidase (COX), NADH and succinate dehydrogenase in comparison to normal cells (122). This effect was related to greater aggressiveness of the tumors. Moreover, it was shown that the mitochondrial complex I NADH dehydrogenase activity is a critical player in the aggressive phenotype in breast cancer through the regulation of NAD^+/NADH redox balance, mTORC1 activity and autophagy (46). The mitochondrial function and its relation with cancer development is a very interesting topic excellently discussed in the review of Vyas et al. (5).

LACTATE AS A KEY MOLECULE IN REGULATION OF THE IMMUNE RESPONSE IN CANCER

The immune system is responsible for protecting the body from damage caused either by pathogens or by tumor cells through the detection and elimination of aberrant cells (128). The presence of neoplastic cells causes the activation of both, the innate and adaptive immune responses in order to maintain homeostasis (129). Nevertheless, tumor cells have developed different mechanisms to evade the immune system including a constant remodeling at the genetic, epigenetic and metabolic levels, in order to resist apoptosis and select tumor variant cells resistant to immune recognition. In addition, TME favors the induction and recruitment of different immune cells and molecules constituting an immunosuppressive environment, favoring the development of the tumor mass (130). It has been shown that metabolic alterations play an important role in cancer development, progression and maintenance (131). As part of the high metabolic rate and reprograming, tumor cells secrete metabolic products as lactate, which is thought to act as an important oncometabolite in the metabolic reprogramming of cancer. In turn, the high levels of secreted lactate promote acidosis in the tumoral environment favoring processes such as metastasis, angiogenesis and importantly, immunosuppression, which has been associated to a worse clinical prognosis (132).

Different cells are involved in the recognition and elimination of tumor cells including natural killer (NK), natural killer T (NKT) cells, macrophages, dendritic cells (DC), macrophages, and lymphocytes (129).

NK cells induce the destruction of stressed cells, cells infected by viruses or bacteria as well as tumor cells (133); this last action performed through their "killer" receptor (KIR) (134). The major histocompatibility class I (MHC-I) complex is recognized by KIR receptors inhibiting the activation of NK cells, however, tumor cells display diminished amounts of MHC-I which in consequence triggers the activation of NK cells, with the subsequent release of their cytoplasmic granules containing granzyme and perforin finally inducing cell lysis (135). Nevertheless, tumor cells inhibit the activation of NK cells triggered by lacking of MCH class I expression through the release of soluble molecules such as MHC class I chain-related protein A (MICA) and MHC class I chain-related protein B (MICB), which bind to the activator receptor (NKG2D) on NK cells surface causing the endocytosis and its subsequent degradation, leading to the inactivation of NK cells (136). In addition to this mechanism for inhibiting NK cells action, it has been demonstrated that the presence of lactate induces the apoptosis of NK cell, since lactate decreases the intracellular pH resulting in mitochondrial dysfunction in colon cancer-derived cells (137). It was also observed that those NK cells that migrate to the tumor cannot regulate intracellular pH, causing mitochondrial stress and subsequent apoptosis.

Using a pancreatic cancer-derived mouse xenografted model, the silencing of LDH caused a reduction of the tumor associated to a better cytolytic activity of NK cells (138). Thus, tumor microenvironment easily affects immune actions by producing lactate, favoring the development of cancer.

NKT are another immune cells with antitumor activity, which recognize glycolipids through the CD1d receptor on the tumor cells, this interaction activate its antitumor action releasing the content of their cytoplasmic granules (perforin and granzyme B) as well as cytokines favoring activation of both, innate and adaptative immune response (139). It has been shown that lactate present in TME, blocks IFNγ, and IL-4 production from NKT cells, since lactate inhibits mTOR signaling due to the inhibition of the nuclear translocation of promyelocytic leukemia zinc-finger (PLZF) avoiding the activation of NKT cells (140). These results are in agreement with those reported recently by Kumar et al. (141), showing that high lactate environment is detrimental for NKT cell survival and proliferation. This indicates that the production of lactate by the tumor microenvironment inhibits the anti-tumor action of NK and NKT cells, promoting tumor development (**Figure 4**).

DCs are antigen-presenting cells, which play a major role in the innate and adaptive immune responses (142). DCs main function relies on its ability to detect and phagocytize pathogens, but also on the recognition of tumor cells, by processing and presenting antigens that finally activates virgin T lymphocytes (143). DCs present the antigen through the MHC-II activating $CD4^+$ T (LT) or helper cells, that depending on the produced

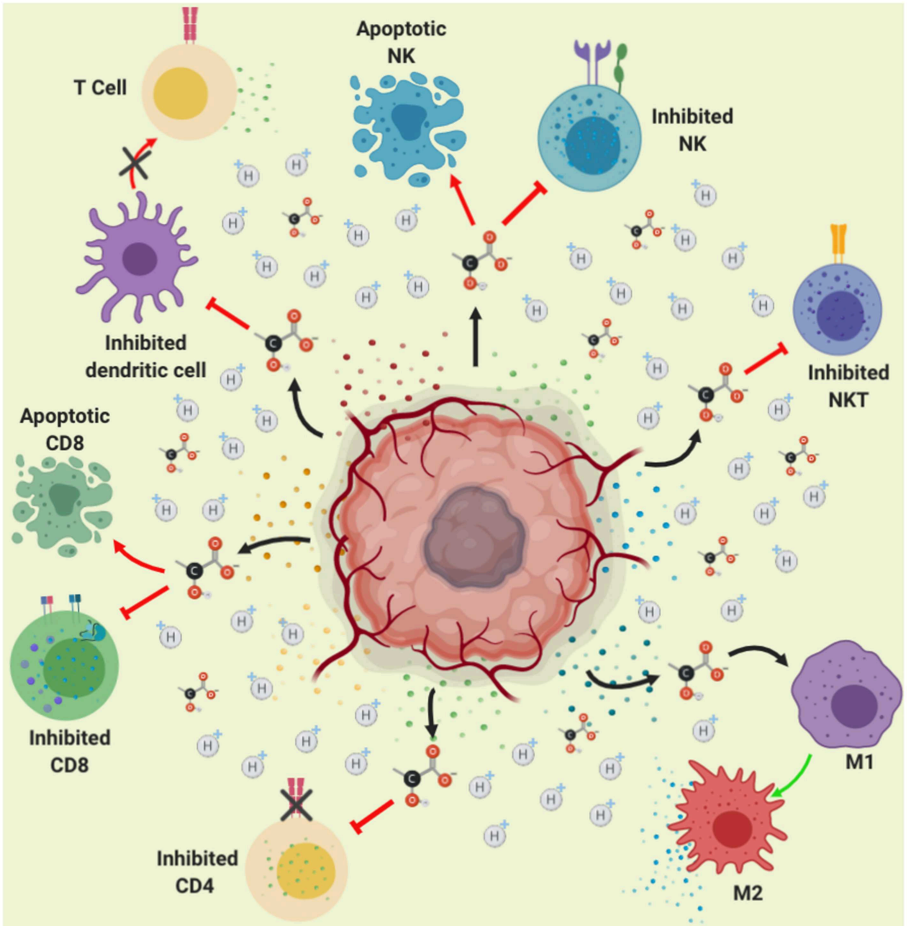

FIGURE 4 | Role of lactate in immune suppression. Lactate secretion by tumor cells promotes acidification of the tumor microenvironment (represented in yellow color). This acidification of the medium, reduces the pH within the immune cells affecting signaling pathways finally causing inhibition of the activation and proliferation of CD4, CD8, NK, NKT, and dendritic cells. Moreover, lactate-induced acidification causes apoptosis in CD8 lymphocytes and NK cells, thus lactate contributes to immune evasion. Furthermore, the acidification of the medium causes the polarization of the macrophages toward the M2 subpopulation, which favors growth, invasion and migration of the tumor.

cytokine environment, these will differentiate into a variety of subpopulations of helper T cells mainly Th1, due to the action of IL-12 and IFN-γ produced by NK, NKT and macrophage cells (144, 145). In addition, DCs present antigens to cytotoxic T lymphocytes (CTL) or CD8, these cells recognize tumor antigen through MHC-I producing the elimination of tumors (146). After the recognition of non-own peptides (such as tumor antigens) through MHC-I, CD8 T cells will be activated causing cytokine release, mainly TNF-α and IFN-γ (147), as well as their cytoplasmic granule (perforin and granzime B) content toward neoplastic cells, it also induces apoptosis through the interaction of dead molecules such as Fas with Fas Ligand present in the tumor cells, causing lysis and apoptosis of the tumor cells (148). The T lymphocytes (CD4 and CD8) play an important role in eliminating tumor cells; this process is known as immune-surveillance (149). The lack of response of the lymphocytes could also be due to the fact that it has been shown that lactate affects the dendritic cells, prevents their differentiation and makes them tolerogenic, leading to an increase in the production of IL-10, a potent immuno-suppressive cytokine (150).

IL-10 is an anti-inflammatory cytokine with potent immune-suppressive action, since it inhibits the production of pro-inflammatory cytokines such as IFNγ, TNFα, IL-1β, and IL-6; moreover, IL-10 prevents DC maturation in part by inhibiting the expression of IL-12, necessary for Activating of type 1 helper T cells and stimulates the production of cytotoxic T cells and NK cells. It also stimulates the production of interferon (151, 152). Also, IL-10 inhibits Th1 differentiation and production of IL-12 (153). In addition, IL-10 inhibits the expression of MCH-I and different co-stimulatory molecules inhibiting T cell activation (154, 155). Several reports indicate an IL-10 increase in serum levels in patients with different types of cancer such as hepatocellular, head and neck, lymphoma, leukemia and melanoma (155). Therefore, over regulation of IL-10 production by tumor cells promotes tumor progression through the escape of immunosurveillance performed by NK,

CD4, and CD8 lymphocytes. In addition, lactate produced by the tumor cell promotes the overexpression of IL-23, present in different types of tumors (colon, breast, stomach, melanoma) (156), the presence of this cytokine promotes expression of IL-17, Matrix metalloprotease 9 (MMP-9), increases angiogenesis and reduces the infiltration of CD8 in the tumor, promoting immunosuppression and tumor growth (157). Thus, increased IL-10 favors tumor microenvironment.

A study quantified lactate levels in the serum of patients with different malignancies (lymphoid malignancies, myeloid malignancies, breast cancer, gastrointestinal cancer, urogenital cancer, sarcoma, lung cancer, melanoma and other types of cancer), finding high levels of lactate, furthermore, the authors demonstrated that lactate inhibits T-cell proliferation and alters the cytokine production of CTLs in cultured CTLs (158), therefore, lactate promotes immunosuppression and the development of cancer through the inhibition of T lymphocytes.

Lactate produced by tumor microenvironment participates in immune escape through an inhibition of lymphocytes activity. As demonstrated in samples of melanoma patients showing that high LDHA expression is associated not only with poor prognosis, reduced disease-free survival, but also with lower expression of T cell markers (159). Moreover, in a melanoma mouse model it was found that tumor-derived lactate reduced the numbers and activity of $CD8^+$ T cells as well as NK cells, both *in vitro* and *in vivo*. This because lactate concentrations above 20 mM caused the apoptosis of T and NK cells, which may explain smaller proportions of T cells and NK cells in tumors with higher concentrations of lactate (159). Similar results were observed recently by Daneshmandi et al. (160), where blocking of LDHA in melanoma tumors effectively enhances infiltration of $CD8^+$ T cells and NK cells in the tumor microenvironment. Interestingly, they also demonstrated that blocking LDHA in tumor cells improves the efficacy of anti-programmed cell death-1 (PD-1) therapy in melanoma (160). Therefore, this is a mechanism used by tumor cells to evade the action of T lymphocytes. Moreover, the acidic pH (6.5) suppress T-cell functions including IL-2 secretion and the activation of T-cell receptors and the treatment with proton pump inhibitor (esomeprazole) delayed cancer progression in tumor bearing mice (161). Recently, it was shown that the acidic pH environment (6.6) blockades the T-cell activation and decreases IFNγ secretion (162).

There is great evidence indicating that lactate promotes immunosuppression thus preventing the recognition of tumor cells and favoring carcinogenesis. Interestingly, lactate has a different effect on macrophages, as demonstrated by Colegio et al. (163) tumor-cell-derived lactate has an important impact in the macrophages polarization and the promotion of tumor growth. This is because lactate induces mainly vascular endothelial growth factor (VEGF) and arginase 1 (Arg1) expression via HIF 1-α, favoring the TAM polarization. Moreover, the upregulation of VEGF and Arg1 in macrophages contributes to the development of cancer since tumor growth is supported by inducing neovascularization and by providing the substrates for cancer cell proliferation. Otherwise, it has been demonstrated that lactate activate human macrophages to a M2 phenotype and stimulate the secretion of Chemokine (C-C motif) ligand 5 (CCL5) by activation of Notch signaling in macrophages. The authors also found that CCL5 increased cell migration and induced cancer cell epithelial to mesenchymal transition in a breast cancer cell model (164). Additionally, it was shown that lactate activates the ERK/STAT3 signaling inducing the M2 macrophage polarization favoring proliferation, migration, and angiogenesis in a breast cancer model (165).

LACTATE IN TUMOR METASTASIS AND THERAPY RESISTANCE

Metastatic dissemination represents a malignant character of cancer with important clinical consequences since the majority of cancer-associated deaths are caused by metastatic disease rather that the primary tumors (166). Intratumor lactate levels can be used as a prognostic factor and a therapy response biomarker. It has been shown that high concentrations of lactate in biopsies of cervical, lung, head and neck, colorectal and breast cancers are associated with an increased risk for developing metastasis, and such levels of lactate indicate a bad prognosis for survival in cancer patients (30, 31, 35, 167) (**Table 1**). In human astrocytomas, a positive correlation between the grade of lesion and high lactate concentration was found using stereotactic brain biopsy specimens (36). Moreover, using imaging bioluminescence from primary cryo-tumor sections of human cancers, lactate concentrations were significantly higher in cervical tumors with metastatic spread (30). Another study encompassing 34 biopsies from patients with cancers of the head and neck, it was demonstrated that elevated tumor lactate concentrations are associated with the subsequent development of nodal or distant metastases (31).

The measurement of intratumor lactate levels using non-invasive methodologies; such as nuclear magnetic resonance (MRS) is currently used (168). In HER2-positive breast cancer lactate can be used as a quantitative and non-invasive biomarker of sensitivity to trastuzumab. Using MRS in a cohort of 39 frozen HER2-positive breast cancer specimens of patients who showed response to trastuzumab, a positive correlation between the transcript levels of HER2 and increased intratumor lactate concentration was found, moreover *in vitro* analyses using HER2-high expression (ZR75.30, SKBR3, BT474, and HCC1954) or HER2-low expression (MDAMB361 and MDAMB453) cell lines, it was found a direct correlation between HER2 transcript levels and lactate content in milieu (169).

Resistance to therapy is frequently developed during the clinical application of antineoplastic agents and is a major obstacle in the treatment of malignant cases (170). A substantial percentage of cancer patients exposed to an antineoplastic agent either does not benefit from the treatment (primary resistance) and shown reduced responsiveness or undergo tumor relapse progression (secondary resistance) (171). This resistance may be due to both, cell-autonomous and non-cell-autonomous mechanisms, TME is important in the initiation and maintenance of non-cell-autonomous drug resistance through various mechanisms including hypoxia, extracellular acidity and production of soluble factors such as lactate (172) (**Figure 1**). The

role of lactate in resistance to therapy has been demonstrated using *in vivo* and *in vitro* models. High lactate concentration in xenografted Nude mice with five human HNCSCC cell lines treated with irradiation (4 Gy) within 6 weeks correlates with radio resistance (37). In NSCLC, it has been shown that lactate is a key molecule involved in resistance to therapy based on tyrosine kinase inhibitors (TKIs), specifically with c-MET receptor tyrosine kinase inhibitor JNJ-605 and the epidermal growth factor receptor (EGFR) inhibitor erlotinib (173). In this work, the authors demonstrated that prolonged treatment with these TKIs induced lactate production by tumor cells, which in turn instructed the TME cells to produce hepatocyte growth factor (HGF), enforcing drug resistance and tumor progression. Targeting tumor lactate metabolism was sufficient to overcome resistance, demonstrating the causative role of lactate in resistance to therapy. Another study tested several metabolic inhibitors including BEZ235, GDC0980 (dual PI3K/mTOR inhibitors), or LY294002 and GDC0942 (PI3K inhibitors) showing an inhibition of cell proliferation of breast cancer cells in high glucose media (54). Nevertheless, when lactate was used as the primary metabolic substrate these cells were completely resistant to these inhibitors, suggesting that cancer cells bypass the need for glycolysis by utilizing lactate and are thus less sensitive to PI3K/mTOR inhibitors.

Due to the role of lactate in tumor initiation and metastatic dissemination previously mentioned, impairing lactate homeostasis is a promising approach for cancer therapeutics and has been implemented in several preclinical and clinical trials, it is also essential to establish a synergy between lactate inhibitors and other adjuvant therapies.

THERAPEUTIC APPROACHES IN LACTATE METABOLISM

Targeting Lactate Production

Glycolytic tumors undergo a metabolic reprogramming transforming themselves into a highly glycolytic and poorly oxidative phenotype with lactate formation as the end product despite normoxic conditions. This high glycolytic metabolism supplies precursors for biomolecules in cellular structure and processes allowing cell survival and proliferation (174, 175). In agreement with the above mentioned with regard to essential role of lactate in tumor development, metastasis and its role in drug resistance, impairing the lactate biogenesis could be a promising approach to cancer therapeutics (**Table 2**).

Several methods have focused in targeting lactate production, for instance Le et al. (68) demonstrated an increase in oxygen consumption, ROS production and late cell death even necrosis, in P493 cells (B-lymphoid cells) after inhibiting the expression of LDHA using siRNAs as well as employing a Gossypol analog as FX11, a direct competitive inhibitor of LDHA. Another compound FK866, that hinders the NAD^+ synthesis through direct inhibition of Nicotinamide Phosphoribosyl transferase (NAMPT) was used. The use of both molecules was toxic for P493 cells either alone or in combination, causing a reduction of mitochondrial membrane potential resulting in profound inhibition of cell proliferation. Tumor xenograft models using P493 (lymphoma) and P198 (pancreatic) cells were performed in order to demonstrate the potential of both compounds in the inhibition of tumorigenesis *in vivo*. FX11 effectively inhibited tumor growth in xenografts derived from both cell lines; the combination of FX11 with FK866 induced tumor regression in the human lymphoma xenograft model. These results showed that LDHA is required for tumor progression where targeting cancer metabolism using small molecules provides a manner for controlling tumor growth.

As part of the responses of tumor metabolic stress, heat shock proteins (HSPs) are rapidly expressed, stress signals include a wide variety of physiological and environmental insults, which are proven to be essential for survival, this protective mechanism is usually referred as "Heat shock response" (HSR). Moreover, there is evidence that a wide range of human cancers exhibit an over-expression of HSPs providing a meaning for cell proliferation, differentiation, invasion, metastasis and evasion of the immune system (187). The HSF-1 transcriptional factor regulates the expression of HSPs but also regulates glucose metabolism by activating the expression of LDHA (188). In order to set the connection between HSR and LDH, an inhibitor of the LDH activity (Oxamate) by direct competition with its natural substrate was used in a hepatocellular carcinoma (HCC) derived cell model. Oxamate, was found to impact the constitutively activated HSR by reducing the levels of the HSP-27,−72, and−90 (181). Additionally, Galloflavin, hindered the ATPase activity of HSP 72 and 90, both compounds resulted in cell senescence. Taken together, the inhibition of LDH could be an efficient way to reduce the constitutively activated HSR in cancer cells by hindering the function of the three major molecular chaperones involved in tumorigenesis.

Targeting lactate metabolism as a therapeutic approach to defeat drug resistance has also been tested in different tumors. For instance, Das et al. (179) induced tamoxifen-resistant breast cancer cell lines (TAM-MCF-7 and -T47D) in order to establish a connection between LDHA and the induced pro-survival mechanism autophagy. The pharmacological and genetic inhibition of LDHA re-sensitized the TAM-resistant breast cancer cells to tamoxifen, but also inhibited the autophagy process therefore increasing cell death. These results provided a link between LDHA and Beclin-1, an important regulator of autophagy, in the induction of the cytoprotective autophagy of TAM-resistant breast cancer cells. Moreover, the depletion of LDHA reverted the EMT-like process attenuating the invasive and migratory properties of TAM-resistant cells. These results reveal that targeting the LDHA enzyme may be a novel strategy to combat glycolytic chemo-resistant cancers.

Given the importance of lactate metabolism in different types of cancers, the discovery and development of new molecules that could inhibit LDHA activity is urgently needed. Only a few molecules have started tests in clinical trials, for this reason there is a trend to optimize existing compounds, such is the case of compound 5, that was used as a template for molecular docking, then the 200 top-ranked compounds with the highest total binding scores were selected, however, only 1 molecule (compound 11: 11c) from 7 candidates was employed

TABLE 2 | Approaches for inhibit lactate production and transport.

Inhibitor(s)	Mechanism of action	Type of cancer or cell/animal model	Research phase	Limitations	References
5 designed peptides (QLYNL, LIYNLL, IYNLLK, KVVYNVA, and KVVYNV)	LDHA tetramerization inhibition, affecting the activity of the enzyme	None	*In silico* modeling	*In vivo* investigation of these peptides on cancer cell lines is needed to evaluate their biological potential	(176)
Compound 24	24c interacts directly with the binding pocket of LDHA affecting the activity of the enzyme	Pancreas carcinoma (MiaPaCa-2)	Pre-clinical	No limitations were shown, indeed 24c did not affect the body weight of the mice, indicating low toxicity of the compound	(177)
1-(Phenylseleno)-4-(Trifluoromethyl) Benzene (PSTMB)	This allosteric inhibitor of LDHA modifies the pyruvate binding site due to conformational changes on the enzyme by non-competition inhibition	Large cell lung cancer (NCI-H460) Breast cancer (MCF-7) Hepatocellular carcinoma (Hep3B) Malignant melanoma (A375) Colorectal adenocarcinoma (HT29) Murine lung cancer cells (LLC)	Pre-clinical	No limitations were shown, even in normal human bronchial epithelial BEAS-2B cells, the cytotoxic effect of PSTMB was limited	(178)
Oxamate siRNA LDHA gene	Oxamate is a non-competitive inhibitor which has same the structure of pyruvate, this compound inhibits LDHA activity Small interfering RNA use to regulate the expression of LDHA gene	Breast cancer (MCF-7 and T47D)	Pre-clinical	No limitations were shown	(179)
Compounds 5 and 11	Both compounds maintain the same hydrogen bond interactions with LDHA, however 11c has extra interactions which could give rise to its inhibitory activity against LDHA	Osteosarcoma (MG-63)	Pre-clinical	No limitations were shown, however further experiments with different cancer models are needed to ensure its biological efficacy	(180)
Oxamate Galloflavin	Oxamate a non-competitive inhibitor hinders LDH activity Galloflavin inhibits human LDH isoforms preferentially binding the free enzyme, without competing with the substrate or cofactor	Liver cancer (PLC/PRF/5)	Pre-clinical	No limitations were shown	(181)
siRNA LDHA gene FX11 FK866	Small interfering RNAs for knocking-down the expression of LDHA gene FX11 is a competitive inhibitor of LDHA FK866 hinders the NAD^+ synthesis through direct inhibition of Nicotinamide Phosphoribosyl transferase (NAMPT)	B-lymphoid cells (P493) Pancreatic cancer (P198)	Pre-clinical	The combination of both compounds was toxic for P493 cells causing a reduction of mitochondrial membrane potential resulting in profound inhibition of cell proliferation In the *in vivo* assay, animals treated only with FX11 did not lose weight or showed any alterations in blood and chemistry studies. However, two of five studied animals treated with FK866 did show mild thrombocytopenia. Remarkably, the combination of FX11 and FK866 increased BUN	(68)
AZD3965	Selective inhibitor of human MCT1 with additional activity against MCT2 This compound hinders lactate transport, consequently increasing intracellular levels followed by glycolytic feedback and increased flux into the TCA cycle	Human diffuse large B-cell lymphomas (HBL-1 and TMD8) Human B-cell lymphoma (WSU-DLCL-2 and SU-DHL10) Lymphoblast (HT) B-cell non-Hodgkin lymphoma (Karpas-422 NHL) Raji Burkitt's lymphoma cells	Pre-clinical	This potent inhibitor of MCT1 showed a reduction in growth of different cell lines especially hematological types. Although the inhibitory effect, some types of cancers express both transporters MCT1 and MCT4, in this regard MCT4 may be continuing the lactate transport suggesting a resistance to the monotherapy	(182)

(Continued)

TABLE 2 | Continued

Inhibitor(s)	Mechanism of action	Type of cancer or cell/animal model	Research phase	Limitations	References
AR-C155858	Selective monocarboxylate transporter (MCT1 and MCT2) which affects lactate uptake in a time dependent manner with slow reversible features	Murine breast cancer cell line, 4T1	Pre-clinical	No limitations were shown	(183)
CHC (α-cyano-4-hydroxycinnamic acid) DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid) Quercetin	CHC inhibits different MCT isoforms, namely MCT1 as a primary target. This compound interacts with the outside proteins of the membrane affecting lactate efflux, consequently arresting glycolysis DIDS is a MCT1 inhibitor, the interaction between one of the isothiocyanate groups of DIDS with a lysine residue of MCT1 could affect the transporter activity Quercetin is a MCT inhibitor, specifically MCT1 and MCT2, the lactate and proton transport promotes intracellular acidification	Colorectal cancer cells (HCT15 and RKO)	Pre-clinical	Tested compounds are wide MCT inhibitors	(184)
BAY-8002	Selective inhibition MCT1 which potently suppress bidirectional lactate transport	Hematopoietic malignancies, Raji, and Daudi Burkitt lymphoma cells	Pre-clinical	A limited antitumor efficacy was observed in the *in vivo* models suggesting a limited effect of the MCT1 blockage. Thus, cells exhibit a capability to adapt to long-term inhibition of MCT1 Only a small proportion of cell lines tested showed a significant reduction of cell viability indicating the necessity for testing MCT1 in clinical tests	(185)
Syrosingopine	Increases intracellular lactate due the inhibition of both MCT transporters (MCT1 and MCT4)	HeLa, HAP1, HL60 cells, liver tumor mouse model	Pre-clinical	No limitations were shown	(186)

for further biological validation (180). 11c maintains the same hydrogen bond interactions as compound 5 in the binding model and exhibits extra hydrogen bond interactions with the residues Asp 194 and Thr 247 in LDHA, which could give rise to its inhibitory activity against LDHA. The *in vitro* assays reveal the potential action of 11c in the metabolism of an osteosarcoma-derived cell line, MG-63. These cells exhibit a dose-response effect to 11c, where lactate formation significantly diminished with the subsequent extracellular acidification rate (ECAR) decrement, consistent with a poor lactate synthesis. In addition, the use of 11c upregulated the oxygen consumption rate (OCR) indicating a metabolic switch from lactate production to pyruvate consumption. In relation to cell proliferation, 11c promoted apoptosis in the same dose dependent manner, thus impacting cell proliferation. Taking together, compound 11 is a new potent LDHA inhibitor, demonstrated by its ability to induce the reprogramming of MG-63 cancer cells metabolism from glycolysis to mitochondrial respiration decreasing cell survival. Nevertheless, further experiments using different types of cancers are needed to ensure its biological efficacy.

As for the optimization of small molecules, compound 24c is a novel potent LDHA inhibitor obtained by a hit-to-lead optimization from an in-house library. 24c interacts directly into the binding pocket of LDHA, forming a direct hydrogen bond interaction with Asn137, Arg168, His192, and Gln99 of the enzyme causing a metabolic alteration by enhancing oxidative phosphorylation and reducing lactate formation in cancer cells, which might contribute to their anti-proliferation effect. In addition, this compound showed a reduction of cell growth as well as apoptosis and cell cycle arrest in a dose dependent manner against MiaPaCa-2 cells derived from pancreas carcinoma. Furthermore, 24c suppressed the tumor growth in the xenograft model. Additionally, the evaluation of the metabolic profile in MiaPaCa-2 cells treated by 24c exhibited a decreasing in ECAR and lactate production but an increased OCR value. Consequently, these observations suggested that 24c could be used as a lead pharmacophore for the development of new potent LDHA inhibitor (177).

Through a screening of novel inhibitors, Kim et al. (178) found several promising selenobenzene compounds with inhibitory effects on LDHA activity. The most potent inhibitor of the activity of LDHA was 1-(phenylseleno)- 4-(trifluoromethyl) benzene (PSTMB), this compound acts as an allosteric inhibitor modifying the active site where pyruvate binds, trough conformational changes that lead to the inhibition of enzymatic activity. Experimental assays indicated that PSTMB inhibited cell proliferation in several tumor cell lines including lung cancer (NCI-H460), breast cancer (MCF-7), hepatocellular carcinoma (Hep3B), malignant melanoma (A375), colorectal adenocarcinoma (HT29) and murine lung cancer cells (LLC). Furthermore, PSTMB incremented ROS generation and reduced the stability of the mitochondria inducing intrinsic pathway-mediated apoptosis of cancer cells. Additionally, LDHA activity and lactate production were clearly reduced by PSTMB under hypoxic and normoxic conditions, this suppression was mainly mediated by the inhibition of the enzyme activity, and not by the regulation of its expression. Summarizing, this novel selenobenzene, PSTMB, was found to be a potent inhibitor of the human LDHA enzyme.

Recently, peptides have been used as new class of drugs for the treatment of different diseases including cancer (189–191). Owing to the protein-protein interaction (PPI), peptides have been used as a novel and powerful tool in drug discovery. Recently, novel peptides aiming to disrupt the subunit association of LDHA during its tetramerization process have been designed through *in silico* methods, designed to impact the activity of the enzyme. These peptides were developed based on its active conformation and the interaction interface of LDHA subunits where the N-terminal arm (residues 5–17) acts as an anchor to maintain the position and distance between the two LDHA subunits. Thus, these new peptides mimic the anchoring of the LDHA subunits avoiding its tetramerization (176). These novel anti-cancer agents designed for therapy have promising advantages like low toxicity, ease of synthesis and high target specificity whereas the classical pharmalogical therapeutics. However, *in vivo* investigation of these peptides and its effects on cancer cell lines is needed to evaluate the biological potential.

Targeting MCTs

The inhibition of the MCTs has also been implemented as a therapeutic strategy. Although there are only few reports inhibiting specifically the MCT, these have showed promising results in different neoplasms.

The AR-C117977 was first identified as an immunomodulatory compound with antiproliferative properties on T lymphocytes, where the MCT1 was identified as its target (192). The AZD3965 is a derivate compound from AR-C117977 with potent inhibition of the MCT1 with additional activity against MCT2. The main action of the compound is the inhibition of lactate transport inducing an acute increase in intracellular lactate levels followed by glycolytic feedback and increased flux into the TCA cycle (182). This compound inhibited the proliferation of several lymphoma cell lines. Even, the combination of AZD3965 with other compounds like inhibitors of GLS1, doxorubicin or rituximab resulted in enhanced inhibition of cell growth and increased cell death in the tested cell lines (182). Nevertheless, the status of MCTs could be contributing to the observed inhibitory effect, thus the evaluation of MCTs expression in the tested models could provide better insights for inhibitory molecules.

The effect of AZD3965 was tested along with AR-C155858 in a murine breast cancer-derived cell line, 4T1 (183). The authors found that both compounds exhibited a time-dependent inhibition of lactate uptake and very importantly, this inhibition was slowly reversible, indicating that such effect could offer potential benefits in cancer treatment. Likewise, Amorim et al. (184) tested the antiproliferative effects of three compounds in colorectal cancer derived cells finding that colorectal cancer cells, HCT15 and RKO decreased its glycolytic rate and enhanced cell death in the presence of any of the probed molecules. Interestingly, the cytotoxic effect exerted by 5 fluoro-uracil was potentiated when using together with those drugs. Moreover, targeting MCT1 and MCT4 with syrosingopine in cell models

from different cancer types, Bejamin et al. (186) found an increase in intracellular lactate in HeLa cells, and the liver mouse model showed reduction in lactate concentration in nodules after syrosingopine treatment. Importantly, an increase in lactate levels in serum from syrosingopine-treated mice and a synergistic effect to metformin anti-properties was shown.

A high throughput examination of over 3 million compounds measuring lactate import-dependent intracellular acidification identified BAY-8002 as a potential MCT1 inhibitor (185). Then, authors showed the antiproliferative properties in different cells lines, where Daudi and Raji cells were most affected by this compound. The *in vivo* testing of BAY-8002 determined its capacity to decrease tumor mass over time using different concentrations ranging from 40 to 160 mg/kg, without affecting body weight. Importantly, chronically exposed cells developed resistance to MCT1 inhibition probably due to the increment of MCT2 and MCT4 expression in resistant cells indicating that different molecular mechanisms could be involved in treatment resistance.

CONCLUDING REMARKS

Lactate is not only considered as a waste product derived from fermentative cell metabolism, but instead is a powerful molecule that contributes to both, the onset and progression of cancer, favoring metastasis and tumor angiogenesis. In tumor microenvironment, lactate establishes metabolic coupling between cancer cells, immune cells and stromal cells, acting as an interchangeable metabolite in the tumor mass. Oxygen availability defines different metabolic phenotypes because their location within the tumor, where hypoxic central areas display a higher lactate concentration. Thus, the development of new tools for quantifying intra-tumoral molecules to trace lactate accumulation and consumption by tumors represents a huge challenge in cancer research.

Lactate participates also in the immune escape through the inhibition of lymphocytes activity and induces the M2 macrophage polarization associated to tumor progression. In addition, it is currently known that metabolic plasticity exhibited by tumors allows the development of treatment resistance mechanisms due to adaptation to metabolic changes, impacting the effect of anti-metabolic drugs. Thus, lactate-induced resistance to therapy represents the major obstacle in the elimination of malignant tumors. For this reason, it is necessary to pursuit for more studies aimed to determine synergistic combinations including lactate dehydrogenase and MCTs inhibitors for developing reliable and effective treatments in cancer.

AUTHOR CONTRIBUTIONS

KC-L and JM-M: conception and design. KC-L, LC-M, DR-H, AG-C, and JM-M: wrote and critically review the manuscript. KC-L, LC-M, DR-H, and JM-M: figure design and elaboration. JM-M: directed manuscript.

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REFERENCES

1. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. (2000) 100:57–70. doi: 10.1016/s0092-8674(00)81683-9
2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
3. Warburg O, Wind F, Negelein E. The metabolims of tumors in the body. *J Gen Physiol*. (1927) 8:519–30. doi: 10.1085/jgp.8.6.519
4. Racker E. Bioenergetics and the problem of tumor growth. *Am Sci*. (1972) 60:56–63.
5. Vyas S, Zaganjor E, Haigis MC. Mitochondria and cancer. *Cell*. (2016) 166:555–66. doi: 10.1016/j.cell.2016.07.002
6. Liu Y, Murray-stewart T, Casero RA, Kagiampakis I, Jin L, Zhang J, et al. Targeting hexokinase 2 inhibition promotes radiosensitization in HPV16 E7-induced cervical cancer and suppresses tumor growth. *Int J Oncol*. (2017) 50:2011–23. doi: 10.3892/ijo.2017.3979
7. Marshall MJ, Neal FE, Goldberg DM. Isoenzymes of hexokinase, 6-phosphogluconate dehydrogenase, phosphoglucomutase and lactate dehydrogenase in uterine cancer. *Br J Cancer*. (1979) 40:380–90. doi: 10.1038/bjc.1979.192
8. Park SI, Suh DS, Kim S, Choi KU, Yoon M. Correlation between biological marker expression and F-fluorodeoxyglucose uptake in cervical cancer measured by positron emission tomography. *Onkologie*. (2013) 36:169–74. doi: 10.1159/000349944
9. Jiao L, Zhang H, Li D, Yang K, Tang J, Li X, et al. Regulation of glycolytic metabolism by autophagy in liver cancer involves selective autophagic. *Autophagy*. (2017) 14:671–84. doi: 10.1080/15548627.2017.1381804
10. Wadsak W, Mitterhauser M. Basics and principles of radiopharmaceuticals for PET/CT. *Eur J Radiol*. (2010) 73:461–9. doi: 10.1016/j.ejrad.2009.12.022
11. Wang L, Xiong H, Wu F, Zhang Y, Wang J, Zhao L, et al. Article hexokinase 2-mediated warburg effect is required for PTEN - and p53 - deficiency-driven prostate cancer growth. *Cell Rep*. (2014) 8:1461–74. doi: 10.1016/j.celrep.2014.07.053
12. Wong N, Ojo D, Yan J, Tang D. PKM2 contributes to cancer metabolism. *Cancer Lett*. (2015) 356:184–91. doi: 10.1016/j.canlet.2014.01.031
13. Mayer A, Schmidt M, Seeger A, Serras AF, Vaupel P, Schmidberger H. GLUT-1 expression is largely unrelated to both hypoxia and the Warburg phenotype in squamous cell carcinomas of the vulva. *BMC Cancer*. (2014) 14:760. doi: 10.1186/1471-2407-14-760
14. Fantin VR, St.-Pierre J, Leder P. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell*. (2006) 9:425–34. doi: 10.1016/j.ccr.2006.04.023
15. Shim H, Dolde C, Lewis BC, Wu C-S, Dang G, Jungmann RA, et al. c-Myc transactivation of LDH-A: implications for tumor metabolism and

growth. *Proc Natl Acad Sci USA*. (1997) 94:6658–63. doi: 10.1073/pnas.94.13.6658
16. Johnson RF, Perkins ND. Nuclear factor-κB, p53, and mitochondria: regulation of cellular metabolism and the Warburg effect. *Trends Biochem Sci*. (2012) 37:317–24. doi: 10.1016/j.tibs.2012.04.002
17. Lu H, Forbes RA, Verma A. Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. *J Biol Chem*. (2002) 277:23111–5. doi: 10.1074/jbc.M202487200
18. Levine AJ, Puzio-Kuter AM. The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. *Science*. (2010) 330:1340–4. doi: 10.1126/science.1193494
19. San-Millán I, Brooks GA. Reexamining cancer metabolism: lactate production for carcinogenesis could be the purpose and explanation of the Warburg Effect. *Carcinogenesis*. (2017) 38:119–33. doi: 10.1093/carcin/bgw127
20. Lyssiotis CA, Kimmelman AC. Metabolic interactions in the tumor microenvironment. *Trends Cell Biol*. (2017) 27:863–75. doi: 10.1016/j.tcb.2017.06.003
21. Talasniemi JP, Pennanen S, Savolainen H, Niskanen L, Liesivuori J. Analytical investigation: assay of d-lactate in diabetic plasma and urine. *Clin Biochem*. (2008) 41:1099–103. doi: 10.1016/J.CLINBIOCHEM.2008.06.011
22. Connor H, Woods HF, Ledingham JG. Comparison of the kinetics and utilisation of D(-)-and L(+)-sodium lactate in normal man. *Ann Nutr Metab*. (1983) 27:481–7. doi: 10.1159/000176723
23. Nalbandian M, Takeda M. Lactate as a signaling molecule that regulates exercise-induced adaptations. *Biology*. (2016) 5:38. doi: 10.3390/biology5040038
24. Gladden LB. Lactate metabolism: a new paradigm for the third millennium. *J Physiol*. (2004) 558:5–30. doi: 10.1113/jphysiol.2003.058701
25. Neufeld EF, Ginsburg V. Carbohydrate metabolism. *Annu Rev Biochem*. (1965) 34:297–312. doi: 10.1146/annurev.bi.34.070165.001501
26. Consoli A, Nurjhan N, Reilly JJ, Bier DM, Gerich JE. Contribution of liver and skeletal muscle to alanine and lactate metabolism in humans. *Am J Physiol Metab*. (2017) 259:E677–84. doi: 10.1152/ajpendo.1990.259.5.e677
27. Brooks GA. *Lactate: Glycolytic end Product and Oxidative Substrate During Sustained Exercise in Mammals—The 'Lactate Shuttle'*. Berlin, Heidelberg: Springer.
28. Brooks GA. Cell-cell and intracellular lactate shuttles. *J Physiol*. (2009) 587:5591–600. doi: 10.1113/jphysiol.2009.178350
29. Brooks GA. The science and translation of lactate shuttle theory. *Cell Metab*. (2018) 27:757–85. doi: 10.1016/j.cmet.2018.03.008
30. Walenta S, Wetterling M, Lehrke M, Schwickert G, Sundfør K, Rofstad EK, et al. High lactate levels predict likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancers. *Cancer Res*. (2000) 60:916–21.
31. Brizel DM, Clough RW, Dewhirst MW, Schroeder T, Walenta S, Mueller-Klieser W, et al. Elevated tumor lactate concentrations predict for an increased risk of metastases in head-and-neck cancer. *Int J Radiat Oncol Biol Phys*. (2001) 51:349–53. doi: 10.1016/S0360-3016(01)01630-3
32. Walenta S, Salameh A, Lyng H, Evensen JF, Mitze M, Rofstad EK, et al. Correlation of high lactate levels in head and neck tumors with incidence of metastasis. *Am J Pathol*. (1997) 150:409–15.
33. Walenta S, Van Chau T, Schroeder T, Lehr HA, Kunz-Schughart LA, Fuerst A, et al. Metabolic classification of human rectal adenocarcinomas: a novel guideline for clinical oncologists? *J Cancer Res Clin Oncol*. (2003) 129:321–6. doi: 10.1007/s00432-003-0450-x
34. Kennedy KM, Scarbrough PM, Ribeiro A, Richardson R, Yuan H, Sonveaux P, et al. Catabolism of exogenous lactate reveals it as a legitimate metabolic substrate in breast cancer. *PLoS ONE*. (2013) 8:e75154. doi: 10.1371/journal.pone.0075154
35. Vlachostergios PJ, Oikonomou KG, Gibilaro E, Apergis G. Elevated lactic acid is a negative prognostic factor in metastatic lung cancer. *Cancer Biomarkers*. (2015) 15:725–34. doi: 10.3233/CBM-150514
36. Nikoobakht M, Shamshiripour P, Azimi Nekoo Z, Fallah Haghmohammadi S. Elevated lactate and total protein levels in stereotactic brain biopsy specimen: potential biomarkers of malignancy and poor prognosis. *Arch Iran Med*. (2019) 22:125–31.
37. Quennet V, Yaromina A, Zips D, Rosner A, Walenta S, Baumann M, et al. Tumor lactate content predicts for response to fractionated irradiation of human squamous cell carcinomas in nude mice. *Radiother Oncol*. (2006) 81:130–5. doi: 10.1016/j.radonc.2006.08.012
38. Tamulevicius P, Streffer C. Metabolic imaging in tumours by means of bioluminescence. *Br J Cancer*. (1995) 72:1102–12. doi: 10.1038/bjc.1995.472
39. Liberti MV, Locasale JW. The warburg effect : how does it benefit cancer cells? *Trends Biochem Sci*. (2016) 41:211–8. doi: 10.1016/j.tibs.2015.12.001
40. Van Der Bliek AM, Sedensky MM, Morgan PG. Cell biology of the mitochondrion. *Genetics*. (2017) 207:843–71. doi: 10.1534/genetics.117.300262
41. DeBerardinis RJ, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, et al. Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc Natl Acad Sci USA*. (2007) 104:19345–50. doi: 10.1073/pnas.0709747104
42. Cruzat V, Rogero MM, Keane KN, Curi R, Newsholme P. Glutamine: metabolism and immune function, supplementation and clinical translation. *Nutrients*. (2018) 10:1–31. doi: 10.3390/nu10111564
43. Reitzer LJ, Wice BM, Kennell D. Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells. *J Biol Chem*. (1979) 254:2669–76.
44. Anderson NM, Mucka P, Kern JG, Feng H. The emerging role and targetability of the TCA cycle in cancer metabolism. *Protein Cell*. (2018) 9:216–37. doi: 10.1007/s13238-017-0451-1
45. Zacksenhaus E, Shrestha M, Liu JC, Vorobieva I, Chung PED, Ju YJ, et al. Mitochondrial OXPHOS induced by RB1 deficiency in breast cancer: implications for anabolic metabolism, stemness, and metastasis. *Trends Cancer*. (2017) 3:768–79. doi: 10.1016/j.trecan.2017.09.002
46. Santidrian AF, Matsuno-yagi A, Ritland M, Seo BB, Leboeuf SE, Gay LJ, et al. Mitochondrial complex I activity and NAD+/NADH balance regulate breast cancer progression. *J Clin Invest*. (2013) 123:1068–81. doi: 10.1172/JCI64264DS1
47. Chen YJ, Mahieu NG, Huang X, Singh M, Crawford PA, Johnson SL, et al. Lactate metabolism is associated with mammalian mitochondria. *Nat Chem Biol*. (2016) 12:937–43. doi: 10.1038/nchembio.2172
48. Hashimoto T, Hussien R, Brooks GA. Colocalization of MCT1, CD147, and LDH in mitochondrial inner membrane of L6 muscle cells: evidence of a mitochondrial lactate oxidation complex. *Am J Physiol Metab*. (2006) 290:E1237–44. doi: 10.1152/ajpendo.00594.2005
49. Hashimoto T, Hussien R, Cho HS, Kaufer D, Brooks GA. Evidence for the mitochondrial lactate oxidation complex in rat neurons: demonstration of an essential component of brain lactate shuttles. *PLoS ONE*. (2008) 3:2915. doi: 10.1371/journal.pone.0002915
50. Gallagher CN, Carpenter KLH, Grice P, Howe DJ, Mason A, Timofeev I, et al. The human brain utilizes lactate via the tricarboxylic acid cycle: a 13C-labelled microdialysis and high-resolution nuclear magnetic resonance study. *Brain*. (2009) 132:2839–49. doi: 10.1093/brain/awp202
51. Hirayama A, Kami K, Sugimoto M, Sugawara M, Toki N, Onozuka H, et al. Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry. *Cancer Res*. (2009) 69:4918–25. doi: 10.1158/0008-5472.CAN-08-4806
52. Hui S, Ghergurovich JM, Morscher RJ, Jang C, Teng X, Lu W, et al. Glucose feeds the TCA cycle via circulating lactate. *Nature*. (2017) 551:115–8. doi: 10.1038/nature24057
53. Sonveaux P, Feron O, Dewhirst MW, Sonveaux P, Végran F, Schroeder T, et al. Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. *J Clin Invest*. (2008) 118:3930–42. doi: 10.1172/JCI36843
54. Park S, Chang CY, Safi R, Liu X, Baldi R, Jasper JS, et al. ERRα-regulated lactate metabolism contributes to resistance to targeted therapies in breast cancer. *Cell Rep*. (2016) 15:323–35. doi: 10.1016/j.celrep.2016.03.026
55. Leithner K, Hrzenjak A, Trötzmüller M, Moustafa T, Köfeler HC, Wohlkoenig C, et al. PCK2 activation mediates an adaptive response to glucose depletion in lung cancer. *Oncogene*. (2015) 34:1044–50. doi: 10.1038/onc.2014.47

56. Faubert B, Li KY, Cai L, Hensley CT, Kim J, Zacharias LG, et al. Lactate metabolism in human lung tumors. *Cell*. (2017) 171:358–71. doi: 10.1016/j.cell.2017.09.019
57. Bok R, Lee J, Sriram R, Keshari K, Sukumar S, Daneshmandi S, et al. The role of lactate metabolism in prostate cancer progression and metastases revealed by dual-agent hyperpolarized ^{13}C MRSI. *Cancers*. (2019) 11:257. doi: 10.3390/cancers11020257
58. Le A, Lane AN, Hamaker M, Bose S, Gouw A, Barbi J, et al. Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. *Cell Metab*. (2012) 15:110–21. doi: 10.1016/j.cmet.2011.12.009
59. Pérez-Escuredo J, Dadhich RK, Dhup S, Cacace A, Van Hée VF, De Saedeleer CJ, et al. Lactate promotes glutamine uptake and metabolism in oxidative cancer cells. *Cell Cycle*. (2016) 15:72–83. doi: 10.1080/15384101.2015.1120930
60. Adeva-Andany M, López-Ojén M, Funcasta-Calderón R, Ameneiros-Rodríguez E, Donapetry-García C, Vila-Altesor M, et al. Comprehensive review on lactate metabolism in human health. *Mitochondrion*. (2014) 17:76–100. doi: 10.1016/j.mito.2014.05.007
61. Liang X, Liu L, Fu T, Zhou Q, Zhou D, Xiao L, et al. Exercise inducible lactate dehydrogenase B regulates mitochondrial function in skeletal muscle. *J Biol Chem*. (2016) 291:25306–18. doi: 10.1074/jbc.M116.749424
62. Dawson DM, Goodfriend TL, Kaplan NO. Lactic dehydrogenases: functions of the two types rates of synthesis of the two major forms can. *Science*. (2013) 143:929–33. doi: 10.2307/1712825
63. Belenky P, Bogan KL, Brenner C. NAD+ metabolism in health and disease. *Trends Biochem Sci*. (2007) 32:12–9. doi: 10.1016/j.tibs.2006.11.006
64. Fu Y, Lan T, Cai H, Lu A, Yu W. Meta-analysis of serum lactate dehydrogenase and prognosis for osteosarcoma. *Medicine*. (2018) 97:1–9. doi: 10.1097/MD.0000000000010741
65. Gan J, Wang W, Yang Z, Pan J, Zheng L, Yin L. Prognostic value of pretreatment serum lactate dehydrogenase level in pancreatic cancer patients. *Medicine*. (2018) 97:e13151. doi: 10.1097/md.0000000000013151
66. Zhang Z, Li Y, Yan X, Song Q, Wang G, Hu Y, et al. Pretreatment lactate dehydrogenase may predict outcome of advanced non small-cell lung cancer patients treated with immune checkpoint inhibitors: a meta-analysis. *Cancer Med*. (2019) 8:1467–73. doi: 10.1002/cam4.2024
67. Hou X, Yuan S, Zhao D, Liu X, Wu X. LDH-A promotes malignant behavior via activation of epithelial-to-mesenchymal transition in lung adenocarcinoma. *Biosci Rep*. (2019) 39:BSR20181476. doi: 10.1042/bsr20181476
68. Le A, Cooper CR, Gouw AM, Dinavahi R, Maitra A, Deck LM, et al. Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. *Proc Natl Acad Sci USA*. (2010) 107:2037–42. doi: 10.1073/pnas.0914433107
69. Chaudhari V, Pradeep G, Prakash N, Mahajan A. Estimation of salivary sialic acid in oral premalignancy and oral squamous cell carcinoma. *Contemp Clin Dent*. (2016) 7:451. doi: 10.4103/0976-237x.194108
70. Saluja TS, Spadigam A, Dhupar A, Syed S. Equating salivary lactate dehydrogenase (LDH) with LDH-5 expression in patients with oral squamous cell carcinoma: an insight into metabolic reprogramming of cancer cell as a predictor of aggressive phenotype. *Tumor Biol*. (2016) 37:5609–20. doi: 10.1007/s13277-015-4415-x
71. Mohajertehran F, Ayatollahi H, Jafarian AH, Khazaeni K, Soukhtanloo M, Shakeri M-T, et al. Overexpression of lactate dehydrogenase in the saliva and tissues of patients with head and neck squamous cell carcinoma. *Rep Biochem Mol Biol*. (2019) 7:1413–9. doi: 10.1002/hed.21618
72. Fan J, Hitosugi T, Chung T-W, Xie J, Ge Q, Gu T-L, et al. Tyrosine phosphorylation of lactate dehydrogenase A is important for NADH/NAD+ redox homeostasis in cancer cells. *Mol Cell Biol*. (2011) 31:4938–50. doi: 10.1128/mcb.06120-11
73. Liu J, Chen G, Liu Z, Liu S, Cai Z, You P, et al. Aberrant FGFR tyrosine kinase signaling enhances the warburg effect by reprogramming LDH isoform expression and activity in prostate cancer. *Cancer Res*. (2018) 78:4459–70. doi: 10.1158/0008-5472.CAN-17-3226
74. Jin L, Chun J, Pan C, Alesi GN, Li D, Magliocca KR, et al. Phosphorylation-mediated activation of LDHA promotes cancer cell invasion and tumour metastasis. *Oncogene*. (2017) 36:3797–806. doi: 10.1038/onc.2017.6
75. Li S, Gao J, Zhuang X, Zhao C, Hou X, Xing X, et al. Cyclin G2 inhibits the warburg effect and tumour progression by suppressing LDHA phosphorylation in glioma. *Int J Biol Sci*. (2019) 15:544–55. doi: 10.7150/ijbs.30297
76. Linster CL, Van Schaftingen E, Hanson AD. Metabolite damage and its repair or pre-emption. *Nat Chem Biol*. (2013) 9:72–80. doi: 10.1038/nchembio.1141
77. Intlekofer AM, DeMatteo RG, Venneti S, Finley LWS, Lu C, Judkins AR, et al. Hypoxia induces production of L-2-hydroxyglutarate. *Cell Metab*. (2015) 22:304–11. doi: 10.1016/j.cmet.2015.06.023
78. Intlekofer AM, Wang B, Liu H, Shah H, Carmona-Fontaine C, Rustenburg AS, et al. L-2-Hydroxyglutarate production arises from noncanonical enzyme function at acidic pH. *Nat Chem Biol*. (2017) 13:494–500. doi: 10.1038/nchembio.2307
79. Wykoff CC, Pugh CW, Harris AL, Maxwell PH, Ratcliffe PJ. The HIF pathway: implications for patterns of gene expression in cancer. *Novartis Found Symp*. (2001) 240:212–25. doi: 10.1002/0470868716.ch15
80. Semenza GL, Jiang BH, Leung SW, Passantino R, Concordat JP, Maire P, et al. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase a gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J Biol Chem*. (1996) 271:32529–37. doi: 10.1074/jbc.271.51.32529
81. Liu Y, Guo JZ, Liu Y, Wang K, Ding W, Wang H, et al. Nuclear lactate dehydrogenase A senses ROS to produce α-hydroxybutyrate for HPV-induced cervical tumor growth. *Nat Commun*. (2018) 9:4429. doi: 10.1038/s41467-018-06841-7
82. Trivedi B, Danforth WH. Effect of pH on the kinetics of frog muscle phosphofructokinase. *J Biol Chem*. (1966) 241:4110–2.
83. Jones R, Morris M. Monocarboxylate transporters: therapeutic targets and prognostic factors in disease. *Clin Pharmacol Ther*. (2016) 100:454–63. doi: 10.1002/cpt.418
84. Garcia CK, Goldstein JL, Pathak RK, Anderson RGW, Brown MS. Molecular characterization of a membrane transporter for lactate, pyruvate, and other monocarboxylates: implications for the Cori cycle. *Cell*. (1994) 76:865–73. doi: 10.1016/0092-8674(94)90361-1
85. Spencer TL, Lehninger AL. L-lactate transport in Ehrlich ascites-tumour cells. *Biochem J*. (1976) 154:405–14. doi: 10.1042/bj1540405
86. Kirk P, Wilson MC, Heddle C, Brown MH, Barclay AN, Halestrap AP. CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. *EMBO J*. (2000) 19:3896–904. doi: 10.1093/emboj/19.15.3896
87. Wilson MC, Meredith D, Manning Fox JE, Manoharan C, Davies AJ, Halestrap AP. Basigin (CD147) is the target for organomercurial inhibition of monocarboxylate transporter isoforms 1 and 4: the ancillary protein for the insensitive MCT2 is embigin (gp70). *J Biol Chem*. (2005) 280:27213–21. doi: 10.1074/jbc.M411950200
88. Gallagher SM, Castorino JJ, Wang D, Philp NJ. Monocarboxylate transporter 4 regulates maturation and trafficking of CD147 to the plasma membrane in the metastatic breast cancer cell line MDA-MB-231. *Cancer Res*. (2007) 67:4182–9. doi: 10.1158/0008-5472.CAN-06-3184
89. Updegraff BL, Zhou X, Guo Y, Padanad MS, Chen PH, Yang C, et al. Transmembrane protease TMPRSS11B promotes lung cancer growth by enhancing lactate export and glycolytic metabolism. *Cell Rep*. (2018) 25:2223-2233.e6. doi: 10.1016/j.celrep.2018.10.100
90. Pinheiro C, Morais-Santos F, Baltazar F, Longatto-Filho A, Garcia EA, Scapulatempo-Neto C, et al. Lactate transporters and vascular factors in HPV-induced squamous cell carcinoma of the uterine cervix. *BMC Cancer*. (2014) 14:1–12. doi: 10.1186/1471-2407-14-751
91. Fang J, Quinones QJ, Holman TL, Morowitz MJ, Wang Q, Zhao H, et al. The H+-linked monocarboxylate transporter (MCT1/SLC16A1): a potential therapeutic target for high-risk neuroblastoma. *Mol Pharmacol*. (2006) 70:2108–15. doi: 10.1124/mol.106.026245
92. Kim Y, Choi JW, Lee JH, Kim YS. Expression of lactate/H+ symporters MCT1 and MCT4 and their chaperone CD147 predicts tumor progression in clear cell renal cell carcinoma: immunohistochemical and the Cancer Genome Atlas data analyses. *Hum Pathol*. (2015) 46:104–12. doi: 10.1016/j.humpath.2014.09.013
93. Curry JM, Tuluc M, Whitaker-Menezes D, Ames JA, Anantharaman A, Butera A, et al. Cancer metabolism, stemness and tumor recurrence : MCT1

and MCT4 are functional biomarkers of metabolic symbiosis in head and neck cancer. *Cell Cycle*. (2013) 12:1371–84. doi: 10.4161/cc.24092
94. Zhang G, Zhang Y, Dong D, Wang F, Ma X, Guan F, et al. MCT1 regulates aggressive and metabolic phenotypes in bladder cancer. *J Cancer*. (2018) 9:2492–501. doi: 10.7150/jca.25257
95. Pinheiro C, Longatto-Filho A, Pereira SMM, Etlinger D, Moreira MAR, Jubé LF, et al. Monocarboxylate transporters 1 and 4 are associated with CD147 in cervical carcinoma. *Dis Markers*. (2009) 26:97–103. doi: 10.3233/DMA-2009-0596
96. Pinheiro C, Miranda-Gonçalves V, Longatto-Filho A, Vicente ALSA, Berardinelli GN, Scapulatempo-Neto C, et al. The metabolic microenvironment of melanomas: prognostic value of MCT1 and MCT4. *Cell Cycle*. (2016) 15:1462–70. doi: 10.1080/15384101.2016.1175258
97. Payen VL, Hsu MY, Rädecke KS, Wyart E, Vazeille T, Bouzin C, et al. Monocarboxylate transporter MCT1 promotes tumor metastasis independently of its activity as a lactate transporter. *Cancer Res*. (2017) 77:5591–601. doi: 10.1158/0008-5472.CAN-17-0764
98. Gerlinger M, Santos CR, Spencer-Dene B, Martinez P, Endesfelder D, Burrell RA, et al. Genome-wide RNA interference analysis of renal carcinoma survival regulators identifies MCT4 as a Warburg effect metabolic target. *J Pathol*. (2012) 227:146–56. doi: 10.1002/path.4006
99. Choi SYC, Ettinger SL, Lin D, Xue H, Ci X, Nabavi N, et al. Targeting MCT4 to reduce lactic acid secretion and glycolysis for treatment of neuroendocrine prostate cancer. *Cancer Med*. (2018) 7:3385–92. doi: 10.1002/cam4.1587
100. Bisetto S, Whitaker-Menezes D, Wilski NA, Tuluc M, Curry J, Zhan T, et al. Monocarboxylate transporter 4 (MCT4) knockout mice have attenuated 4NQO induced carcinogenesis; a role for MCT4 in driving oral squamous cell cancer. *Front Oncol*. (2018) 8:1–13. doi: 10.3389/fonc.2018.00324
101. Ippolito L, Morandi A, Giannoni E, Chiarugi P. Lactate: a metabolic driver in the tumour landscape. *Trends Biochem Sci*. (2019) 44:153–66. doi: 10.1016/j.tibs.2018.10.011
102. Morandi A, Giannoni E, Chiarugi P. Nutrient exploitation within the tumor-stroma metabolic crosstalk. *Trends Cancer*. (2016) 2:736–46. doi: 10.1016/j.trecan.2016.11.001
103. García-Cañaveras JC, Chen L, Rabinowitz JD. The tumor metabolic microenvironment: lessons from lactate. *Cancer Res*. (2019) 2019:3276. doi: 10.1158/0008-5472.can-18-3726
104. Estrella V, Chen T, Lloyd M, Wojtkowiak J, Cornnell HH, Ibrahim-Hashim A, et al. Acidity generated by the tumor microenvironment drives local invasion. *Cancer Res*. (2013) 73:1524–35. doi: 10.1158/0008-5472.CAN-12-2796
105. Lee SH, McIntyre D, Honess D, Hulikova A, Pacheco-Torres J, Cerdán S, et al. Carbonic anhydrase IX is a pH-stat that sets an acidic tumour extracellular pH *in vivo*. *Br J Cancer*. (2018) 119:622–30. doi: 10.1038/s41416-018-0216-5
106. Pastorek J, Pastoreková S, Callebaut I, Mornon JP, Zelník V, Opavský R, et al. Cloning and characterization of MN, a human tumor-associated protein with a domain homologous to carbonic anhydrase and a putative helix-loop-helix DNA binding segment. *Oncogene*. (1994) 9:2877–88.
107. Büscheck F, Fraune C, Simon R, Kluth M, Hube-Magg C, Möller-Koop C, et al. Aberrant expression of membranous carbonic anhydrase IX (CAIX) is associated with unfavorable disease course in papillary and clear cell renal cell carcinoma. *Urol Oncol Semin Orig Investig*. (2018) 36:531.e19–25. doi: 10.1016/j.urolonc.2018.08.015
108. Panisova E, Kery M, Sedlakova O, Brisson L, Debreova M, Sboarina M, et al. Lactate stimulates CA IX expression in normoxic cancer cells. *Oncotarget*. (2017) 8:77819–35. doi: 10.18632/oncotarget.20836
109. Klier M, Schüler C, Halestrap AP, Sly WS, Deitmer JW, Becker HM. Transport activity of the high-affinity monocarboxylate transporter MCT2 is enhanced by extracellular carbonic anhydrase IV but not by intracellular carbonic anhydrase II. *J Biol Chem*. (2011) 286:27781–91. doi: 10.1074/jbc.M111.255331
110. Jamali S, Klier M, Ames S, Felipe Barros L, McKenna R, Deitmer JW, et al. Hypoxia-induced carbonic anhydrase IX facilitates lactate flux in human breast cancer cells by non-catalytic function. *Sci Rep*. (2015) 5:1–16. doi: 10.1038/srep13605
111. Ames S, Pastorekova S, Becker HM. The proteoglycan-like domain of carbonic anhydrase IX mediates non-catalytic facilitation of lactate transport in cancer cells. *Oncotarget*. (2018) 9:27940–57. doi: 10.18632/oncotarget.25371
112. Forero-Quintero LS, Ames S, Schneider HP, Thyssen A, Boone CD, Andring JT, et al. Membrane-anchored carbonic anhydrase IV interacts with monocarboxylate transporters via their chaperones CD147 and GP70. *J Biol Chem*. (2019) 294:593–607. doi: 10.1074/jbc.RA118.005536
113. Hensley CT, Faubert B, Yuan Q, Lev-Cohain N, Jin E, Kim J, et al. Metabolic heterogeneity in human lung tumors. *Cell*. (2016) 164:681–94. doi: 10.1016/j.cell.2015.12.034
114. Ullah MS, Davies AJ, Halestrap AP. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1α-dependent mechanism. *J Biol Chem*. (2006) 281:9030–7. doi: 10.1074/jbc.M511397200
115. Dimmer KS, Friedrich B, Lang F, Deitmer JW, Bröer S. The low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells. *Biochem J*. (2000) 350:219–27. doi: 10.1042/bj3500219
116. Baek GH, Tse YF, Hu Z, Cox D, Buboltz N, McCue P, et al. MCT4 defines a glycolytic subtype of pancreatic cancer with poor prognosis and unique metabolic dependencies. *Cell Rep*. (2014) 9:2233–49. doi: 10.1016/j.celrep.2014.11.025
117. Pavlova NN, Thompson CB. Perspective The emerging hallmarks of cancer metabolism. *Cell Metab*. (2016) 23:27–47. doi: 10.1016/j.cmet.2015.12.006
118. Bonuccelli G, Tsirigos A, Whitaker-Menezes D, Pavlides S, Pestell RG, Chiavarina B, et al. Ketones and lactate "fuel" tumor growth and metastasis: evidence that epithelial cancer cells use oxidative mitochondrial metabolism. *Cell Cycle*. (2010) 9:3506–14. doi: 10.4161/cc.9.17.12731
119. Shan T, Chen S, Chen X, Lin WR, Li W, Ma J, et al. Cancer-Associated fibroblasts enhance pancreatic cancer cell invasion by remodeling the metabolic conversion mechanism. *Oncol Rep*. (2017) 37:1971–9. doi: 10.3892/or.2017.5479
120. Martinez-Outschoorn UE, Lin Z, Trimmer C, Flomenberg N, Wang C, Pavlides S, et al. Cancer cells metabolically "fertilize" the tumor microenvironment with hydrogen peroxide, driving the Warburg effect: implications for PET imaging of human tumors. *Cell Cycle*. (2011) 10:2504–20. doi: 10.4161/cc.10.15.16585
121. Kimmelman AC, White E. Autophagy and tumor metabolism. *Cell Metab*. (2017) 25:1037–43. doi: 10.1016/j.cmet.2017.04.004
122. Whitaker-Menezes D, Martinez-Outschoorn UE, Flomenberg N, Birbe RC, Witkiewicz AK, Howell A, et al. Hyperactivation of oxidative mitochondrial metabolism in epithelial cancer cells *in situ*: visualizing the therapeutic effects of metformin in tumor tissue. *Cell Cycle*. (2011) 10:4047–64. doi: 10.4161/cc.10.23.18151
123. Luo M, Luo Y, Mao N, Huang G, Teng C, Wang H, et al. Cancer-associated fibroblasts accelerate malignant progression of non-small cell lung cancer via connexin 43-formed unidirectional gap junctional intercellular communication. *Cell Physiol Biochem*. (2018) 51:315–36. doi: 10.1159/000495232
124. Zhou W, Xu G, Wang Y, Xu Z, Liu X, Xu X, et al. Oxidative stress induced autophagy in cancer associated fibroblast enhances proliferation and metabolism of colorectal cancer cells. *Cell Cycle*. (2017) 16:73–81. doi: 10.1080/15384101.2016.1252882
125. Sun K, Tang S, Hou Y, Xi L, Chen Y, Yin J, et al. Oxidized ATM-mediated glycolysis enhancement in breast cancer-associated fibroblasts contributes to tumor invasion through lactate as metabolic coupling. *EBio Med*. (2019) 41:370–83. doi: 10.1016/j.ebiom.2019.02.025
126. Fiaschi T, Marini A, Giannoni E, Taddei ML, Gandellini P, De Donatis A, et al. Reciprocal metabolic reprogramming through lactate shuttle coordinately influences tumor-stroma interplay. *Cancer Res*. (2012) 72:5130–40. doi: 10.1158/0008-5472.CAN-12-1949
127. Fu Y, Liu S, Yin S, Niu W, Xiong W, Tan M, et al. The reverse Warburg effect is likely to be an Achilles' heel of cancer that can be exploited for cancer therapy. *Oncotarget*. (2017) 8:57813–25. doi: 10.18632/oncotarget.18175
128. Davies M. New modalities of cancer treatment for NSCLC: focus on immunotherapy. *Cancer Manag Res*. (2014) 6:63–75. doi: 10.2147/CMAR.S57550
129. Gonzalez H, Hagerling C, Werb Z. Roles of the immune system in cancer: from tumor initiation to metastatic progression. *Genes Dev*. (2018) 32:1267–84. doi: 10.1101/GAD.314617.118

130. Liu Y, Cao X. Immunosuppressive cells in tumor immune escape and metastasis. *J Mol Med*. (2016) 94:509–22. doi: 10.1007/s00109-015-1376-x
131. Dang CV. Links between metabolism and cancer. *Genes Dev*. (2012) 26:877–90. doi: 10.1101/gad.189365.112
132. Dhup S, Kumar Dadhich R, Ettore Porporato P, Sonveaux P. Multiple biological activities of lactic acid in cancer: influences on tumor growth, angiogenesis and metastasis. *Curr Pharm Des*. (2012) 18:1319–30. doi: 10.2174/138161212799504902
133. Paul S, Lal G. The molecular mechanism of natural killer cells function and its importance in cancer immunotherapy. *Front Immunol*. (2017) 8:e1124. doi: 10.3389/fimmu.2017.01124
134. Pegram HJ, Andrews DM, Smyth MJ, Darcy PK, Kershaw MH. Activating and inhibitory receptors of natural killer cells. *Immunol Cell Biol*. (2011) 89:216–24. doi: 10.1038/icb.2010.78
135. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol*. (2009) 9:495–502. doi: 10.1038/ni1581.Up
136. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature*. (2002) 419:734–8. doi: 10.1038/nature01112
137. Harmon C, Robinson MW, Hand F, Almuaili D, Mentor K, Houlihan DD, et al. Lactate-mediated acidification of tumor microenvironment induces apoptosis of liver-resident NK cells in colorectal liver metastasis. *Cancer Immunol Res*. (2019) 7:335–46. doi: 10.1158/2326-6066.cir-18-0481
138. Husain Z, Huang Y, Seth P, Sukhatme VP. Tumor-derived lactate modifies antitumor immune response: effect on myeloid-derived suppressor cells and NK cells. *J Immunol*. (2013) 191:1486–95. doi: 10.4049/jimmunol.1202702
139. Bae EA, Seo H, Kim IK, Jeon I, Kang CY. Roles of NKT cells in cancer immunotherapy. *Arch Pharm Res*. (2019) 42:543–8. doi: 10.1007/s12272-019-01139-8
140. Xie D, Zhu S, Bai L. Lactic acid in tumor microenvironments causes dysfunction of NKT cells by interfering with mTOR signaling. *Sci China Life Sci*. (2016) 59:1290–6. doi: 10.1007/s11427-016-0348-7
141. Kumar A, Pyaram K, Yarosz EL, Hong H, Lyssiotis CA, Giri S, et al. Enhanced oxidative phosphorylation in NKT cells is essential for their survival and function. *Proc Natl Acad Sci USA*. (2019) 116:7439–48. doi: 10.1073/pnas.1901376116
142. Gupta S. Role of dendritic cells in innate and adaptive immune response in human aging. *Exp Gerontol*. (2014) 54:47–52. doi: 10.1016/j.exger.2013.12.009
143. Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol*. (1991) 9:271–96. doi: 10.1146/annurev.iy.09.040191.001415
144. Kim B, Kim TH. Fundamental role of dendritic cells in inducing Th2 responses. *Korean J Intern Med*. (2018) 33:483–9. doi: 10.3904/kjim.2016.227
145. Deligeoroglou E, Giannouli A, Athanasopoulos N, Karountzos V, Vatopoulou A, Dimopoulos K, et al. HPV infection: immunological aspects and their utility in future therapy. *Infect Dis Obstet Gynecol*. (2013) 2013:540850. doi: 10.1155/2013/540850
146. Scott M, Nakagawa M, Moscicki AB. Cell-mediated immune response to human papillomavirus infection. *J Allergy Clin Immunol*. (2001) 8:209–20. doi: 10.1128/CDLI.8.2.209-220.2001
147. Leone P, Shin EC, Perosa F, Vacca A, Dammacco F, Racanelli V. MHC class i antigen processing and presenting machinery: organization, function, and defects in tumor cells. *J Natl Cancer Inst*. (2013) 105:1172–87. doi: 10.1093/jnci/djt184
148. Andersen MH, Schrama D, Thor Straten P, Becker JC. Cytotoxic T cells. *J Invest Dermatol*. (2006) 126:32–41. doi: 10.1038/sj.jid.5700001
149. Burnet M. Cancer; a biological approach. I. The processes of control. *Br Med J*. (1957) 1:779–86. doi: 10.1136/bmj.1.5022.779
150. Nasi A, Fekete T, Krishnamurthy A, Snowden S, Rajnavölgyi E, Catrina AI, et al. Dendritic cell reprogramming by endogenously produced lactic acid. *J Immunol*. (2013) 191:3090–9. doi: 10.4049/jimmunol.1300772
151. Hsieh C, Macatonia S, Tripp C, Wolf S, O'Garra A, Murphy K. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science*. (1993) 260:547–9. doi: 10.1126/science.8097338
152. Wang KS, Frank DA, Ritz J. Interleukin-2 enhances the response of natural killer cells to interleukin-12 through up-regulation of the interleukin-12 receptor and STAT4. *Blood*. (2000) 95:3183–90. doi: 10.1182/blood.v95.10.3183.010k36_3183_3190
153. Gu Y, Yang J, Ouyang X, Liu W, Li H, Yang J, et al. Interleukin 10 suppresses Th17 cytokines secreted by macrophages and T cells. *Eur J Immunol*. (2008) 38:1807–13. doi: 10.1002/eji.200838331
154. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. I Nterleukin−10 and the I Nterleukin−10 R eceptor. *Annu Rev Immunol*. (2001) 19:683–765. doi: 10.1146/annurev.immunol.19.1.683
155. Zhao S, Wu D, Wu P, Wang Z, Huang J, Gao JX. Serum IL-10 predicts worse outcome in cancer patients: a meta-analysis. *PLoS ONE*. (2015) 10:1–15. doi: 10.1371/journal.pone.0139598
156. Langowski JL, Zhang X, Wu L, Mattson JD, Chen T, Smith K, et al. IL-23 promotes tumour incidence and growth. *Nature*. (2006) 442:461–5. doi: 10.1038/nature04808
157. Shime H, Yabu M, Akazawa T, Kodama K, Matsumoto M, Seya T, et al. Tumor-secreted lactic acid promotes IL-23/IL-17 proinflammatory pathway. *J Immunol*. (2008) 180:7175–83. doi: 10.4049/jimmunol.180.11.7175
158. Fischer K, Hoffmann P, Voelkl S, Meidenbauer N, Ammer J, Edinger M, et al. Inhibitory effect of tumor cell-derived lactic acid on human T cells. *Blood*. (2007) 109:3812–9. doi: 10.1182/blood-2006-07-035972
159. Brand A, Singer K, Koehl GE, Kolitzus M, Schoenhammer G, Thiel A, et al. LDHA-associated lactic acid production blunts tumor immunosurveillance by T and NK cells. *Cell Metab*. (2016) 24:657–71. doi: 10.1016/j.cmet.2016.08.011
160. Daneshmandi S, Wegiel B, Seth P. Blockade of lactate dehydrogenase-A (LDH-A) improves efficacy of anti-programmed cell death-1 (PD-1) therapy in melanoma. *Cancers*. (2019) 11:450. doi: 10.3390/cancers11040450
161. Calcinotto A, Filipazzi P, Grioni M, Iero M, De Milito A, Ricupito A, et al. Modulation of microenvironment acidity reverses anergy in human and murine tumor-infiltrating T lymphocytes. *Cancer Res*. (2012) 72:2746–56. doi: 10.1158/0008-5472.CAN-11-1272
162. Pilon-Thomas S, Kodumudi KN, El-Kenawi AE, Russell S, Weber AM, Luddy K, et al. Neutralization of tumor acidity improves antitumor responses to immunotherapy. *Cancer Res*. (2016) 76:1381–90. doi: 10.1158/0008-5472.CAN-15-1743
163. Colegio OR, Chu NQ, Szabo AL, Chu T, Rhebergen AM, Jairam V, et al. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature*. (2014) 513:559–63. doi: 10.1038/nature13490
164. Lin S, Sun L, Lyu X, Ai X, Du D, Su N, Li H, Zhang L, Yu J, Yuan S. Lactate-activated macrophages induced aerobic glycolysis and epithelial-mesenchymal transition in breast cancer by regulation of CCL5-CCR5 axis: a positive metabolic feedback loop. *Oncotarget*. (2017) 8:110426–43. doi: 10.18632/oncotarget.22786
165. Mu X, Shi W, Xu Y, Xu C, Zhao T, Geng B, et al. Tumor-derived lactate induces M2 macrophage polarization via the activation of the ERK/STAT3 signaling pathway in breast cancer. *Cell Cycle*. (2018) 17:428–38. doi: 10.1080/15384101.2018.1444305
166. Lambert AW, Pattabiraman DR, Weinberg RA. Emerging biological principles of metastasis. *Cell*. (2017) 168:670–91. doi: 10.1016/j.cell.2016.11.037
167. Walenta S, Schroeder T, Mueller-Klieser W. Lactate in solid malignant tumors: potential basis of a metabolic classification in clinical oncology. *Curr Med Chem*. (2012) 11:2195–204. doi: 10.2174/0929867043364711
168. Matsumura A, Isobe T, Takano S, Kawamura H, Anno I. Non-invasive quantification of lactate by proton MR spectroscopy and its clinical applications. *Clin Neurol Neurosurg*. (2005) 107:379–84. doi: 10.1016/j.clineuro.2004.10.009
169. Castagnoli L, Iorio E, Dugo M, Koschorke A, Faraci S, Canese R, et al. Intratumor lactate levels reflect HER2 addiction status in HER2-positive breast cancer. *J Cell Physiol*. (2019) 234:1768–79. doi: 10.1002/jcp.27049
170. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer*. (2013) 13:714–26. doi: 10.1038/nrc3599
171. Amoedo ND, Obre E, Rossignol R. Drug discovery strategies in the field of tumor energy metabolism: limitations by metabolic flexibility and metabolic resistance to chemotherapy. *Biochim Biophys Acta Bioenerg*. (2017) 1858:674–85. doi: 10.1016/j.bbabio.2017.02.005

172. Qu Y, Dou B, Tan H, Feng Y, Wang N, Wang D. Tumor microenvironment-driven non-cell-autonomous resistance to antineoplastic treatment. *Mol Cancer*. (2019) 18:1–16. doi: 10.1186/s12943-019-0992-4
173. Apicella M, Giannoni E, Fiore S, Ferrari KJ, Fernández-Pérez D, Isella C, et al. Increased lactate secretion by cancer cells sustains non-cell-autonomous adaptive resistance to MET and EGFR targeted therapies. *Cell Metab*. (2018) 28:848-865.e6. doi: 10.1016/j.cmet.2018.08.006
174. DeBerardinis RJ, Chandel NS. Fundamentals of cancer metabolism. *Sci Adv*. (2016) 2:e1600200. doi: 10.1126/sciadv.1600200
175. Feng Y, Xiong Y, Qiao T, Li X, Jia L, Han Y. Lactate dehydrogenase A: a key player in carcinogenesis and potential target in cancer therapy. *Cancer Med*. (2018) 7:6124–36. doi: 10.1002/cam4.1820
176. Jafary F, Ganjalikhany MR, Moradi A, Hemati M, Jafari S. Novel peptide inhibitors for lactate dehydrogenase A (LDHA): a survey to inhibit LDHA activity via disruption of protein-protein interaction. *Sci Rep*. (2019) 9:1–13. doi: 10.1038/s41598-019-38854-7
177. Zhou Y, Tao P, Wang M, Xu P, Lu W, Lei P, et al. Development of novel human lactate dehydrogenase A inhibitors: high-throughput screening, synthesis, and biological evaluations. *Eur J Med Chem*. (2019) 177:105–15. doi: 10.1016/j.ejmech.2019.05.033
178. Kim EY, Chung TW, Han CW, Park SY, Park KH, Jang SB, et al. A novel lactate dehydrogenase inhibitor, 1-(phenylseleno)-4-(trifluoromethyl) benzene, suppresses tumor growth through apoptotic cell death. *Sci Rep*. (2019) 9:1–12. doi: 10.1038/s41598-019-40617-3
179. Das CK, Parekh A, Parida PK, Bhutia SK, Mandal M. Lactate dehydrogenase A regulates autophagy and tamoxifen resistance in breast cancer. *Biochim Biophys Acta Mol Cell Res*. (2019) 1866:1004–18. doi: 10.1016/j.bbamcr.2019.03.004
180. Fang A, Zhang Q, Fan H, Zhou Y, Yao Y, Zhang Y, Huang X. Discovery of human lactate dehydrogenase A (LDHA) inhibitors as anticancer agents to inhibit the proliferation of MG-63 osteosarcoma cells. *MedChemComm*. (2017) 8:1720–6. doi: 10.1039/c7md00222j
181. Manerba M, Di Ianni L, Govoni M, Roberti M, Recanatini M, Di Stefano G. LDH inhibition impacts on heat shock response and induces senescence of hepatocellular carcinoma cells. *Eur J Pharm Sci*. (2017) 105:91–8. doi: 10.1016/j.ejps.2017.05.015
182. Curtis NJ, Mooney L, Hopcroft L, Michopoulos F, Whalley N, Zhong H, et al. Pre-clinical pharmacology of AZD3965, a selective inhibitor of MCT1: DLBCL, NHL and Burkitts lymphoma anti-tumor activity. *Oncotarget*. (2017) 8:69219–36. doi: 10.18632/oncotarget.18215
183. Guan X, Rodriguez-Cruz V, Morris ME. Cellular uptake of MCT1 inhibitors AR-C155858 and AZD3965 and their effects on MCT-mediated transport of L-lactate in murine 4T1 breast tumor cancer cells. *AAPS J*. (2019) 21:5. doi: 10.1208/s12248-018-0279-5
184. Amorim R, Pinheiro C, Miranda-Gonçalves V, Pereira H, Moyer MP, Preto A, et al. Monocarboxylate transport inhibition potentiates the cytotoxic effect of 5-fluorouracil in colorectal cancer cells. *Cancer Lett*. (2015) 365:68–78. doi: 10.1016/j.canlet.2015.05.015
185. Quanz M, Bender E, Kopitz C, Grünewald S, Schlicker A, Schwede W, et al. Preclinical efficacy of the novel monocarboxylate transporter 1 inhibitor BAY-8002 and associated markers of resistance. *Mol Cancer Ther*. (2018) 17:2285–96. doi: 10.1158/1535-7163.MCT-17-1253
186. Benjamin D, Robay D, Hindupur SK, Pohlmann J, Colombi M, El-Shemerly MY, et al. Dual inhibition of the lactate transporters MCT1 and MCT4 is synthetic lethal with metformin due to NAD+ depletion in cancer cells. *Cell Rep*. (2018) 25:3047-3058.e4. doi: 10.1016/j.celrep.2018.11.043
187. Ciocca DR, Calderwood SK. Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. *Cell Stress Chaperones*. (2005) 10:86–103. doi: 10.1379/csc-99r.1
188. Zhao YH, Zhou M, Liu H, Ding Y, Khong HT, Yu D, et al. Upregulation of lactate dehydrogenase A by ErbB2 through heat shock factor 1 promotes breast cancer cell glycolysis and growth. *Oncogene*. (2009) 28:3689–701. doi: 10.1038/onc.2009.229
189. La Manna S, Di Natale C, Florio D, Marasco D. Peptides as therapeutic agents for inflammatory-related diseases. *Int J Mol Sci*. (2018) 19:1–18. doi: 10.3390/ijms19092714
190. Henninot A, Collins JC, Nuss JM. The current state of peptide drug discovery: back to the future? *J Med Chem*. (2018) 61:1382–14. doi: 10.1021/acs.jmedchem.7b00318
191. Kurrikoff K, Aphkhazava D, Langel Ü. The future of peptides in cancer treatment. *Curr Opin Pharmacol*. (2019) 47:27–32. doi: 10.1016/j.coph.2019.01.008
192. Murray CM, Hutchinson R, Bantick JR, Belfield GP, Benjamin AD, Brazma D, et al. Monocarboxylate transporter Mctl is a target for immunosuppression. *Nat Chem Biol*. (2005) 1:371–6. doi: 10.1038/nchembio744

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REVIEW
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The Metabolic Landscape of Lung Cancer: New Insights in a Disturbed Glucose Metabolism

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Metabolism encompasses the biochemical processes that allow healthy cells to keep energy, redox balance and building blocks required for cell development, survival, and proliferation steady. Malignant cells are well-documented to reprogram their metabolism and energy production networks to support rapid proliferation and survival in harsh conditions via mutations in oncogenes and inactivation of tumor suppressor genes. Despite the histologic and genetic heterogeneity of tumors, a common set of metabolic pathways sustain the high proliferation rates observed in cancer cells. This review with a focus on lung cancer covers several fundamental principles of the disturbed glucose metabolism, such as the "Warburg" effect, the importance of the glycolysis and its branching pathways, the unanticipated gluconeogenesis and mitochondrial metabolism. Furthermore, we highlight our current understanding of the disturbed glucose metabolism and how this might result in the development of new treatments.

Keywords: lung cancer, glucose, metabolism, genetic alterations, targeting metabolism

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INTRODUCTION

The metabolic alterations of cancer cells, that distinguish them from healthy cells, are recognized as one of the ten hallmarks of cancer. An altered metabolism helps cancer cells to sustain high proliferative rates even in a hostile environment resulting from a poor vascularization, which limits the supply of oxygen (O_2) and essential nutrients (1).

In the 1920s, Otto Warburg postulated that tumor cells consume glucose and excrete lactate at a significantly higher rate compared to healthy resting cells (2). Even in normoxic conditions, proliferating cells, such as cancer cells, rely on fermentation, i.e., glycolysis resulting in the generation of lactate via fermentation of pyruvate. The increased reduction of pyruvate to lactate and the passage of glycolytic intermediates into diverse biosynthetic pathways reduces the available concentration of pyruvate to form acetyl-CoA and to drive the tricarboxylic acid (TCA) cycle. In contrast with the original hypothesis of Warburg, the mitochondrial metabolism remains vital for both the production of ATP and the supply of biosynthetic intermediates (3). The TCA cycle or Krebs cycle is a mitochondrial pathway where acetyl-CoA undergoes a condensation reaction with oxaloacetate (OAA) to form carbon dioxide (CO_2). In successive oxidation reactions, the coenzymes NAD^+ and FAD are reduced and subsequently used to drive the generation of the

majority of ATP by oxidative phosphorylation (OXPHOS). Although the Warburg effect is often found in malignant tumors, OXPHOS still has a significant contribution to the energy supply of at least some cancers (4, 5). Furthermore, metabolic intermediates are deviated toward biosynthetic processes operational in growing and proliferating malignant cells. To compensate for the ongoing drainage of TCA cycle metabolites into anabolic pathways, glutamine is often used in cancer cells as a carbon source to replenish TCA cycle intermediates (6, 7).

In this review, we focus on the altered glucose metabolism in lung cancer cells. As lung cancer is by far the leading cause of cancer death with limited curative treatment options, detailed understanding of the dysregulated glucose metabolism and its associated signaling pathways may help us to design more efficient treatment regimens (8, 9).

GLYCOLYSIS: ATP AND BUILDING BLOCKS

During glycolysis, each molecule of glucose is broken down in ten steps to two molecules of pyruvate resulting in a net gain of two molecules of NADH and two ATP. In the presence of O_2, healthy cells further oxidize pyruvate to CO_2 through the mitochondrial located oxidative pathways, i.e., the TCA cycle and OXPHOS. Starting from one molecule of glucose, the combined action of the pathways mentioned above, generally known as aerobic respiration, results in the production of water as well as at least 32 ATP molecules. Under anaerobic conditions, pyruvate is reduced to lactate by lactate dehydrogenase (LDH), and lactate is secreted in the extracellular space by monocarboxylate transporters (MCT). Unlike healthy cells, lung cancer cells metabolize glucose via lactic acid fermentation even in the presence of sufficient O_2. This metabolic condition received a plethora of names, such as aerobic fermentation, aerobic glycolysis or Warburg effect (10). Otto Warburg observed that cancer cells generate ATP through a non-oxidative pathway, i.e., glycolysis with the generation of lactic acid, even in normoxic conditions, and attributed this to mitochondrial dysfunction. To emphasize this process in the presence of O_2, the historical concept of Warburg has led to the misleading term "aerobic glycolysis." In our opinion, the term "aerobic fermentation" as coined by Warburg himself as "a property of all growing cancer cells" seems more appropriate to denote the fermentation in the presence of O_2 (2). Aerobic fermentation is nowadays seen as a hallmark of rapid cell proliferation even in a non-cancerous context (11). As compared to aerobic respiration, (an)aerobic fermentation produces a 16-fold lower amount of ATP per glucose consumed, making it an inefficient way of generating ATP. However, under the non-limiting supply of glucose, a ∼15 times higher glycolytic flux can be reached as compared to TCA cycle flux and consequently, a drastic increase in ATP production rate in aerobic fermentation (12). After the phosphorylation of glucose by hexokinase (HK), glucose-6-phosphate can no longer leave the cell. This combined activity of glucose uptake and its subsequent phosphorylation forms the basis for Positron Emission Tomography (PET) imaging in which an injected radioactive glucose analog (^{18}F-FDG) is detected in higher concentrations in lung cancer tissue than in healthy tissues (13, 14). Currently, metabolic imaging with ^{18}F-FDG-PET is regarded as a standard of care in the management of lung cancer (15, 16). The high intracellular concentrations of glucose-6-phosphate (glucose-6-P) are indispensable to maintain high glycolytic activity, and thus upregulation of HK and the glucose transporter GLUT are essential. The upregulation of the isoform GLUT1 and the relation with the uptake of ^{18}F-FDG have been demonstrated in lung cancer tissue, as well as overexpression of the HK2 isoform (17, 18). Glucose-6-phosphate has to continue along the glycolytic pathway to result in the final product pyruvate in aerobic, or lactate in anaerobic conditions (**Figure 1**). The upregulation of almost all glycolytic enzymes has been demonstrated, including HK2 and phosphofructokinase 1 (PFK1) that catalyzes the committed step in glycolysis namely, the phosphorylation of fructose-6-phosphate into fructose-1,6-bisphosphate (19). Fructose-1,6-bisphosphate is subsequently converted into dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) by aldolase (ALDO). In contrast with GAP, DHAP is not on the direct pathway of glycolysis. To prevent loss of this three-carbon fragment, and thus ATP, DHAP is isomerized to GAP by triose-phosphate isomerase (TPI). The resulting GAP is oxidized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH*)* into 1,3-bisphosphoglycerate (1,3-BPG). As this reaction is at the expense of NAD^+, the NADH formed by this reaction must be continuously re-oxidized to NAD^+ for glycolysis to continue. Hence, the fate of lactate production from pyruvate finds its rationale in this recycling process. The importance of this reaction is demonstrated by a decreased survival and proliferation of lung cancer cells during the inhibition of LDH (20). Excretion of lactate through MCT4 transporters does not only result in the acidification of the microenvironment, but also modulates the immune cell function and promotes invasion and metastasis (21). The microenvironment in which lung cancer cells live is heterogeneous because of ineffective tumor vascularization. As a consequence, cancer cells may be subject to hypoxia and nutrient deprivation. Interestingly, swapping of lactate between hypoxic and oxygenated cells has been reported (22–24). Using MCT1 transporters, normoxic lung cancer cells can remove lactate from the microenvironment and convert it to pyruvate for further oxidation, conserving glucose for use by the hypoxic cells. In contrast with the initial hypothesis of Warburg, a majority of human cancers, including lung cancer, produces ATP through OXPHOS (25). Besides for ATP production, a high glycolytic rate is imperative to support cancer cell proliferation by supplying building blocks to duplicate the cell biomass and genome at each cell division (26). In this context, the Warburg effect or aerobic fermentation has been hypothesized to support the biosynthetic requirements of uncontrolled proliferation rather than ATP generation. The excess glycolytic carbon is deviated to multiple anabolic pathways that branch off from the glycolytic pathway (**Figure 1**).

A remarkable enzyme that supports the metabolism in lung cancer cells is pyruvate kinase (PK). PK catalyzes the transfer of phosphate from phosphoenolpyruvate (PEP) to ADP to produce

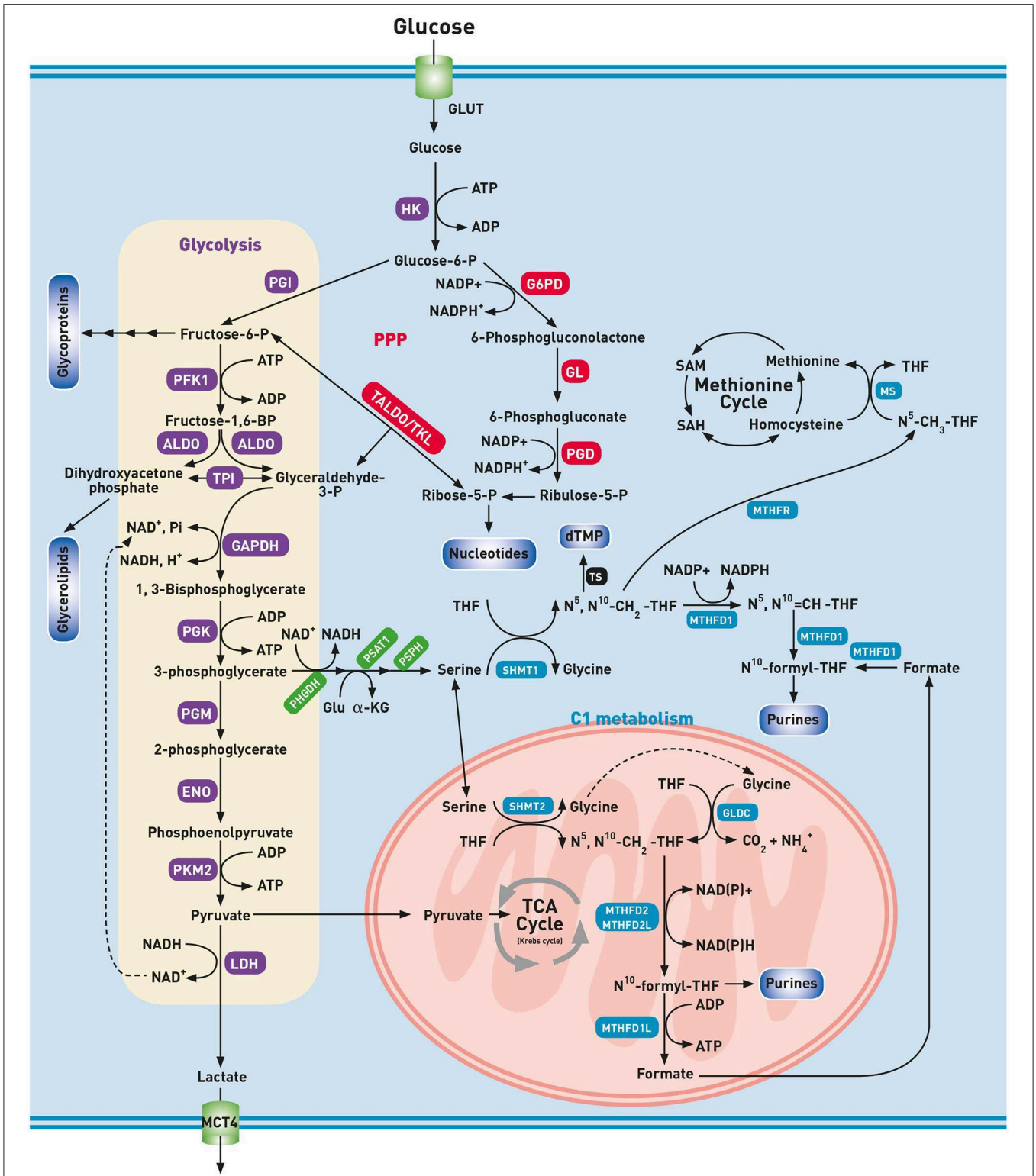

FIGURE 1 | Glycolysis and biosynthetic pathways emanating from glycolysis. ALDO, aldolase; dTMP, deoxythymidine monophosphate; ENO, enolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GL, gluconolactonase; Glu, glutamine; GLUT, glucose transporter; G6PD, glucose-6-phosphate 1-dehydrogenase; GLDC, glycine cleavage system P protein; HK, hexokinase; LDH, lactate dehydrogenase; MCT4, monocarboxylate transporter 4; MS, methionine synthase; MTHFD, methylenetetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; NH_4^+, ammonia; N^5-CH_3-THF, methyl-tetrahydrofolate; N^5N^{10}-CH_2-THF, methylene-tetrahydrofolate; N^5N^{10}-CH=THF, methenyl-tetrahydrofolate; N^{10}-formyl-THF, formyl-tetrahydrofolate; PFK1, phosphofructokinase 1; PGM, phosphoglycerate mutase; PGD, 6-phosphogluconate dehydrogenase; PGI, phosphoglucoisomerase; PGK, phosphoglycerate kinase; PHGDH, phosphoglycerate

(Continued)

FIGURE 1 | dehydrogenase; PKM2, pyruvate kinase M2; PPP, pentose phosphate pathway; PSAT1, phosphoserine aminotransferase 1; PSPH, phosphoserine phosphatase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SHMT, serine hydroxyl-methyltransferase; TALDO, transaldolase; THF, tetrahydrofolate; TKL, transketolase; TPI, triose phosphate isomerase; TS, thymidylate synthetase. Glycolysis (purple), One-carbon metabolism (blue), PPP (red), Serine biosynthesis (green), Other pathways (black).

ATP and pyruvate. PK comprises four isoenzymes (L, R, M1, and M2) derived from two genes. Cancer cells prefer expressing the PKM2 form by alternative splicing. The isoenzyme PKM2 occurs in a dimeric or tetrameric form. The tetrameric form has a high affinity to PEP and is present in normal proliferating cells. In contrast, the dimeric form is defined by a lower affinity to PEP. Lung cancer cells are characterized by expression of a dimeric form of PKM2 which implies that all glycolytic intermediates preceding PKM2 activity accumulate and are directed into biosynthetic processes, such as nucleotide-, lipid- and serine/glycine synthesis which stimulates tumor proliferation as demonstrated in **Figure 1** (27–29).

METABOLIC PATHWAYS EMANATING FROM GLYCOLYSIS

The Pentose Phosphate Pathway (PPP)

The PPP consists of two phases: a reversible non-oxidative phase and an irreversible oxidative phase. Overexpression and upregulation of two enzymes of the oxidative phase, i.e., glucose-6-phosphate 1-dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (PGD), contributes to increased production of NADPH and ribose-5-phosphate in lung cancer (30). NADPH is a principal reducing agent that is employed in biosynthetic pathways, such as the synthesis of fatty acids, cholesterol and nucleotides. Furthermore, NADPH is oxidized during the reduction of oxidized glutathione (GSSG) to glutathione (GSH), which is essential for the detoxification of reactive oxygen species (ROS). To keep hypoxia-induced ROS due to aberrant vascularization in balance, reduced glutathione and thus NADPH is required (31). Ribose-5-phosphate is an essential building block of coenzymes as well as purine and pyrimidine nucleotides. In contrast with healthy cells, the non-oxidative phase of the PPP seems to be important in lung cancer cells (32–34). The glycolytic intermediates fructose-6-phosphate (fructose-6-P) and GAP are diverted toward ribose-5-phosphate production by transaldolase and transketolase (35). Transketolase-like-protein 1 (TKTL1) protein, a transketolase associated with the condition of aerobic fermentation is overexpressed in lung cancer cells resulting in a higher amount of ribose-5-phosphate (ribose-5-P) than needed for de novo synthesis of purines and pyrimidines (33, 34).

The Hexosamine Biosynthetic Pathway (HBP)

Fructose-6-phosphate can branch off from the glycolytic pathway as a substrate in the HBP. The upregulated import of both glucose and glutamine results in an increased flux through the HBP and an increased level of its end product UDP-GlcNAc (36). UDP-GlcNAc is an essential metabolite for synthesis of many glycoconjugates, such as glycosaminoglycans, glycolipids, and glycoproteins. Lung cancer cells exhibits striking alterations in glycosylation but their complete description is out of the scope of this review, and Lemjabbar-Alaoui et al. described this extensively (37). O-GlcNAcylation, i.e., the enzymatic addition of the N-acetylglucosamine moiety of UDP-GlcNAc to the hydroxyl groups of serine and threonine residues, is of particular interest in lung cancer. As UDP-GlcNAc is the end product of the HBP, a pathway that makes direct use of glucose and glutamine inputs, the O-GlcNAcylation is modulated by nutrient availability and thereby acts as a nutrient sensor and metabolic regulator (38). The process of O-GlcNAcylation is regulated by O-GlcNAc-transferase (OGT) and its opponent O-GlcNAcase (OGA). Mi et al. demonstrated an elevated expression of OGT and an increased O-GlcNAcylation in lung cancer tissue. However, there was significant difference in OGA levels between cancer tissue and adjacent healthy tissue (39).

O-GlcNAcylation, an epigenetic modification of cellular proteins, oncogenes, and tumor suppressor genes, can significantly impact tumor growth, proliferation, invasion, and metastasis (40). For instance, the oncogene c-MYC is frequently expressed at constitutive high levels. Once activated by an extracellular tyrosine kinase, the degradation of c-MYC is regulated by phosphorylation of specific sites. Increased O-GlcNAcylation of the threonine site competes with its phosphorylation, resulting in the stabilization of c-MYC and sustained transcription of genes involved in the tumorigenesis. On the enzymatic level, O-GlcNAcylation is a modulator of several glycolytic enzymes (41). As an example, glycosylation of PFK1 is triggered under hypoxic conditions, and its inactivation redirects the flux of glucose from glycolysis to the PPP, thereby providing reducing power to, among other things, prevent ROS toxicity (42).

The Serine–Glycine Pathway and One-Carbon Metabolism

An amount of glycolytic 3-phosphoglycerate (3-PG), is siphoned into serine and glycine metabolism, which provides carbon units for the one-carbon metabolism. Serine is incorporated into the head-groups phosphatidylserine and sphingolipids and is an abundant constituent of proteins (43). The serine biosynthesis pathway uses three subsequent enzymes to convert 3-PG into serine (**Figure 1**) (44). The increased expression of phosphoglycerate dehydrogenase (PHGDH) and the upregulation of both phosphoserine aminotransferase 1 (PSAT1) and phosphoserine phosphatase (PSPH) highlight the importance of the serine biosynthesis pathway in lung cancer biology (45, 46). Serine is the primary substrate for the so-called one-carbon cycle (47). The one-carbon metabolism, that includes both the folate and methionine cycles, is a complex metabolic network based on the biochemical

reactions of folate components. A pivotal reaction of the folate cycle is the conversion of serine to glycine by serine hydroxyl-methyltransferase enzymes (cytosolic SHMT1 and mitochondrial SHMT2). This reaction generates glycine and N^5,N^{10} methylenetetrahydrofolate (N^5,N^{10}-CH_2-THF) which is the first one-carbon donor in the folate cycle. The knockdown of SHMT results in cell cycle arrest and cell death, suggesting that SHMT plays a crucial role in lung cancer (48). The cleavage of glycine into CO_2 and NH_4^+ by a decarboxylase (GLDC) of the glycine cleavage system (GCS) likewise results in the production of N^5,N^{10}-CH_2-THF. The GCS results in significant changes in both the glycolysis and serine/glycine metabolism of lung cancer patients, leading to changes in pyrimidine metabolism and cancer cell proliferation (46, 49, 50). Lung cancer cells can use N^5,N^{10}-CH_2-THF in several ways: (i) as a one-carbon donor for the first step of thymidylate synthesis; (ii) as a substrate for N^5,N^{10}-CH_2-THF dehydrogenase 1 (MTHFD1) or the mitochondrial tandem enzyme MTHFD2L/MTHFD2 to produce N^{10}-formyl-THF, a one-carbon donor for purine synthesis; or (iii) by N^5,N^{10}-CH_2-THF reductase (MTHFR) to generate N^5-CH_3-THF. This N^5-CH_3-THF donates its methyl group generating methionine and THF. This reaction couples the folate cycle with the methionine cycle and can be considered as the first reaction of the methionine cycle. When the resulting THF is converted into N^5,N^{10}-CH_2-THF by SHMT, the folate cycle is closed. Methionine is the precursor of S-adenosylmethionine (SAM), a methyl donor that plays a role in both DNA and histone methylation. As reported by Mentch et al., intermediary metabolites and cofactors in one-carbon metabolism and SAM metabolism determine the DNA and histone methylation status (51). Promoter hypermethylation plays a significant role in cancer through transcriptional silencing of growth inhibitors, such as tumor suppressor genes. Together with the folate metabolites provided by SHMT-mediated reactions, SAM is vital in maintaining a regular methylation pattern and DNA stability in lung cancer (50–52). In contrast with genetic mutations, epigenetic modifications are reversible. For instance, DNA and histone methylation can be removed by α-ketoglutarate (α-KG) demethylases. The high uptake of glucose and glutamine in proliferative cells results in higher intracellular concentrations of α-KG. However, the glucose and glutamine addiction of malignant cells may end in regional depletion of both nutrients, and thus in a decrease of the α-KG concentration, resulting in the inhibition of demethylation (53). In contrast with this observation, where cell metabolites and enzymes modulate epigenetic phenomena, epigenetic modifications at metabolic genes, such as acylation or O-GlcNAcylation may affect cell metabolism. A detailed description of the link between metabolism and epigenetic changes is out of the scope of this review, and has been described extensively by Yu et al. (54). Summarized, it seems that epigenetic modifications and cellular metabolism interact with each other and that their relationship is reciprocal. Indeed, the enhanced aerobic glycolysis has a disruptive effect on tumor suppressor genes and oncogenes resulting in genomic instability. Loss of genes that are involved in the repair of DNA results in dysregulation of the mitochondrial energy production resulting in metabolic instability. In the theory of Davies et al. the interaction between genomic and metabolic instability enables pre-cancerous cells to obtain a malignant phenotype (55).

After donation of its methyl group, SAM becomes S-adenosylhomocysteine (SAH), which is subsequently converted to homocysteine. Finally homocysteine is either converted back to methionine resulting in a full turn of the cycle or enters the transsulfuration pathway to form cysteine. Cysteine can be incorporated into proteins or can be used in the formation of glutathione (52).

THE ROLE OF REACTIONS OF THE GLUCONEOGENESIS

The discovery that the activation of the gluconeogenesis pathway, until recently thought to be restricted to kidney and liver cells, also occurs in lung cancer cells, unfolds an unanticipated metabolic flexibility of cancerous cells (**Figure 2**) (56). Malignant cells are adapted to upregulate the glycolytic pathway at high rates. Consequently, glucose levels may drop in less perfused tumor areas. The decreased availability of glucose significantly reduces the metabolic flow via glycolysis. This reduction in glycolytic flux may result in a drop of cellular intermediates required for the biosynthesis of building blocks unless other pathways generate these glycolytic intermediates. Whereas, both the gluconeogenesis and glycolytic pathway generate identical intermediates, enhancement of either pathway could increase the supply of building blocks for cell growth. Recently, Vincent et al. described an alternative pathway in lung carcinoma cells involving phosphoenolpyruvate carboxykinase 2 (PEPCK2), a mitochondrial gluconeogenesis enzyme (57). In healthy cells, the gluconeogenesis pathway results in the production of glucose from non-carbohydrate carbon substrates. Under the condition of glucose starvation, the amino acid glutamine can maintain the TCA cycle function. Indeed, glucose-deprived malignant cells use glutamine as an anaplerotic substrate to generate α-ketoglutarate (α-KG) and subsequent TCA cycle intermediates (58). Glutamine-derived oxaloacetate is converted into PEP by mitochondrial PEPCK2, and this glutamine-derived PEP may be used for anabolic purposes (57). Indeed, conversion of PEP into 3-PG by enolase (ENO) and phosphoglyceromutase (PGM) might result in a deviation from the gluconeogenic pathway into the biosynthesis of serine, glycine, glutathione and purine nucleotides. Glutamine-derived PEP may also fuel other biosynthetic pathways that are, in normal conditions, supported by glucose, including the conversion of 1,3-BPG into glycerol for the lipid biosynthesis and utilization of GAP by the non-oxidative branch of the PPP to produce ribose-5-phosphate (59). Recently, Louis et al. detected a higher concentration of glucose and a lower level of alanine in the plasma of lung cancer patients through nuclear magnetic resonance (NMR) metabolomics (25). These findings suggest the role of a compensatory gluconeogenesis to sustain high glucose levels in plasma to support the ongoing glycolysis in cancer cells. Here, in contrast with the rescue pathway proposed by Vincent et al., the source of glucose is the gluconeogenesis of healthy cells.

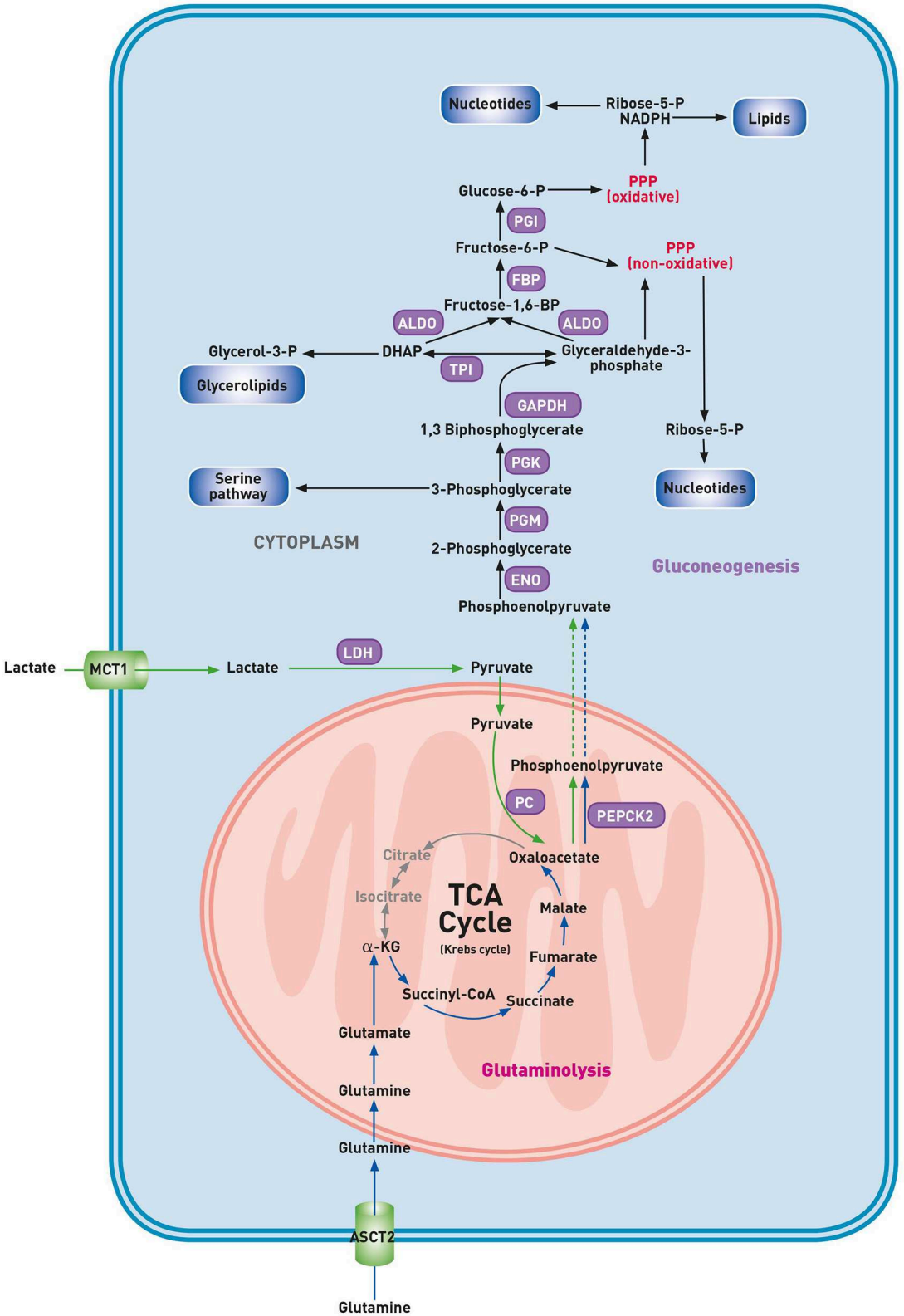

FIGURE 2 | Gluconeogenesis pathway in glucose deprived lung cancer cells. α-KG, α-ketoglutarate; ALDO, aldolase; ASCT2, alanine-serine-cysteine-transporter 2; DHAP, dihydroxyacetone phosphate; ENO, enolase; FBP, fructose bisphosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate

(Continued)

FIGURE 2 | dehydrogenase; MCT1, monocarboxylate transporter 1; PC, pyruvate carboxylase; PEPCK2, phosphoenolpyruvate carboxykinase 2; PGM, phosphoglycerate mutase; PGI, phosphoglucoisomerase; PGK, phosphoglycerate kinase; PPP, pentose phosphate pathway; TPI, triose phosphate isomerase. Gluconeogenesis pathway (purple), lactic carbon (green arrows), glutaminolytic carbon (blue arrows).

THE ROLE OF THE TCA CYCLE AND OXIDATIVE PHOSPHORYLATION

In contrast with the original hypothesis of Warburg, cancer cells have functional mitochondria that act as biosynthetic hubs. Respiration, oxidative metabolism and other mitochondrial pathways are required by malignant cells for tumor growth (3, 60). An important metabolic pathway that occurs in the mitochondrial matrix is the TCA cycle or Krebs cycle. The TCA cycle is composed of biochemical reactions that oxidize fuel sources to provide ATP, support the synthesis of macromolecules and regulate the cellular redox balance. Moreover, the TCA cycle provides precursors of various amino acids. When TCA cycle intermediates, such as glucose- and glutamine-derived α-KG, are diverted for synthesis of macromolecules and ATP they need to be replaced to permit the sustained function of the TCA cycle by anaplerosis. This process is accomplished via two major pathways: glutaminolysis and carboxylation of pyruvate to OAA via pyruvate carboxylase (PC). As this review focuses on the disturbed glucose metabolism, we refer the interested reader to our recently published review that describes the role of glutamine in lung cancer (7). An important step in the TCA cycle is the conversion of isocitrate to α-KG by isocitrate dehydrogenases (IDH) and thereafter to succinate and fumarate by succinate dehydrogenase (SDH) and fumarase (FH), respectively. Mutations in genes encoding for IDH, FH, and the SCD complex lead to an altered metabolism, i.e., accumulation of TCA cycle metabolites, that enhances cell transformation by epigenetic alterations (61). Mutations in IDH1 and IDH2 are found in 1% of NSCLC and result in the conversion of α-KG to 2-hydroxyglutarate (62). This oncometabolite is considered as a competitive inhibitor of multiple dioxygenase enzymes that use α-KG as a cofactor, such as histone demethylases and TET (ten-eleven translocation) proteins resulting in DNA and histone methylation alterations and epigenetic changes altering gene expression (61). In addition, both TET2 and 2-hydroxyglutarate block differentiation in hematopoietic cells. Inactivating mutations of SDH and FH have been identified in several cancers and result in accumulation of succinate and fumarate, respectively. Succinate and fumarate are capable of inhibiting multiple α-KG dependent dioxygenases. Due to inhibition of prolyl-hydroxylases, HIF1 accumulates in SDH and FH mutant tumors and promotes metabolic rewiring of the glucose metabolism.

The voltage-dependent anion channel (VDAC1) is considered as the mitochondrial gatekeeper. The VDAC1 is the main transport channel for metabolites and its overexpression in many cancers indicates that this mitochondrial pore contributes to the metabolic phenotype of cancer cells (63). Along the regulation of the metabolic and energetic homeostasis, VDAC1 functions as a regulator of the redox balance by its capacity to transport ROS. In addition, the mitochondrial pore is involved in the process of apoptosis by interaction with inhibitors of cell death and the release of apoptotic proteins. For example, binding between VDAC1 and HK2 leads not only to a metabolic benefit but also results in the inhibition of apoptosis offering the cell not only a proliferative advantage but also protection against chemotherapy induced cell death. Downregulation of VDAC1 expression in cancer may impair the exchange of metabolites between the cytosol and the mitochondria leading to inhibition of growth and proliferation of cancer cells and their ability to evade apoptosis. The OXPHOS pathway effectively generates ATP by electron transport through several protein complexes across the mitochondrial membrane. As previously described, OXPHOS is often downregulated in hypoxic cancer tissue to limit the production of ROS by the mitochondrial respiratory chain. Warburg proposed that a decreased OXPHOS induced the enhanced glycolysis due to mitochondrial defects. This concept has been applied to all types of cancer cells without appropriate experimental evaluation. However, recently, Moreno-Sanchez described the contribution of OXPHOS in lung cancer and several other cancers. In contrast with previous assumptions, the majority of ATP in cancer cells is produced during OXPHOS (64). Indeed, studies by Hensley et al. and Davidson et al. reveal that both glycolysis and mitochondrial OXPHOS are elevated in non-small cell lung tumors (65, 66). Many other authors nowadays also support the idea that mitochondrial OXPHOS might actually be suppressed as a result of the dominating strong upregulation of the glycolysis, rather than being initially impaired as stated by Warburg. This means that OXPHOS might serve as an additional rescue energy alternative in cancer cells, in case of glycolysis inhibition (67, 68). The other way around, OXPHOS can also be preferred for energy production in normoxic conditions in order to spare glucose which can be used in an hypoxic environment.

Lactate, produced by glycolysis in both cancer cells and carcinoma-associated fibroblasts (CAFs), is converted to pyruvate and enters the mitochondria of aerobic lung cancer cells to undergo OXPHOS to generate ATP (69). This lactate shuttling, mainly via MCT1 and MCT4, is one important way how cancer tissue keeps the interplay between glycolytic and oxidative cells in balance (22). A plausible explanation might be found in the heterogeneity of lung tumors. They show to exhibit both the glycolytic and oxidative metabolic phenotype between different regions inside the same tumor (65). It seems that cancer cells of the same tumor can be divided into subgroups, often depending on their microenvironment: highly glycolytic with lower OXPHOS in hypoxic conditions and the other way around where nutrients are rather low (68). Strikingly, some lung tumors that have acquired resistance against targeted therapy also seem to switch to elevated OXPHOS activity, leaving it vulnerable for inhibition (70).

Since different types of cancer rely on the OXPHOS pathway for their development, OXPHOS inhibition is a target of several cancer therapy studies (71). For example, NSCLC tumors with LKB1 (liver kinase B) tumor suppressor mutation are shown to be sensitive to phenformin, as it shuts down oxygen consumption in these cells by inhibition of the protein complex I of the oxidative respiratory chain. Instead of reprogramming to using glycolysis for ATP generation, LKB1 mutated NSCLC cells are shown to exhibit an OXPHOS-driven phenotype (72).

GENETIC REGULATION OF LUNG CANCER METABOLISM

Lung cancer cells often harbor mutations in genes and pathways, such as the PI3K (phosphoinositide-3-kinase)-AKT-mTOR (mammalian target of rapamycin) pathway, the oncogenes RAS, c-MYC, and HIF-1 (hypoxia inducible factor), and the tumor suppressor gene TP53 (tumor protein) (73–78). These cell signaling pathways are implicated in the metabolism by securely regulating the capacity of cells to obtain access to nutrients and subsequently process these compounds.

PI3K-AKT-mTOR Pathway

The PI3K-AKT-mTOR pathway, one of the signaling pathways most frequently altered in cancer, is an essential regulator of metabolism, coordinating the uptake and fate of glucose (74, 75, 79). The PI3K-AKT-mTOR pathway can be aberrantly activated by multiple factors including oncogenic genomic alterations in e.g., PI3K, PTEN (phosphatase and tensin homolog), AKT, TSC (tuberous sclerosis complex), LKB1, and mTOR (80). The binding of ligands, such as epidermal growth factor, to receptor tyrosine kinases, results in dimerization of the receptors which stimulates the receptor's intrinsic cytoplasmatic kinase activity, leading to auto- and transphosphorylation on tyrosine residues, which serves as docking sites of several proteins and enzymes. Recruitment of PI3K to the membrane results in the phosphorylation of the membrane compound phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3). The serine/threonine kinase AKT is recruited to the plasma membrane along with PI3K-dependent kinase 1 which has been recruited and activated by PIP_3. Phosphorylation of specific threonine and serine residues by PI3K-dependent kinase 1 and mTORC2 is essential for complete AKT activation. Once activated, AKT potentially phosphorylates many proteins which explains its broad range of downstream effects in angiogenesis, apoptosis, differentiation, and proliferation. In contrast, PTEN is a phosphatase that reduces the intracellular levels of PIP_3 and functions as a tumor suppressor by inhibition of the AKT signaling cascade. AKT also fulfills a critical role in the uptake and metabolism of glucose by promoting the transcription of several glycolytic enzymes, such as HK, PFK1, and recruitment of GLUTs to the cell membrane (81, 82). While overexpression of nutrient transporters can help cells to harvest scarce blood-born nutrients, it has become recognized that malignant cells acquire the capacity to bypass the blood circulation and obtain nutrients by scavenging macromolecules from the microenvironment i.e., extrinsic scavenging. In contrast to autophagy or intrinsic scavenging, extrinsic scavenging can maintain survival and promote growth (83). Macropinocytosis begins with the activation of RAC1, a small GTPase, and a cell division control protein that produces ruffles that form circular cups. Closure of these cups depends on both PIP_3 production and RAC1 inhibition. Inactivation or loss of PTEN, elevates the intracellular PIP_3 levels which results in the stimulation of the uptake of macropinosomes by murine fibroblasts (83, 84). Furthermore, PTEN inhibition in these fibroblasts allowed them to grow even in a nutrient-depleted medium in a manner that depends on macropinocytosis. Whether other tumor types with reduced PTEN activity, such as lung cancer, use macropinocytosis to support growth, requires further research.

Downstream of PI3K and PTEN, activated AKT inhibits TSC2 via phosphorylation. Inactive TSC2 is uncapable to bind RHEB, which enables its activation of mTORC1 initiating its effect on downstream proteins that play a role in protein translation. Activation of mTOR can drive metabolic processes through the regulation of metabolic gene expression. These processes include glucose import and glycolysis via HIF-1, and the PPP (nucleotide biosynthesis and reducing equivalents for fatty acid synthesis) through sterol regulatory element-binding proteins (SREBPs).

RAS-RAF-MEK-MAPK Pathway

The RAS family encodes four membrane-bound proteins that are involved in signal transduction underlying diverse cellular activities, such as differentiation, growth, migration, proliferation, and survival (85). Activation of RAS proteins at the cell membrane by growth factors results in the binding of effector molecules, formation of signaling complexes and initiation of a cascade of intracellular signaling pathways including the RAS-RAF-MEK-MAPK-and PI3K-AKT-mTOR pathway. RAS proteins alternate between GTP- and GDP-bound conformations, where the GTP-bound conformation represents the active state. Oncogenic mutants function by preventing hydrolysis of GTP, thereby generating highly active RAS molecules resulting in uncontrolled growth and malignant transformation. Activating (K)RAS mutations are prevalent in ∼15–20% of NSCLC and 30–50% of the adenocarcinoma subtype (73). KRAS mutations are mutually exclusive to EGFR mutations and predict resistance to EGFR TKI and chemotherapy (86, 87). Another RAS effector family is PI3K, which implicates that some of the effects of RAS may be mediated through the PI3K-AKT-mTOR pathway. Indirectly, activating RAS mutations results in the upregulation of many glycolytic enzymes and transporters (55). RAS-transformed cancer cells overcome limitations of nutrients by scavenging extracellular fluid and macromolecules (e.g., albumin, extracellular matrix proteins, necrotic cell debris, …) by generating large vesicles i.e., macropinosomes. The building blocks that make up these macromolecules can be released after degradation and used for the generation of ATP and biosynthetic purposes. In analogy with KRAS-driven pancreatic cancer cells, KRAS-mutated lung cancer cells also exhibit constitutive macropinocytosis. However, *in vitro* findings show that KRAS-driven lung cancer cells degrade less albumin

than isogenic lines derived from the pancreas. This observation raises the possibility that changed characteristics of the tissue of origin also control scavenging in cells with identical genomes (88). Though this intriguing result, an important caveat of this study is the *ex vivo* monitoring, which may not reflect how these cells behave within tissues. Indeed, other pathways that modulate the macropinocytic flux may be affected by both the tumor micro-environment and the mutational load. Additional studies are indispensable to ascertain whether the same KRAS-mutation leads to different amounts of macropinocytic flux in different tissue types.

c-MYC

The MYC proto-oncogene members are targets of RAS and PI3K-AKT-mTOR signaling and critical regulators of numerous downstream pathways, such as apoptosis, differentiation, and proliferation (89). The MYC oncogene family is frequently deregulated in both NSCLC and SCLC. Activation of MYC members often occurs through amplification although excess MYC expression can also result from retroviral promotor insertion, chromosomal translocation, activation of enhancers within the MYC gene or mutations of upstream signaling pathways that enhance MYC stability (90). Concerning metabolic reprogramming, the c-MYC transcription factor promotes expression of glycolytic target genes (GLUT, HK, PFK1, and ENO) and LDH contributing directly to the Warburg effect (91, 92). MCT4, another c-MYC target extrudes lactic acid produced from glucose. It is particularly notable that c-MYC not only drives the expression of glycolytic enzymes but also favor specific mRNA splice variants, such as PKM2 over PKM1. As a consequence, c-MYC-driven accumulation of glycolytic intermediates fuels pathways that share intermediates with glycolysis, such as the PPP and the one-carbon metabolism (92). Besides, c-MYC induces expression of enzymes involved in the synthesis of nucleotide metabolism, including SHMT, which allows glycolytic carbon units to be used in the synthesis of purines and pyrimidines (92–94). Furthermore, c-MYC is also involved in the induction of pyruvate dehydrogenase kinase-1 (PDK1), an enzyme that participates in the regulation of the pyruvate dehydrogenase complex (PDH). This enzyme catalyzes the decarboxylation of pyruvate to acetyl-CoA, thereby linking glycolysis to the TCA cycle. PDK1 inhibits PDH by phosphorylation, resulting in increased conversion of pyruvate to lactate, and limiting the entry of glycolytic carbon substrates into the TCA cycle (95, 96).

HIF-1

The transcription factor HIF is a heterodimeric complex composed of an unstable oxygen-dependent α-unit and a stable oxygen-insensitive β-unit. Under normal O_2 conditions, the α-subunit of HIF is hydroxylated by prolyl-dehydroxylases, allowing recognition and ubiquitination by the Von Hippel Lindau ubiquitin ligase, which labels them for rapid degradation (97). In hypoxia, prolyl-dehydroxylases are inactive as they require O_2 as an essential cofactor. In the nucleus, the stabilized HIF α-subunit dimerizes with HIF-1β and induces the transcription of many genes involved in proliferation, apoptosis, and angiogenesis (98). HIF-1 expression is absent in healthy lung tissue in contrast with cancerous lung tissue, where increased levels of HIF-1 are documented (76, 77). The significant metabolic effect of HIF-1 is to trigger the switch from OXPHOS to anaerobic glycolysis. HIF1 induces the expression of GLUT and upregulates many genes affecting glucose metabolism, such as HK, PGI, ALDO, PGK1, PDK1, ENO, PKM2, and LDH (98–100). Furthermore, HIF-1 participates in the synthesis of serine and the one-carbon metabolism by transactivation of PHGDH and SHMT, which both increase NADPH generation and defense against ROS under hypoxic conditions (101, 102).

TP53

In lung cancer, TP53 is a commonly inactivated tumor suppressor gene. TP53 encodes a protein, p53, that prevents the accumulation of genetic damage during mitosis. In response to cellular stress, p53 induces the expression of genes that regulate cell cycle checkpoints, resulting in G1 arrest and DNA repair or apoptosis (103). Wild type TP53 inhibits transcription of glucose transporters, promotes the expression of Tumor Protein 53-Induced Glycolysis and Apoptosis Regulator (TIGAR), and inhibits the transcription of glycolytic enzymes like PGM (104). By decreasing the level of fructose-2,6-bisphosphate, TIGAR decreases the activity of PFK1, the key enzyme of glycolysis (105). Wild type TP53 supports the expression of PTEN, which inhibits the PI3K pathway, thereby suppressing glycolysis. Additionally, wild type TP53 promotes OXPHOS by activating the transcription of cytochrome c oxidase assembly protein 2 (SCO2), which is required for the assembly of the cytochrome oxidase complex of the electron transport chain. Mutations or deletions in TP53 in cancers result in the stimulation of glucose transport and glycolysis by expression of PGM and inhibition of TIGAR. Wild type TP53 also suppresses the oxidative phase of the PPP by directly binding to G6PD and repressing the enzyme activity. Cancer-associated mutations in p53 have been shown to result in loss of the ability to block G6PD activity, resulting in an increased PPP flux and glycolysis (106).

THERAPEUTIC IMPLICATIONS OF TARGETING THE METABOLIC HALLMARK OF CANCER

Treatment of lung cancer is moving toward the design of drugs that specifically target aberrant pathways involved in carcinogenesis (107). The increased dependence of lung cancer cells on fermentation provides a biochemical basis for the development of antineoplastic treatments that preferentially target cancer cells by pharmacological inhibition of anaerobic glycolysis. One of the advantages of metabolism-based therapeutics over gene-based therapies are the standard shifts in metabolism observed in cancers derived from many tissues. Indeed, the mechanisms underlying cancer development are incredibly complex, and genetic alterations are heterogeneous even in a specific cancer type. As a consequence, targeting a single gene is difficult and an alternative strategy is to take advantage of the fundamental difference between cancer cells

and their regular counterparts. In the past decades, it has become increasingly evident that many metabolic pathways are altered in cancer cells (3, 50, 104, 108). According to Altenberg et al., glucose transporters and glycolytic enzymes are overexpressed in 24 different types of cancer, including lung cancer (19). As previously described, the disturbed glucose metabolism is driven by signal pathways and transcription factors. Inhibition of these pathways and more downstream targets, such as glucose transporters, glycolytic (iso)enzymes, or the mitochondrial pore (VDAC1), provides a tempting avenue for the development of new anti-cancer drugs. Several inhibitors (**Table 1**) of glycolytic enzymes and transporters are in (pre)clinical development, however only inhibitors of IDH have reached approved status. Nevertheless, there are disadvantages to a metabolism-based approach as well. Since the identical metabolic pathways are necessary for the cell division and survival of all cells, metabolism-based treatment face a major hurdle of non-specific toxicity. Immune cells, such as cytotoxic T lymphocytes, are often found in the tumor microenvironment and immune stimulation leads toward an increased demand for glucose. The glycolytic pathway does not only support the proliferation of immune cells but is also crucial for their functional activity, such as the production of cytokines and ATP (144). Therefore, activated immune cells might be expected to be vulnerable to glycolytic inhibition, resulting in immune suppression which is concerning because reactivation of the suppressed immune system has become a first line treatment in PD-L1 positive NSCLC (145, 146). A pitfall in the trials planned to test drugs targeting metabolism is the lack of knowledge of the metabolic pathways because no metabolic profiling has been performed before the initiation of therapy. Indeed, although the aerobic fermentation is the most observed phenotype, it is not a universal trait of all human tumors. In addition, due to the metabolic plasticity exhibited by cancer cells, it is not unexpected that tumor cells could develop resistance to inhibition of a specific pathway through upregulation of alternative pathways. As previously mentioned, continued functioning of the TCA cycle requires the replenishment of intermediates that are diverted for synthesis of ATP and macromolecules. The increased uptake of the anaplerotic substrate glutamine and its metabolic conversion products glutamate and α-KG contribute to the biosynthesis of all cellular constituents. Therefore, concurrent inhibition of the glutaminolysis pathway using small molecules, such as BPTES, compound 968 or CB-839 may be a valuable treatment strategy (7).

Glucose Restriction and Diabetes Control

Both hyperinsulinemia and hyperglycemia are predictors of cancer incidence and worse survival in patients with various cancers as demonstrated by retrospective studies (147–150). It is unknown whether the reduction in insulin levels can affect tumors that are already present. Carbohydrate restriction and pharmacological approaches to reduce the levels of insulin may result in the development of insulin-dependent diabetes in euglycemic subjects and thus in increased glucose levels and overfeeding of tumor cells.

Recently, Ohkuma et al. published a large systematic review that confirmed the higher risk of cancer in diabetics (147). The activation of the IGFR1-IR-PI3K-AKT-mTOR pathway through hyperglycemia and hyperinsulinemia has been suggested as a cause of carcinogenesis. Indeed, binding of insulin and IGF to their receptor tyrosine kinase results in autophosphorylation of the receptors and activation of the PI3K-AKT-mTOR pathway. In addition, mTOR is negatively affected through activation of AMPK, which can also be achieved by dietary restriction (151). This previously described hyperactivation of the IGFR1-IR pathway does not occur through genetic mutations, but co-existence of cancer-associated mutations in these pathways may result in an even more pronounced promotion of growth and survival in malignant cells (152). Masur et al. showed that diabetogenic glucose concentrations compared to physiological levels resulted in different expression of genes that promote adhesion, migration, and proliferation in several cancer cell lines (153). The addition of insulin to the glucose-enriched culture medium further increased the rate of proliferation and promoted activation of the PI3K-AKT-mTOR pathway (153). It could be hypothesized that high glucose and the resulting release of insulin provides additional stimuli for neoplastic cells. However, as demonstrated by Louis et al., cancer leads to increased gluconeogenesis that is fueled by glycerol from lipolysis and alanine from rhabdomyolysis. As a consequence, higher levels of glucose are available for cancer cells, resulting in fat loss and muscle wasting, both hallmarks of cancer cachexia. As sarcopenia is related to a poor prognosis and a substantial loss in the quality of life, carbohydrate restriction has no established role in the treatment or prevention of cancer (154, 155). A switch from carbohydrate metabolism to fatty acid metabolism by diets poor in carbohydrates and rich in fats, i.e., ketogenic diets, may result in anti-cachectic effects. Based on the ability of healthy cells to use ketones as energy source, ketogenic diets have been proposed to treat glioblastomas (156). In general, the current phase I and II studies are hampered by poor accrual and compliance, and until present, no randomized controlled trials have been terminated to study the potential effects of a ketogenic diet on tumor growth and survival.

Inhibition of Glucose Transport

Targeting GLUTs could be an efficient anticancer approach since tumor cells depend on increased utilization of glucose. This difference in glucose addiction between cancer and healthy cells provides a therapeutic window by which glucose uptake in cancer cells can be efficaciously suppressed with significantly less toxic effects in healthy cells. Inhibition of glucose importers is equivalent to the inhibition of the entire glycolytic pathway. Cancer cells will have to use other transport mechanisms, such as macropinocytosis or other metabolic fuels, such as glutamine, to compensate for the shortage of glucose. Although it is possible to acquire these compensation mechanisms, such adaptations are more complicated then bypassing the inhibition of a single enzyme in the glycolytic pathway (88). Based on physiological requirements for glucose, different isoforms of GLUTs are expressed in various cell types. In cancer, GLUT1 and GLUT3 are the most relevant transporters. GLUT1 is a

TABLE 1 | Some inhibitors of glycolytic enzymes and transporters which are in (pre)clinical development.

Target	Drug	References	Remark
GLUT	Fasentin, phloretin, STF-31, WZB117	(109–112)	Preclinical models
HK	Lonidamine	(113–119)	Only one study with survival benefit
	2-deoxyglucose	(120, 121)[1]	Activation of proapoptotic pathways, probably an only role in combination with chemotherapeutic treatments
	Bromopyruvate	(122–126)	Rapid inactivation, venous irritation, lack of crossing blood-brain barrier prevents its clinical development.
			Role in the restoration of chemo susceptibility
PFKFB	3PO	(127)	Preclinical models
	PFK158	(128)	NCT02044861
GAPDH	Bromopyruvate	(124, 126, 129)	Rapid inactivation, venous irritation, lack of crossing blood-brain barrier prevents its clinical development.
			Role in the restoration of chemosusceptibility
PKM2	Shikonin	(130)	Inhibitor PKM2
			Both activators and inhibitors of PKM2 could be beneficial dependent on oxygen levels in cancer cells
LDH	FX11	(131)	Inhibition progression human lymphoma and pancreatic xenografts
	Quinoline-3-sulfonamide	(132)	Unacceptable pharmacokinetic profile preventing further investigation *in vivo* models
	Oxamate	(133)	Role in the restoration of chemosusceptibility
	GNE-140	(134)	High potency, modest permeability and a low plasma protein binding
	PSTMB	(135)	Induction of apoptosis in lung cancer cell lines
PDK	Dichloroacetate	(136)	Phase 2 trial in brain cancer NCT00540176
		(137)	Low potency, a requirement of high doses resulting in significant toxicities
			Preclinical in lung cancer NCT01029925 Terminated due to higher than expected risk/safety concerns.
	AZD7545	(138)	
MCT1	AZD3965		Currently tested in phase 1 clinical trial (NCT01791595)
IDH	Enasidenib	(139)	Approved in relapsed/refractory IDH2 mutant AML
	Ivosedinib	(140)	Approved in relapsed/refractory IDH1 mutant AML
			NCT02989857 (Phase 3 in IDH-mutant cholangiocarcinoma)
			NCT03343197 (Phase 1 in IDH-mutated glioma)
	GSK864		Preclinical, potent IDH1 inhibitor
	GSK321		Preclinical, potent IDH1 inhibitor
VDAC1	Lonidamine	(118)	Preclinical, induction of apoptosis
	SiRNA	(141–143)	Rewiring of tumor cell metabolism, reduction of cancer stem cell levels and induction of differentiation in cell lines and xenografts of glioblastoma, lung cancer and breast cancer

fundamental transporter expressed in almost all cell types, and its upregulation in cancer cells is well-documented (17, 19). Unlike GLUT1, GLUT3 is expressed primarily in tissues with high energy demand to supplement GLUT1. Several inhibitors of glucose transporters, such as fasentin, phloretin, STF-31, and WZB117 have already been discovered, and experiments with preclinical models demonstrated their anticancer effects (109–112). For example, as demonstrated by Liu et al., the treatment of lung cancer cells with WZB117 did not only result in decreased levels of GLUT1 protein but also in a decline in the concentration of intracellular ATP and glycolytic enzymes (112). Furthermore, these authors demonstrated that intraperitoneal injection of WZB117 resulted in a significant reduction of tumor volume *in vivo* in a nude mouse xenograft model.

[1]https://clinicaltrials.gov/ct2/show/NCT00633087

Research by Wood et al. documented that fasentin not only partially inhibited glucose transport but also broke down the resistance of caspase activation which usually is seen in cells that are resistant to antineoplastic treatment (110). Despite these exciting findings, inhibitors of GLUTs have not yet entered clinical trials.

Inhibition of Hexokinase (HK)

In addition to the inhibition of glucose transport, the glycolytic pathway can be inhibited at the enzymatic level. Lonidamine is a selective inhibitor of the soluble and mitochondrial-bound HK2 iso-enzyme, which is present in malignant cells but not in healthy cells and is effective in the treatment of diverse cancer cells (113–115). However, the combination of lonidamine and chemotherapy did not improve the time to progression in breast cancer patients, and its hepatoxicity resulted in early

termination of clinical trials (116, 117). The inhibition of HK2 by lonidamine leads to decreased glucose phosphorylation, which results in lower concentrations of glucose-6-phosphate and as a consequence, results in a reduction of glycolytic intermediates and the PPP. Furthermore, in cancer cells, HK2 associates with the voltage-dependent anion channel (VDAC1), located on the outer mitochondrial membrane, to protect malignant cells from mitochondrial membrane permeabilization. Ravagnan et al. showed that supernatants of mitochondria that were processed with lonidamine contain several factors, including cytochrome C, capable of inducing apoptosis (118). These findings indicate that lonidamine acts through the opening of the mitochondrial permeability transition pore. Indeed, targeting VDAC1 by small molecules or VDAC1-based peptides that interfere with anti-apoptotic proteins results in the induction of apoptosis, making VDAC1 an interesting target to overcome resistance to chemotherapy. Furthermore, strategies using specific small interfering RNA (siRNA) in glioblastoma cells lines and xenografts resulted in a rewiring of tumor cell metabolism, a reduction of cancer stem cell levels and induced differentiation into neuron- and astrocyt-like like cells (141). Similar results, regardless of cell origin and genetic mutational burden, were obtained in lung cancer and breast cancer cell lines and in mouse xenografts (142, 143). As demonstrated by Arif et al., VDAC1 depletion resulted in depletion of transcription factors coordinating cell metabolism, such as c-MYC and HIF-1, finally leading to differentiation, independent of cell type and genetic alterations (142). Therefore, VDAC1 is an interesting target for treating various cancers.

Encouraging data of phase 1 and 2 trials have led to testing lonidamine in several phase 3 trials in several cancers including lung cancer Unfortunately, these results were rather disappointing as only one study detected a statistically significant higher response rate and better survival in patients treated with lonidamine-containing regimens. The glucose analog 2-deoxyglucose, another inhibitor of HK2, demonstrated promising effects in preclinical models (157). Despite the results, its success as a single glycolysis inhibitor has become controversial as the drug activates multiple pro-survival pathways in cancer cells and studies in prostate cancer documented insignificant effects on tumor growth[1] (120). Combination therapy of paclitaxel and 2-deoxyglucose in a NSCLC xenograft model resulted in a remarkable reduction in tumor growth than when compared with either agent alone (121). This observation presents a rationale for the initiation of clinical trials using chemotherapy in combination with 2-deoxyglucose, in order to increase their clinical effectiveness.

Inhibition of Phosphofructokinase Isoforms (PFK)

As previously described, the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate by PFK1 is the committed rate-limiting step of glycolysis. Fructose-2,6-bisphosphate is a potent activator of PFK1. The concentration of fructose-2,6-bisphosphate is determined by a family of bifunctional enzymes PFK-2/FBP (PFKFB) which consists of four iso-enzymes. The high kinase/phosphatase ratio of the iso-enzyme encoded by the PFKFB3 gene, results in sustained high glycolytic rates. As in colon cancer, loss of PTEN, stabilization of HIF-1, and activation of RAS in lung cancer cells, converge to increase the activity of PFKFB3. The small-molecule inhibitor 3PO inhibits the PFKFB3 iso-enzyme through competition with fructose-6-phosphate without inhibition of PFK1 activity. *In vitro*, 3PO attenuates the proliferation of several human cancer cells and exhibits selective cytostatic activity to RAS-mutated epithelial lung cancer cell lines relative to their healthy counterparts (127). *In vivo*, the administration of 3PO reduces growth of lung adenocarcinoma cells. The optimization of this class led to a more potent inhibitor of PFKFB3, i.e., PFK158. *In vitro*, PFK158 results in a decreased uptake of glucose and the release of lactate as well as induction of apoptosis in gynecologic cancer cell lines (128). Furthermore, PKF158 treatment sensitizes chemoresistant cells and induces cell death. These findings indicate that chemotherapy in combination with PFK158 may have a role in the treatment of chemoresistant cancer. Safety and toxicity studies in animals have demonstrated that PFK158 is well-tolerated with a good therapeutic index, lending further support for a phase 1 clinical trial in patients with metastatic solid malignancies (NCT02044861).

Inhibition of GAPDH

The glycolytic enzyme GAPDH plays a critical role in the cellular redox balance by the generation of NADH, which is involved in the regulation of ROS and in biosynthetic processes of macromolecules. Apart from its glycolytic function, tumor-specific roles of GAPDH include chemoresistance, metastatic potential, protection of cancer cells from apoptosis, and cell cycle regulation (158–160). Given the central role of GAPDH, its inhibition triggers a cascade that may lead to cell death. Under normal conditions, degradation of accumulated GAP and DHAP results in the formation of the cytotoxic metabolite methylglyoxal, which enters the glyoxalase system to undergo detoxification. However, in the presence of oxidative stress and glutathione depletion, the glyoxalase system fails to detoxify the cytotoxic metabolite resulting in apoptosis (161). Several GAPDH inhibitors have been tested in cell cultures and animal models for their efficacy (162). However, the ubiquitous nature of GAPDH and the resulting systemic toxicity needs to be addressed in clinical trials. A promising GAPDH inhibitor is the pyruvate analog 3-bromopyruvate. Bromopyruvate is a powerful anti-cancer agent that not only interferes with the process of glycolysis but also impacts the TCA- and folate cycle (122, 163). Unfortunately, the molecule faces many biochemical and practical problems, such as rapid inactivation by the thiol groups of e.g., glutathione and venous irritation during infusion (164). Lack of early tumor response, the resistance of cells rich in glutathione, the lack of crossing the blood-brain barrier, and the phenomenon of enhanced permeability and retention prevents the approval of 3-bromopyruvate in clinical trials. Notwithstanding the induction of apoptosis in breast cancer cell lines, bromopyruvate was observed to trigger autophagy, which increased resistance to bromopyruvate treatment (123, 129). In colon cancer, bromopyruvate treatment rendered resistant cells susceptible to 5-fluorouracil and oxaliplatin (124). Malignant cells, treated with bromopyruvate, were observed to have a larger uptake of chemotherapeutic drugs resulting in a

restoration of susceptibility to these drugs. Overexpression of drug-expelling ATP-binding cassette transporters (ABC) prevents accumulation of chemotherapeutic drugs into cancer cells, eventually leading to drug resistance. Since these transporters are dependent on ATP production through enhanced glycolysis, inhibition of the glycolytic pathway with bromopyruvate may restore the susceptibility of malignant cells to chemotherapy.

Pyruvate Kinase (PK): Inhibitors or Activators?

The discovery that the expression of PKM2 results in a growth advantage for malignant cells raised the hypothesis that the enzyme could be an interesting target for cancer treatment. The inhibition of PKM2 may result in the accumulation of glycolytic intermediates that feed biosynthetic pathways resulting in tumor proliferation. As demonstrated by Anastasiou et al., oxidative stress results in the oxidation of PKM thereby suppressing its activity and supporting the diversion of glycolytic intermediates into the PPP resulting in the generation of NADPH and restoration of the redox balance (165). Activators of PKM2 could be interesting cancer drugs, mainly when administered in combination with treatments that disrupt the cellular redox balance, such as radiotherapy and chemotherapeutics. In contrast, other investigators demonstrated that inhibition of PKM2 increases cell death in mouse xenograft models (166). This discrepancy may result from different cellular responses to variable degrees of hypoxia (167). Mild hypoxia results in the production of hydrogen peroxide, which ultimately promotes signaling pathways that are critical for the response to hypoxia. In this setting, oxidation of PKM2 leads to inactivation of the glycolytic flux and increased flow through the PPP. As a result, the production of NADPH prevents the accumulation of ROS and oxidative damage. During severe hypoxia, the O_2 supply to the electron transport chain becomes compromised, resulting in a reduction of mitochondrial ATP production and hydrogen peroxide. As a consequence, cancer cells depend on the PK activity for the production of ATP. In conclusion, depending on the degree of hypoxia, both PKM2 activators and inhibitors could be beneficial. Indeed, in severely hypoxic cells PKM2 inhibitors may prevent ATP production, whereas PKM activators may result in oxidative damage in cells with moderate O_2 levels. Shikonin is a potent and specific inhibitor of PKM2. Incubation of lung cancer cells with shikonin resulted in a reduced glycolytic rate as manifested by decreased glucose consumption and lactate production (130).

Inhibition of Pyruvate Dehydrogenase Kinase (PDK)

PDKs and PDH are mitochondrial enzymes that determine the proportion between the Warburg effect and aerobic respiration (168). As overexpression of PDKs has been detected in several human cancer samples and has been associated with a dismal prognosis in several other cancers, new drugs that inhibit PDKs may be used to treat a variety of cancers and may provide a new kind of antineoplastic class (96). In addition, the low expression of PDK in normal tissue may spare healthy cells and adverse effects may be minimal. Several PDK inhibitors have been reported, although their clinical efficacy needs confirmation. Dichloroacetate (DCA) has been identified as an activator of PDH by inhibition of PDK activity and has successfully entered into phase 2 trials in treating brain tumor patients (136). The consequences of DCA on lung cancer cells and animal models were explored in detail by Bonnet et al. who demonstrated that administration of DCA resulted in a shift from glycolysis to OXPHOS (137). Furthermore, this shift in metabolism led to higher levels of ROS and a decreased mitochondrial membrane potential in lung and several other malignancies without any effect on standard cell lines. The activation of the mitochondrial function resulted in apoptosis due to the efflux of pro-apoptotic mediators from the mitochondria. Despite these encouraging results, the application of DCA in the treatment of cancer is plagued by its low potency and the need for high dosages to exhibit therapeutic effects, resulting in toxicities, such as peripheral neurological toxicity (169). Due to high risk/safety concerns, the NCT01029925 trial investigating the response rate of DCA in patients with recurrent and advanced NSCLC was closed prematurely. Therefore, clinical trials with more potent and selective PDKs inhibitors, such as AZD7545 are of significant importance (138).

Inhibition of Lactate Dehydrogenase a (LDH-A)

LDH-A has an essential role in perpetuating a high rate of glycolysis by the regeneration of NAD^+ making it a potential therapeutic target. Inhibition of LDH-A by the small molecule inhibitor FX11 increased non-productive mitochondrial respiration, leading to reduced ATP levels, increased O_2 consumption, ROS production, and cell death. In addition, the molecule inhibited the progression of lymphoma and other cancer xenografts (131). In combination with FK866, another metabolic inhibitor that inhibits NAD^+ synthesis, FX11 can induce lymphoma regression. Quinoline 3-sulfonamide, another LDH-A inhibitor, has been studied in multiple cancer cell lines by Billiard et al. (132). LDH-A inhibition resulted in increased intracellular concentrations of glycolytic and TCA cycle intermediates, consistent with blockage of glycolysis and enhanced TCA cycle activity, respectively. However, the unacceptable pharmacokinetic profile, i.e., the low *in vivo* clearance and the low oral bioavailability, prevents further use *in vivo*. To improve the cellular potency of LDH inhibitors, structure based designs, such as substitution of the hydroxylactam core, were utilized to create a novel series of LDH-A inhibitors. This strategy resulted in the discovery of GNE-140, a molecule that inhibits proliferation in several cancer cell lines and mice. The combination of high potency, modest permeability and a low plasma protein binding makes it a promising metabolic drug (134). More recently, Kim et al. demonstrated that the inhibitory concentration of PSTMB was significantly lower than that of other LDH-A inhibitors which may result in less toxicity (135). These authors demonstrated that PSTMB induces apoptosis in lung cancer

cell lines, through induction of ROS production. In breast cancer, it was demonstrated that LDH-A plays a vital role in taxol resistance. Treatment of breast cancer cell lines with the LDH-A inhibitor oxamate and taxol resulted in a synergistic inhibitory effect on taxol resistant cancer cells by promoting apoptosis in these cells (133). This result provides evidence for the future development and use of metabolic therapies to overcome chemoresistance.

Monocarboxylate Transport Inhibitors

Depending upon the isoform of MCT, lactate could be imported (MCT1) or exported (MCT4). Intracellular trapping of lactate will result in intracellular acidification, causing cell death. Recent studies with AZD3965, a potent, selective inhibitor of the MCT1 have demonstrated that the drug inhibits the transport of lactate and cell growth in cancer cells. The drug is currently tested in a phase 1 clinical trial that enrolls patients with advanced solid tumors or lymphoma that are refractory to conventional treatment or for which no conventional therapy exists. In addition, the disruption of lactate/H^+ symporters has also been studied via genetic tools. Marchiq et al. studied the effect of knocking out the BASIGIN (BSG) and MCT4 genes on the metabolism of colon adenocarcinoma and glioblastoma cells (170). In their study, the authors found a strong reduction of the rate of glycolysis as expected. However, upon inhibition of MCT1 by the MCT1 inhibitor AR-C155858, the cells O_2 consumption increased, thus indicating a rapid shift from glycolysis to OXPHOS. The authors went one step further and showed that the disruption of MCT4 and BSG sensitized the glycolytic tumor cells to phenformin, an inhibitor of mitochondrial complex I. Due to the rapid decrease in cellular ATP by disrupting both glycolysis as well as OXPHOS, cell death by "metabolic catastrophe" was observed. This observation confirmed their larger dependency on OXPHOS following the disruption of glycolysis. Similar shifts toward OXPHOS were later reported in cancer cells following disruption of glucose-6-phosphate isomerase and LDHs as covered in a mini-review by Ždralević et al. (171).

Inhibition of Mutant Isocitrate Dehydrogenase

As mentioned before, mutations in IDH iso-enzymes result in the production of the oncometabolite 2-hydroxyglutarate, which has been linked to the interference with metabolic and epigenetic alterations responsible for cellular differentiation. Recently, the IDH1 inhibitor enasidenib, and IDH2 inhibitor ivosidenib, were approved in the treatment of patients with acute myeloid leukemia (AML) (172). GSK864 and GSK321 are promising potent inhibitors of IDH1 but have not yet entered clinical trials. Existing clinical and preclinical data in hematologic and solid tumors and the potential limitations of treatment were recently discussed by Golub et al. (172).

CONCLUSIONS

Metabolic instability caused by environmental influences or perturbations in certain enzymes and substrates may result in mutations in oncogenes and tumor suppressor genes, leading to activation or inhibition of signaling pathways and transcriptional networks which account for the metabolic reprogramming observed in cancer cells. These metabolic adaptations are mandatory for the requirements of rapidly dividing cells: a rapid ATP generation to maintain energy status, an increased biosynthesis of biomolecules and the maintenance of the cellular redox balance. The metabolic phenotype of lung cancer cells is characterized by increased glucose uptake and glycolytic activity. However, new insights reveal the importance of other glucose-related pathways, such as gluconeogenesis, the TCA cycle and OXPHOS. Specific variations in the metabolism of cancer depend not only on the genetic alterations but also on environmental factors, such as vascularization and the supply of oxygen and nutrients. Targeting the metabolic differences between cancer and healthy cells may turn into a novel, promising anticancer strategy. Several recent studies have focused on targeting the cellular metabolic pathways in cancer cells. However, pharmacologic studies are primarily carried out using cell lines or xenograft models. To avoid the same types of toxicity that plague the current chemotherapeutic regimens, the toxic effects of inhibiting glycolytic enzymes in healthy cells needs further investigation. Besides, due to the metabolic plasticity exhibited by cancer cells, cancer cells could develop resistance to inhibition of a particular pathway through upregulation of alternative pathways, such as glutaminolysis and OXPHOS or through interaction with neighboring cells that may also provide precursors for their metabolic needs.

AUTHOR CONTRIBUTIONS

KV: original draft. G-JG, LM, MT, ED, J-PN, WG, and PA: review and editing.

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REFERENCES

1. Anastasiou D. Tumour microenvironment factors shaping the cancer metabolism landscape. *Br J Cancer.* (2017) 116:277–86. doi: 10.1038/bjc.2016.412
2. Warburg O. On the origin of cancer cells. *Science.* (1956) 123:309–14. doi: 10.1126/science.123.3191.309
3. Ahn CS, Metallo CM. Mitochondria as biosynthetic factories for cancer proliferation. *Cancer Metab.* (2015) 3:1. doi: 10.1186/s40170-015-0128-2

4. Moreno-Sanchez R, Rodriguez-Enriquez S, Marin-Hernandez A, Saavedra E. Energy metabolism in tumor cells. *FEBS J.* (2007) 274:1393–418. doi: 10.1111/j.1742-4658.2007.05686.x
5. Zu XL, Guppy M. Cancer metabolism: facts, fantasy, and fiction. *Biochem Biophys Res Commun.* (2004) 313:459–65. doi: 10.1016/j.bbrc.2003.11.136
6. Mohamed A, Deng X, Khuri FR, Owonikoko TK. Altered glutamine metabolism and therapeutic opportunities for lung cancer. *Clin Lung Cancer.* (2014) 15:7–15. doi: 10.1016/j.cllc.2013.09.001
7. Vanhove K, Derveaux E, Graulus GJ, Mesotten L, Thomeer M, Noben JP, et al. Glutamine addiction and therapeutic strategies in lung cancer. *Int J Mol Sci.* (2019) 20:E252. doi: 10.3390/ijms20020252
8. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin.* (2015) 65:87–108. doi: 10.3322/caac.21262
9. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer.* (2015) 136:E359–86. doi: 10.1002/ijc.29210
10. Vazquez A. *A Historical View of Overflow Metabolism.* Glasgow: Academic Press (2018).
11. Abdel-Haleem AM, Lewis NE, Jamshidi N, Mineta K, Gao X, Gojobori T. The emerging facets of non-cancerous warburg effect. *Front Endocrinol (Lausanne).* (2017) 8:279. doi: 10.3389/fendo.2017.00279
12. Shestov AA, Lee SC, Nath K, Guo L, Nelson DS, Roman JC, et al. ^{13}C MRS and LC-MS flux analysis of tumor intermediary metabolism. *Front Oncol.* (2016) 6:135. doi: 10.3389/fonc.2016.00135
13. Medina RA, Owen GI. Glucose transporters: expression, regulation and cancer. *Biol Res.* (2002) 35:9–26. doi: 10.4067/S0716-97602002000100004
14. Smith TA. FDG uptake, tumour characteristics and response to therapy: a review. *Nucl Med Commun.* (1998) 19:97–105. doi: 10.1097/00006231-199802000-00002
15. Vansteenkiste J, Crino L, Dooms C, Douillard JY, Faivre-Finn C, Lim E, et al. 2nd ESMO Consensus Conference on Lung Cancer: early-stage non-small-cell lung cancer consensus on diagnosis, treatment and follow-up. *Ann Oncol.* (2014) 25:1462–74. doi: 10.1093/annonc/mdu089
16. Schrevens L, Lorent N, Dooms C, Vansteenkiste J. The role of PET scan in diagnosis, staging, and management of non-small cell lung cancer. *Oncologist.* (2004) 9:633–43. doi: 10.1634/theoncologist.9-6-633
17. Higashi K, Ueda Y, Sakurai A, Mingwang X, Xu L, Murakami M, et al. Correlation of Glut-1 glucose transporter expression with [^{18}F]FDG uptake in non-small cell lung cancer. *Eur J Nucl Med.* (2000) 27:1778–85. doi: 10.1007/s002590000367
18. Mamede M, Higashi T, Kitaichi M, Ishizu K, Ishimori T, Nakamoto Y, et al. [^{18}F]FDG uptake and PCNA, Glut-1, and Hexokinase-II expressions in cancers and inflammatory lesions of the lung. *Neoplasia.* (2005) 7:369–79. doi: 10.1593/neo.04577
19. Altenberg B, Greulich KO. Genes of glycolysis are ubiquitously overexpressed in 24 cancer classes. *Genomics.* (2004) 84:1014–20. doi: 10.1016/j.ygeno.2004.08.010
20. Ooi AT, Gomperts BN. Molecular pathways: targeting cellular energy metabolism in cancer via inhibition of SLC2A1 and LDHA. *Clin Cancer Res.* (2015) 21:2440–4. doi: 10.1158/1078-0432.CCR-14-1209
21. Romero-Garcia S, Moreno-Altamirano MM, Prado-Garcia H, Sanchez-Garcia FJ. Lactate contribution to the tumor microenvironment: mechanisms, effects on immune cells and therapeutic relevance. *Front Immunol.* (2016) 7:52. doi: 10.3389/fimmu.2016.00052
22. Sonveaux P, Vegran F, Schroeder T, Wergin MC, Verrax J, Rabbani ZN, et al. Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. *J Clin Invest.* (2008) 118:3930–42. doi: 10.1172/JCI36843
23. Schulze A, Harris AL. How cancer metabolism is tuned for proliferation and vulnerable to disruption. *Nature.* (2012) 491:364–73. doi: 10.1038/nature11706
24. Faubert B, Li KY, Cai L, Hensley CT, Kim J, Zacharias LG, et al. Lactate metabolism in human lung tumors. *Cell.* (2017) 171:358–71 e9. doi: 10.1016/j.cell.2017.09.019
25. Louis E, Adriaensens P, Guedens W, Bigirumurame T, Baeten K, Vanhove K, et al. Detection of lung cancer through metabolic changes measured in blood plasma. *J Thorac Oncol.* (2016) 11:516–23. doi: 10.1016/j.jtho.2016.01.011
26. Liberti MV, Locasale JW. The Warburg effect: how does it benefit cancer cells? *Trends Biochem Sci.* (2016) 41:211–8. doi: 10.1016/j.tibs.2015.12.001
27. Israelsen WJ, Vander Heiden MG. Pyruvate kinase: function, regulation and role in cancer. *Semin Cell Dev Biol.* (2015) 43:43–51. doi: 10.1016/j.semcdb.2015.08.004
28. Wu W, Zhao S. Metabolic changes in cancer: beyond the Warburg effect. *Acta Biochim Biophys Sin (Shanghai).* (2013) 45:18–26. doi: 10.1093/abbs/gms104
29. Mazurek S. Pyruvate kinase type M2: a key regulator of the metabolic budget system in tumor cells. *Int J Biochem Cell Biol.* (2011) 43:969–80. doi: 10.1016/j.biocel.2010.02.005
30. Martin-Bernabe A, Cortes R, Lehmann SG, Seve M, Cascante M, Bourgoin-Voillard S. Quantitative proteomic approach to understand metabolic adaptation in non-small cell lung cancer. *J Proteome Res.* (2014) 13:4695–704. doi: 10.1021/pr500327v
31. Liou GY, Storz P. Reactive oxygen species in cancer. *Free Radic Res.* (2010) 44:479–96. doi: 10.3109/10715761003667554
32. Boros LG, Lee PW, Brandes JL, Cascante M, Muscarella P, Schirmer WJ, et al. Nonoxidative pentose phosphate pathways and their direct role in ribose synthesis in tumors: is cancer a disease of cellular glucose metabolism? *Med Hypotheses.* (1998) 50:55–9. doi: 10.1016/S0306-9877(98)90178-5
33. Schultz H, Kahler D, Branscheid D, Vollmer E, Zabel P, Goldmann T. TKTL1 is overexpressed in a large portion of non-small cell lung cancer specimens. *Diagn Pathol.* (2008) 3:35. doi: 10.1186/1746-1596-3-35
34. Fritz P, Coy JF, Murdter TE, Ott G, Alscher MD, Friedel G. TKTL-1 expression in lung cancer. *Pathol Res Pract.* (2012) 208:203–9. doi: 10.1016/j.prp.2012.01.007
35. Patra KC, Hay N. The pentose phosphate pathway and cancer. *Trends Biochem Sci.* (2014) 39:347–54. doi: 10.1016/j.tibs.2014.06.005
36. Ferrer CM, Sodi VL, Reginato MJ. O-GlcNAcylation in cancer biology: linking metabolism and signaling. *J Mol Biol.* (2016) 428:3282–94. doi: 10.1016/j.jmb.2016.05.028
37. Lemjabbar-Alaoui H, McKinney A, Yang YW, Tran VM, Phillips JJ. Glycosylation alterations in lung and brain cancer. *Adv Cancer Res.* (2015) 126:305–44. doi: 10.1016/bs.acr.2014.11.007
38. Ruan HB, Singh JP, Li MD, Wu J, Yang X. Cracking the O-GlcNAc code in metabolism. *Trends Endocrinol Metab.* (2013) 24:301–9. doi: 10.1016/j.tem.2013.02.002
39. Mi W, Gu Y, Han C, Liu H, Fan Q, Zhang X, et al. O-GlcNAcylation is a novel regulator of lung and colon cancer malignancy. *Biochim Biophys Acta.* (2011) 1812:514–9. doi: 10.1016/j.bbadis.2011.01.009
40. Jozwiak P, Forma E, Brys M, Krzeslak A. O-GlcNAcylation and metabolic reprograming in cancer. *Front Endocrinol (Lausanne).* (2014) 5:145. doi: 10.3389/fendo.2014.00145
41. Yi W, Clark PM, Mason DE, Keenan MC, Hill C, Goddard WA III, et al. Phosphofructokinase 1 glycosylation regulates cell growth and metabolism. *Science.* (2012) 337:975–80. doi: 10.1126/science.1222278
42. Sola-Penna M, Da Silva D, Coelho WS, Marinho-Carvalho MM, Zancan P. Regulation of mammalian muscle type 6-phosphofructo-1-kinase and its implication for the control of the metabolism. *IUBMB Life.* (2010) 62:791–6. doi: 10.1002/iub.393
43. Mullarky E, Lairson LL, Cantley LC, Lyssiotis CA. A novel small-molecule inhibitor of 3-phosphoglycerate dehydrogenase. *Mol Cell Oncol.* (2016) 3:e1164280. doi: 10.1080/23723556.2016.1164280
44. Yang M, Vousden KH. Serine and one-carbon metabolism in cancer. *Nat Rev Cancer.* (2016) 16:650–62. doi: 10.1038/nrc.2016.81
45. Zhu J, Ma J, Wang X, Ma T, Zhang S, Wang W, et al. High expression of PHGDH predicts poor prognosis in non-small cell lung cancer. *Transl Oncol.* (2016) 9:592–9. doi: 10.1016/j.tranon.2016.08.003
46. Zhang WC, Shyh-Chang N, Yang H, Rai A, Umashankar S, Ma S, et al. Glycine decarboxylase activity drives non-small cell lung cancer tumor-initiating cells and tumorigenesis. *Cell.* (2012) 148:259–72. doi: 10.1016/j.cell.2012.02.024
47. Newman AC, Maddocks ODK. One-carbon metabolism in cancer. *Br J Cancer.* (2017) 116:1499–504. doi: 10.1038/bjc.2017.118
48. Paone A, Marani M, Fiascarelli A, Rinaldo S, Giardina G, Contestabile R, et al. SHMT1 knockdown induces apoptosis in lung cancer

cells by causing uracil misincorporation. *Cell Death Dis.* (2014) 5:e1525. doi: 10.1038/cddis.2014.482
49. Tedeschi PM, Markert EK, Gounder M, Lin H, Dvorzhinski D, Dolfi SC, et al. Contribution of serine, folate and glycine metabolism to the ATP, NADPH and purine requirements of cancer cells. *Cell Death Dis.* (2013) 4:e877. doi: 10.1038/cddis.2013.393
50. Amelio I, Cutruzzola F, Antonov A, Agostini M, Melino G. Serine and glycine metabolism in cancer. *Trends Biochem Sci.* (2014) 39:191–8. doi: 10.1016/j.tibs.2014.02.004
51. Lu F, Zhang HT. DNA methylation and nonsmall cell lung cancer. *Anat Rec (Hoboken).* (2011) 294:1787–95. doi: 10.1002/ar.21471
52. Shuvalov O, Petukhov A, Daks A, Fedorova O, Vasileva E, Barlev NA. One-carbon metabolism and nucleotide biosynthesis as attractive targets for anticancer therapy. *Oncotarget.* (2017) 8:23955–77. doi: 10.18632/oncotarget.15053
53. Pan M, Reid MA, Lowman XH, Kulkarni RP, Tran TQ, Liu X, et al. Regional glutamine deficiency in tumours promotes dedifferentiation through inhibition of histone demethylation. *Nat Cell Biol.* (2016) 18:1090–101. doi: 10.1038/ncb3410
54. Yu X, Ma R, Wu Y, Zhai Y, Li S. Reciprocal regulation of metabolic reprogramming and epigenetic modifications in cancer. *Front Genet.* (2018) 9:394. doi: 10.3389/fgene.2018.00394
55. Davies P, Demetrius LA, Tuszynski JA. Implications of quantum metabolism and natural selection for the origin of cancer cells and tumor progression. *AIP Adv.* (2012) 2:11101. doi: 10.1063/1.3697850
56. Leithner K. PEPCK in cancer cell starvation. *Oncoscience.* (2015) 2:805–6. doi: 10.18632/oncoscience.252
57. Vincent EE, Sergushichev A, Griss T, Gingras MC, Samborska B, Ntimbane T, et al. Mitochondrial phosphoenolpyruvate carboxykinase regulates metabolic adaptation and enables glucose-independent tumor growth. *Mol Cell.* (2015) 60:195–207. doi: 10.1016/j.molcel.2015.08.013
58. Harvey A, Ferrier D. Tricarboxylic acid cycle. In: Harvey RA, editor. *Biochemistry*. 5th Edn. Philadelphia, PA: Lippincott Williams & Wilkins (2011). p. 109–16.
59. Leithner K, Hrzenjak A, Trotzmuller M, Moustafa T, Kofeler HC, Wohlkoenig C, et al. PCK2 activation mediates an adaptive response to glucose depletion in lung cancer. *Oncogene.* (2015) 34:1044–50. doi: 10.1038/onc.2014.47
60. DeBerardinis RJ, Chandel NS. Fundamentals of cancer metabolism. *Sci Adv.* (2016) 2:e1600200. doi: 10.1126/sciadv.1600200
61. Kaelin WG Jr, McKnight SL. Influence of metabolism on epigenetics and disease. *Cell.* (2013) 153:56–69. doi: 10.1016/j.cell.2013.03.004
62. Toth LN, de Abreu FB, Tafe LJ. Non-small cell lung cancers with isocitrate dehydrogenase 1 or 2 (IDH1/2) mutations. *Hum Pathol.* (2018) 78:138–43. doi: 10.1016/j.humpath.2018.04.014
63. Shoshan-Barmatz V, Ben-Hail D, Admoni L, Krelin Y, Tripathi SS. The mitochondrial voltage-dependent anion channel 1 in tumor cells. *Biochim Biophys Acta.* (2015) 1848:2547–75. doi: 10.1016/j.bbamem.2014.10.040
64. Moreno-Sanchez R, Rodriguez-Enriquez S, Saavedra E, Marin-Hernandez A, Gallardo-Perez JC. The bioenergetics of cancer: is glycolysis the main ATP supplier in all tumor cells? *Biofactors.* (2009) 35:209–25. doi: 10.1002/biof.31
65. Hensley CT, Faubert B, Yuan Q, Lev-Cohain N, Jin E, Kim J, et al. Metabolic heterogeneity in human lung tumors. *Cell.* (2016) 164:681–94. doi: 10.1016/j.cell.2015.12.034
66. Davidson SM, Papagiannakopoulos T, Olenchock BA, Heyman JE, Keibler MA, Luengo A, et al. Environment impacts the metabolic dependencies of ras-driven non-small cell lung cancer. *Cell Metab.* (2016) 23:517–28. doi: 10.1016/j.cmet.2016.01.007
67. Fantin VR, St-Pierre J, Leder P. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell.* (2006) 9:425–34. doi: 10.1016/j.ccr.2006.04.023
68. Smolkova K, Plecita-Hlavata L, Bellance N, Benard G, Rossignol R, Jezek P. Waves of gene regulation suppress and then restore oxidative phosphorylation in cancer cells. *Int J Biochem Cell Biol.* (2011) 43:950–68. doi: 10.1016/j.biocel.2010.05.003
69. Pavlides S, Whitaker-Menezes D, Castello-Cros R, Flomenberg N, Witkiewicz AK, Frank PG, et al. The reverse Warburg effect: aerobic glycolysis in cancer associated fibroblasts and the tumor stroma. *Cell Cycle.* (2009) 8:3984–4001. doi: 10.4161/cc.8.23.10238
70. Hirpara J, Eu JQ, Tan JKM, Wong AL, Clement MV, Kong LR, et al. Metabolic reprogramming of oncogene-addicted cancer cells to OXPHOS as a mechanism of drug resistance. *Redox Biol.* (2018) 17:101076. doi: 10.1016/j.redox.2018.101076
71. Ashton TM, McKenna WG, Kunz-Schughart LA, Higgins GS. Oxidative phosphorylation as an emerging target in cancer therapy. *Clin Cancer Res.* (2018) 24:2482–90. doi: 10.1158/1078-0432.CCR-17-3070
72. Shackelford DB, Abt E, Gerken L, Vasquez DS, Seki A, Leblanc M, et al. LKB1 inactivation dictates therapeutic response of non-small cell lung cancer to the metabolism drug phenformin. *Cancer Cell.* (2013) 23:143–58. doi: 10.1016/j.ccr.2012.12.008
73. Rodenhuis S, Slebos RJ. Clinical significance of ras oncogene activation in human lung cancer. *Cancer Res.* (1992) 52:2665s–9s.
74. Massarelli E, Papadimitrakopoulou VA. Phosphatidykinosital 3-kinase and mammalian target of rapamycin pathway in non-small-cell lung cancer. *J Thorac Oncol.* (2012) 7:S379–82. doi: 10.1097/JTO.0b013e31826df0f0
75. Santarius T, Shipley J, Brewer D, Stratton MR, Cooper CS. A census of amplified and overexpressed human cancer genes. *Nat Rev Cancer.* (2010) 10:59–64. doi: 10.1038/nrc2771
76. Maxwell PH, Pugh CW, Ratcliffe PJ. Activation of the HIF pathway in cancer. *Curr Opin Genet Dev.* (2001) 11:293–9. doi: 10.1016/S0959-437X(00)00193-3
77. Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene.* (2010) 29:625–34. doi: 10.1038/onc.2009.441
78. Kishimoto Y, Murakami Y, Shiraishi M, Hayashi K, Sekiya T. Aberrations of the p53 tumor suppressor gene in human non-small cell carcinomas of the lung. *Cancer Res.* (1992) 52:4799–804.
79. Yu JS, Cui W. Proliferation, survival and metabolism: the role of PI3K/AKT/mTOR signalling in pluripotency and cell fate determination. *Development.* (2016) 143:3050–60. doi: 10.1242/dev.137075
80. Janku F, Yap TA, Meric-Bernstam F. Targeting the PI3K pathway in cancer: are we making headway? *Nat Rev Clin Oncol.* (2018) 15:273–91. doi: 10.1038/nrclinonc.2018.28
81. Elstrom RL, Bauer DE, Buzzai M, Karnauskas R, Harris MH, Plas DR, et al. Akt stimulates aerobic glycolysis in cancer cells. *Cancer Res.* (2004) 64:3892–9. doi: 10.1158/0008-5472.CAN-03-2904
82. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. *Nat Rev Cancer.* (2011) 11:85–95. doi: 10.1038/nrc2981
83. Finicle BT, Jayashankar V, Edinger AL. Nutrient scavenging in cancer. *Nat Rev Cancer.* (2018) 18:619–33. doi: 10.1038/s41568-018-0048-x
84. Kim SM, Nguyen TT, Ravi A, Kubiniok P, Finicle BT, Jayashankar V, et al. PTEN deficiency and AMPK activation promote nutrient scavenging and anabolism in prostate cancer cells. *Cancer Discov.* (2018) 8:866–83. doi: 10.1158/2159-8290.CD-17-1215
85. Takashima A, Faller DV. Targeting the RAS oncogene. *Expert Opin Ther Targets.* (2013) 17:507–31. doi: 10.1517/14728222.2013.764990
86. Eberhard DA, Johnson BE, Amler LC, Goddard AD, Heldens SL, Herbst RS, et al. Mutations in the epidermal growth factor receptor and in KRAS are predictive and prognostic indicators in patients with non-small-cell lung cancer treated with chemotherapy alone and in combination with erlotinib. *J Clin Oncol.* (2005) 23:5900–9. doi: 10.1200/JCO.2005.02.857
87. Pao W, Wang TY, Riely GJ, Miller VA, Pan Q, Ladanyi M, et al. KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. *PLoS Med.* (2005) 2:e17. doi: 10.1371/journal.pmed.0020017
88. Commisso C, Davidson SM, Soydaner-Azeloglu RG, Parker SJ, Kamphorst JJ, Hackett S, et al. Macropinocytosis of protein is an amino acid supply route in Ras-transformed cells. *Nature.* (2013) 497:633–7. doi: 10.1038/nature12138
89. Adhikary S, Eilers M. Transcriptional regulation and transformation by Myc proteins. *Nat Rev Mol Cell Biol.* (2005) 6:635–45. doi: 10.1038/nrm1703
90. Meyer N, Penn LZ. Reflecting on 25 years with MYC. *Nat Rev Cancer.* (2008) 8:976–90. doi: 10.1038/nrc2231
91. Miller DM, Thomas SD, Islam A, Muench D, Sedoris K. c-Myc and cancer metabolism. *Clin Cancer Res.* (2012) 18:5546–53. doi: 10.1158/1078-0432.CCR-12-0977

92. Stine ZE, Walton ZE, Altman BJ, Hsieh AL, Dang CV. MYC, metabolism, and cancer. *Cancer Discov.* (2015) 5:1024–39. doi: 10.1158/2159-8290.CD-15-0507
93. Nikiforov MA, Chandriani S, O'Connell B, Petrenko O, Kotenko I, Beavis A, et al. A functional screen for Myc-responsive genes reveals serine hydroxymethyltransferase, a major source of the one-carbon unit for cell metabolism. *Mol Cell Biol.* (2002) 22:5793–800. doi: 10.1128/MCB.22.16.5793-5800.2002
94. Liu YC, Li F, Handler J, Huang CR, Xiang Y, Neretti N, et al. Global regulation of nucleotide biosynthetic genes by c-Myc. *PLoS ONE.* (2008) 3:e2722. doi: 10.1371/journal.pone.0002722
95. Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab.* (2006) 3:177–85. doi: 10.1016/j.cmet.2006.02.002
96. Zhang W, Zhang SL, Hu X, Tam KY. Targeting tumor metabolism for cancer treatment: is pyruvate dehydrogenase kinases (PDKs) a viable anticancer target? *Int J Biol Sci.* (2015) 11:1390–400. doi: 10.7150/ijbs.13325
97. West JB. Physiological effects of chronic hypoxia. *N Engl J Med.* (2017) 376:1965–71. doi: 10.1056/NEJMra1612008
98. Schito L, Semenza GL. Hypoxia-inducible factors: master regulators of cancer progression. *Trends Cancer.* (2016) 2:758–70. doi: 10.1016/j.trecan.2016.10.016
99. Semenza GL, Roth PH, Fang HM, Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J Biol Chem.* (1994) 269:23757–63.
100. Courtnay R, Ngo DC, Malik N, Ververis K, Tortorella SM, Karagiannis TC. Cancer metabolism and the Warburg effect: the role of HIF-1 and PI3K. *Mol Biol Rep.* (2015) 42:841–51. doi: 10.1007/s11033-015-3858-x
101. Ye J, Fan J, Venneti S, Wan YW, Pawel BR, Zhang J, et al. Serine catabolism regulates mitochondrial redox control during hypoxia. *Cancer Discov.* (2014) 4:1406–17. doi: 10.1158/2159-8290.CD-14-0250
102. Samanta D, Park Y, Andrabi SA, Shelton LM, Gilkes DM, Semenza GL. PHGDH expression is required for mitochondrial redox homeostasis, breast cancer stem cell maintenance, and lung metastasis. *Cancer Res.* (2016) 76:4430–42. doi: 10.1158/0008-5472.CAN-16-0530
103. Breuer RH, Postmus PE, Smit EF. Molecular pathology of non-small-cell lung cancer. *Respiration.* (2005) 72:313–30. doi: 10.1159/000085376
104. DeBerardinis RJ. Is cancer a disease of abnormal cellular metabolism? New angles on an old idea. *Genet Med.* (2008) 10:767–77. doi: 10.1097/GIM.0b013e31818b0d9b
105. Kruiswijk F, Labuschagne CF, Vousden KH. p53 in survival, death and metabolic health: a lifeguard with a licence to kill. *Nat Rev Mol Cell Biol.* (2015) 16:393–405. doi: 10.1038/nrm4007
106. Jiang P, Du W, Wang X, Mancuso A, Gao X, Wu M, et al. p53 regulates biosynthesis through direct inactivation of glucose-6-phosphate dehydrogenase. *Nat Cell Biol.* (2011) 13:310–6. doi: 10.1038/ncb2172
107. Mayekar MK, Bivona TG. Current landscape of targeted therapy in lung cancer. *Clin Pharmacol Ther.* (2017) 102:757–64. doi: 10.1002/cpt.810
108. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
109. Chan DA, Sutphin PD, Nguyen P, Turcotte S, Lai EW, Banh A, et al. Targeting GLUT1 and the Warburg effect in renal cell carcinoma by chemical synthetic lethality. *Sci Transl Med.* (2011) 3:94ra70. doi: 10.1126/scitranslmed.3002394
110. Wood TE, Dalili S, Simpson CD, Hurren R, Mao X, Saiz FS, et al. A novel inhibitor of glucose uptake sensitizes cells to FAS-induced cell death. *Mol Cancer Ther.* (2008) 7:3546–55. doi: 10.1158/1535-7163.MCT-08-0569
111. Wu KH, Ho CT, Chen ZF, Chen LC, Whang-Peng J, Lin TN, et al. The apple polyphenol phloretin inhibits breast cancer cell migration and proliferation via inhibition of signals by type 2 glucose transporter. *J Food Drug Anal.* (2018) 26:221–31. doi: 10.1016/j.jfda.2017.03.009
112. Liu Y, Cao Y, Zhang W, Bergmeier S, Qian Y, Akbar H, et al. A small-molecule inhibitor of glucose transporter 1 downregulates glycolysis, induces cell-cycle arrest, and inhibits cancer cell growth *in vitro* and *in vivo*. *Mol Cancer Ther.* (2012) 11:1672–82. doi: 10.1158/1535-7163.MCT-12-0131
113. Gatzemeier U1 CF, Häussinger K, Kaukel E, Koschel G, Martinelli G, Neuhauss R, von Pawel J. Phase III trial with and without lonidamine in non-small cell lung cancer. *Semin Oncol.* (1991) 18:42–8.
114. Di Cosimo S, Ferretti G, Papaldo P, Carlini P, Fabi A, Cognetti F. Lonidamine: efficacy and safety in clinical trials for the treatment of solid tumors. *Drugs Today (Barc).* (2003) 39:157–74. doi: 10.1358/dot.2003.39.3.799451
115. De Lena M, Lorusso V, Latorre A, Fanizza G, Gargano G, Caporusso L, et al. Paclitaxel, cisplatin and lonidamine in advanced ovarian cancer. A phase II study. *Eur J Cancer.* (2001) 37:364–8. doi: 10.1016/S0959-8049(00)00400-7
116. Berruti A, Bitossi R, Gorzegno G, Bottini A, Alquati P, De Matteis A, et al. Time to progression in metastatic breast cancer patients treated with epirubicin is not improved by the addition of either cisplatin or lonidamine: final results of a phase III study with a factorial design. *J Clin Oncol.* (2002) 20:4150–9. doi: 10.1200/JCO.2002.08.012
117. Granchi C, Minutolo F. Anticancer agents that counteract tumor glycolysis. *ChemMedChem.* (2012) 7:1318–50. doi: 10.1002/cmdc.201200176
118. Ravagnan L, Marzo I, Costantini P, Susin SA, Zamzami N, Petit PX, et al. Lonidamine triggers apoptosis via a direct, Bcl-2-inhibited effect on the mitochondrial permeability transition pore. *Oncogene.* (1999) 18:2537–46. doi: 10.1038/sj.onc.1202625
119. Cervantes-Madrid D, Romero Y, Duenas-Gonzalez A. Reviving lonidamine and 6-diazo-5-oxo-L-norleucine to be used in combination for metabolic cancer therapy. *Biomed Res Int.* (2015) 2015:690492. doi: 10.1155/2015/690492
120. Zhong D, Xiong L, Liu T, Liu X, Liu X, Chen J, et al. The glycolytic inhibitor 2-deoxyglucose activates multiple prosurvival pathways through IGF1R. *J Biol Chem.* (2009) 284:23225–33. doi: 10.1074/jbc.M109.005280
121. Maschek G, Savaraj N, Priebe W, Braunschweiger P, Hamilton K, Tidmarsh GF, et al. 2-deoxy-D-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers *in vivo*. *Cancer Res.* (2004) 64:31–4. doi: 10.1158/0008-5472.CAN-03-3294
122. Shoshan MC. 3-Bromopyruvate: targets and outcomes. *J Bioenerg Biomembr.* (2012) 44:7–15. doi: 10.1007/s10863-012-9419-2
123. Zhang Q, Zhang Y, Zhang P, Chao Z, Xia F, Jiang C, et al. Hexokinase II inhibitor, 3-BrPA induced autophagy by stimulating ROS formation in human breast cancer cells. *Genes Cancer.* (2014) 5:100–12. doi: 10.18632/genesandcancer.9
124. Chong D, Ma L, Liu F, Zhang Z, Zhao S, Huo Q, et al. Synergistic antitumor effect of 3-bromopyruvate and 5-fluorouracil against human colorectal cancer through cell cycle arrest and induction of apoptosis. *Anticancer Drugs.* (2017) 28:831–40. doi: 10.1097/CAD.0000000000000517
125. Ganapathy-Kanniappan S, Kunjithapatham R, Geschwind JF. Anticancer efficacy of the metabolic blocker 3-bromopyruvate: specific molecular targeting. *Anticancer Res.* (2013) 33:13-20.
126. Azevedo-Silva J, Queiros O, Baltazar F, Ulaszewski S, Goffeau A, Ko YH, et al. The anticancer agent 3-bromopyruvate: a simple but powerful molecule taken from the lab to the bedside. *J Bioenerg Biomembr.* (2016) 48:349–62. doi: 10.1007/s10863-016-9670-z
127. Clem B, Telang S, Clem A, Yalcin A, Meier J, Simmons A, et al. Small-molecule inhibition of 6-phosphofructo-2-kinase activity suppresses glycolytic flux and tumor growth. *Mol Cancer Ther.* (2008) 7:110–20. doi: 10.1158/1535-7163.MCT-07-0482
128. Mondal S, Roy D, Sarkar Bhattacharya S, Jin L, Jung D, Zhang S, et al. Therapeutic targeting of PFKFB3 with a novel glycolytic inhibitor PFK158 promotes lipophagy and chemosensitivity in gynecologic cancers. *Int J Cancer.* (2019) 144:178–89. doi: 10.1002/ijc.31868
129. Kwiatkowska E, Wojtala M, Gajewska A, Soszynski M, Bartosz G, Sadowska-Bartosz I. Effect of 3-bromopyruvate acid on the redox equilibrium in non-invasive MCF-7 and invasive MDA-MB-231 breast cancer cells. *J Bioenerg Biomembr.* (2016) 48:23–32. doi: 10.1007/s10863-015-9637-5
130. Chen J, Xie J, Jiang Z, Wang B, Wang Y, Hu X. Shikonin and its analogs inhibit cancer cell glycolysis by targeting tumor pyruvate kinase-M2. *Oncogene.* (2011) 30:4297–306. doi: 10.1038/onc.2011.137
131. Le A, Cooper CR, Gouw AM, Dinavahi R, Maitra A, Deck LM, et al. Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. *Proc Natl Acad Sci USA.* (2010) 107:2037–42. doi: 10.1073/pnas.0914433107

132. Billiard J, Dennison JB, Briand J, Annan RS, Chai D, Colon M, et al. Quinoline 3-sulfonamides inhibit lactate dehydrogenase A and reverse aerobic glycolysis in cancer cells. *Cancer Metab.* (2013) 1:19. doi: 10.1186/2049-3002-1-19
133. Zhou M, Zhao Y, Ding Y, Liu H, Liu Z, Fodstad O, et al. Warburg effect in chemosensitivity: targeting lactate dehydrogenase-A re-sensitizes taxol-resistant cancer cells to taxol. *Mol Cancer.* (2010) 9:33. doi: 10.1186/1476-4598-9-33
134. Purkey HE, Robarge K, Chen J, Chen Z, Corson LB, Ding CZ, et al. Cell active hydroxylactam inhibitors of human lactate dehydrogenase with oral bioavailability in mice. *ACS Med Chem Lett.* (2016) 7:896–901. doi: 10.1021/acsmedchemlett.6b00190
135. Kim EY, Chung TW, Han CW, Park SY, Park KH, Jang SB, et al. A novel lactate dehydrogenase inhibitor, 1-(Phenylseleno)-4-(Trifluoromethyl) benzene, suppresses tumor growth through apoptotic cell death. *Sci Rep.* (2019) 9:3969. doi: 10.1038/s41598-019-40617-3
136. Dunbar EM, Coats BS, Shroads AL, Langaee T, Lew A, Forder JR, et al. Phase 1 trial of dichloroacetate (DCA) in adults with recurrent malignant brain tumors. *Invest New Drugs.* (2014) 32:452–64. doi: 10.1007/s10637-013-0047-4
137. Bonnet S, Archer SL, Allalunis-Turner J, Haromy A, Beaulieu C, Thompson R, et al. A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. *Cancer Cell.* (2007) 11:37–51. doi: 10.1016/j.ccr.2006.10.020
138. Morrell JA, Orme J, Butlin RJ, Roche TE, Mayers RM, Kilgour E. AZD7545 is a selective inhibitor of pyruvate dehydrogenase kinase 2. *Biochem Soc Trans.* (2003) 31:1168–70. doi: 10.1042/bst0311168
139. Kim ES. Enasidenib: first global approval. *Drugs.* (2017) 77:1705–11. doi: 10.1007/s40265-017-0813-2
140. Dhillon S. Ivosidenib: first global approval. *Drugs.* (2018) 78:1509–16. doi: 10.1007/s40265-018-0978-3
141. Arif T, Krelin Y, Nakdimon I, Benharroch D, Paul A, Dadon-Klein D, et al. VDAC1 is a molecular target in glioblastoma, with its depletion leading to reprogrammed metabolism and reversed oncogenic properties. *Neuro Oncol.* (2017) 19:951–64. doi: 10.1093/neuonc/now297
142. Arif T, Amsalem Z, Shoshan-Barmatz V. Metabolic reprograming via silencing of mitochondrial VDAC1 expression encourages differentiation of cancer cells. *Mol Ther Nucleic Acids.* (2019) 17:24–37. doi: 10.1016/j.omtn.2019.05.003
143. Arif T, Paul A, Krelin Y, Shteinfer-Kuzmine A, Shoshan-Barmatz V. Mitochondrial VDAC1 silencing leads to metabolic rewiring and the reprogramming of tumour cells into advanced differentiated states. *Cancers (Basel).* (2018) 10:E499. doi: 10.3390/cancers10120499
144. O'Neill LA, Kishton RJ, Rathmell J. A guide to immunometabolism for immunologists. *Nat Rev Immunol.* (2016) 16:553–65. doi: 10.1038/nri.2016.70
145. Reck M, Rodriguez-Abreu D, Robinson AG, Hui R, Csoszi T, Fulop A, et al. Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. *N Engl J Med.* (2016) 375:1823–33. doi: 10.1056/NEJMoa1606774
146. Renner K, Singer K, Koehl GE, Geissler EK, Peter K, Siska PJ, et al. Metabolic hallmarks of tumor and immune cells in the tumor microenvironment. *Front Immunol.* (2017) 8:248. doi: 10.3389/fimmu.2017.00248
147. Ohkuma T, Peters SAE, Woodward M. Sex differences in the association between diabetes and cancer: a systematic review and meta-analysis of 121 cohorts including 20 million individuals and one million events. *Diabetologia.* (2018) 61:2140–54. doi: 10.1007/s00125-018-4664-5
148. Yu OH, Suissa S. Identifying causes for excess mortality in patients with diabetes: closer but not there yet. *Diabetes Care.* (2016) 39:1851–3. doi: 10.2337/dci16-0026
149. Derr RL, Ye X, Islas MU, Desideri S, Saudek CD, Grossman SA. Association between hyperglycemia and survival in patients with newly diagnosed glioblastoma. *J Clin Oncol.* (2009) 27:1082–6. doi: 10.1200/JCO.2008.19.1098
150. Wolpin BM, Meyerhardt JA, Chan AT, Ng K, Chan JA, Wu K, et al. Insulin, the insulin-like growth factor axis, and mortality in patients with nonmetastatic colorectal cancer. *J Clin Oncol.* (2009) 27:176–85. doi: 10.1200/JCO.2008.17.9945
151. Jiang W, Zhu Z, Thompson HJ. Dietary energy restriction modulates the activity of AMP-activated protein kinase, Akt, and mammalian target of rapamycin in mammary carcinomas, mammary gland, and liver. *Cancer Res.* (2008) 68:5492–9. doi: 10.1158/0008-5472.CAN-07-6721
152. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer.* (2008) 8:915–28. doi: 10.1038/nrc2536
153. Masur K, Vetter C, Hinz A, Tomas N, Henrich H, Niggemann B, et al. Diabetogenic glucose and insulin concentrations modulate transcriptome and protein levels involved in tumour cell migration, adhesion and proliferation. *Br J Cancer.* (2011) 104:345–52. doi: 10.1038/sj.bjc.6606050
154. Kimura M, Naito T, Kenmotsu H, Taira T, Wakuda K, Oyakawa T, et al. Prognostic impact of cancer cachexia in patients with advanced non-small cell lung cancer. *Support Care Cancer.* (2015) 23:1699–708. doi: 10.1007/s00520-014-2534-3
155. Collins J, Noble S, Chester J, Coles B, Byrne A. The assessment and impact of sarcopenia in lung cancer: a systematic literature review. *BMJ Open.* (2014) 4:e003697. doi: 10.1136/bmjopen-2013-003697
156. Maurer GD, Brucker DP, Bahr O, Harter PN, Hattingen E, Walenta S, et al. Differential utilization of ketone bodies by neurons and glioma cell lines: a rationale for ketogenic diet as experimental glioma therapy. *BMC Cancer.* (2011) 11:315. doi: 10.1186/1471-2407-11-315
157. Maher JC, Krishan A, Lampidis TJ. Greater cell cycle inhibition and cytotoxicity induced by 2-deoxy-D-glucose in tumor cells treated under hypoxic vs aerobic conditions. *Cancer Chemother Pharmacol.* (2004) 53:116–22. doi: 10.1007/s00280-003-0724-7
158. Epner DE, Partin AW, Schalken JA, Isaacs JT, Coffey DS. Association of glyceraldehyde-3-phosphate dehydrogenase expression with cell motility and metastatic potential of rat prostatic adenocarcinoma. *Cancer Res.* (1993) 53:1995–7.
159. Colell A, Ricci JE, Tait S, Milasta S, Maurer U, Bouchier-Hayes L, et al. GAPDH and autophagy preserve survival after apoptotic cytochrome c release in the absence of caspase activation. *Cell.* (2007) 129:983–97. doi: 10.1016/j.cell.2007.03.045
160. Phadke MS, Krynetskaia NF, Mishra AK, Krynetskiy E. Glyceraldehyde 3-phosphate dehydrogenase depletion induces cell cycle arrest and resistance to antimetabolites in human carcinoma cell lines. *J Pharmacol Exp Ther.* (2009) 331:77–86. doi: 10.1124/jpet.109.155671
161. Thornalley PJ, Rabbani N. Glyoxalase in tumourigenesis and multidrug resistance. *Semin Cell Dev Biol.* (2011) 22:318–25. doi: 10.1016/j.semcdb.2011.02.006
162. Ganapathy-Kanniappan S, Kunjithapatham R, Geschwind JF. Glyceraldehyde-3-phosphate dehydrogenase: a promising target for molecular therapy in hepatocellular carcinoma. *Oncotarget.* (2012) 3:940–53. doi: 10.18632/oncotarget.623
163. Paiardini A, Tramonti A, Schirch D, Guiducci G, di Salvo ML, Fiascarelli A, et al. Differential 3-bromopyruvate inhibition of cytosolic and mitochondrial human serine hydroxymethyltransferase isoforms, key enzymes in cancer metabolic reprogramming. *Biochim Biophys Acta.* (2016) 1864:1506–17. doi: 10.1016/j.bbapap.2016.08.010
164. El Sayed SM. Enhancing anticancer effects, decreasing risks and solving practical problems facing 3-bromopyruvate in clinical oncology: 10 years of research experience. *Int J Nanomedicine.* (2018) 13:4699–709. doi: 10.2147/IJN.S170564
165. Anastasiou D, Poulogiannis G, Asara JM, Boxer MB, Jiang JK, Shen M, et al. Inhibition of pyruvate kinase M2 by reactive oxygen species contributes to cellular antioxidant responses. *Science.* (2011) 334:1278–83. doi: 10.1126/science.1211485
166. Goldberg MS, Sharp PA. Pyruvate kinase M2-specific siRNA induces apoptosis and tumor regression. *J Exp Med.* (2012) 209:217–24. doi: 10.1084/jem.20111487
167. Hamanaka RB, Chandel NS. Targeting glucose metabolism for cancer therapy. *J Exp Med.* (2012) 209:211–5. doi: 10.1084/jem.20120162

168. Kaplon J, Zheng L, Meissl K, Chaneton B, Selivanov VA, Mackay G, et al. A key role for mitochondrial gatekeeper pyruvate dehydrogenase in oncogene-induced senescence. *Nature.* (2013) 498:109–12. doi: 10.1038/nature12154
169. Yang Y SP, Cheng C, Wang D, Yang P, Zhang F, Li T, Lu A, Zhao Y. Novel N-phenyl dichloroacetamide derivatives as anticancer reagents: design, synthesis and biological evaluation. *Eur J Med Chem.* (2010) 45:4300–6. doi: 10.1016/j.ejmech.2010.06.032
170. Marchiq I, Le Floch R, Roux D, Simon MP, Pouyssegur J. Genetic disruption of lactate/H^+ symporters (MCTs) and their subunit CD147/BASIGIN sensitizes glycolytic tumor cells to phenformin. *Cancer Res.* (2015) 75:171–80. doi: 10.1158/0008-5472.CAN-14-2260
171. Zdralevic M, Marchiq I, de Padua MMC, Parks SK, Pouyssegur J. Metabolic plasiticy in cancers-distinct role of glycolytic enzymes GPI, LDHs or membrane transporters MCTs. *Front Oncol.* (2017) 7:313. doi: 10.3389/fonc.2017.00313
172. Golub D, Iyengar N, Dogra S, Wong T, Bready D, Tang K, et al. Mutant isocitrate dehydrogenase inhibitors as targeted cancer therapeutics. *Front Oncol.* (2019) 9:417. doi: 10.3389/fonc.2019.00417

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Autophagy Machinery as a Promising Therapeutic Target in Endometrial Cancer

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Endometrial cancer is the fourth most frequent neoplasia for women worldwide, and over the past two decades it incidence has increased. The most common histological type of endometrial cancer is endometrioid adenocarcinoma, also known as type 1 endometrial cancer. Endometrioid endometrial cancer is associated with diverse epidemiological risk factors including estrogen use, obesity, diabetes, cigarette smoking, null parity, early menarche, and late menopause. Clinical effectiveness of chemotherapy is variable, indicating that novel molecular therapies against specific cellular processes associated to cell survival and resistance to therapy, such as autophagy, urged to ameliorate the rates of success in endometrial cancer treatment. Autophagy (also known as macroautophagy) is a specialized mechanism that maintains cell homeostasis which is activated in response to cellular stressors including nutrients deprivation, amino acids starvation, hypoxia, and metabolic stress to prolong cell survival via lysosomal degradation of cytoplasmic macromolecules and organelles. However, in human cancer cells, autophagy has a controversial function due to its dual role as self-protective or apoptotic. Conventional antitumor therapies including hormones, chemotherapy and ionizing radiation, may activate autophagy as a pro-survival tumor response contributing to treatment resistance. Intriguingly, if autophagy continues above reversibility of cell viability, autophagy can result in apoptosis of tumor cells. Here, we have reviewed the mechanisms of autophagy described in endometrial cancers, including the role of PI3K/AKT/mTOR, AMPK-mTOR, and p53 signaling pathways that trigger or inhibit the process and thus representing potential molecular targets in therapeutic clinical approaches. In addition, we discussed the recent findings indicating that autophagy can be modulated using repurposing drugs which may leads to faster experimentation and validation, as well as more easy access of the medications to patients. Finally, the promising role of dietary compounds and microRNAs in autophagy modulation is also discussed. In conclusion, although the research about autophagy is scarce but ongoing in endometrial cancer, the actual findings highlight the promising usefulness of novel molecules for directing targeted therapies.

Keywords: endometrial cancer, autophagy, AKT/mTOR pathway, microRNAs, therapy

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ENDOMETRIAL CANCER

Endometrial cancer is the fourth most frequent neoplasia for women worldwide (1). The incidence of endometrial cancer has increased around than 20%, and currently, ∼1 in 37 women will develop endometrial cancer during their lifetime (2, 3). The age of onset of endometrial cancer is typically in postmenopausal women, although in the last decade the incidence in young women has dramatically increased as a result of earlier-onset obesity and hyperinsulinemia (4). The mean age at cancer diagnosis is 63 years. Endometrial cancer is originated in the uterine epithelium and can be classified into diverse histological subtypes: (i) endometrioid endometrial cancer (EEC, or Type I), (ii) serous endometrial cancer (SEC, or Type II), (iii) clear cell endometrial cancer (CCEC, or Type II), and (iv) mixed endometrial cancer and uterine carcinoma (USC), which have different clinical and molecular features, as well as prognosis and therapeutic regimen (5). Type I tumors are the most frequent subtype representing about 70% of diagnosed cases, they are low grade and associated to estrogen stimulation, whereas type II tumors are generally high grade, estrogen-independent, less common, clinically aggressive, metastatic, and exhibit a increased risk of relapse after chemotherapy. Type II tumors accounts for 10% of endometrial cancers, but it's related with poor prognosis and 40% deaths (2, 5). However, a large study of the Epidemiology of Endometrial Cancer Consortium USA suggested that the etiology of type II tumors may not be completely estrogen independent (6). Endometrioid endometrial cancer is associated with diverse epidemiological risk factors including unopposed estrogen use, obesity, diabetes, cigarette smoking, null parity, early menarche and late menopause (6, 7). Increased risk for endometrial cancer development is associated in less extend with: (i) Lynch syndrome (2–6% of ECs) caused by monoallelic germline mutation in MLH1, MSH2, MSH6, or PMS2 mismatch repair genes (8), (ii) polymerase proofreading-associated polyposis attributed to germline mutations of the DNA polymerase-delta 1 (POLD1) or DNA polymerase-epsilon (POLE) (9), and Cowden syndrome caused by tumor suppressor PTEN mutations (10). Combinatory therapy with carboplatin and paclitaxel is the main front-line chemotherapy in endometrial cancer (11). These agents are characterized by its capacity to generate DNA damage and bock the proper polymerization of actin microtubules in cytoskeleton, respectively (12). However, its clinical effectiveness is variable, indicating that novel molecular therapies against specific cellular processes associated to cell survival and therapy resistance, such as autophagy, urged to ameliorate the rates of success in endometrial cancer therapies.

AUTOPHAGY: MECHANISMS AND FUNCTIONS

Autophagy (also known as macroautophagy) is a highly specialized and evolutionarily conserved process that maintains cell homeostasis (13). Autophagy is activated through a specific transcriptional program (see below) in response to continuous cellular stressors including nutrients deprivation, amino acids starvation, hypoxia, and metabolic stress (14). This self-eating cellular mechanism prolongs survival under diverse stressors via lysosomal degradation of cytoplasmic macromolecules and organelles (15). The autophagic flux is characterized by: (i) the enclose of intracellular cytoplasmic components, macromolecular complexes, long-lived soluble and aggregated proteins, and cellular organelles in vesicles dubbed as autophagosomes, (ii) the degradation of cytoplasmic constituents by fusion of autophagosomes in the lysosomes, and (iii) the reuse of ATP and other molecules for cell biosynthesis. Two main features distinguish the catabolic autophagy: (i) basal autophagy which carried out a key function in homeostasis by reducing the pool of lasting proteins and organelles. Normal cells activate basal autophagy in response to diverse stressors as a temporary cell survival mechanism (16), (ii) induced autophagy in disease conditions, for instance in human cancer cells autophagy has a controversial and complicated function due to its dual role as self-protective or apoptotic. It has been reported that antitumor therapies including hormones, chemotherapy and ionizing radiation, may activate autophagy as a pro-survival tumor response contributing to treatment resistance. Intriguingly, if autophagy continues beyond reversibility of cell viability, autophagy can result in apoptosis of tumor cells (17). The dual role of autophagy during carcinogenesis difficult the efforts to understand how to modulate it to achieve successful treatments, suggesting that genetic mutational background and tumor cell type specific knowledge should be required (18).

In mammalian cells, autophagy depends on the function of the core autophagy proteins (ATG) which initiate the assembly of the omegasome at reticulum endoplasmic, followed by the phagophore formation and later the autophagosomes which fused with lysosomes (19). Briefly, the molecular autophagic pathway can be divided in three steps: (i) after a stimulus, such as nutrients starvation, the ATG proteins are assembled into functional ULK 1/2 and PtdIns3P protein complexes, which are engaged to inner membranes to begin the formation of the omegasome (20). The ULK complex is formed by diverse proteins including ULK1, ULK2, ATG13, RBCC1/FIP200, and ATG101; whereas the class III lipid kinase complex I is formed by ATG14, BECN1/Beclin 1, PIK3R4/p150, and PIK3C3/VPS34. Initiation of autophagy begins when the ULK complex is activated by AMPK kinase (or repressed by mTORC1) at the endoplasmic reticulum membranes that have been previously marked by ATG9 resulting in the formation of the omegasome. Remarkably, there are evidences suggesting that Golgi apparatus is important for the production of ATG9-containing vesicles (named the ATG compartment visualized as small vesicles and tubules) that nucleate the membranous structures shaping the omegasome in order to merge with the phagophore (21). (ii) Then, the phosphatidylinositol 3-kinase (PtdIns3-kinase) complex is recruited at the reticulum endoplasmic generating curved structures that contain PtdIns3P favoring the recruitment of PtdIns3K complex I, and also facilitating the assembly of PtdIns3P and gathering of WIPI2B, and the E3-like complex (ATG12–ATG5-ATG16L1). The Ptdlns3k complex I consist of phosphatidylinositol 3-phosphate (PtdIns3P), the WIPI proteins;

two ubiquitin-like conjugation complexes: (i) one conjugates ATG12 to ATG5 together with ATG16L1 (ATG7, ATG10), and (ii) other that lipidated the Atg8 proteins (ATG7, ATG3, LC3A, GABARAPL1, and GABARAPL2/GATE-16), and ATG9. (iii) Finally, the phagophore is closed; the double-membrane autophagosomes matures and then take place the SNAREs-mediated fusion with lysosomes to degrade its contents (22).

Autophagy has been conceived as a process where intense vesicular trafficking leading to recycling of cytosolic components is the common characteristic, recent evidences showed that transcriptional control of their main molecular players represents also a major regulatory event. Transcription factors TFEB, MiT, and fork-head box members (FOXO) like FOXO1, FOXO3, FOXO4 regulate the expression of diverse autophagy genes including ATG4, ATG12, BECN1, LC3, BNIP3, LC3, ULK1, ULK2, and VPS34 (23, 24). For instance, autophagy flux is controlled by TFEB transcription factor which after phosphorylation is retained in cytosol resulting in inhibition of target genes expression. Conversely, after nutrient starvation the TFEB dephosphorylation causes translocation to the nucleus where it binds to target gene promoters involved in autophagy initiation (*BECN1, WIPI1, ATG9B*, and *NRBF2*) autophagosome membrane elongation (*GABARAP, MAP1LC3B*, and *ATG5*), substrate capture (*SQSTM1*), and autophagosomes trafficking and fusion with lysosomes (*UVRAG, RAB7*). This fine tuning of autophagy genes expression is regulated by mTOR and AKT kinases. In addition p53 transcription factor also controls the expression of key genes for autophagy induction (LKB1, ULK1/2), and autophagosome maturation (ATG4, ATG7, and ATG10) (25).

MUTATIONS IN AUTOPHAGY GENES IN ENDOMETRIAL CANCER

In an outstanding paper, Lebovitz and coworkers reported a Pan-cancer study in patient samples and reveal frequent mutations in autophagy genes in endometrial cancer (26). Using data from The Cancer Genome Atlas 211 autophagy-related genes were surveyed for alterations in DNA sequence and mRNA expression. Authors found somatic mutations in a number if autophagy genes including RB1CC1/FIP200, WDR45/WIPI4, ULK4, and ATG7 in endometrial carcinoma and clear cell renal carcinoma. Remarkably, endometrial carcinomas showed a high number of mutations in ATG4C, RB1CC1/FIP200, and ULK4 genes. Also, common mTOR sequence alterations including C1483F and S2215Y hotspot mutations were detected. Moreover, mutations were also observed in endometrioid endometrial tumors including patients that carried out double mutations for PTEN and mTOR genes. Moreover, a truncating mutation (R1321*) in RB1CC1 gene was found suggesting autophagy induction may be compromised in patients with type I tumors. In contrast, type II serous tumors exhibited a significant increase of the autophagy inductor CDKN2A.

TARGETING SIGNALING PATHWAYS CONTROLLING AUTOPHAGY IN CANCER ENDOMETRIAL

Diverse approaches to target genes or signaling pathways controlling autophagy are focused in intervention of the autophagic flux. The autophagic flux assays measure autophagic system's degradation activity (27). These methods track the formation and accumulation of autophagosomes, as well as their fusion with lysosomes and the degradation of their content in them. Immunoblotting and immunofluorescence analysis allows the indirect assessment of the number of autophagosomes based on the abundance of MAP1LC3/LC3-II protein. LC3 is known for incorporating into the autophagosome membrane; also a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which in turn is recruited to autophagosomal membranes. Other proteins, including p62/SQSTM1 (sequestrosome 1), contain domains that interact with LC3 and serve as adapters between ubiquitinated protein structures and damaged organelles and autophagic machinery. Another important marker of autophagic flux is p62 protein which is located in the autophagosome and is continuously degraded, observing reduced levels when autophagy is activated and an accumulation of p62 when autophagy is inhibited. Immunofluorescence also allows following of autophagy and formation of autophagosomes using antibodies or expression vectors fused to autophagic proteins. An example is vector GFP-LC3; in basal circumstances the GFP-LC3 protein is uniformly distributed in the cytoplasm, however when autophagy is activated, GFP-LC3 is recruited to the phagosomal membrane, generating punctual signals fluorescents in the cell. Finally, transmission electron microscopy (TEM), considered as the gold standard in many autophagy research applications, has the advantage of allowing a direct assessment of autophagosomes in cells (28).

Autophagy Therapies Related to AKT-mTOR Pathway Inhibition

The aberrant activation of phosphoinositide 3-kinase (PI3K) and the mammalian target of rapamycin (mTOR) signaling occur frequently during the progression of endometrial malignancies. The PI3K/AKT/mTOR signaling is pivotal in regulation of gene transcription, invasion, proliferation, cell survival, and central in the metabolism through regulation of enzymes like Glyoxalase 2 (Glo2) and glyoxalase 1 (Glo1) promoting cancer progression (29, 30). Master regulator mTOR is a serine/threonine kinase with pivotal roles in autophagy that function downstream of PI3K/AKT pathway (31). Under normal cell conditions mTOR impairs autophagy by phosphorylation of mAtg13 and ULK1; whereas in the opposite way after rapamycin stimulation or nutrients starvation, mTOR kinase is repressed (32). Next, we will summarize diverse studies that describe the regulation of autophagic activity through mTOR signaling pathway. Lin et al. (33) reported that oncogene FAM83B expression was significantly higher in endometrial cancer cell lines and

tissues relative to normal tissues, and it was associated with myometrial invasion, poor survival, and FIGO II-IV stages (33). Functional analysis showed that FAM83B knockdown leads to the suppression of PI3K/AKT/mTOR pathway, while stimulates autophagy. Furthermore, activation of the pathway turned back the effects of FAM83B silencing-induced autophagy and cancer hallmarks in endometrial cancer cells (33).

In another study, Kanda and colleagues demonstrated that glucagon-like peptide-1 receptor (GLP-1R) agonist liraglutide stimulates autophagy via AMPK pathway in endometrial cancer cells. Authors first showed that treatments with GLP-1R agonist significantly inhibited Ishikawa endometrial cancer cells growth and induced apoptosis. Interestingly, liraglutide also induced autophagy characterized by accumulation of cytoplasmic autophagosomes, higher LC3 expression, decreased p62 levels, and an increased phosphorylated ratio of AMPK kinase (34).

On the other hand, functional links between resistance to chemotherapy and autophagy have been dilucidated. Progestins have been used as conservative endocrine treatment in young early endometrial adenocarcinoma patients (35). However, up to 30% of patients showed progestin treatment resistance. Using in-home generated progestin-resistant cells, Liu and coworkers showed that resistant cells acquired increased cell proliferation and resistance to autophagy revealed by a decrease in beclin-1, ATG3, ATG5, and LC3B protein expression relative to parental no resistant cells (36). These changes were associated with PI3K/AKT/mTOR signaling activation. Moreover, treatment of cells with RAD001, a novel mTOR inhibitor, resulted in low mTOR phosphorylation and suppressed the cell proliferation of progestin-resistant endometrial cancer cells by activating autophagy. These data suggested that mTOR can be a therapeutic agent associated to progestin-resistance and autophagy in endometrial cancer (37). In a preclinical study it was reported that RAD001 (known as everolimus), an mTOR inhibitor, suppressed tumor growth and cell proliferation and promoted an additive cytotoxic effect when use together other chemotherapy agents. The underlying mechanisms of RAD001 treatment is based on the inhibition of AKT and mTOR phosphorylation (**Figure 1**), associated to up-regulation of LC3II protein in Ishikawa (IK) and HEC-1A endometrial cancer cells lines. In addition, an increase in cell death was found after RAD001 treatments in combination with paclitaxel, and interestingly these effects were suppressed after autophagy inhibition (37).

Another effective compound used in endometrial cancer cells is ABTL0812, a fatty acid-derived molecule which may targets the PI3K/AKT/mTOR axis (38). ABTL0812 is an alternative for first line therapy focused in autophagy. Evaluation of its efficacy into IK, AN3CA, and HEC-1A endometrial cancer cell lines showed that ABTL0812 reduced cell viability, promoted the activation of the pro-apoptotic pathway and autophagy induction by TRIB3 overexpression (a negative regulator of AKT1) promoting downregulation of mTOR resulting in autophagy activation (**Figure 1**) and the transformation of soluble LC3 (LC3-I) to a lipidated form (LC3-II) (38). This molecular mechanism places ABTL0812 as an excellent first-line treatment.

On the other hand, diverse antitumor therapies that have abilities to stimulate autophagy are the combinational use of mTOR inhibitors and proteins related with immune system like interleukins. Natural killer (NK) cells functions are important for sustained tumor growth and therapy response (39) and recently it was shown that both effects exerted by NK cells were enhanced by interleukin (IL)-27. The evidence indicates that IL-27 secreted by endometrial cells may trigger the activation of NK cells in a co-culture system. Related with immune regulation, the Rapamycin is an immunosuppressant and specific inhibitor of mTOR and it has been show that exposure to rapamycin synergistically activates the cytotoxicity of NK cells associated to overexpression of IL-27 (**Figure 1**). Importantly, this cytotoxicity was favored by the stimulation of rapamycin-mediated autophagy, a signal that was amplified by IL-27, further promoting a suppression of endometrial cancer progression. However, IL-27 could not directly impair cell death and the growth of endometrial cancer cells, but in combination with rapamycin and cisplatin amplifies these effects (40). This knowledge about autophagy activation by mTOR inhibitors and immune system participation is a novel promising direction for endometrial cancer therapies.

Cisplatin (CDDP) and related platinum salts-based molecules are cytotoxic drugs that directly damage the double strand of DNA, inhibiting DNA replication, impairing cellular mitosis and inducing cancer cells death (41). The mechanisms underlying the effects induced by CDDP include the production of reactive oxygen species (ROS), the peroxidation of lipids, activation of p53 signaling, cell cycle arrest, and activation of intrinsic and extrinsic pathways of apoptosis (42). Lin et al. (33) reported that cisplatin modulated the autophagy flux in the endometrial cancer cell line Ishikawa through inhibition of the PI3K/AKT/mTOR signaling. Total and phosphorylated PI3K, AKT, and mTOR proteins were downregulated after treatment with CDDP. Moreover, after cisplatin intervention the number of autophagosomes was augmented relative to untreated controls. Also, the treatment of Ishikawa cells with a PI3K activator, IGF1, partially reversed the effect of CDDP on cell autophagy. These data indicate that conventional cytotoxic therapies may activates autophagy in endometrial cancer cells.

Autophagy Therapies Related to p53 Pathway

The mismatch repair (MMR) system play a pivotal role in repairing the DNA polymerase errors including diverse types of base mismatches. The MMR machinery activates also cell cycle checkpoints and apoptotic responses following some types of DNA damage including those caused by Sn1-methylating agents N-methyl-N-nitro-N-nitroso guanidine and N-methyl-N-nitrosourea and cisplatin, 5-fluorouracil, temozolomide, and 6-TG (43). The p53 gene is tumor suppressor which plays a key role in safeguarding the genome integrity and it's also an integrator of diverse stressors, such as DNA damage, hypoxia, cell cycle arrest, and programmed cell death (44). After incubation with methylating agents N-methyl-N-nitro-N-nitroso guanidine and N-methyl-N-nitroso urea, the MMR machinery binds to O6-methyl guanine adducts triggering the induction of p53 and apoptosis (45). Zeng et al. (46) analyzed the roles of MMR and p53 signaling in activation of autophagy and apoptosis

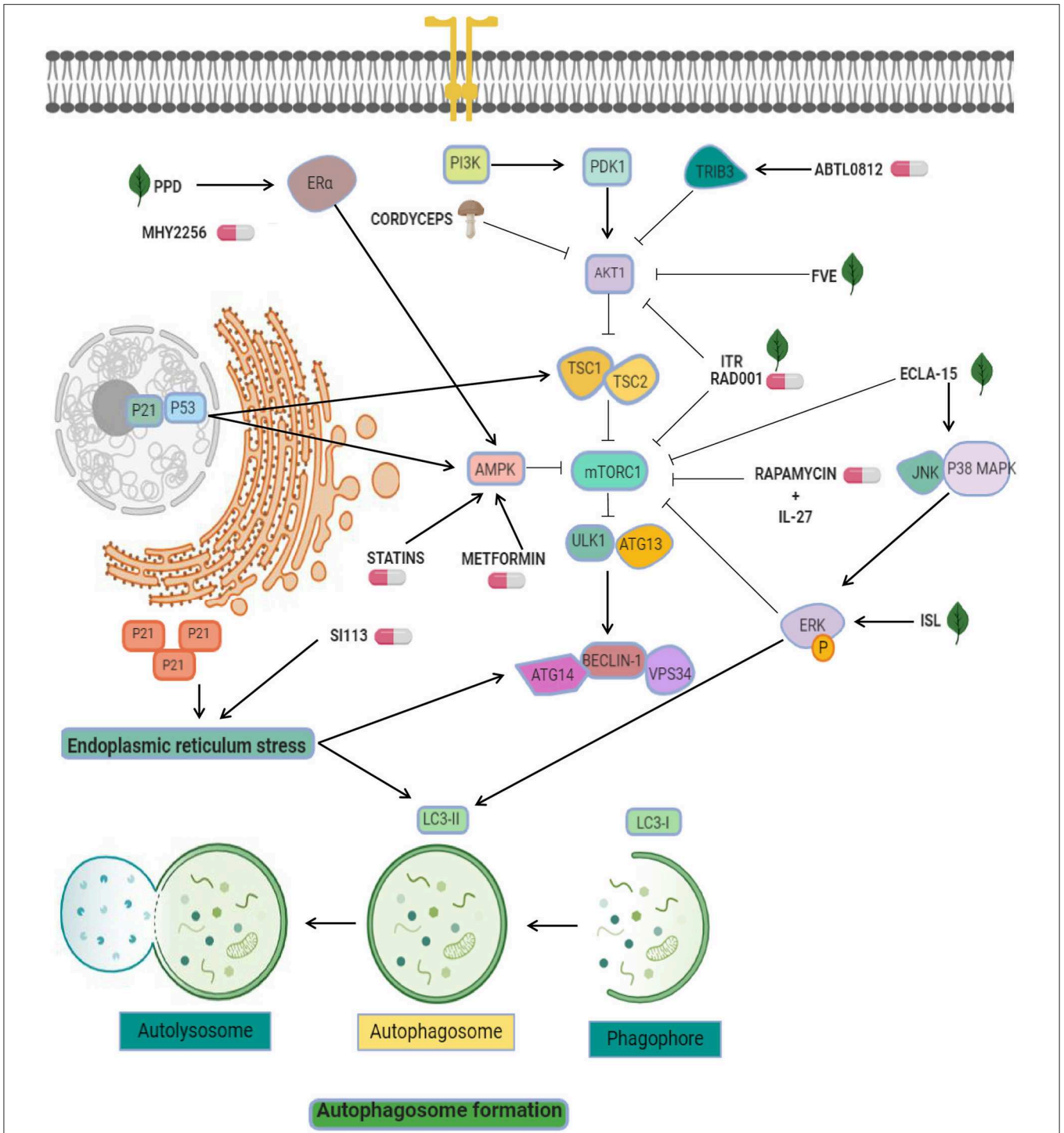

FIGURE 1 | Targeting of pro-autophagy factors in endometrial cancer. Pro-autophagic chemical (pills) or natural compounds (leaf) tested in endometrial cancer cells. AKT inhibitors are used as the main strategy to activate autophagy, and include ABTL0812, FVE, ITR, RAD001, and cordyceps. Other strategies exploit the enhancement of mTOR inhibitors effectiveness through the modulation of AMPK, ERK, and ER-alpha using agents such as ecla-15, ISL, MYH2256, metformin, and statins. Statins and SI113 may induce cytosolic expression of p21, which leads to endoplasmic reticulum stress and autophagy.

using MLH1–/MLH1+ colorectal cancer cells (HCT116) as well as MSH2–/MSH2+ endometrial cancer cells (HEC59) after exposure to 6-thioguanine (6-TG). Authors found that MMR repair pathway was required for 6-TG-indiced autophagy, and that p53 tumor suppressor was pivotal for transducing signals from MMR to the autophagic pathway. MSH2 protein was

essential for induction of autophagy after 6-TG ^ treatments in endometrial HEC59 (MSH2+, MMR+) cancer cells. Moreover, Atg5 knockdown resulted in enhanced cell death in HCT116 (MLH1+, MMR+) cells following MMR repair of 6-TG damage. In addition, the induction of autophagy resulted in inhibition of apoptosis in response to 6-TG damage, maybe by degradation of damaged mitochondria (46).

EPIGENETIC INHIBITORS AS MODULATORS OF AUTOPHAGY IN ENDOMETRIAL CANCER

A link between histone deacetylases inhibitors and mTOR pathway came from a recent study in endometrial stromal sarcomas which are rare tumors representing <1% of all uterine malignancies (47). Histone deacetylases (HDACs) and histone acetyl transferases (HATs) are enzymes that catalyze the modification of histone proteins inducing changes in chromatin structure and function. An increase in histones acetylation in a specific gene promoter results in activation of expression of oncogenes. Therefore, HDACs play pivotal roles in the development of diverse human tumors; thus HDAC targeting with inhibitors represents a promising approach in cancer therapies (48). Another HDAC inhibitor is the suberoylanilide hydroxamic acid (SAHA) which blocks HDACs activity inducing cell differentiation, cell cycle arrest, and cell death of cancer cells (49). In ESS-1 cells, the suppression of mTOR by SAHA leads to impairment in cell proliferation (50). Recent data showed that increased phosphorylation of the S6 ribosomal protein S6rp was involved in autophagy activation. Phosphorylation of p70S6K kinase (Ser235/236) which in turns phosphorylates S6rp regulating in this way the synthesis of proteins involved in cell growth and cell cycle progression in ESS-1 cells but not in HESCs (51). ESS-1 cells were more sensitive to SAHA inhibitor than normal endometrial stromal cells, and significantly inhibited the proliferation of ESS-1 cells by inducing cell cycle arrest and activating autophagic process. The data suggested that activation of autophagy by modulating HDACs activity could have clinical potential in treatment of uterine and endometrial sarcomas (52).

On the other hand, recent evidence has implicated p53 as an unexpected player in autophagy regulation via apoptosis activation (53). Several studies have shown that HDACs inhibitors have a potential clinical value in endometrial cancer therapy. For instance, it was showed that MHY2256, an inhibitor of sirtuin (SIRT) protein, has antitumor effects inducing the acetylation of p53 protein in estrogen positive breast cancer cells (54). Further, the same group reported that MHY2256 treatment in endometrial cancer cells enhanced late apoptosis and significantly reduced tumor growth in a mouse xenograft model (55). The anticancer activity autophagy-associated in Ishikawa cells was observed at low concentrations of MHY2256 leading to increases of LC3-II and autophagy-related protein 5 (ATG5), furthermore MYH2256 significantly increased the red florescence acidic vesicular organelles (55). There is a possibility that the mechanism of autophagy activation was related to cell cycle regulators p21 and acetylated p53 (**Figure 1**). Some reports highlight this possibility. For example, it was reviewed that p53 mediated autophagy-regulation by increased of tuberous sclerosis complex 2 (TSC2) and AMPK, resulting in suppressing mTOR and the activating ULK1 complex (53). Also, p21 induced autophagy and senescence in breast cancer (56), and quinacrine showed antitumor effects by inducing p21-dependent autophagy in HCT-116 colorectal cancer cells (57). These data suggested that Sirtuin inhibitors may modulates autophagy and could be an interesting therapeutic tool in cancer.

ENDOPLASMIC RETICULUM-RELATED THERAPIES IN ENDOMETRIAL CANCER

Endoplasmic reticulum (ER)-stress is a powerful trigger of autophagy. It has been reported that ER stress leads to upregulation of genes related to autophagy activation, including ATG8, ATG14, and facilitate the formation of the phagophore and autophagosomes through ER-membrane bound ATG9 (58). Interestingly, some therapeutic molecules have been shown to activate autophagy through this pathway in endometrial cancer cells. For instance, the serine/threonine protein kinase SGK1 showed anticancer activity because its ability to controls oxidative and ER stresses. SI113 compound has been recently identified as a potent and selective inhibitor SGK1 and AKT kinases activity with the ability to trigger the autophagic process. In particular, treatment with SI113 in endometrial cancer cells promoted the increase in LC3B-II and beclin1 levels (59). Activation of autophagy appears to be connected with induction of apoptosis and the cleavage of both PARP and caspase-9 proteins. Furthermore, these effects were related to the activation of ER stress GRP78 and CHOP proteins (**Figure 1**). These promising effects place the inhibitor as a promising therapeutic approach in endometrial cancer (59).

AUTOPHAGY MODULATION USING REPURPOSING COMPOUNDS IN ENDOMETRIAL CANCER THERAPY

Drug repurposing (also referred to as repositioning or redirection) refers to the utilization of current therapeutic medications originally developed for one specific health condition to treat alternative indications such as cancer, which leads to easy access of these agents for patients. In addition, the utilization of these drugs reduces the cost associated to development of novel oncologic agents (60). For instance, epidemiologic studies indicated that dimethylbiguanide metformin, an orally administered medication commonly used to maintain low blood glucose in individuals with non-insulin-dependent diabetes mellitus, have also significant chemopreventive effects by decreasing the risk to develop diverse human cancers including colon, pancreatic, breast, and prostate (61), and recent evidence shows that it is also able to stimulate autophagy based on activation of AMP-activated protein kinase (AMPK). On this topic, three reports described the up-regulation of AMPK, p-AMPK, LC3II, and beclin1 after metformin treatment in endometrial cancer cells (62–64). AMPK

is an important factor involved in the inhibition of tumor growth and autophagy triggering. Two mechanisms have been proposed for modulation of autophagy: (i) the mTOR inhibition by AMPK, and (ii) the activation of ULK1 to induce autophagic processes (65). Another investigations combined metformin with natural agents such as ginseng saponin protopanaxadiol (PPD) resulting in enhanced anti-tumor effects induced by metformin and unexpectedly reducing the levels of estrogen receptor alpha. It has been shown that estrogen stimulated the cell viability and blocks the cell death and autophagy of Ishikawa and RL95-2 cells, and that combinations of protopanaxadiol and metformin effectively revers the aforementioned cellular effects (64).

Chloroquine is a therapeutic molecule widely used to treat malaria disease. It's now known that chloroquine inhibits autophagy by a mechanisms associated to the increase of lysosomal pH, and its antitumor effects have been documented in brain, breast, lung, and colon cancers (66) as well as in endometrial cancer cells. Interestingly, chloroquine also has been reported to exert anticancer effects through autophagy-independent mechanisms such as lysosomal accumulation, mitochondrial disintegration, selective necrosis of tumoral cells, normalizing tumor vasculature, and reducing tumor hypoxia, causing the cancer cell growth inhibition, cell death, and an increase in the therapy responses (67, 68). Direct effects of chloroquine in autophagy have been reported by Fukuda and coworkers which showed that chloroquine intervention of resistant endometrial cancer cells inhibited autophagy and partially restored its sensitivity to cisplatin (69). The suppression of autophagy using chloroquine increased both cisplatin and paclitaxel-induced cell death in HEC-1A and JEC endometrial cancer cells; furthermore the sensitivity to cisplatin was increased after chloroquine intervention. As previously observed, ROS generation following the treatment with these drugs mediate the autophagic process by activating of ERK, AMPK, and JNK signaling, and impairs mTOR pathway (**Figure 2**) (69). These data highlight the chemosensitization effects of chloroquine associated to autophagy inhibition in endometrial cancer cells.

One of the most attractive approaches in oncologic treatments is the combination of autophagy inhibitors such as sorafenib with agents like chloroquine to synergize their antitumor effects. Sorafenib is a FDA-approved broad-spectrum kinase inhibitor which was initially used as an inhibitor of RAF1 but it also has inhibitory effects on BRAF kinase (70). Sorafenib is commonly used in patients with renal cell and hepatocellular carcinoma, and recently it has been suggested as therapeutic in endometrial carcinoma (3, 71). However, increased resistance to sorafebib limited its clinical utilization in patients. Eritja and coworkers demonstrated that sorafenib resistance was associated to autophagy endometrial cancer cells. This compound activates a MAPK/JNK-dependent protective autophagic mechanism in endometrial cancer after therapeutic stress (3). The evidence supported that sorafenib exposure in endometrial cancer cells promoted the modification of LC3B- I to LC3B-II, which was accompanied by apparition of autophagic structures. However, in conditions of autophagy, the silencing of Beclin 1 using siRNAs and chloroquine sensitized endometrial cancer cells to sorafenib treatment (**Figure 2**). *In vivo* studies showed that targeting autophagy resulted in enhanced sorafenib cytotoxicity and suppressed tumor growth and pulmonary metastasis. These results grant novel insights about the role of sorafenib in the activation of a protective autophagic response as a new strategy for therapeutic intervention in endometrial cancer (3).

Another valuable approach related to the inhibition of autophagy as a therapy in endometrial cancer is the use of bortezomib in combination with platinum-based chemotherapy. Bortezomib is a novel inhibitor of the 26S proteasome, which exhibit anticancer properties in diverse types of human neoplasias including colon, breast, ovarian, and prostate cancer (72–74). Its molecular mechanism focuses on the inhibition NF-κB pathway resulting in augmented sensitivity of cells to chemotherapy via apoptosis activation (65). It has been shown that sustained activation of ERK may inhibit the autophagy process (**Figures 1**, **2**) (75, 76). In this context it could be explained that bortezomib inhibits the fusion of lysosome and autophagosome promoting p62 accumulation at the autophago-lysomal stage in endometrial cancer Ishikawa cells. Interestingly platinum-based chemotherapy activates autophagy in ovarian cancer cells resistant to cisplatin and bortezomib block the cisplatin induced autophagy stimulating the chemotherapy efficacy in ovarian cancer (77).

Anti-autophagic approaches using repurposing compounds are also related to arterial hypertension medications such as nifedipine, an L-type calcium channel antagonist that suppress the cell proliferation of diverse types of cancer (78). The effect of nifedipine on HEC-1A endometrial cancer cells was the suppression of cell proliferation and triggering of apoptosis. Furthermore, Nifedipine also induced autophagy and staining analysis revealed that the formation of autophagic GFP-LC3-II was stimulated by nifedipine treatment. Interestingly, the autophagy inhibitor 3-MA combined with nifedipine activated cell death indicating that autophagy may promotes the cell survival associated to Beclin1 and mTOR functions (79).

The combination of chloroquine and paclitaxel (Taxol) has become a promising strategy. Paclitaxel acts by binding to β-tubulin thereby inhibiting microtubule depolymerization in cytoskeleton, and consequently resulting in cell cycle arrest at G2/M stage and cell death (80). Surprisingly, it has also been shown to be an autophagy activator in diverse types of cancers (81, 82). Paclitaxel exposure in endometrial cancer HEC-1A and JEC cells induce autophagy-related events such as augmented LC3-II/LC3-I ratio and low p62 abundance (83). Perhaps the mechanism of paclitaxel-induced autophagy is related with the generation of intracellular ROS. In previous studies, it has been described that anticancer agents can promote the generation of ROS and in turns activates autophagy after turning on ERK, JNK, and AMPK transducers (**Figure 2**) (83, 84). An interesting effect in sensitivity of endometrial carcinoma cells to chemotherapy was observed when autophagy was inactivated through knockdown of Beclin 1 and by treatment with chloroquine. Also, combined intervention with chloroquine and paclitaxel leads to autophagy abrogation and high proportion of HEC-1A and JEC cell death (83). These findings suggested that approaches based on the inhibition of autophagy can open

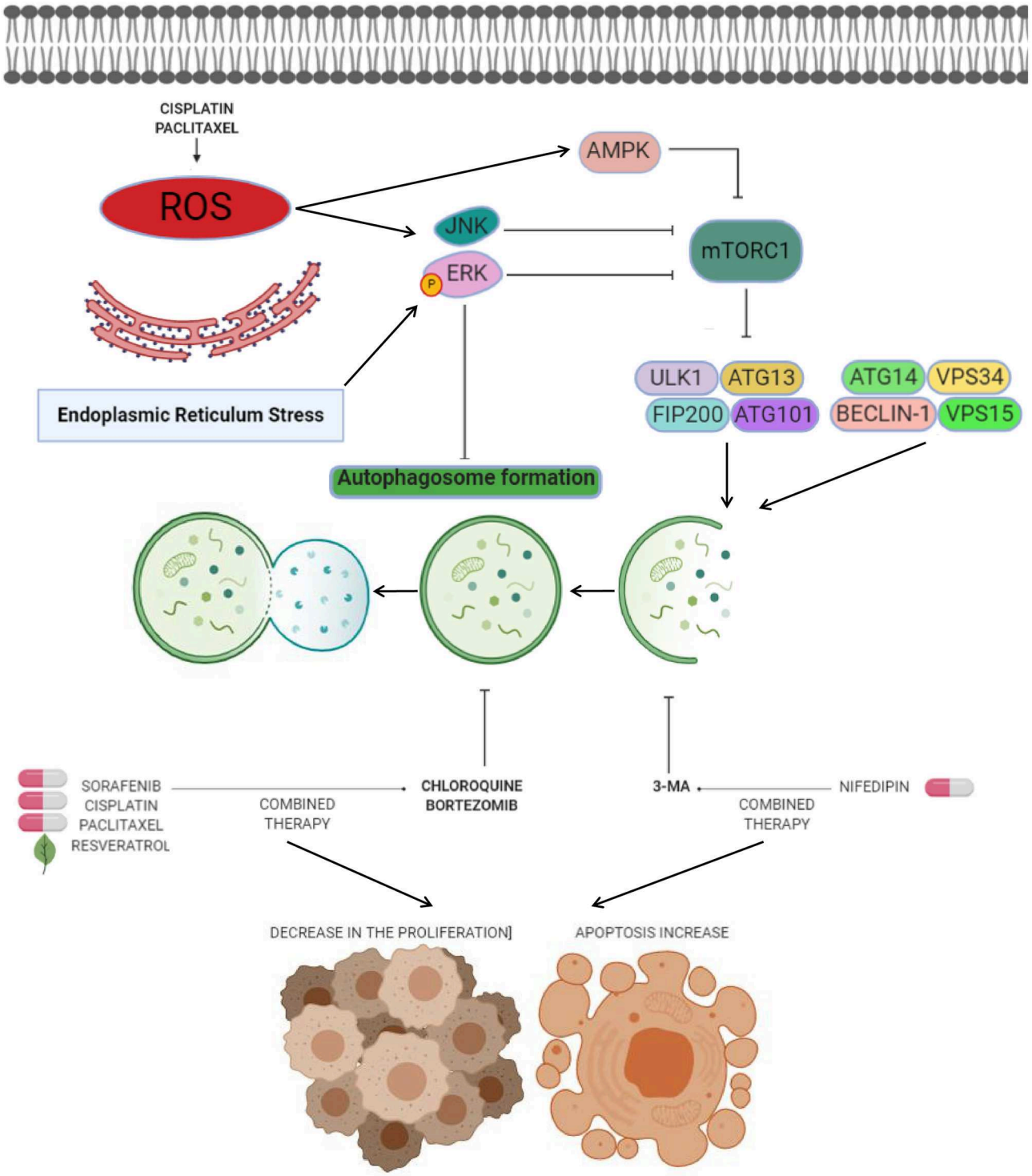

FIGURE 2 | Anti-autophagic approaches in endometrial cancer. Anti-autophagic chemicals (bold letters), autophagy inducing chemicals (pills), or natural compounds (leaf) tested in endometrial cancer cells. The production of reactive oxygen species (ROS) after cisplatin and paclitaxel therapies mediates the autophagy process. ROS have been shown to induce autophagy by several processes including activation of ERK and JNK transducers, as well as inhibition of mTOR signaling by AMPK. Novel strategies for therapeutic intervention in endometrial cancer are based on the suppression of autophagy through use of agents such as CQ or 3-MA to promotes cell death and decreases in cell proliferation when combine with agents like sorafenib, cisplatin, paclitaxel, resveratrol, and nifedipin. Other agents such as bortezomib block cisplatin-induced autophagy and stimulate the cytotoxic effects of chemotherapy on cancer cells.

new paths to improve the paclitaxel efficiency in endometrial cancer therapy.

AUTOPHAGY AND CHEMOTHERAPY RESISTANCE

Novel clues about the relationships between the cellular mechanisms underlying drug resistance, stemness, and autophagy in endometrial cancer were provided in a study by Ran et al. (85). Using CD133+/CD44+ cancer stem cells-like isolated from the JEC endometrial cancer cell line they found an increase in autophagy relative to parental endometrial cells. Moreover, autophagy inhibition was associated with the inhibition of stem cells-like phenotype, specifically diminished spheroids formation and enhanced sensitivity to paclitaxel. These data support the notion that stemness phenotype and activation of pro-survival autophagy are both related to chemoresistance of cancer stem-cells. In addition it was found that the estrogen induced gene (EIG121) promoted both autophagy and cell survival in the subpopulation of CD133+/CD44+ cells and normal endometrial cancer cells (85).

TARGETING AUTOPHAGY WITH NATURAL DIETARY COMPOUNDS

Several natural compounds exhibit promising effectiveness against endometrial cancer cells in *in vitro* studies, and therefore have been proposed as attractive therapeutic agents in aggressive endometrial cancers. These compounds have a direct impact on several metabolic pathways, as the autophagy, but their role in cancer is contradictory due it has a dual function in survival and cell death. Nevertheless, autophagy targeting can be exploited as a new therapeutic target to contribute with development of alternative and more effective treatments in endometrial cancer. Natural compounds provide opportunities for combinatorial therapies which may affects at the same time multiple targets to achieve a higher effectiveness in comparison to that of single molecule-based drugs.

Hedgehog signaling pathway inhibitors have been shown to successfully impair proliferation of endometrial cancer cells (86), and mTOR inhibitors have also been investigated for their therapeutic potential in endometrial cancers (87). Itraconazole is a common antifungal agent that inhibited Hedgehog and AKT/mTOR signaling transducers as well as WNT/β-catenin signaling (88) and showed a dose- and time-dependent suppression of cell proliferation in human endometrial cancer cell lines (89). Tsubamoto and coworkers reported that this fungal agent also suppressed the proliferation of AN3-CA, HEC-1A, and Ishikawa cells, but did not suppress GLI1 or GLI2 transcription, downstream effectors of the Hedgehog pathway in HEC-50B or SNG-II cells (90). Moreover, itraconazole also inhibited the expression of signaling proteins in HEC-1A and AN3-CA cells, and upregulated the microtubule-associated protein 1A/1B-light chain 3-II. In Ishikawa, HEC-50B and SNG-II cells, the ATP-binding cassette transporter A1 (ABCA1) protein expression was suppressed following itraconazole treatment (90). ABCA1 regulates cholesterol efflux across the plasma membrane. Itraconazole treatment also appears to inhibit the growth of cancer cells by blocking the activation of AKT/mTOR signaling. In addition, the consistent activation of AKT was associated with higher rates of lipid raft formation, while its abrogation impeded AKT activation, and the intracellular cholesterol trafficking to the plasma membrane in human umbilical vein endothelial cells, thereby suppressing mTOR (91).

The isoliquiritigenin (ISL) is a licorice flavonoid with anti-oxidant, anti-inflammatory, and tumor suppression effects. In telomerase-immortalized endometrial stromal (T-HESCs), Ishikawa, HEC-1A, and RL95-2 cells the treatment with ISL resulted in a reduction of cell growth and survival in a dose- and time-dependent way (92). ISL also inhibited the proliferation and induced the arrest of cell cycle (G1 and G2/M phase) through activation of p53/p21 signaling. It also promoted apoptosis associated with the activation of caspase-3, caspase-7, and PARP, and promoted the autophagy by an accumulation of LC3II protein and higher levels of phosphorylated p-ERK. The ERK-dependent autophagic activity was associated with the LC3 induction and the conversion of LC3I to LC3II (93, 94). ISL also suppressed the growth of HEC-1A-LUC xenograft tumors and suppressed the expression of nuclear PCNA. These effects were accompanied by increased caspase-7, p62, and LC3B protein expression in tumor tissues suggesting that it could be a potential anti-cancer drug candidate (95).

Resveratrol is a polyphenolic compound derived from red wine and red fruits with well-documented anti-tumor effects. Also it exhibits the ability to enhance autophagy by an increase of LC3-II accumulation, and suppressed the proliferation of Ishikawa cells in lower dose (20 μM) than other cancer cell (96). Resveratrol treatment also increased the expression of p-AMPKα that has been associated with its ability to induce apoptosis (97), and repressed autophagy by the inhibition of mTOR signaling pathway (98, 99). The activation of AMPK by resveratrol also counteracts the inhibition of the mTOR-dependent autophagy (thereby resulting in autophagy promotion). The combinatory treatment of resveratrol with chloroquine markedly suppressed the cell growth and suppressed apoptosis, compared with the resveratrol treatment alone. The inhibition of autophagy process after silencing of ATG5 or ATG7 genes using siRNAs effectively activated the cell death in endometrial cancer cells (**Figure 1**).

Fucus vesiculosus (brown seaweed) extracts (FVE) appears to have display anti-cancer effects in estrogen receptor (ER)-dependent and -independent way in HEC-1-B and RL95-2 endometrial cancer cell lines by a competitive inhibition of estradiol (E2) binding to the estrogen receptor (100, 101). FVE inhibited aromatase enzyme activity *in vitro* and a co-treatment with E2 reduced the estrogen receptor activation by 50%, inhibited endogenous E2 and significantly decreased viability of cells. FVE also induced the expression of apoptotic (CASP6; APAF1, FANCG, XIAP, MED1), autophagy (ATG10, GABARAP) proteins, and BRAF, PIK3R4, PRKAA1, PRKACB, PRKAR1A, PRKAR2A, and MAP3K14 kinases. In addition altered morphological features in the cells suggested active apoptosis and autophagy, evidencing the effects of FVE as an autophagic-mediator of apoptosis, associated to low

phosphorylation of proteins from PI3K/Akt/mTOR signaling (**Figure 1**). It has been reported that FVE may also be effective in the therapy of breast, ovarian, and endometrial cancers. These findings suggested that FVE may achieve a protective effect against estrogen-dependent cancers (102).

Triterpenoids echinocystic acid and its glycosides isolated from *Eclipta prostrata* exhibits diverse protective activities in malaria, HIV, and cancer diseases. They also showed anti-venom, antioxidant and anti-inflammatory abilities (103–106). Isolate compounds have cytotoxic effects in endometrial cancer cells, in that way, eclalbasaponin II (one glucose moiety) > echinocystic acid >> eclalbasaponin I (two glucose moiety) suggesting that the cytotoxic activity of oleanane-type triterpenoids was associated with the sugar moiety at the C-3 position and the free carboxyl at C-28 (107). Eclalbasaponin II treatment induced apoptosis in a caspase-independent manner (type II programmed cell death) in ovarian cancer cells by an increase in the sub G1 population. Also, it was found an increase in acidic vesicular organelle content and an in the levels of LC3-II. Moreover, eclalbasaponin II also activated MAP kinase JNK and p38 proteins and repressed the mTOR pathway (**Figure 1**). Therefore, these perennial herb-derivate compounds exhibit anti- tumor activities in ovarian and endometrial cancer cells (108).

Three fungi *Cordyceps Sinensis* (Cordy), *Ganoderma lucidum* (Reishi), and *Agaricus Blazi Murill* (ABM) extracts have biological activities and numerous pharmacological effects and are commonly utilized in traditional Chinese medicine as adjuvant in cancer therapies (109–111). Cordy and Reishi extracts has been also used in leukemia, ovarian, breast, prostate, and gastric cancer (112–115). Crude extracts derived from Cordy and ABM/Reishi had an inhibitory effect on cell viability, proliferation, and suppression of cell growth of Ishikawa, Hec-1A, and AN3-CA cells by suppression of p-AKT (116). Remarkably, Beclin-1 phosphorylation by p-AKT was essential for autophagy and anchorage-independent growth (117). The low levels of p-AKT lead to higher autophagy and endometrial cancer cells death. In tumors, the p-AKT level PI3K/AKT pathway has been associated with cisplatin-resistance (118) and unlike other fungi, Cordy and ABM/Reishi extracts did not affects the interplay between endometrial cancer cells and NK-cells *in vitro* (**Figure 1**).

NON-CODING RNAs REGULATING AUTOPHAGY

Recent studies strongly suggested the participation of microRNAs (miRNAs) in the progression of endometrial cancer, at least in part, through autophagy modulation. MiRNAs are small non-coding RNAs of 25 nucleotides length. These tiny RNAs function as master regulators of gene expression at posttranscriptional level by blocking translation and activating the degradation of specific transcripts or mRNAs in cellular cytoplasmic P-bodies (119). Alterations in the miRNAs abundance have been found during early and late stages of endometrial cancers. For example, Wang and Liu demonstrated that miR-101-3p activates the autophagy in endometrial cancer cells by binding and subsequent downregulation of EZH2 mRNA (120). Some miRNAs, such as miR-218 and miR-205, have been demonstrated to modulate chemoresistance in endometrial cancer (121, 122). Particularly, miR-218 was downregulated in paclitaxel-resistant endometrial cancer cells relative to non-resistant parental cells, whereas upregulation of miR-218 leads to sensitization of paclitaxel-resistant endometrial cancer cells to paclitaxel. In addition, miR-218 repressed autophagy by targeting HMGB1 in paclitaxel-resistant cells (121). On the other hand, Zhuo and colleagues demonstrated that miR-205 was upregulated in a progesterone-resistant (PR) sub-cell line and induced apoptosis through repressing autophagy process by targeting tumor suppressor PTEN through AKT/mTOR signaling in endometrial cancer (122). Finally, a recent study showed the participation of the HOX Transcript Antisense RNA (HOTAIR) in the regulation of the resistance to cisplatin in endometrial cancer cells. HOTAIR controlled autophagy by modulating the expression of Beclin-1, multidrug resistance (MDR), and P-glycoprotein (PGP) proteins (123). Thus, these initial reports showed that miRNAs and long non-coding RNAs may participate in the process of endometrial tumorigenesis and development by influencing autophagy.

CONCLUSIONS

Various roles of autophagy have emerged in the scientific literature and the relationship between autophagy and cancer is enormously controversial due to its dual roles depending on the context and tumor environment. From the biological view of cancer, even particularly in endometrial cancer, it remains controversial whether autophagy is a tumor suppressor process (by stopping the cell cycle, activating apoptosis, decreasing proliferation) or oncogenic process (promoting cell survival against insults caused by chemotherapy agents). Endometrial cancer is the fourth most frequent neoplasia for women worldwide. Combinatory therapy with carboplatin and paclitaxel is the main front-line chemotherapy in endometrial cancer. However, its clinical effectiveness is variable, indicating that novel molecular therapies against specific cellular processes associated to cell survival and therapy resistance urged to ameliorate the rates of success in endometrial cancer therapies. Autophagy represents in this context a potential cellular target for the development of new therapeutic agents. Although the knowledge of autophagy mechanisms in endometrial cancer are limited, important recent findings in cell lines and patients greatly help us to visualize the potential intervention of these human carcinomas in order to ameliorate the rates of successful therapy for patients. As we have reviewed in this work, various signaling pathways such as PI3K-AKT-mTOR, AMPK-mTOR, and p53 trigger or inhibit the autophagy process and can be used as potential molecular targets in therapeutic approaches in two ways: (i) inhibiting autophagy to promote sensitization of endometrial tumor cells in response to chemotherapy agents such as cisplatin and paclitaxel, and (ii) its activation in some endometrial cancer cell lines is related with low cell proliferation, migration, invasion, and activation of apoptosis. However, despite the discrepancies

in these studies, we believe that variables such as tumor stage, alteration in the balance of cell signaling, epigenetics, cell cycle, and even mutations in autophagy genes could be playing important roles that lead the results of the investigations at both ends: oncogenic and tumor suppressor. Of interest are the findings that autophagy can be modulated using repurposing compounds which may leads to faster experimentation and validation, and access of these drugs to patients. Hence, although the research about autophagy is ongoing, the actual studies highlight the potential usefulness of novel molecules and proteins for directed targeted therapies in endometrial cancer.

AUTHOR CONTRIBUTIONS

SN-O, DG-R, JP-R, YS-V, LM, RM-V, and CL-C wrote all the manuscript sections. SN-O and JP-R draw the figures. DG-R, LM, and CL-C conceived and designed the review.

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REFERENCES

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin*. (2015) 65:87–108. doi: 10.3322/caac.21262
2. Sorosky JI. Endometrial cancer. *Obstet Gynecol*. (2012) 120:383–97. doi: 10.1097/AOG.0b013e3182605bf1
3. Eritja N, Chen BJ, Rodríguez-Barrueco R, Santacana M, Gatius S, Vidal A, et al. Autophagy orchestrates adaptive responses to targeted therapy in endometrial cancer. *Autophagy*. (2017) 4:608–24. doi: 10.1080/15548627.2016.1271512
4. Moore K, Brewer MA. Endometrial cancer: is this a new disease? *Am Soc Clin Oncol*. (2017) 37:435–42. doi: 10.14694/EDBK_175666
5. Urick ME, Bell DW. Clinical actionability of molecular targets in endometrial cancer. *Nat Rev Cancer*. (2019) 19:510–21. doi: 10.1038/s41568-019-0177-x
6. Setiawan VW, Yang HP, Pike MC, McCann SE, Yu H, Xiang YB, et al. Type I and II endometrial cancers: have they different risk factors? *J Clin Oncol*. (2013) 10:2607–18. doi: 10.1200/JCO.2012.48.2596
7. National Cancer Institute. *Cancer Stat Facts: Endometrial Cancer*. Bethesda, MD (2015).
8. Lynch HT, Snyder CL, Shaw TG, Heinen CD, Hitchins MP. Milestones of Lynch syndrome: 1895–2015. *Nat Rev Cancer*. (2015) 15:181–94. doi: 10.1038/nrc3878
9. Briggs S, Tomlinson I. Germline and somatic polymerase ε and δ mutations define a new class of hypermutated colorectal and endometrial cancers. *J Pathol*. (2013) 230:148–53. doi: 10.1002/path.4185
10. Ngeow J, Stanuch K, Mester JL, Barnholtz-Sloan JS, Eng C. Second malignant neoplasms in patients with Cowden syndrome with underlying germline PTEN mutations. *J Clin Oncol*. (2014) 10:1818–24. doi: 10.1200/JCO.2013.53.6656
11. Bestvina CM, Fleming GF. Chemotherapy for endometrial cancer in adjuvant and advanced disease settings. *Oncologist*. (2016) 21:1250–9. doi: 10.1634/theoncologist.2016-0062
12. Graziele FdS, Samarina RW, Gisele M. Carboplatin: molecular mechanisms of action associated with chemoresistance. *Braz J Pharm Sci*. (2014) 50:693–701. doi: 10.1590/S1984-82502014000400004
13. Bishop E, Bradshaw TD. Autophagy modulation: a prudent approach in cancer treatment? *Cancer Chemother Pharmacol*. (2018) 82:913–22. doi: 10.1007/s00280-018-3669-6
14. Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell*. (2004) 6:463–77. doi: 10.1016/S1534-5807(04)00099-1
15. Høyer-Hansen M, Jäättelä M. Autophagy: an emerging target for cancer therapy. *Autophagy*. (2008) 4:574–80. doi: 10.4161/auto.5921
16. Chen N, Karantza V. Autophagy as a therapeutic target in cancer. *Cancer Biol Ther*. (2011) 11:157–68. doi: 10.4161/cbt.11.2.14622
17. Buytaert E, Callewaert G, Vandenheede JR, Agostinis P. Deficiency in apoptotic effectors Bax and Bak reveals an autophagic cell death pathway initiated by photodamage to the endoplasmic reticulum. *Autophagy*. (2006) 2:238–40. doi: 10.4161/auto.2730
18. Servante J, Estranero J, Meijer L, Layfield R, Grundy R. Chemical modulation of autophagy as an adjunct to chemotherapy in childhood and adolescent brain tumors. *Oncotarget*. (2018)16:35266–77. doi: 10.18632/oncotarget.26186
19. Yu L, Chen Y, Tooze SA. Autophagy pathway: cellular and molecular mechanisms. *Autophagy*. (2018) 14:207–15. doi: 10.1080/15548627.2017.1378838
20. Axe EL, Walker SA, Manifava M, Chandra P, Roderick HL, Habermann A, et al. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *J Cell Biol*. (2008) 182:685–701. doi: 10.1083/jcb.200803137
21. Yamamoto H, Kakuta S, Watanabe TM, Kitamura A, Sekito T, Kondo-Kakuta C, et al. Atg9 vesicles are an important membrane source during early steps of autophagosomes formation. *J Cell Biol*. (2012) 198:219–33. doi: 10.1083/jcb.201202061
22. Jahreiss L, Menzies FM, Rubinsztein DC. The itinerary of autophagosomes: from peripheral formation to kiss-and-run fusion with lysosomes. *Traffic*. (2008) 9:574–87. doi: 10.1111/j.1600-0854.2008.00701.x
23. Palmieri M, Pal R, Nelvagal HR, Lotfi P, Stinnett GR, Seymour ML, et al. MTORC1-independent TFEB activation via Akt inhibition promotes cellular clearance in neurodegenerative storage diseases. *Nat Comm*. (2017) 8:14338. doi: 10.1038/ncomms15793
24. Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, Del Piccolo P, et al. FoxO3 controls autophagy in skeletal muscle *in vivo*. *Cell Metab*. (2007) 6:458–71. doi: 10.1016/j.cmet.2007.11.001
25. Di Malta C, Cinque L, Settembre C. Transcriptional regulation of autophagy: mechanisms and diseases. *Front Cell Dev Biol*. (2019) 7:114. doi: 10.3389/fcell.2019.00114
26. Lebovitz CB, Robertson AG, Goya R, Jones SJ, Morin RD, Marra MA. Cross-cancer profiling of molecular alterations within the human autophagy interaction network. *Autophagy*. (2015) 11:1668–87. doi: 10.1080/15548627.2015.1067362
27. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy*. (2016) 12:1–222. doi: 10.1080/15548627.2015.1100356
28. Du Toit A, Hofmeyr JS, Gniadek TJ, Loos B. Measuring autophagosome flux. *Autophagy*. (2018) 14:1060–71. doi: 10.1080/15548627.2018.1469590
29. Slomovitz BM, Coleman RL. The PI3K/AKT/mTOR pathway as a therapeutic target in endometrial cancer. *Clin Cancer Res*. (2012) 1:5856–64. doi: 10.1158/1078-0432.CCR-12-0662
30. Talesa VN, Ferri I, Bellezza G, Love HD, Sidoni A, Antognelli C. Glyoxalase 2 is involved in human prostate cancer progression as part of a mechanism driven by PTEN/PI3K/AKT/mTOR signaling with involvement of PKM2 and ERα. *Prostate*. (2017), 77:196–210. doi: 10.1002/pros.23261
31. Hassan B, Akcakanat A, Holder AM, Meric-Bernstam F. Targeting the PI3-kinase/Akt/mTOR signaling pathway. *Surg Oncol Clin N Am*. (2013) 22:641–64. doi: 10.1016/j.soc.2013.06.008
32. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol*. (2011) 13:132–41. doi: 10.1038/ncb2152
33. Lin Q, Chen H, Zhang M, Xiong H, Jiang Q. Knocking down FAM83B inhibits endometrial cancer cell proliferation and metastasis by silencing

the PI3K/AKT/mTOR pathway. *Biomed Pharmacother*. (2019) 115:108939. doi: 10.1016/j.biopha.2019.108939
34. Kanda R, Hiraike H, Wada-Hiraike O, Ichinose T, Nagasaka K, Sasajima Y, et al. Expression of the glucagon-like peptide-1 receptor and its role in regulating autophagy in endometrial cancer. *BMC Cancer*. (2018) 1:657. doi: 10.1186/s12885-018-4570-8
35. Benshushan A. Endometrial adenocarcinoma in young patients: evaluation and fertility-preserving treatment. *Eur J Obstet Gynecol Reprod Biol*. (2004)1:132–7. doi: 10.1016/j.ejogrb.2004.05.015
36. Liu H, Zhang L, Zhang X, Cui Z. PI3K/AKT/mTOR pathway promotes progestin resistance in endometrial cancer cells by inhibition of autophagy. *Onco Targets Ther*. (2017) 6:2865–71. doi: 10.2147/OTT.S95267
37. Wang H, Li D, Li X, Ou X, Liu S, Zhang Y, et al. Mammalian target of rapamycin inhibitor RAD001 sensitizes endometrial cancer cells to paclitaxel-induced apoptosis via the induction of autophagy. *Oncol Lett*. (2016) 12:5029–35. doi: 10.3892/ol.2016.5338
38. Felip I, Moiola CP, Megino-Luque C, Lopez-Gil C, Cabrera S, Solé-Sánchez S, et al. Therapeutic potential of the new TRIB3-mediated cell autophagy anticancer drug ABTL0812 in endometrial cancer. *Gynecol Oncol.* (2019) 153:425–35. doi: 10.1016/j.ygyno.2019.03.002
39. Soloski MJ. Recognition of tumor cells by the innate immune system. *Curr Opin Immunol*. (2001). 13: 154–62. doi: 10.1016/S0952-7915(00)00198-9
40. Zhou WJ, Chang KK, Wu K, Yang HL, Mei J, Xie F, et al. Rapamycin synergizes with cisplatin in antiendometrial cancer activation by improving IL-27-stimulated cytotoxicity of NK cells. *Neoplasia*. (2018) 20:69–79. doi: 10.1016/j.neo.2017.11.003
41. Dasari S, Tchounwou PB. Cisplatin in cancer therapy: molecular mechanisms of action. *Eur J Pharmacol*. (2014) 5:364–78. doi: 10.1016/j.ejphar.2014.07.025
42. Zhu H, Luo H, Zhang W, Shen Z, Hu X, Zhu X. Molecular mechanisms of cisplatin resistance in cervical cancer. *Drug Des Devel Ther*. (2016) 7:1885–95. doi: 10.2147/DDDT.S106412
43. O'Brien V, Brown R. Signalling cell cycle arrest and cell death through the MMR system. *Carcinogenesis*. (2006) 27:682–92. doi: 10.1093/carcin/bgi298
44. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. *Nature*. (2000) 16:307–10. doi: 10.1038/35042675
45. Zou L, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science*. (2003) 6:1542–8. doi: 10.1126/science.1083430
46. Zeng X, Yan T, Schupp JE, Seo Y, Kinsella TJ. DNA mismatch repair initiates 6-thioguanine-induced autophagy through p53 activation in human tumor cells. *Clin Cancer Res*. (2007) 15:1315–21. doi: 10.1158/1078-0432.CCR-06-1517
47. Mansi JL, Ramachandra S, Wiltshaw E, Fisher C. Endometrial stromal sarcomas. *Gynecol Oncol*. (1990) 36:113–8. doi: 10.1016/0090-8258(90)90120-A
48. Minucci S, Pelicci PG. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nat Rev Cancer*. (2006) 6:38–51. doi: 10.1038/nrc1779
49. O'Connor OA, Heaney ML, Schwartz L, Richardson S, Willim R, MacGregor-Cortelli B, et al. Clinical experience with intravenous and oral formulations of the novel histone deacetylase inhibitor suberoylanilide hydroxamic acid in patients with advanced hematologic malignancies. *J Clin Oncol*. (2006) 1:166–73. doi: 10.1200/JCO.2005.01.9679
50. Kawamata N, Chen J, Koeffler HP. Suberoylanilide hydroxamic acid (SAHA;vorinostat) suppresses translation of cyclin D1 in mantle cell lymphoma cells. *Blood*. (2007) 1:2667–73. doi: 10.1182/blood-2005-11-026344
51. Iwenofu OH, Lackman RD, Staddon AP, Goodwin DG, Haupt HM, Brooks JS. Phospho-S6 ribosomal protein: a potential new predictive sarcoma marker for targeted mTOR therapy. *Mod Pathol*. (2008) 21:231–7. doi: 10.1038/modpathol.3800995
52. Hrzenjak A, Kremser ML, Strohmeier B, Moinfar F, Zatloukal K, Denk H. SAHA induces caspase-independent, autophagic cell death of endometrial stromal sarcoma cells by influencing the mTOR pathway. *J Pathol*. (2008) 216:495–504. doi: 10.1002/path.2434
53. Mrakovcic M, Fröhlich LF. p53-mediated molecular control of autophagy in tumor cells. *Biomolecules*. (2018) 21:E14. doi: 10.3390/biom8020014
54. Park EY, Woo Y, Kim SJ, Kim DH, Lee EK, De U, et al. Anticancer effects of a new SIRT inhibitor, MHY2256, against human breast cancer MCF-7 cells via regulation of MDM2-p53 binding. *Int J Biol Sci*. (2016) 6:1555–67. doi: 10.7150/ijbs.13833
55. De U, Son JY, Sachan R, Park YJ, Kang D, Yoon K, et al. A new synthetic histone deacetylase inhibitor, MHY2256, induces apoptosis and autophagy cell death in endometrial cancer cells via p53 acetylation. *Int J Mol Sci*. (2018) 19:E2743. doi: 10.20944/preprints201808.0146.v1
56. Capparelli C, Chiavarina B, Whitaker-Menezes D, Pestell TG, Pestell RG, Hulit J, et al. CDK inhibitors (p16/p19/p21) induce senescence and autophagy in cancer-associated fibroblasts, "fueling" tumor growth via paracrine interactions, without an increase in neo-angiogenesis. *Cell Cycle*. (2012). 11:3599–610. doi: 10.4161/cc.21884
57. Mohapatra P, Preet R, Das D, Satapathy SR, Choudhuri T, Wyatt MD, et al. Quinacrine-mediated autophagy and apoptosis in colon cancer cells is through a p53- and p21-dependent mechanism. *Oncol Res*. (2012) 20:81–91. doi: 10.3727/096504012X13473664562628
58. Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell*. (2000)101:249–58. doi: 10.1016/S0092-8674(00)80835-1
59. Conza D, Mirra P, Calì G, Tortora T, Insabato L, Fiory F, et al. The SGK1 inhibitor SI113 induces autophagy, apoptosis, and endoplasmic reticulum stress in endometrial cancer cells. *J Cell Physiol*. (2017) 232:3735–43. doi: 10.1002/jcp.25850
60. Pantziarka P. Scientific advice–is drug repurposing missing a trick? *Nat Rev Clin Oncol*. (2017) 14:455–6. doi: 10.1038/nrclinonc.2017.69
61. Wright JL, Stanford JL. Metformin use and prostate cancer in Caucasian men: results from a population-based case-control study. *Cancer Causes Control*. (2009) 20:1617–22. doi: 10.1007/s10552-009-9407-y
62. Zhuo Z, Wang A, Yu H. Metformin targeting autophagy overcomes progesterone resistance in endometrial carcinoma. *Arch Gynecol Obstet*. (2016) 294:1055–61. doi: 10.1007/s00404-016-4148-0
63. Takahashi A, Kimura F, Yamanaka A, Takebayashi A, Kita N, Takahashi K, et al. Metformin impairs growth of endometrial cancer cells via cell cycle arrest and concomitant autophagy and apoptosis. *Cancer Cell Int*. (2014) 14:53. doi: 10.1186/1475-2867-14-53
64. Gu CJ, Cheng J, Zhang B, Yang SL, Xie F, Sun JS, et al. Protopanaxadiol and metformin synergistically inhibit estrogen-mediated proliferation and anti-autophagy effects in endometrial cancer cells. *Am J Transl Res*. (2017) 9:4071–82.
65. Hanna RK, Zhou C, Malloy KM, Sun L, Zhong Y, Gehrig PA, et al. Metformin potentiates the effects of paclitaxel in endometrial cancer cells through inhibition of cell proliferation and modulation of the mTOR pathway. *Gynecol Oncol*. (2012) 125:458–69. doi: 10.1016/j.ygyno.2012.01.009
66. Solomon VR, Lee H. Chloroquine and its analogs: a new promise of an old drug for effective and safe cancer therapies. *Eur J Pharmacol*. (2009) 625:220–33. doi: 10.1016/j.ejphar.2009.06.063
67. Maes H, Kuchnio A, Carmeliet P, Agostinis P. How to teach an old dog new tricks: autophagy-independent action of chloroquine on the tumor vasculature. *Autophagy*. (2014) 10:2082–4. doi: 10.4161/auto.36259
68. Harhaji-Trajkovic L, Arsikin K, Kravic-Stevovic T, Petricevic S, Tovilovic G, Pantovic A, et al. Chloroquine-mediated lysosomal dysfunction enhances the anticancer effect of nutrient deprivation. *Pharm Res*. (2012) 29:2249–63. doi: 10.1007/s11095-012-0753-1
69. Fukuda T, Oda K, Wada-Hiraike O, Sone K, Inaba K, Ikeda Y, et al. The anti-malarial chloroquine suppresses proliferation and overcomes cisplatin resistance of endometrial cancer cells via autophagy inhibition. *Gynecol Oncol*. (2015) 137:538–45. doi: 10.1016/j.ygyno.2015.03.053
70. Wilhelm SM, Carter C, Tang L, Wilkie D, McNabola A, Rong H, et al. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res*. (2004) 64:7099–109. doi: 10.1158/0008-5472.CAN-04-1443
71. Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, Siebels M, et al. Sorafenib in advanced clear-cell renal-cell carcinoma. *N Engl J Med*. (2007) 356:125–34. doi: 10.1056/NEJMoa060655

72. Richardson PG, Anderson KC. Bortezomib: a novel therapy approved for multiple myeloma. *Clin Adv Hematol Oncol.* (2003) 1:596–600.
73. Periyasamy-Thandavan S, Jackson WH, Samaddar JS, Erickson B, Barrett JR, Raney L, et al. Bortezomib blocks the catabolic process of autophagy via a cathepsin-dependent mechanism, affects endoplasmic reticulum stress and induces caspase-dependent cell death in antiestrogen-sensitive and resistant ER+ breast cancer cells. *Autophagy*. (2010) 6:19–35. doi: 10.4161/auto.6.1.10323
74. Befani CD, Vlachostergios PJ, Hatzidaki E, Patrikidou A, Bonanou S, Simos G, et al. Bortezomib represses HIF-1alpha protein expression and nuclear accumulation by inhibiting both PI3K/Akt/TOR and MAPK pathways in prostate cancer cells. *J Mol Med*. (2012)90:45–54. doi: 10.1007/s00109-011-0805-8
75. Martinez-Lopez N, Athonvarangkul D, Mishall P, Sahu S, Singh R. Autophagy proteins regulate ERK phosphorylation. *Nat Commun*. (2013) 4:2799. doi: 10.1038/ncomms3799
76. Corcelle E, Djerbi N, Mari M, Nebout M, Fiorini C, Fenichel P, et al. Control of the autophagy maturation step by the MAPK ERK and p38: lessons from environmental carcinogens. *Autophagy*. (2007) 3:57–9. doi: 10.4161/auto.3424
77. Kao C, Chao A, Tsai CL, Chuang WC, Huang WP, Chen GC, et al. Bortezomib enhances cancer cell death by blocking the autophagic flux through stimulating ERK phosphorylation. *Cell Death Dis*. (2014) 5:e1510. doi: 10.1038/cddis.2014.468
78. Zhang J, Ren C, Chen L, Navedo MF, Antos LK, Kinsey SP, et al. Knockout of Na+/Ca2+exchanger in smooth muscle attenuates vasoconstriction and L-type Ca2+ channel current and lowers blood pressure. *Am J Physiol Cric Physiol*. (2010) 298:H472–83. doi: 10.1152/ajpheart.00964.2009
79. Bao XX, Xie BS, Li Q, Li XP, Wei LH, Wang JL. Nifedipine induced autophagy through Beclin1 and mTOR pathway in endometrial carcinoma cells. *Chin Med J*. (2012) 125:3120–6. doi: 10.3760/cma.j.issn.0366-6999.2012.17.028
80. Sparreboom A, Van Tellingen O, Nooijen WJ, Beijnen JH. Preclinical pharmacokinetics of paclitaxel and docetaxel. *Anticancer Drugs*. (1988) 9:1–17. doi: 10.1097/00001813-199801000-00001
81. Zhang Q, Si S, Schoen S, Chen J, Jin XB, Wu G. Suppression of autophagy enhances preferential toxicity of paclitaxel to folliculin deficient renal cancer cells. *J Exp Clin Cancer Res*. (2013) 32:99. doi: 10.1186/1756-9966-32-99
82. Kim HJ, Lee SG, Kim YJ, Park JE, Lee KY, Yoo YH, et al. Cytoprotective role of autophagy during paclitaxelinduced apoptosis in Saos-2 osteosarcoma cells. *Int J Oncol*. (2013) 42:1985–92. doi: 10.3892/ijo.2013.1884
83. Liu S, Li X. Autophagy inhibition enhances sensitivity of endometrial carcinoma cells to paclitaxel. *Int J Oncol*. (2015) 46:2399–408. doi: 10.3892/ijo.2015.2937
84. Miki H, Uehara N, Kimura A, Sasaki T, Yuri T, Yoshizawa K, et al. Resveratrol induces apoptosis via ROS-triggered autophagy in human colon cancer cells. *Int J Oncol*. (2012) 40:1020–8. doi: 10.3892/ijo.2012.1325
85. Ran X, Zhou P, Zhang K. Autophagy plays an important role in stemness mediation and the novel dual function of EIG121 in both autophagy and stemness regulation of endometrial carcinoma JEC cells. *Int J Oncol*. (2017) 51:644–56. doi: 10.3892/ijo.2017.4047
86. Feng YZ, Shiozawa T, Miyamoto T, Kashima H, Kurai M, Suzuki A, et al. Overexpression of hedgehog signaling molecules and its involvement in the proliferation of endometrial carcinoma cells. *Clin Cancer Res*. (2007) 13:1389–98. doi: 10.1158/1078-0432.CCR-06-1407
87. Colombo N, Creutzberg C, Amant F, Bosse T, González-Martín A, Ledermann J, et al. ESMO-ESGO-ESTRO Endometrial Consensus Conference Working Group. ESMO-ESGO-ESTRO consensus conference on endometrial cancer: diagnosis, treatment and follow-up. *Radiother Oncol*. (2015) 117:559–81. doi: 10.1016/j.radonc.2015.11.013
88. Nishiya N. *WNT/β-Catenin Signal Inhibitory Action of Azole Antifungal Drugs*. (2014) Available online at: https://www.iwate-med.ac.jp/wp-content/uploads/biotech2014nishiya.pdf (accessed September 15, 2019).
89. Inoue K, Tsubamoto H, Sakata K, Sakane R, Hao H, Hirota S, et al. Expression of hedgehog signals and growth inhibition by itraconazole in endometrial cancer. *Anticancer Res*. (2016) 36:149–53. doi: 10.1158/1538-7445.AM2016-1703
90. Tsubamoto H, Inoue K, Sakata K, Ueda T, Takeyama R, Shibahara H, et al. Itraconazole inhibits Akt/mTOR signalling and proliferation in endometrial cancer cells. *Anticancer Res*. (2017) 37:515–9. doi: 10.21873/anticanres.11343
91. Xu J, Dang Y, Ren YR, Liu JO. Cholesterol trafficking is required for mTOR activation in endothelial cells. *Proc Natl Acad Sci USA*. (2010) 107:4764–9. doi: 10.1073/pnas.0910872107
92. Kim YG, Ramachandran S, Kim DC, Hong YB, Kim EH, Kwon SH, et al. Isoliquiritigenin inhibited cell proliferation and triggered apoptosis in human endometrial cancer cell line. *J Women's Med*. (2010) 3:89–95. doi: 10.5468/jwm.2010.3.3.89
93. Roberts PJ, Der CJ. Targeting the Raf-MEK-ERK mitogen activated protein kinase cascade for the treatment of cancer. *Oncogene*. (2007) 26:3291–310. doi: 10.1038/sj.onc.1210422
94. Sivaprasad U, Basu A. Inhibition of ERK attenuates autophagy and potentiates tumour necrosis factor-α-induced cell death in MCF-7 cells. *J Cell Mol Med*. (2008) 12:1265–71. doi: 10.1111/j.1582-4934.2008.00282.x
95. Wu CH, Chen HY, Wang CW, Shieh TM, Huang TC, Lin LC, et al. Isoliquiritigenin induces apoptosis and autophagy and inhibits endometrial cancer growth in mice. *Oncotarget*. (2016) 7:73432–47. doi: 10.18632/oncotarget.12369
96. Fukuda T, Oda K, Wada-Hiraike O, Sone K, Inaba K, Ikeda Y, et al. Autophagy inhibition augments resveratrol-induced apoptosis in ishikawa endometrial cancer cells. *Oncol Lett*. (2016)12:2560–6. doi: 10.3892/ol.2016.4978
97. Chen S, Zhou N, Zhang Z, Li W, Zhu W. Resveratrol induces cell apoptosis in adipocytes via AMPK activation. *Biochem Biophys Res Commun*. (2015) 457:608–13. doi: 10.1016/j.bbrc.2015.01.034
98. Miyasaka A, Oda K, Ikeda Y, Wada Hiraike O, Kashiyama T, Enomoto A, et al. Anti tumor activity of olaparib, a poly (ADP ribose) polymerase (PARP) inhibitor, in cultured endometrial carcinoma cells. *BMC Cancer*. (2014) 14:179. doi: 10.1186/1471-2407-14-179
99. Oda K, Ikeda Y, Kawana K, Osuga Y, Fujii T. mTOR signaling in endometrial cancer: from a molecular and therapeutic point of view. *Curr Obstet Gynecol Rep*. (2015) 4:1–10. doi: 10.1007/s13669-014-0103-x
100. Skibola CF. The effect of Fucus vesiculosus, an edible brown seaweed, upon menstrual cycle length and hormonal status in three pre-menopausal women: a case report. *BMC Complement Altern Med*. (2004) 4:10. doi: 10.1186/1472-6882-4-10
101. Skibola CF, Curry JD, VandeVoort C, Conley A, Smith MT. Brown kelp modulates endocrine hormones in female Sprague–Dawley rats and in human luteinized granulosa cells. *J Nutr*. (2005) 135:296–300. doi: 10.1093/jn/135.2.296
102. Zhang J, Riby JE, Conde L, Grizzle WE, Cui X, Skibola CF. A *Fucus vesiculosus* extract inhibits estrogen receptor activation and induces cell death in female cancer cell lines. *BMC Complement Altern Med*. (2016) 16:151. doi: 10.1186/s12906-016-1129-6
103. Lirdprapamongkol K, Kramb JP, Chokchaichamnankit D, Srisomsap C, Surarit R, Sila-Asna M, et al. Juice of *Eclipta prostrata* inhibits cell migration *in vitro* and exhibits anti-angiogenic activity *in vivo*. *In Vivo*. (2008) 22: 363–8.
104. Chaudhary H, Dhuna V, Singh J, Kamboj SS, Seshadri S. Evaluation of hydroalcoholic extract of *Eclipta alba* for its anticancer potential: an *in vitro* study. *J Ethnopharmacol*. (2011)136:363–7. doi: 10.1016/j.jep.2011.04.066
105. Tewtrakul S, Subhadhirasakul S, Cheenpracha S, Karalai C. HIV-1 protease and HIV-1 integrase inhibitory substances from *Eclipta prostrata*. *Phytother Res*. (2007) 21:1092–5. doi: 10.1002/ptr.2252
106. Pithayanukul P, Laovachirasuwan S, Bavovada R, Pakmanee N, Suttisri R. Antivenom potential of butanolic extract of *Eclipta prostrata* against Malayan pit viper venom. *J Ethnopharmacol*. (2004) 90:347–52. doi: 10.1016/j.jep.2003.10.014
107. Chwalek M, Lalun N, Bobichon H, Ple K, Voutquenne-Nazabadioko L. Structure- activity relationships of some hederagenin diglycosides: haemolysis, cytotoxicity and apoptosis induction. *Biochim Biophys Acta*. (2006) 1760: 1418–27. doi: 10.1016/j.bbagen.2006.05.004
108. Cho YJ, Woo JH, Lee JS, Jang DS, Lee KT, Choi JH. Eclalbasaponin II induces autophagic and apoptotic cell death in human ovarian cancer cells. *J Pharmacol*. (2016)132:6–14. doi: 10.1016/j.jphs.2016.02.006

109. Patel S, Goyal A. Recent developments in mushrooms as anti-cancer therapeutics: a review. *3 Biotech*. (2012) 2:1–15. doi: 10.1007/s13205-011-0036-2
110. Lindequist U, Niedermeyer TH, Jülich WD. The pharmacological potential of mushrooms. *Evid Based Complement Alternat Med*. (2005) 2:285–99. doi: 10.1093/ecam/neh107
111. Lima CU, Cordova CO, Nóbrega Ode T, Funghetto SE, Karnikowski MG. Does the *Agaricus blazei* Murill mushroom have properties that affect the immune system? An integrative review. *J Med Food*. (2011) 14:2–8. doi: 10.1089/jmf.2010.0017
112. Zhao S, Ye G, Fu G, Cheng JX, Yang BB, Peng C. *Ganoderma lucidum* exerts anti-tumor effects on ovarian cancer cells and enhances their sensitivity to cisplatin. *Int J Oncol*. (2011) 38:1319–27. doi: 10.3892/ijo.2011.965
113. Jiang J, Slivova V, Harvey K, Valachovicova T, Sliva D. *Ganoderma lucidum* suppresses growth of breast cancer cells through the inhibition Akt/NF-κB signaling. *Nutr Cancer*. (2004) 49:209–16. doi: 10.1207/s15327914nc4902_13
114. Jiang J, Sliva D. Novel medicinal mushroom blend suppresses growth and invasiveness of human breast cancer cells. *Int J Oncol*. (2010) 37:1529–36. doi: 10.3892/ijo_00000806
115. Calviño E, Manjón JL, Sancho P, Tejedor MC, Herráez A, Diez JC. *Ganoderma lucidum* induced apoptosis in NB4 human leukemia cells: involvement of Akt and Erk. *J Ethnopharmacol*. (2010) 128:71–8. doi: 10.1016/j.jep.2009.12.027
116. Hahne JC, Meyer SR, Dietl J. et al. The effect of Cordyceps extract and a mixture of *Ganoderma lucidum*/Agaricus Blazi Murill extract on human endometrial cancer cell lines *in vitro*. *Int J Oncol*. (2014) 45:373–82. doi: 10.3892/ijo.2014.2414
117. Wang RC, Wei Y, An Z, Zou Z, Xiao G, Bhagat G, et al. Akt-mediated regulation of autophagy and tumorigenesis through Beclin 1 phosphorylation. *Science*. (2012) 338:956–9. doi: 10.1126/science.1225967
118. Hahne JC, Honig A, Meyer SR, Gambaryan S, Walter U, Wischhusen J, et al. Downregulation of AKT reverses platinum resistance of human ovarian cancers *in vitro*. *Oncol Rep*. (2012) 28:2023–8. doi: 10.3892/or.2012.2041
119. Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer*. (2006) 6:259–69. doi: 10.1038/nrc1840
120. Wang C, Liu B. miR-101-3p induces autophagy in endometrial carcinoma cells by targeting EZH2. *Arch Gynecol Obstet*. (2018) 297:1539–48. doi: 10.1007/s00404-018-4768-7
121. Ran X, Yang J, Liu C, Zhou P, Xiao L, Zhang K. MiR-218 inhibits HMGB1 mediated autophagy in endometrial carcinoma cells during chemotherapy. *Int J Clin Exp Pathol*. (2015) 8:6617–26.
122. Zhuo Z, Yu H. miR-205 inhibits cell growth by targeting AKT-mTOR signaling in progesterone-resistant endometrial cancer Ishikawa cells. *Oncotarget*. (2017) 8:28042–51. doi: 10.18632/oncotarget.15886
123. Sun MY, Zhu JY, Zhang CY, Zhang M, Song YN, Rahman K, et al. Autophagy regulated by lncRNA HOTAIR contributes to the cisplatin-induced resistance in endometrial cancer cells. *Biotechnol Lett*. (2017) 39:1477–84. doi: 10.1007/s10529-017-2392-4

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The Phytosterol Peniocerol Inhibits Cell Proliferation and Tumor Growth in a Colon Cancer Xenograft Model

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Objective: This study aimed to evaluate the cytotoxic activity of peniocerol against human colon cancer cell lines and its antitumor effect *in vivo* in a xenograft model using *nu/nu* mice.

Materials and Methods: SW-620, HCT-15, and HCT-116 colon cancer cell lines were treated with peniocerol for cytotoxicity by crystal violet technique. Cell apoptosis induction was detected by flow cytometry, and the antitumor activity of peniocerol was evaluated in a xenograft model of HCT-116 in *nu/nu* mice. After treatment, the effect of peniocerol was analyzed in histological sections of tumors by immunohistochemistry using DAPI, anti-PCNA, and PARP-1 antibodies.

Results: Peniocerol inhibited cell growth and induced apoptosis *in vitro* in a time and dose-dependent manner. Besides, peniocerol administration (30 or 15 mg/kg) inhibited tumor growth and induced apoptosis in the xenograft mice. The lack of peniocerol toxicity was proved by a biochemical blood analysis of healthy *nu/nu* mice administrated with this sterol.

Conclusions: Our results proved that peniocerol induces apoptosis *in vitro* and *in vivo* assays.

Keywords: phytosterol, cytotoxicity, antiproliferative, antitumor, xenograft, apoptosis, colon cancer

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INTRODUCTION

Colorectal cancer (CC) is second cancer with the highest mortality rate worldwide, responsible for more than 880 thousand deaths, and is the third most common cancer, with almost 2 million incidents in both sexes and all ages (1). Although several chemotherapeutic options are available, 5-fluorouracil (5-FU) is still the base drug for the treatment of colorectal cancer in combination with other anticancer agents. For instance, the combination of oxaliplatin, 5-FU, and leucovorin is used after tumor surgery for patients undergoing treatment with curative intent for stage III (2). However, the drug with the desirable activity and adequate toxicity has not been developed yet. Therefore, searching for better antitumor drugs is not concluded. In this sense, the so-called natural

products are a rich source of bioactive compounds that could be considered as prototypes against this cancer.

Phytosterols are plant sterols, structural components of the cell membrane that participate in the regulation of fluidity and permeability associated with the membrane (3). They are byproducts of a complex isoprenoid biosynthesis pathway through the squalene (4). Phytosterols belong to a family of more than 200 different compounds. The most common are β-sitosterol, campesterol, and stigmasterol (3). They have demonstrated protection against various chronic diseases such as cardiovascular, liver, diabetes, and different types of cancer (5–9). Cancer prevention studies show that a diet rich in phytosterols can reduce the risk of different types of cancer (10). For example, the intake of β-sitosterol, an anti-inflammatory agent (11), can prevent colon cancer (12, 13). In this context, we have previously published the anti-inflammatory activity *in vivo* and the cytotoxic activity *in vitro* against human breast and colon cancer cells of the peniocerol, a sterol isolated from the cactus *Myrtillocactus geometrizans* (Mart Ex Pfeiff) Console (14, 15). In this article, we show the apoptotic activity of the peniocerol both *in vitro* against the colon cancer cell line HCT-116, as well as its antitumor effect *in vivo*.

MATERIALS AND METHODS

Extraction and Isolation

Peniocerol (3β, 6α-diol-cholest-8-ene) was isolated from *Myrtillocactus geometrizans* and purified as previously described (15). Copies of the original spectra are obtainable from the author.

Cell Lines and Cell Culture

Colon carcinoma cells SW-620, HCT-15, and HCT-116 were obtained from the American Type Culture Collection (ATCC). Cells were cultured in proper media (DMEM, DMPQ8-1L) as previously reported.

Animals

Male *nu/nu* mice, 6–8 weeks old were used. The animals were housed, fed, and maintained following the recommendations of the ethics committee. The Animal Research Committee approved the experimental procedures and were carried out in accordance to the Guidelines for the Care and Use of Animals of the Bioterium Laboratory of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ), Mexico City, Mexico.

Solutions

Working solutions of peniocerol (25 mg/mL) were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA. Cat. D4540–100 mL) and stored at −20°C. The peniocerol dilutions (80, 40, 20, and 10 μM) were prepared with DMEM and the DMSO concentration was <0.2%. Cisplatin (Sigma-Aldrich, St. Louis, MO, USA. Cat. 479306-1G), was included as a positive control (1 mg/kg), dissolved in DMEM. For *in vivo* experiments, 25 mg/mL concentrated peniocerol solutions were prepared, dissolved in sesame oil and 5% DMSO and cisplatin 10 mg/kg in saline solution. All solutions were prepared on the day of administration.

Cytotoxicity

The cells HCT-15, HCT-116, and SW-620 were seeded in 48-well-plates at a density of 4×10^4 cells per cm^2 in DMEM plus 10% FBS. The cells were incubated for 24 h in an atmosphere of 5% CO_2 and 95% humidity at 37°C. After 24 h the cells were treated with serial concentrations of peniocerol (80, 40, 20, and 10 μM). Cell viability was evaluated at 24, 48, and 72 h. Cisplatin was used as a positive control at concentrations <10 μM. Medium plus DMSO was included as a negative control. After incubation, the cells were fixed with DMEM, 2% FBS, and 1.1% glutaraldehyde for 15 min at room temperature. Subsequently, the fixation medium was removed from the cells, allowed to dry, stained with 200 μL of violet crystal for 15 min, the violet crystal was removed and finally, the stained protein was solubilized with 500 μL of 10% acetic acid. Optical density values were determined at a wavelength of 595 nm. A dose-response curve was plotted for each compound and the IC_{50} was estimated using the Excel statistical program using linear regression. The tests were carried out in quadruplicate in three independent experiments.

Flow Cytometric Detection of Apoptotic Cells

Apoptotic cell death was determined using flow cytometry with the identification of Annexin V and propidium iodide markers (GTX85591, GeneTex). HCT-116 colon cancer cells were seeded in 6-well-plates with a density of 1.5×10^5 cells per well. The cells were incubated for 24 h in an atmosphere of 5% CO_2 and 95% humidity at 37°C. After 24 h the cells were treated with vehicle, peniocerol (20 μM) and camptothecin (2 μM) as a positive control (each treatment was done in triplicate), at different times (24, 48, and 72 h). After incubation times, the cells were harvested with trypsin, washed with PBS and centrifuged at 1,500 rpm for 5 min. The cell aggregate was resuspended in 500 μL of binding buffer, then 5 μL of Annexin V-FITC, 5 μL of propidium iodide were added and finally incubated for 5 min, according to the manufacturer's instructions. The cells were analyzed using a FACS Can flow cytometer from the National Flow Cytometry Laboratory, 10,000 cells were analyzed with the BD Cell Quest Pro Software program.

Toxicity Assessment in *nu/nu* Mice

Lethal dose 50 (LD_{50}) determination was performed using two groups of three female mice each. One group was treated with 125 mg/kg and the second with 62.5 mg/kg of peniocerol. Food and water were administered up to 4 h after treatment. Mortality was observed during the first 4 h. The LD_{50} was determined by the formula $LD_{50} = (M0 + M1)/2$ where M0 is the dose that does not cause the death of any mouse in the group and M1 is the dose that causes the death of at least one mouse in the group (16). The determination of the doses that did not induce toxic effects was carried out using female *nu/nu* mice distributed in groups of three mice. Peniocerol was administered intraperitoneally (i.p.) in two treatment schemes, once a week, and three times a week both for 21 days (**Figure 3A**). The weight and behavior of the

mice were monitored every third day during treatment. Mice were sacrificed on day twenty-one. Blood tests were performed in the Departamento de Patología de la Facultad de Medicina Veterinaria y Zootecnia, UNAM, Mexico City, México.

Antitumor Activity Evaluation in Xenografted Mice

Male *nu/nu* mice were distributed in seven groups of 6 mice each. The animals were xenografted with 1.5×10^6 HCT-116 cells resuspended in 100 μL of PBS and inoculated via subcutaneous in the right flank from the back of the animal. The treatments were administered (i.p.) in two schemes, once a week or three times a week, both for 21 days. The treatments started when the tumors reached a volume of 50 mm^3. The groups were organized as follows:

1. Negative control: 5% DMSO dissolved in sesame oil, once a week.
2. Negative control: 5% DMSO dissolved in sesame oil, three times a week.
3. Positive control: cisplatin 4 mg/kg once a week.
4. Positive control: cisplatin 2 mg/kg, three times a week.
5. Peniocerol: 30 mg/kg once a week.
6. Peniocerol: 15 mg/kg once a week.
7. Peniocerol: 15 mg/kg three times per week.

The weight of the mice and the tumor growth were measured three times a week. Tumor volume was calculated using the formula $V = \pi/6 \times (\text{larger diameter} \times [\text{smaller diameter}]^2)$ (17). The experiment was carried out for 21 days, at the end of the experiment, the animals were weighed, euthanized and the tumors were extracted, fixed in formalin, and embedded in paraffin.

Histologic Evaluation of Tumors

Paraffin-embedded tumors were cut in histologic sections of 5 μm thick and used for subsequent analysis with hematoxylin-eosin, DAPI, and immunohistochemistry. The samples were analyzed in an Olympus IX71 microscope, with the QImaging program at the Microscopy Unit of the Biomedical Research Institute, UNAM, Mexico.

Hematoxylin-Eosin Staining (H-E)

Tissue slides were exposed at a temperature of 60°C for 15–30 min and rehydrated with the following solution for 5 min: Xylol (twice), ethanol/xylol 50:50, 100% ethanol, 96% ethanol, 80% ethanol, 70% ethanol, 50% ethanol, distilled water, and PBS. The cell membrane was subsequently permeabilized with a 0.5% Triton solution x-100 for 30 min, followed by two washes with PBS of 5 min each. One hundred fifty microliter of hematoxylin was added to the sample for 2 min, washed with distilled water, and covered with eosin for 20 s, washed with distilled water and assembled.

4′,6-Diamidino-2-Phenylindole (DAPI) Immunofluorescence

The dewaxing and rehydrated of tissues were performed as previously described for H-E staining. Then, the membrane was permeabilized with a 0.5% Triton solution x-100 for 30 min, followed by two washes with PBS of 5 min each. The kit used was Vectashield mounting medium for fluorescence with DAPI from Vector Laboratories.

Nuclear Antigen of Proliferation Cells (PCNA) Determination

The dewaxing and rehydrated of tissues were performed as previously described for H-E staining. The membrane was permeabilized with a 0.5% Triton solution x-100 for 30 min, followed by two washes with PBS of 5 min each. Antigen exposure was carried out heating the samples in a 0.25 mM sodium citrate solution at pH 6.2 for 20 min in a microwave oven. Then, samples were left at room temperature for 20 min and slides were washed twice with PBS for 5 min. Endogenous peroxidase was inhibited by exposing the tissues to 3% H_2O_2 for 30 min, followed by 2 washes with PBS for 5 min each. The cell membrane permeabilization was performed with a 0.5% Triton solution x-100 for 30 min, followed by two washes with PBS of 5 min each. Subsequently, the non-specific signal was blocked by incubation with 1% H_2O_2 + 5% albumin, in PBS for 30 min, followed by one wash with PBS of 5 min. The slides were incubated with the primary anti-PCNA antibody (sc-25280, Santa Cruz Biotechnology, INC). Incubation was performed overnight at 4°C, followed by two washes with PBS for 5 min. The slides were incubated with the secondary antibody for 60 min at 37°C (anti-mouse IgG for PCNA, GTX77315, GeneTex). The detection of immunohistochemical signals was performed with diaminobenzidine (DAB) for 10 min, the excess was removed with distilled water. Couterstain: Harris hematoxylin staining was performed for 3–5 min on each tissue, then the excess was removed with distilled water. It was covered with Li_2CO_3 for 15 s; then the excess was removed with distilled water. Once the samples dried completely, the assembly was carried out with EcoMount from Biocare Medical and covered with a coverslip. Qualitative analysis of PCNA positive cells was carried out using the ImageJ-win64 image processing package.

Poly(ADP-Ribose) Polymerase (PARP-1) Determination

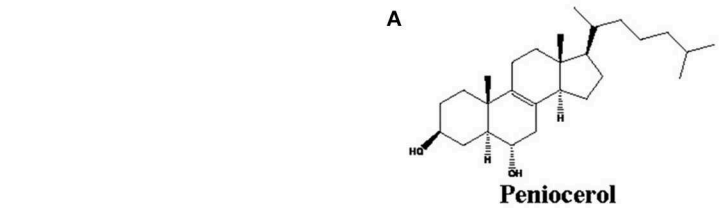
The dewaxing and rehydrated of tissues were performed as previously described for H-E staining. The membrane was permeabilized with a 0.5% Triton solution x-100 for 30 min, followed by two washes with PBS of 5 min each. Antigen exposure was carried out, heating the samples in a 0.25 mM sodium citrate solution at pH 6.2 for 20 min in a microwave oven. Then, the samples were left at room temperature for 20 min, and slides were washed twice with PBS for 5 min. Endogenous peroxidase was inhibited by exposing the tissues to 3% H_2O_2 for 30 min, followed by two washes with PBS for 5 min each.

The cell membrane permeabilization was performed with a 0.5% Triton solution x-100 for 30 min, followed by two washes with PBS of 5 min each. Subsequently, the non-specific signal was blocked by incubation with 1% H_2O_2 + 5% albumin in PBS for 30 min, followed by one wash with PBS of 5 min. The slides were incubated with the primary anti-PARP-1 antibody (sc-8007, Santa Cruz Biotechnology, INC). Incubation was performed

overnight at 4°C, followed by two washes with PBS for 5 min. The slides were incubated with the secondary antibody for 60 min at 37°C (anti-mouse IgG for PARP-1, GTX77315, GeneTex). The detection of immunohistochemical signals was performed with diaminobenzidine (DAB) for 10 min. The excess was removed with distilled water. Couterstain: Harris hematoxylin staining was performed for 3–5 min on each tissue, then the excess was removed with distilled water. It was covered with Li_2CO_3 for 15 s; then, the excess was removed with distilled water. Once the samples dried completely, the assembly was carried out with EcoMount from Biocare Medical and covered with a coverslip. Qualitative analysis of PARP-1 positive and claved-PARP-1 cells was carried out using the ImageJ-win64 image processing package.

Data Analysis

Each experiment was performed in triplicate. Data are presented as mean ± standard deviation (SD) of three independent experiments. Statistical differences were determined using the GraphPad Prism 6.0 software program (GraphPad Software Inc., La Jolla, CA). Comparisons between the treated and control groups were made in the *t*-test of unpaired data and the two-way ANOVA. All comparisons are made concerning untreated controls. A statistical difference in $^*P < 0.05$ was considered.



IC_{50} (μM) at 24 hours		
Cell line	Treatment	
	Peniocerol	Cisplatin
HCT-15	48.89±0.95	12.14±0.5
HCT-116	39.14±0.1	2.87±0.3
SW-620	47.19±0.31	13.58±0.4

IC_{50} (μM) at 48 hours		
Cell line	Treatment	
	Peniocerol	Cisplatin
HCT-15	34.72±0.4	8.54±0.3
HCT-116	18.68±0.35	0.87±0.1
SW-620	23.10±0.18	10.14±0.9

IC_{50} (μM) at 72 hours		
Cell line	Treatment	
	Peniocerol	Cisplatin
HCT-15	23.57±0.19	3.46±0.8
HCT-116	13.20±0.0.04	0.34±0.05
SW-620	19.59±0.1	4.32±0.5

FIGURE 1 | Cytotoxic activity of peniocerol in the colon cancer cell lines SW-620, HCT-116, and HCT-15. **(A)** Chemical structure of peniocerol. **(B)** Cell viability graphs represented in percentage with respect to the ascending results of peniocerol at different times, with their respective tables showing the average IC_{50} of each cell line, peniocerol and cisplatin as a positive control. The bars in the graphs represent the standard deviation of three independent experiments per quadruplicate and in the tables the averages ± SD of three independent experiments per quadruplicate are represented.

RESULTS

Cytotoxicity of Peniocerol

The chemical structure of peniocerol is shown in **Figure 1A**. The cytotoxic activity of peniocerol was assessed using the crystal violet cell staining technique. The HCT-116 cells were the most susceptible to peniocerol (**Figure 1B**), in comparison to the other two cell lines. Therefore, the HCT-116 line was used for subsequent experiments.

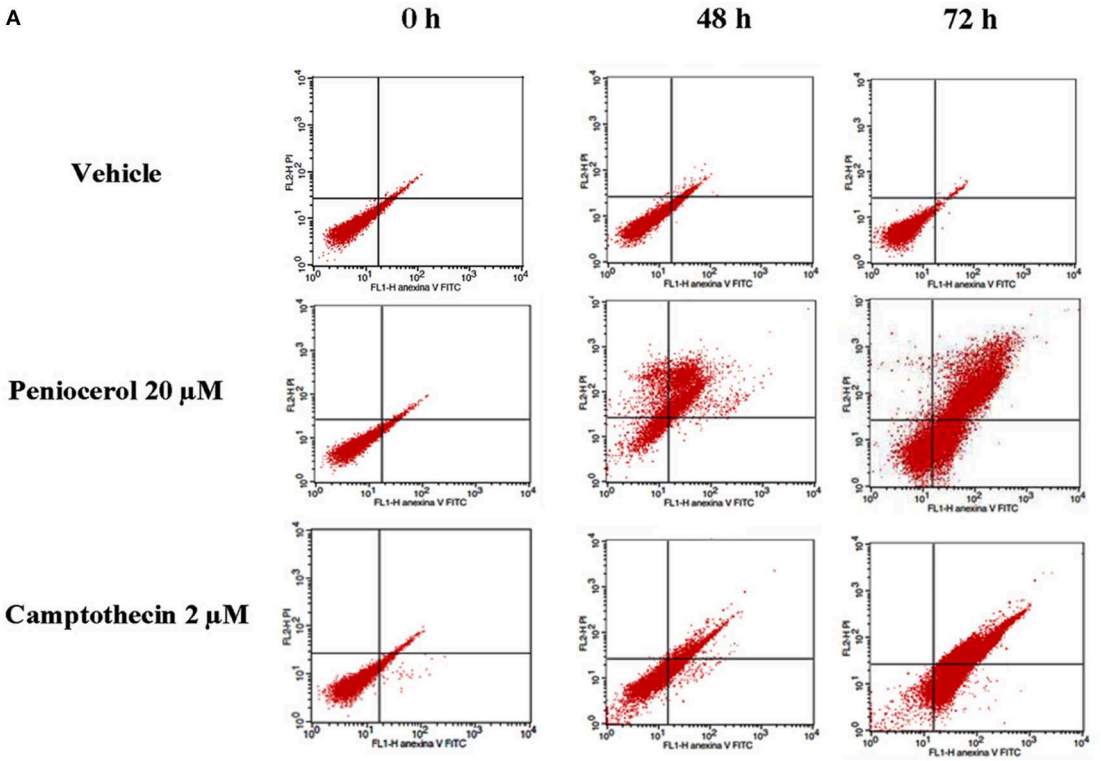
Detection of Apoptosis

HCT116 cells treated with peniocerol experimented apoptosis in a time-dependent manner. As shown in **Figure 2A**, cell death was observed after 48 h of treatment and increased after 72 h, 67.21 ± 0.24, and 72.32 ± 0.04%, respectively. The level of apoptosis induced by peniocerol was similar to that produced by camptothecin (**Figure 2B**). These results are totally in agreement with those previously obtained where it was proved, through cleavage of PARP-1 and flow cytometry, that this sterol induced apoptosis in several human cancer lines (14, 18).

Toxicity of Peniocerol of Peniocerol Was Evaluated in Female *nu/nu* Mice, Using Two Different Schemes of Treatment

We first evaluated the effect of peniocerol administrated once a week during 21 days at 15 or 30 mg/kg doses. Our results showed that no significant modification in body weight were noticed compare with those of animal control. As shown in **Figure 3B**, when mice received peniocerol three times a week at different doses, also no significant differences in weight were detected. In contrast, the administration of cisplatin one time a week produced a significant decrease in body weight compared with the negative control ($p < 0.0001$). The weight of mice treated with cisplatin was reduced by 42%, which suggests a toxic effect of the positive control. Administration of 15 mg/kg of peniocerol

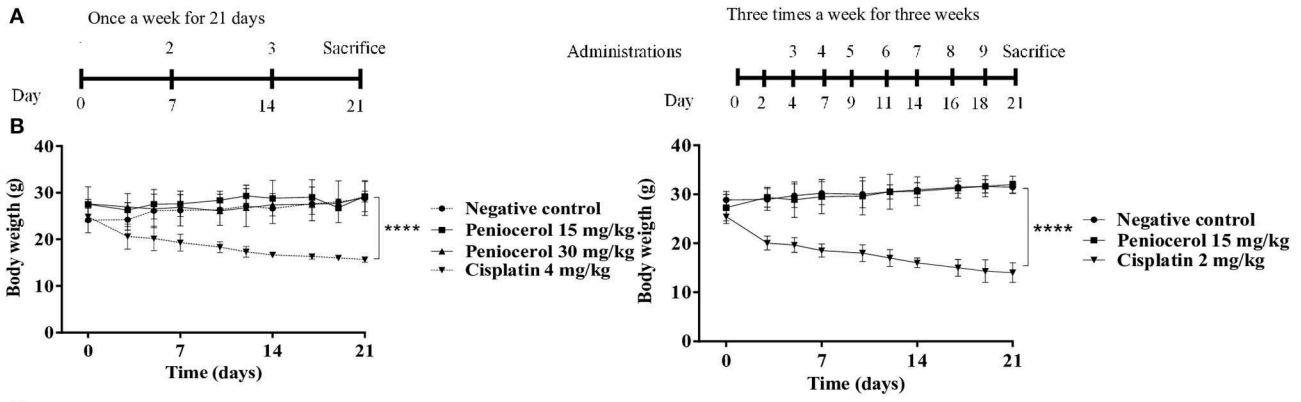
A



B

Apoptotic cells (%)				
Treatment	**0 h**	**24 h**	**48 h**	**72 h**
Vehicle	8.57±0.1%	9.44±0.1%	12.72±0.02%	2.71±0.02%
Peniocerol 20µM	8.67±0.3%	26.2±0.37%	67.21±0.24%	72.32±0.04%
Camptothecin 2 µM	8.53±0.2%	32.74±0.1%	47.33±0.2%	95.53±0.31%

FIGURE 2 | *In vitro* evaluation of apoptosis induction by peniocerol in the HCT-116 cell line. **(A)** Representative dot plots of HCT-116 cells treated with vehicle, peniocerol 20 µM and camptothecin 2 µM. **(B)** Table of percentages of early and late apoptosis induced by peniocerol. The numbers represent the average of three independent experiments ± SD.



C

Systemic analysis of peniocerol in *nu/nu* mice							
Biochemical analysis	Reference [13]	Negative control	Cisplatin		Peniocerol		
			4 mg/kg	2 mg/kg 3 times/week	15 mg/kg	30 mg/kg	15 mg/kg 3 times/week
Leukocytes ($x10^9$/L)	3.2 - 7.0	4.57 ± 0.5	2.06 ± 0.12*	1.61 ± 0.23*	3.77 ± 0.25	3.6 ± 0.56	3.23 ± 0.23
Lymphocytes ($x10^9$/L)	3.16 - 7.8	3.17 ± 0.65	0.52 ± 0.04*	0.52 ± 0.08*	3.58 ± 0.39	3.37 ± 0.25	3.57 ± 0.21
Erythrocytes ($x10^{12}$/L)	7.1 - 10.2	7.17 ± 1.04	7.37 ± 0.46	7.14 ± 0.26	7.43 ± 0.45	7.23 ± 0.85	7.63 ± 0.32
Hemoglobin (g/L)	149 -170	152.33 ± 10.5	115.33 ± 1.53	113.33 ± 1.53	155 ± 13	151 ± 14.73	153 ± 8.54
Glucose (mmol/L)	6.6 - 8.5	7.6 ± 1.39	8.56 ± 0.15	7.83 ± 0.45	7.93 ± 0.40	8 ± 0.8	8.17 ± 0.49
Urea (mmol/L)	2.6 - 3.5	3.07 ± 0.67	7.57 ± 0.4	9.06 ± 0.45	2.53 ± 0.25	2.83 ± 0.61	3.33 ± 0.15
Creatinine (mmol/L)	8.8 - 26.5	24.33 ± 2.08	29.06 ± 1.9	43.33 ± 0.68*	23.33 ± 2.52	24.33 ± 2.08	25 ± 3
Alanine transferase (U/L)	46 - 55	52.33 ± 2.52	62.89 ± 1.21*	75.06 ± 1.68*	49 ± 4.58	48.33 ± 3.21	51.67 ± 0.58
Aspartate transferase (U/L)	85 - 101	92.33 ± 2.52	151.25 ± 1.35	197.32 ± 1.18*	96 ± 2	94.67 ± 2.51	93.67 ± 1.53

FIGURE 3 | Toxicity evaluation of peniocerol in *nu/nu* mice. **(A)** Treatment scheme: Once a week for 3 weeks, the administrations were performed on days 0, 7, and 14; Three times a week for 3 weeks, administrations were performed, on days 0, 2, 4, 7, 9, 11, 14, 16, and 18. In both schemes, the animals were slaughtered on day 21. **(B)** Toxicity graphs: Once a week for 3 weeks, doses 30 mg/kg and 15 mg/kg of peniocerol, 4 mg/kg of cisplatin and as a negative control (sesame oil + DMSO 5%); three times a week for 3 weeks, doses 30 mg/kg and 15 mg/kg of peniocerol, 2 mg/kg of cisplatin and as a negative control the vehicle (sesame oil + 5% DMSO). The weight of the mice was monitored three times a week for 21 days. The results shown are the mean ± standard deviation of the monitoring of three mice. The significant difference $^{****}p < 0.0001$ was compared to the negative control (*t*-test). **(C)** Table of the systemic analysis of peniocerol in mice administered once a week and three times a week for 21 days. The data shown are the mean values ± SD of three mice per group. Significant difference $^{*}p < 0.05$ compared to the vehicle (*t*-test).

three times a week for 21 days did not produce a change in the weight of the animals as compared with the negative control. On the contrary, cisplatin administered in the lower dose (2 mg/kg) three times a week induced a significant decrease in body weight compared with the negative control ($p < 0.0001$).

To further analyze the potential toxic effect of peniocerol in healthy mice, we performed a biochemical blood analysis including glucose, hemoglobin, urea, creatinine, alanine transferase, and aspartate transferase, and a cell count including leukocytes, lymphocytes and erythrocytes (**Figure 3C**). The analysis revealed that peniocerol did not produce any change in blood biochemical and cellular components compared with those observed in mice that received the vehicle. In sharp contrast, administration of cisplatin resulted in a significant decrease in leukocytes, lymphocytes, and hemoglobin ($p < 0.05$) and an increase in the concentrations of liver enzymes alanine transferase and aspartate transferase ($p < 0.05$). These results suggest that peniocerol (15 mg/kg and 30 mg/kg) is less toxic than cisplatin, and that healthy mice tolerate it well.

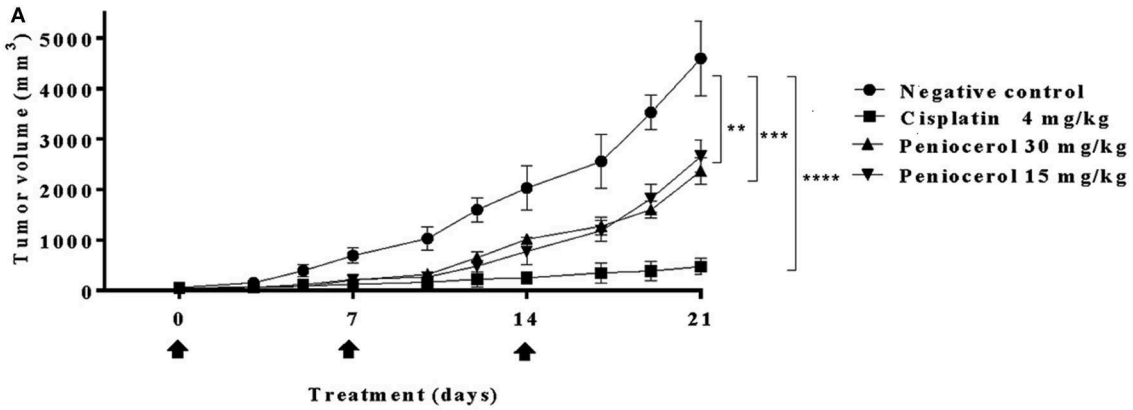
Anti-tumor Activity of Peniocerol

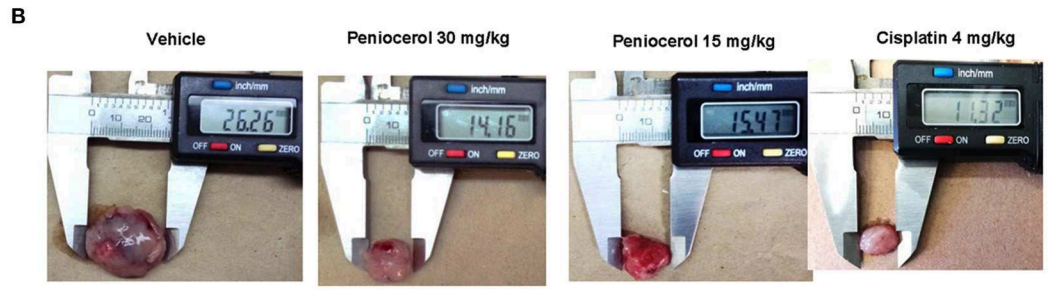
To test the potential effect of peniocerol on tumor growth *in vivo*, we established a xenograft model in mice using the HCT-116 cell line. When the tumors reached a mean of 50 mm^3 mice were treated with peniocerol (15 or 30 mg/kg doses) one time a week for 21 days, cisplatin (4 mg/kg) also one time a week for 21 days. As seen in **Figure 4A**, both concentrations of the peniocerol induced a significant reduction of tumor volume at the end of the experiment ($p < 0.01$). As expected, treatment with cisplatin-induced a highly significant reduction of tumor volume ($p < 0.0001$). At the finale of the experiment the remaining tumor masses were dissected and weighed, the results presented in **Figures 4B,C** demonstrated that peniocerol induced a reduction of tumor weight of 75.2 and 76.4% when administered at 30 or 15 mg/kg, respectively.

Our results suggest that peniocerol has a significant anti-tumor effect on mice, so we decided to investigate whether an increase of frequency of administration of the lower doses evaluated of peniocerol would produce a major effect. Administration of peniocerol at 15 mg/kg doses three times a week for 21 days induced a significant decrement of tumor volume compared to that produced by the previously administration ($p < 0.05$; **Figure 5A**). However, it did not reach the level of inhibition produced by treatment with cisplatin (**Figures 5B,C**).

Immunohistological Analysis of Tumors

Tissue slides were obtained from tumors dissected after treatment. H-E showed a precise modification of cell morphology that can be observed by their amorphous and condensed shape in





C

Tumor weight	
Treatment	**g±SD**
Negative control	4.35±0.37
Peniocerol 30 mg/kg	1.08±0.24****
Peniocerol 15 mg/kg	1.03±0.26****
Cisplatin 2 mg/kg	0.24±0.11****

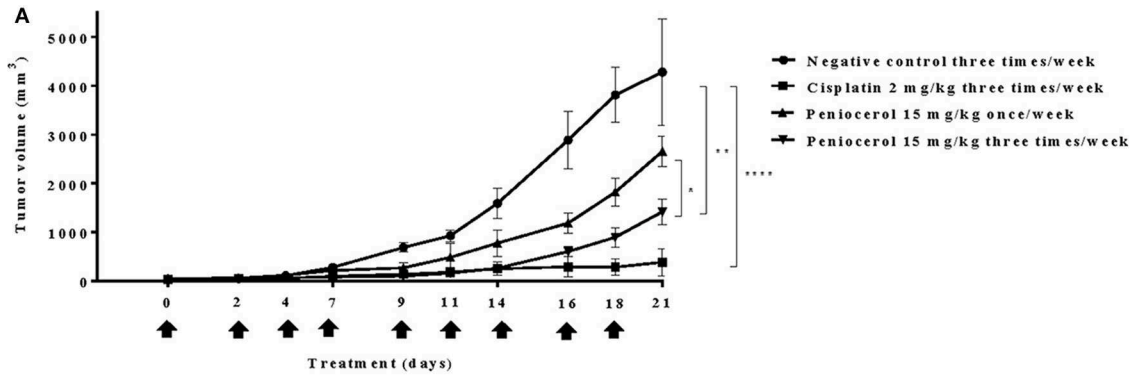
FIGURE 4 | Antitumor activity evaluation of peniocerol once a week for 21 days administration in *nu/nu* mice. Treatment scheme: Once a week for 21 days, treatment started when the tumors reached a volume of approximate 50 mm^3, on days 0, 7, and 14. The animals were sacrificed on day 21. **(A)** Antitumor activity graphs. Groups of six *nu/nu* mice inoculated with 1.5 × 10^6 HCT-116 cells were treated once a week for 21 days with 30 mg/kg and 15 mg/kg of peniocerol, 4 mg/kg of cisplatin and the vehicle (sesame oil + 5% DMSO), on days 0, 7, and 14 (black arrows). The tumors were measured three times per week. The bars indicate the SD of the mean $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ compared to the vehicle (ANOVA and *t*-test). **(B)** Photographs of the tumors at the end of the experiment. **(C)** Table of tumor weights ± SD at the end of the experiment.

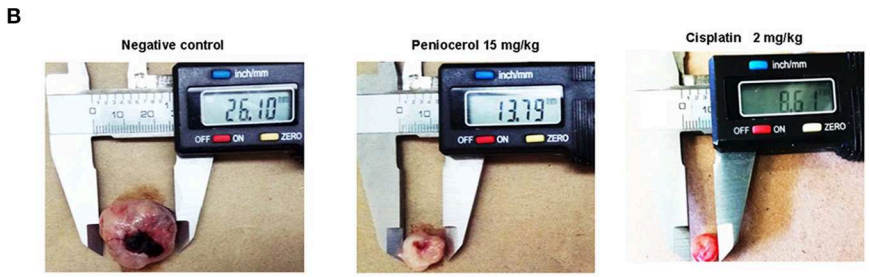
the tumor samples that were treated with peniocerol (30 and 15 mg/kg, once a week and 15 mg/kg three times a week), compared to the positive control (**Figure 6A**).

Furthermore, the nuclei tissues were stained with DAPI. As shown in **Figure 6B**, nuclei with condensation or fragmentation, indicative of apoptosis, can be seen in peniocerol treatments.

To determine if peniocerol was able to inhibit cell proliferation, tumor slides were incubated with an anti-PCNA. Results of specific immunostaining indicate that both doses 30 and 15 mg/kg once a week and 15 mg/kg three times a week of peniocerol, decreased the number of positive cells compared to the negative control (**Figure 6C**). The results show that peniocerol significantly inhibited ($p < 0.0001$) the expression of PCNA at all three doses compared to the positive control. In the doses of 30 mg/kg and 15 mg/kg administered once a week, 150.07 ± 17.14 and 143.1 ± 34.7 labeled cells were detected respectively; while the dose of 15 mg/kg administered three times a week, 95.57 ± 18.85 cells were marked, compared with the negative control that was 328.3 ± 64.07 labeled cells (**Figure 7A**).

The induction of apoptosis in histological sections was determined with the specific immune staining of anti-PARP-1 (**Figure 6D**). The results show that independently of the peniocerol doses, there is a significant decrease ($p < 0.0001$) in the expression of PARP-1 in the nucleus, compared to the vehicle (78.34 ± 3.32%). On the contrary, in the expression of cleaved PARP-1, the doses of 30 mg/kg (54.96 ± 2.28%) and 15 mg/kg (59.96 ± 9.09%) once a week, and 15 mg/kg three times a week (61.7 ± 1.56%), significantly increase the percentage compared to the vehicle (5.64 ± 2.35%). Peniocerol treatments effected similarly to that of cisplatin (**Figure 7B**).





C

Tumor weight	
Treatment	**g±SD**
Negative control	4.34±0.48
Peniocerol 15 mg/kg	0.78±0.12****
Cisplatin 2 mg/kg	0.23±0.10****

FIGURE 5 | Antitumor activity evaluation of peniocerol three times a week for 21 day administration in *nu/nu* mice. Treatment scheme: Three times a week for 21 days, a total of nine administrations were performed, when the tumor reached an approximate volume of 50 mm^3, on days 0, 2, 4, 7, 9, 11, 14, 16, and 18. The animals were sacrificed on day 21. **(A)** Antitumor activity graph. Groups of six *nu/nu* mice inoculated with 1.5×10^6 HCT-116 cells were treated three times a week for 21 days, the doses tested were 30 mg/kg and 15 mg/kg of peniocerol, 2 mg/kg of cisplatin and as a negative control the vehicle was used (sesame oil + 5% DMSO). The size of the tumors was measured three times per week. The bars indicate the standard deviation of the mean $^*p < 0.01$, $^{**}p < 0.001$, and $^{****}p < 0.0001$ compared to the vehicle (ANOVA and *t*-test). **(B)** Photographs of the tumors at the end of the experiment. **(C)** Table of tumor weights ± SD at the end of the experiment.

DISCUSSION

The National Cancer Institute (NCI, USA), has regulated the natural products antineoplastic activity to select potential compounds. Although it is not a formal rule, they are considered active when $ED_{50} \leq 4\,\mu g/ml$ (19, 20). Under this premise, many compounds of natural origin, including phytosterols, would be considered inactive. However, there seems to be a probable relationship between the anti-inflammatory properties of some secondary metabolites such as the phytosterols, and their antitumor effects *in vivo* (21, 22). Our research group published the exceptional anti-inflammatory activity of peniocerol and its modest cytotoxic activity against the HCT-15 and the MCF-7 cancer cell lines *in vitro* (14). Consequently, we evaluated the antitumor activity of peniocerol in xenografted mice.

Although peniocerol showed cytotoxic activity on the colon cancer lines, HCT-15, HCT-116, and SW-620, the HCT-116 line was the most susceptible, so it was chosen for the next experiments. The level of apoptosis induced by peniocerol was similar to that produced by camptothecin *in vitro* (**Figure 2B**). In earlier studies, we proved that peniocerol might trigger both the caspase-dependent and caspase-independent apoptotic pathways (18).

To determine the toxicity of peniocerol female *nu/nu* mice were used. Our results showed that at 15 or 30 mg/kg doses of peniocerol under three or once a week administration, there was no significant modification in body weight compared with the untreated mice. It is worth to note that the cis-platin group showed until 42% of body weight loss (**Figures 3A,B**).

Biochemical blood analysis was performed to evaluate the potential effect of peniocerol in healthy animals. The analysis revealed that peniocerol did not produce any change in blood biochemical and cellular components compared with those observed in mice that received the vehicle. This analysis also reaffirms the toxicity so substantial that cisplatin induces in mice. For example, the administration of cisplatin resulted in a significant increase of alanine- and aspartate transferase as well as the decrease of hemoglobin, lymphocytes, and leukocytes. These

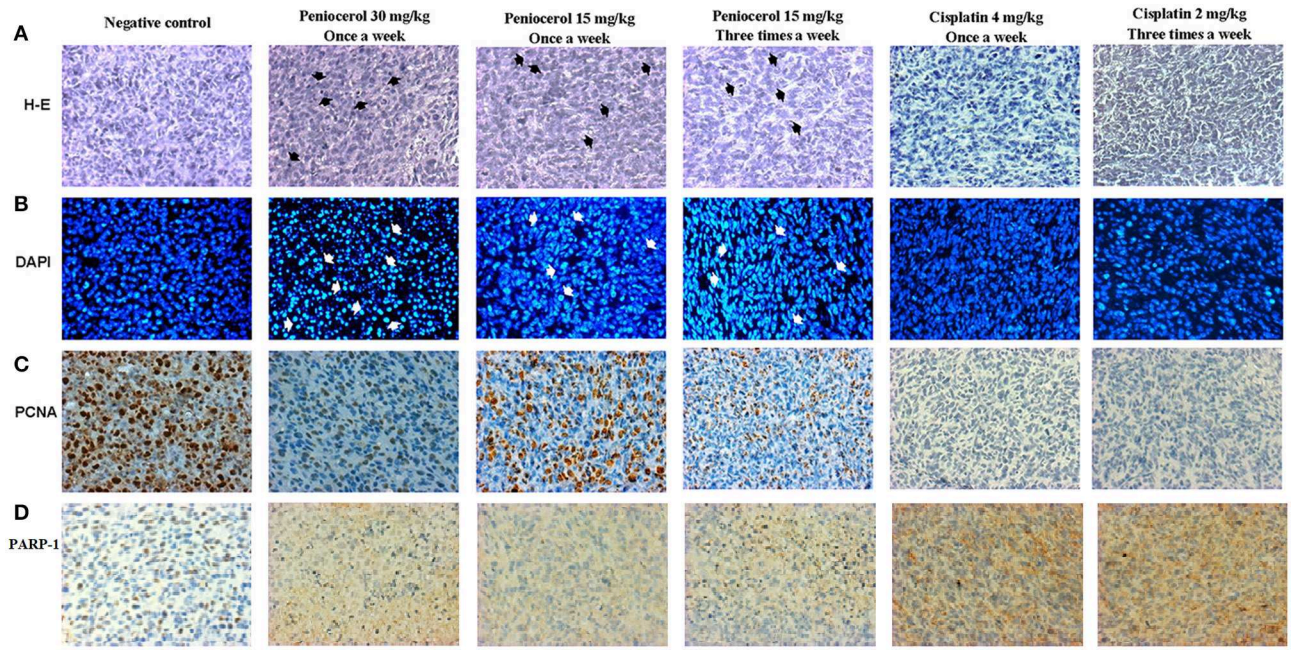


FIGURE 6 | Tissues photomicrographs obtained from xenografted tumors with HCT-116 cells stained with; **(A)** hematoxylin-eosin (H-E) (Black arrows indicate amorphous and condensed shape). **(B)** 4′,6-diamidino-2-phenylindole (DAPI) (White arrows indicate condensation and fragmentation of the nuclei), **(C)** specific immunostaining for proliferating cell nuclear antigen (PCNA). The brown marks show the PCNA positive cells. **(D)** Specific immunostaining for poly(ADP-ribose) polymerase 1 (PARP-1) (brown nucleus) and Cleaved-PARP-1 (brown cytoplasm). The text at the top indicates the treatment for each experimental group. The acquisition of the images was done at 20x on the microscopic scale in the Q capture pro 5 QImaging capture software.

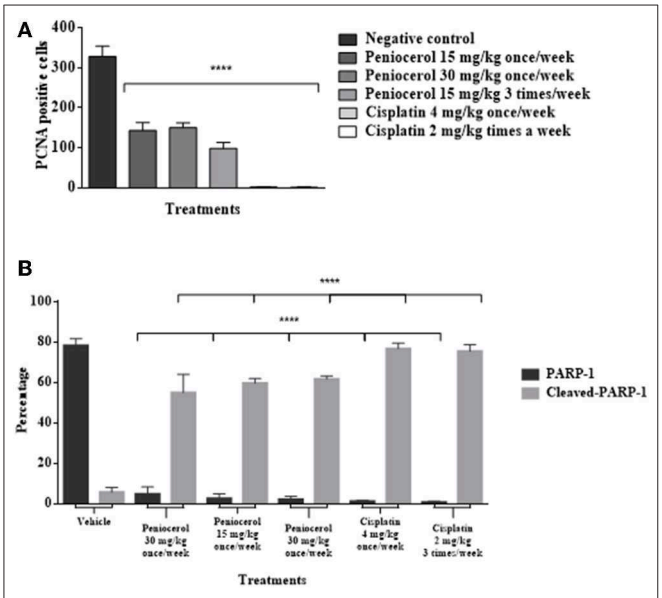


FIGURE 7 | (A) Antiproliferative effect of peniocerol on the PCNA cell proliferation marker in the tissues of xenografted tumors of HCT-116 cells. **(B)** Apoptotic cell death effect of peniocerol on the PARP-1 and Claved-PARP-1 marker in the tissues of xenografted tumors of HCT-116 cells. The bars represent the average of positive cells ± standard deviation of three tissues analyzed by each experimental group. The data were analyzed and compared against the negative control ****$p < 0.0001$ (*t*-test).

results showed that in spite that both compounds showed similar antitumoral activity in the three times a week administration, their toxicity is quite different (**Figure 3C**).

The results registered that a higher frequency of administration of peniocerol improved its antitumor activity (**Figure 5**) and could suggest that the frequency of administration is more significant than the dose. Several studies have shown that dietary intake of phytosterols reduces the risk of suffering from diverse types of cancer. In experimental studies *in vivo* of ovarian, breast, colon and others neoplasia, was observed that the consumption of β-sitosterol or mixed phytosterols in diet, reduced the number of animals with tumors, or reduced the size of tumors (23–28). In epidemiological studies, the intake of β-sitosterol and stigmasterol was associated with lower risks of esophageal (29) and ovarian cancers (30), respectively. Moreover, it was reported that in female populations with minimal risk of breast cancer have a greater consume of phytosterols in the diet than those at high risk (31, 32).

Our findings show that the peniocerol tumor growth inhibition is related to an antiproliferative effect and induction of apoptosis. The PCNA is used in clinics as a classic marker of cell proliferation as a diagnostic and prognostic tool (33). PARP-1 is an abundant and ubiquitous nuclear enzyme related to DNA repair (34); its overexpression is linked to the development of some types of cancer. Therefore, PARP-1 inhibition selectively ends several types of tumorigenic cells (35). The significant

decrease in the expression of PCNA in the tumor samples shows a condensation and fragmentation of the nuclei, thus an antiproliferative action (**Figures 6C**, **7A**). Moreover, a significant decrease of PARP-1 in the nucleus and a significant increase of cleaved PARP-1 in the cytoplasm (**Figures 6D**, **7B**) indicates apoptosis cell death.

In summary, the administration of peniocerol with a higher frequency, but with a lower dose provides a greater therapeutic effect, suggesting the possibility to develop an anticancer drug from this phytosterol.

DATA AVAILABILITY STATEMENT

Most of the data (**Figures 1**–**7**) used to support the findings of this study are included within the article. Copies of the peniocerol spectra are available upon request to the responsible author.

ETHICS STATEMENT

The Animal Research Committee approved the experimental procedures and were carried out in accordance to the Guidelines for the Care and Use of Animals of the Bioterium Laboratory of the National Institute of Medical Sciences and Nutrition Salvador Zubirán, México.

AUTHOR CONTRIBUTIONS

MM-V contributed to the conception, writing, and discussion of the article. BC-G, contributed in all the experiments that were performed as well as in the discussion of the article. NJ-H contributed substantially in the conduct of animal experiments as well as in the writing and discussion of the manuscript. LR-Z contributed to the conduct of molecular biology experiments as well as the writing and discussion of the article. ZT-S contributed to the performance of some of the experiments. AZ-D contributed significantly to the discussion on molecular biology of the article. All authors discussed, reviewed, and approved the final version of the manuscript to be published.

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REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A, et al. *Global and Regional Estimates of the Incidence and Mortality for 38 Cancers: GLOBOCAN*. Lyon: International Agency for Research on Cancer; World Health Organization (2018). Available online at: https://gco.iarc.fr/ (accessed cited September 10, 2019).
2. André T, Boni C, Navarro M, Tabernero J, Hickish T, Topham C, et al. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. *J Clin Oncol*. (2009) 27:3109–16. doi: 10.1200/JCO.2008.20.6771
3. Bacchetti T, Masciangelo S, Bicchiega V, Bertoli E, Ferretti G. Phytosterols, phytostanols and their esters: from natural to functional foods. *Mediterr J Nutr Metab*. (2011) 4:165–72. doi: 10.1007/s12349-010-0049-0
4. Nes WD, Song ZH, Dennis AL, Zhou WX, Nam J, Miller MB. Biosynthesis of phytosterols - kinetic mechanism for the enzymatic C methylation of sterols. *J Biol Chem*. (2003) 278:34505–16. doi: 10.1074/jbc.M3033 59200
5. Jones PJ, AbuMweis SS. Phytosterols as functional food ingredients: linkages to cardiovascular disease and cancer. *Curr Opin Clin Nutr Metab Care*. (2009) 12:147–51. doi: 10.1097/MCO.0b013e328326770f
6. Plat J, Hendrikx T, Bieghs V, Jeurissen ML, Walenbergh SM, Van Gorp PJ, et al. Protective role of plant sterol and stanol esters in liver inflammation: insights from mice and humans. *PLoS ONE*. (2014) 9:e110758. doi: 10.1371/journal.pone.0110758
7. Shirwaikar A, Punitha ISR, Upadhye M, Dhiman A. Antidiabetic activity of alcohol root extract of Holostemma annulare in NIDDM rats. *Pharm Biol*. (2007) 45:440–5. doi: 10.1080/13880200701388989
8. Misawa E, Tanaka M, Nomaguchi K, Nabeshima K, Yamada M, Toida T, et al. Oral ingestion of aloe vera phytosterols alters hepatic gene expression profiles and ameliorates obesity-associated metabolic disorders in zucker diabetic fatty rats. *J Agric Food Chem*. (2012) 60:2799–806. doi: 10.1021/jf204465j
9. Ramprasath VR, Awad AB. Role of phytosterols in cancer prevention and treatment. *J AOAC Int*. (2015) 98:735–8. doi: 10.5740/jaoacint.SGERamprasath
10. Woyengo TA, Ramprasath VR, Jones PJH. Anticancer effects of phytosterols. *Eur J Clin Nutr*. (2009) 63:813–20. doi: 10.1038/ejcn.2009.29
11. Gupta MB, Nath R, Srivastava N, Shanker K, Kishor K, Bhargava KP. Anti-inflammatory and antipyretic activities of beta-sitosterol. *Planta Med*. (1980) 39:157–63. doi: 10.1055/s-2008-1074919
12. Choi YH, Kong KR, Kim YA, Jung KO, Kil JH, Rhee SH, et al. Induction of bax and activation of caspases during beta-sitosterol-mediated apoptosis in human colon cancer cells. *Int J Oncol*. (2003) 23:1657–62. doi: 10.3892/ijo.23.6.1657
13. Cruz Rojas MC, Delgado Buenrostro NL, Revuelta Miranda ME. (2009). *Guía Básica Ilustrada para el manejo de animales de laboratorio: Ratón, rata, hámster, jerbo, cobayo y conejo*. México: Universidad Nacional Autónoma de México; Facultad de Estudios Superiores Cuautitlán.
14. Bolaños-Carrillo MA, Ventura-Gallegos JL, Saldívar-Jiménez AD, Zentella-Dehesa A, Martínez-Vázquez M. Effect of sterols isolated from *Myrtillocactus geometrizans* on growth inhibition of colon and breast cancer cells. *Evid Based Complement Alternat Med*. (2015) 2015:589350. doi: 10.1155/2015/589350
15. Salazar JR, Martínez-Vázquez M, Cespedes CL, Ramírez-Apan T, Nieto-Camacho A, Rodríguez-Silverio J, et al. Anti-inflammatory and cytotoxic activities of chichipegenin, peniocerol and macdougallin isolated from *Myrtillocactus geometrizans* (Mart. ex Pfeiff.) Con. *Z Naturforsch C J Biosci*. (2011) 66:24–30. doi: 10.1515/znc-2011-1-204
16. Chinedu E, Arome D, Ameh FS. A new method for determining acute toxicity in animal models. *Toxicol Int*. (2013) 20:224–6. doi: 10.4103/0971-6580.121674

17. Shanmugan MK, Rajendran P, Li F, Nema T, Vali S, Abbasi T, et al. Ursolic acid inhibits multiple cell survival pathways leading to suppression of growth of prostate cancer xenograft in nude mice. *J Mol*. (2011) 89:713–27. doi: 10.1007/s00109-011-0746-2
18. Via LD, García-Argáeza AN, Martínez-Vázquez M, Grancarac S, Martinisc P, Toninelloc A. Mitochondrial permeability transition as target of anticancer drugs. *Curr Pharm Des*. (2014) 20:223–44. doi: 10.2174/13816128113199990033
19. Sánchez-Monroy MB, Jacobo-Herrera NJ, Zentella-Dehesa A, Hernández-Téllez B, Martínez-Vázquez M. Masticadienonic and 3α-OH masticadienoic acids induce apoptosis and inhibit cell proliferation and tumor growth in prostate cancer xenografts *in vivo*. *Molecules*. (2017) 22:1–12. doi: 10.3390/molecules22091479
20. Alonso CAJ, Villareal ML, Salazar OLA, Gómez SM, Domínguez F, García CA. Mexican medicinal plants used for cancer treatment: pharmacological, phytochemical and ethnobotanical studies. *J Ethnopharmacol*. (2011) 133:945–72. doi: 10.1016/j.jep.2010.11.055
21. Loizou S, Lekakis I, Chrousos GP, Moutsatsou P. Beta-sitosterol exhibits anti-inflammatory activity in human aortic endothelial cells. *Mol Nutr Food Res*. (2010) 54:551–8. doi: 10.1002/mnfr.200900012
22. Kangsamakin T, Chaithongyot S, Wootthichairangsan C, Hanchaina R, Tangshewinsirikul C, Svasti J. Lupeol and stigmasterol suppress tumor angiogenesis and inhibit cholangiocarcinoma growth in mice via downregulation of tumor necrosis factor-α. *PLoS ONE*. (2017) 12:e0189628. doi: 10.1371/journal.pone.0189628
23. Raicht RF, Cohen EP, Fazzini EP, Sarwal AN, Takahashi M. Protective effect of plant sterols against chemically induced colon tumors in rats. *Cancer Res*. (1980) 40:403–5.
24. Awad AB, Garcia MD, Fink CS. Effect of dietary phytosterols on rat tissue lipids. *Nutr Cancer*. (1997) 29:212–6. doi: 10.1080/01635589709514626
25. Deschner EE, Cohen BI, Raicht RF. The kinetics of the protective effect of beta-sitosterol against MNU-induced colonic neoplasia. *J Cancer Res Clin Oncol*. (1982) 103:49–54. doi: 10.1007/BF00410305
26. Rubis B, Paszel A, Kaczmarek M, Rudzinska M, Jelen H, Rybczynska M. Beneficial or harmful influence of phytosterols on human cells? *Br J Nutr*. (2008) 100:1183–91. doi: 10.1017/S0007114508981423
27. Ju YH, Clausen LM, Allred KF, Almada AL, Helferich WG. Beta-sitosterol, beta-sitosterol glucoside, and a mixture of beta-sitosterol and beta-sitosterol glucoside modulate the growth of estrogen-responsive breast cancer cells *in vitro* and in ovariectomized athymic mice. *J Nutr*. (2004) 134:1145–51. doi: 10.1093/jn/134.5.1145
28. Awad AB, Hernandez AY, Fink CS, Mendel SL. Effect of dietary phytosterols on cell proliferation and protein kinase C activity in rat colonic mucosa. *Nutr Cancer*. (1997) 27:210–5. doi: 10.1080/01635589709514527
29. De Stefani E, Boffetta P, Ronco AL, Brennan P, Deneo-Pellegrini H, Carzoglio JC, et al. Plant sterols and risk of stomach cancer: a case-control study in Uruguay. *Nutr Cancer*. (2000) 37:140–4. doi: 10.1207/S15327914NC372_4
30. McCann SE, Freudenheim JL, Marshall JR, Graham S. Risk of human ovarian cancer is related to dietary intake of selected nutrients, phytochemicals and food groups. *J Nutr*. (2003) 133:1937–42. doi: 10.1093/jn/133.6.1937
31. Ling WH, Jones PJ. Dietary phytosterols: a review of metabolism, benefits and side effects. *Life Sci*. (1995) 57:195–206. doi: 10.1016/0024-3205(95)00263-6
32. Messina M, Barnes S. The role of soy products in reducing risk of cancer. *J Natl Cancer Inst*. (1991) 83:541–6. doi: 10.1093/jnci/83.8.541
33. Estrada AC, Syrovets T, Pitterle K, Lunov O, Büchele B, Schimana PJ, et al. Tirucallic acids are novel pleckstrin homology domain-dependent Akt inhibitors inducing apoptosis in prostate cancer cells. *Mol Pharmacol*. (2010) 73:378–87. doi: 10.1124/mol.109.060475
34. Curtin NJ. PARP inhibitors for cancer therapy. *Expert Rev Mol Med*. (2005) 7:1–20. doi: 10.1017/S146239940500904X
35. Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly (ADP-ribose) polymerase. *Nature*. (2005) 434:913–7. doi: 10.1038/nature03443

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REVIEW
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Pathogenetic, Prognostic, and Therapeutic Role of Fatty Acid Synthase in Human Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is one of the most common solid tumors worldwide, characterized by clinical aggressiveness, resistance to conventional chemotherapy, and high lethality. Consequently, there is an urgent need to better delineate the molecular pathogenesis of HCC to develop new preventive and therapeutic strategies against this deadly disease. Noticeably, emerging evidence indicates that proteins involved in lipid biosynthesis are important mediators along the development and progression of HCC in humans and rodents. Here, we provide a comprehensive overview of: (a) The pathogenetic relevance of lipogenic proteins involved in liver carcinogenesis, with a special emphasis on the master fatty acid regulator, fatty acid synthase (FASN); (b) The molecular mechanisms responsible for unrestrained activation of FASN and related fatty acid biosynthesis in HCC; (c) The findings in experimental mouse models of liver cancer and their possible clinical implications; (d) The existing potential therapies targeting FASN. A consistent body of data indicates that elevated levels of lipogenic proteins, including FASN, characterize human hepatocarcinogenesis and are predictive of poor prognosis of HCC patients. Pharmacological or genetic blockade of FASN is highly detrimental for the growth of HCC cells in both *in vitro* and *in vivo* models. In conclusion, FASN is involved in the molecular pathogenesis of HCC, where it plays a pivotal role both in tumor onset and progression. Thus, targeted inhibition of FASN and related lipogenesis could be a potentially relevant treatment for human HCC.

Keywords: hepatocellular carcinoma, *de novo* lipogenesis, FASN, tumor metabolism, precision medicine

INTRODUCTION: HUMAN HEPATOCELLULAR CARCINOMA

Human hepatocellular carcinoma (HCC) is one of the most frequent and pernicious solid tumors, ranking fifth in incidence and second in lethality worldwide (1–3). Albeit the prevalence of HCC is highest in Eastern Asia and sub-Saharan Africa, where the HBV chronic infection is endemic and the food is contaminated by the mycotoxin aflatoxin B1, its incidence is rapidly rising also in Western Europe and North America (1–3). In the latter areas, however, this escalation in HCC occurrence cannot be entirely explained by the established causal relationship linking chronic hepatitis B or C infection, or ethanol consumption, to hepatocarcinogenesis. Indeed, at least one

quarter of HCC cases remains idiopathic (1–3). In the last decade, non-alcoholic fatty liver disease (NAFLD) has emerged for its potential etiopathogenetic role in liver cancer development, especially in industrialized countries. Numerous case-control studies indicate in fact that HCC patients with cryptogenic cirrhosis display clinical and demographic characteristics suggestive of NAFLD, when compared with HCC patients of viral or alcoholic etiology (3–6). In particular, it has been shown that the increased incidence of HCC in the United States over the past few decades has occurred in parallel with the epidemic of NAFLD (3–6). The latter condition is characterized by the excessive accumulation of lipids in the liver and is associated with obesity, insulin resistance, and type 2 diabetes, often evolving into HCC (3–6).

Regardless of the causative agent, most HCC patients are diagnosed with an advanced disease, precluding the employment of potentially curative therapies, including liver transplantation or partial liver resection (1–3). In addition, molecularly based treatments provided negligible benefits in terms of survival in HCC patients, with the multi-kinase inhibitors Sorafenib and Regorafenib being the only drugs able to extend the life expectancy by ∼2/3 months (7–9). Consequently, new therapeutic approaches aimed at restraining the growth of advanced HCC are highly needed. For this purpose, the molecular pathogenesis of HCC should be better elucidated to identify critical targets whose inhibition might hamper liver tumor development and/or progression.

THE "LIPOGENIC PHENOTYPE"

Deregulated lipid biosynthesis (commonly referred to as "*de novo* lipogenesis" or "*de novo* lipid synthesis") plays an important pathogenetic role in the development of various metabolic diseases, such as diabetes mellitus, obesity, and the metabolic syndrome. In addition, emerging evidences indicate that metabolism reprogramming, including aberrant lipogenesis, is a widespread phenomenon in most cancer types (10–12).

From the historical point of view, the scientific work of the German biochemist and Nobel Prize laureate Otto Warburg, who has been dealing with this issue for several decades since the 1920s, can be considered a pioneer work in this field (13, 14). The starting point was his observation that tumor cells metabolize glucose into lactate under aerobic conditions, while not using the energetically more plausible route of oxidative decarboxylation by the citric acid cycle for energy production. This observation is nowadays well-known as the "Warburg effect" or "Warburg phenomenon" (13, 14). One plausible explanation for this apparently paradoxical event is that glycolysis, although significantly less efficient for energy production than aerobic decarboxylation, can produce adenosine triphosphate (ATP) about 100 times faster than mitochondrial respiration would (14). Consequently, the tumor cell can provide sufficient energy for the accelerated metabolic processes along carcinogenesis. In addition, through the Warburg phenomenon, a reservoir of important metabolic intermediates available for amino acid synthesis and pentose phosphate production—indispensable prerequisites for ensuring adequate protein and DNA synthesis—is generated (14). Furthermore, elevated aerobic glycolysis results in a growth advantage for the most proliferating tumor cells within their microenvironment (14). The immediate consequence of increased glycolysis is the accumulation of the pyruvic acid (pyruvate) metabolite. While most of the pyruvate is converted into lactate and eliminated via the cell membrane, some of the pyruvate is instead converted to acetyl-CoA. In contrast to the normal cell, acetyl-coA represents the primary substrate of the *de novo* lipid synthesis in tumor cells (14).

As normal tissues can cover most of their lipid requirements via dietary lipids coming from the blood circulation, *de novo* lipogenesis does not play a significant role in the metabolism of these cell types; as a result, the expression of lipogenic enzymes is low (10–12). In striking contrast, a universal up-regulation of lipid synthesis occurs in tumor cells (10–12). Importantly, the latter phenomenon is only occasionally associated with a change in cellular morphological properties that are detectable by light microscopy (namely, lipid accumulation in tumor cells that, consequently, appear enlarged, and with a clear cytoplasm) (10–12). Most frequently, indeed, aberrant lipogenesis results in marked alterations of various molecular and metabolic processes, including intracellular signal transduction, and gene expression. At the molecular level, increased lipogenesis is primarily recognizable by the fact that numerous enzymes involved in lipid metabolism (lipogenic enzymes) display strong activity and high expression in tumor cells (10–12). In particular, this refers to the coordinated upregulation of the key enzymes involved in the conversion of glucose into fatty acids, such as ATP citrate lyase (ACLY), acetyl-CoA carboxylase (ACAC), fatty acid synthase (FASN), malate enzyme (ME), and stearoyl-CoA-desaturase 1 (SCD1). Each of these enzymes exhibits a pivotal function in the series of events leading to aberrant lipid biosynthesis. Specifically: (a) ACLY converts citrate from the citrate cycle to acetyl-CoA; (b) ACAC synthesizes malonyl-CoA starting from acetyl-CoA; (c) FASN, starting from malonyl-CoA and consuming acetyl-CoA and NADPH, synthesizes the saturated fatty acid palmitate (palmitic acid) and other saturated long-chain fatty acids; (d) ME catalyzes the production of the reducing NADPH necessary for the synthesis of long-chain fatty acids; and (e) SCD1 converts saturated fatty acids into unsaturated fatty acids, which serve as substrates for the synthesis of triglycerides, cholesterol esters, and phospholipids (10–12, 14, 15). The major steps of *de novo* lipogenesis are summarized in **Figure 1**.

The requirement of lipids for proliferating tumor cells is high for several reasons. First, lipids represent the building blocks necessary for cell membrane production and, consequently, cell duplication (10–12, 14, 15). Second, the newly synthesized fatty acids are used, if needed, to provide additional energy through the β-oxidation. Third, lipids serve as anchors for selective protein transport to the membrane and as precursors for the synthesis of "lipid second messenger" molecules (10–12, 14, 15).

Based on these data, it is obviously not surprising that most epithelial tumors exhibit an increased *de novo* lipid synthesis and an associated upregulation of the lipogenic enzymes. These include carcinomas of the breast, colorectal, prostate, urinary

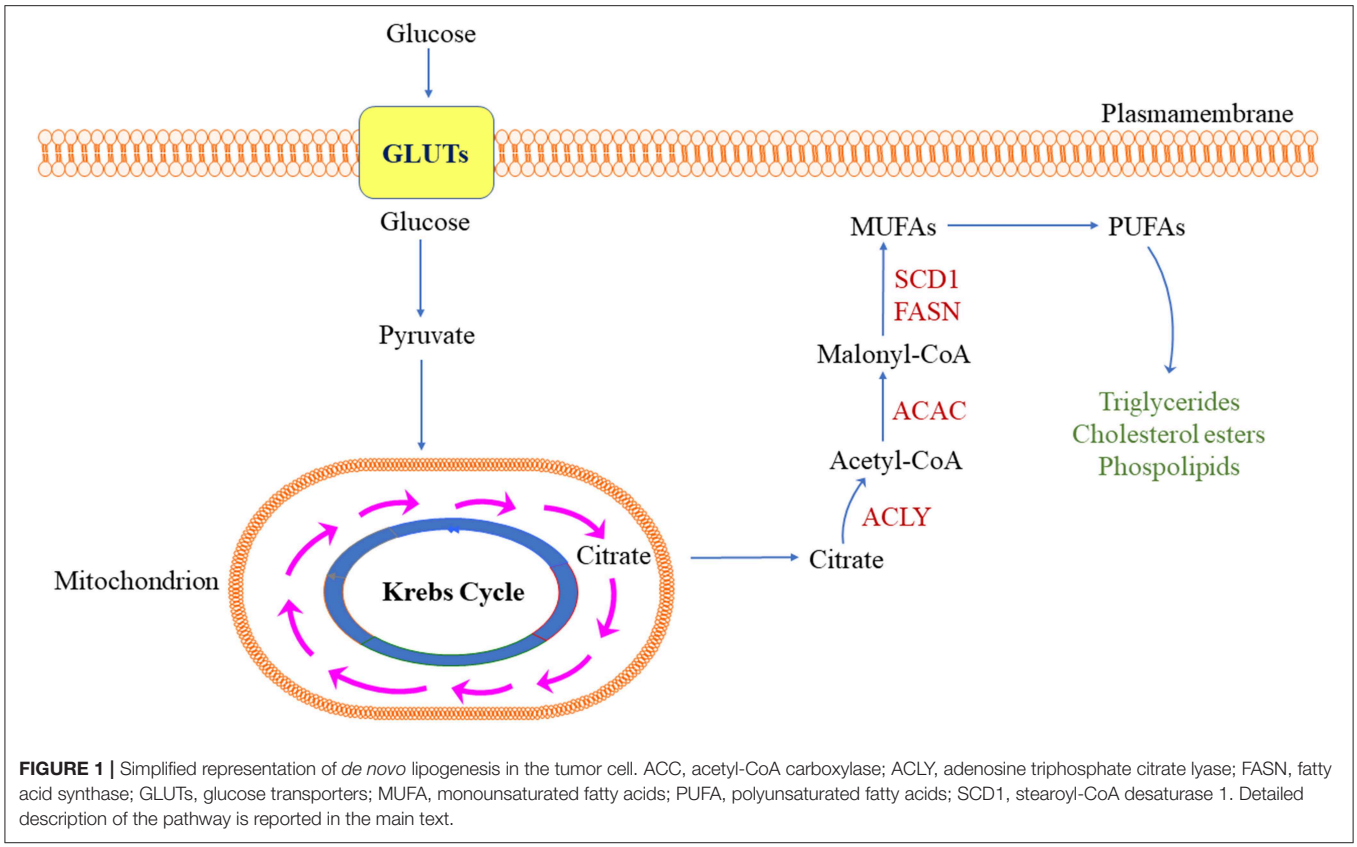

FIGURE 1 | Simplified representation of *de novo* lipogenesis in the tumor cell. ACC, acetyl-CoA carboxylase; ACLY, adenosine triphosphate citrate lyase; FASN, fatty acid synthase; GLUTs, glucose transporters; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SCD1, stearoyl-CoA desaturase 1. Detailed description of the pathway is reported in the main text.

system, ovary, upper gastrointestinal tract, lung, and oral cavity (10–12). Furthermore, it is well-established that tumor cells display increased ACLY expression and activity. Of note, suppression of ACLY by either small interfering RNA molecules (siRNAs) or the pharmacological inhibitor SB-204990 blunts the proliferation and survival of carcinoma cells both *in vitro* and *in vivo* (16). These intriguing findings are in line with the observation that ACAC, FASN, and SCD1 are up-regulated in numerous malignancies at both the transcriptional and protein level, and their inactivation by treatment with specific siRNAs or small molecular inhibitors significantly restrains tumor cell proliferation and survival (10–12, 14, 15). Altogether, these data suggest that *de novo* lipid synthesis as well as the activation of lipogenic proteins and enzymes are critical for the growth of tumor cells.

FATTY ACID SYNTHASE IN PHYSIOLOGY AND CANCER

As reported above, fatty acid synthase (FASN) is the critical enzyme responsible for *de novo* fatty acid synthesis (10–12). Specifically, FASN catalyzes the reaction leading to the generation of palmitate and 16-carbon long fatty acid from acetyl-CoA and malonyl-CoA (10–12). Palmitate is a 16 carbon saturated fatty acid that is a major component of cell membranes and human breast milk, and is incorporated into triglycerides for energy storage. In addition, palmitate is a substrate in the palmitoylation of membrane proteins and acts as a precursor in the synthesis of complex lipids, including cholesterol and glycerophospholipids (10–12).

FASN consists of seven functional domains: acyl carrier protein, malonyl/acetyltransferase, ketoacyl synthase, ketoacyl reductase, dehydrase, enoyl reductase, and thioesterase (17, 18). In humans, FASN is encoded by the *FASN* gene and composed of two identical 272 kDa multifunctional polypeptides, in which the seven domains form a single bond (17). The human *FASN* gene locus is located at chromosome 17 (17q25.3) (10).

FASN is mainly expressed in the cytosol of healthy liver, adipose, brain, cycling endometrium, and lactating mammary gland cells; in these tissues and organs, lipogenesis is a crucial physiological process (10–12).

In cancer, multiple studies have shown that FASN is strongly upregulated in tumors from breast, prostate, colorectal, bladder, ovary, and lung, especially when characterized by clinical aggressiveness, poor prognosis, and resistance to therapy. In contrast, corresponding non-tumor tissues adjacent to the tumor generally express low levels of FASN protein (10–12). However, increased FASN expression has also been detected in some benign and pre-neoplastic lesions of the prostate, breast, lung, stomach, colon, and cutaneous nevi (10–12). Furthermore, investigations conducted in breast, pancreatic, and colorectal tumors showed that cancer patients exhibit elevated levels of FASN in the serum.

Once again, FASN levels in patients' serum directly correlate with an adverse outcome (10–12).

Additional evidence linking FASN to cancer comes from experimental models. For instance, *in vitro* ectopic overexpression of *FASN* in breast cancer cells was found to promote lipogenesis along with augmented cell growth and proliferation (19). Also, transgenic overexpression of *Fasn* in mice triggered the development of prostate epithelial neoplasia, albeit it was not sufficient to induce invasive tumors *per se* (20). Further studies with immortalized prostate epithelial cells (iPrEC) suggested that, in addition to the *Fasn* expression, co-expression of androgen receptor was required for invasive adenocarcinoma development (20). Altogether, this body of evidence indicates a unique association between FASN expression and tumor development and/or progression.

FATTY ACID SYNTHASE IN HEPATOCELLULAR CARCINOMA: EVIDENCE FROM HUMAN DISEASE AND EXPERIMENTAL MODELS

The contribution of unrestrained lipogenesis to the development of hepatocellular carcinoma (HCC) and its progression as well as the molecular mechanisms contributing to the aberrant lipid biosynthesis are starting to be understood. Despite the mounting evidence concerning the importance of aberrant lipid biosynthesis in carcinogenesis, the first studies on this phenomenon in human HCC are relative recent. In a small study (21), overexpression of the mRNA of the main lipogenic enzymes (FASN, ACAC, ACLY and SCD1) was described in HCC when compared with non-neoplastic liver counterparts. In addition, the sterol regulatory element-binding protein 1 transcription factor (SREBP1), a major inducer of lipogenesis, has been identified as a negative prognostic factor in liver cancer (22). Also, an *in vitro* study demonstrated that inhibition of FASN significantly affects the growth of human HCC cell lines in a p53-independent manner (23). Based on these intriguing observations, several studies into the pathogenetic relevance of *de novo* lipid synthesis in human HCC have been initiated, especially focusing on the molecular pathways that drive this event.

In a pioneering investigation, we analyzed the levels of the critical lipogenic proteins in a large human HCC collection (24). In particular, the HCC cohort used could be differentiated into two distinct subgroups based on patient survival after partial liver resection: a group of HCC with less aggressive biological behavior or HCCB (defined as survival longer than 3 years) and one with higher aggressive behavior or HCCP (defined as survival time shorter than 3 years) (24). Intriguingly, a simultaneous upregulation of all relevant enzymes of the lipogenic metabolism was observed in HCC when compared with non-tumorous surrounding liver tissues (24). These included the enzymes responsible for fatty acids production (FASN, ACAC, ACLY, ME, and SCD1) as well as the enzymes for cholesterol biosynthesis [SREBP2, 3-hydroxymethylglutaryl-CoA reductase (HMGCR), mevalonate kinase (MVK), and squalene synthetase (SQS)]. Concomitantly, their upstream inducers [carbohydrate-responsive element-binding protein (chREBP), SREBP1, liver X receptor β (LXR-β)] were upregulated. Of note, the highest levels of lipogenic enzymes were detected in HCC with poorer prognosis (HCCP) (24). It is noteworthy to underline that the content of the chemical end products of the respective lipid synthesis (fatty acids, triglycerides, and cholesterol) changed in an analogous manner (24). Thus, these data indicate increased lipogenesis during development and progression of HCC in humans. Subsequent investigations showed that the induction of unrestrained lipogenesis was the result of both transcriptional and post-transcriptional mechanisms (24). Specifically, in addition to the aforementioned transcription factors (chREBP, SREBP1, and LXR-β), we detected a prominent induction of the ubiquitin-specific peptidase 2a (USP2a) (24). This protein is involved in the inhibition of proteasome-induced degradation of FASN, thus inducing stabilization and increased half-life of the latter (25). Similarly, v-akt murine thymoma viral oncogene homologous (AKT) was found to inhibit the ubiquitination of SREBP1 by phosphorylation-dependent mechanisms (24). These findings indicate that presumably a complex program involving several pathways converge to increase lipid biosynthesis in human HCC.

Since it is established that the AKT/mTOR pathway is a prominent inducer of *de novo* lipogenesis in various tissues and organs (26, 27), our group investigated whether this also applies to human HCC. As expected, an increased induction of activated (phosphorylated) AKT, mTOR, and the mTOR effector RPS6, was detected from surrounding liver tissues to HCC, especially HCCP, when compared to normal liver (24). The importance of the AKT/mTOR signaling in lipogenesis was further substantiated in human HCC cell lines, where overexpression of myristoylated/activated AKT led to a rapid increase in cell growth and a reduction in apoptosis. This change in proliferation kinetics was paralleled by a sharp increase in lipid synthesis and up-regulation of lipogenic enzymes in AKT-overexpressing cells (24). Conversely, there was a robust inhibition of cell growth associated with a decrease in lipogenesis and a reduction in the content of lipogenic proteins when AKT was selectively suppressed in HCC cell lines (24). At the molecular level, activation of lipogenesis was dependent on an intact mTOR complex1 (mTORC1)/RPS6 signaling pathway, as the addition of the mTORC1 inhibitor rapamycin or the targeted inactivation of RPS6 by specific siRNA impaired cell growth in the same cell lines (24). The functional importance of the AKT/mTOR pathway in HCC aberrant lipogenesis and FASN induction was substantiated in a recent investigation from Zhao et al. (28). These authors confirmed the relationship between FASN and the AKT/mTOR cascade in HCC cell lines; furthermore, they identified the loss of the microRNA (miR) 1207-5p as a critical mechanism leading to unconstrained AKT/mTOR signaling pathway and FASN activity in human liver cancer (28). Alternatively, activation of the AKT/mTOR/FASN axis might be triggered by upregulation of the basigin/CD147 protooncogene, a molecular event often detected in human hepatocarcinogenesis (29, 30). Taken together, these data indicate that the AKT/mTOR pathway plays a

leading role in the activation of lipogenesis in human HCC. The identified molecular mechanisms triggering unrestrained FASN activity and lipogenesis in HCC are summarized in **Figure 2**.

In light of these important premises, we determined the requirement of FASN and *de novo* lipogenesis in hepatocarcinogenesis *in vivo*, using genetic approaches. To achieve this goal, we employed conditional FASN knockout (KO) mice (31) and various oncogene driven HCC models, such as AKT and AKT/c-Met mice. Previous data from our group showed that hydrodynamic transfection of an activated form of AKT (myristoylated/myr-AKT) triggers upregulation of FASN, aberrant *de novo* lipid synthesis, and HCC development after long latency in mice (24). To determine whether FASN expression is necessary for myr-AKT driven liver tumor development, we hydrodynamically injected myr-AKT and Cre recombinase (AKT/Cre mice) into conditional FASNfl/fl mice (32). Of note, while AKT overexpression in control mice resulted in HCC development within 22–28 weeks post-injection, none of the AKT/Cre mice exhibited pre-neoplastic and neoplastic lesions. Equivalent results were achieved following overexpression of myr-AKT in liver-specific FASN KO mice (AlbCre; FASNfl/fl mice) (32). The anti-neoplastic effect resulting from FASN ablation in AKT/Cre mice was presumably due to the downregulation of rapamycin-insensitive companion of mTOR (Rictor), the critical member of the mammalian target of rapamycin complex 2 (mTORC2) (27), which is responsible for activation of the AKT protooncogene via phosphorylation. The relevance of Rictor in this process was further demonstrated by the finding that genetic depletion of Rictor in hepatocytes prevented myr-AKT driven hepatocarcinogenesis in mice (32). The crucial role of FASN in hepatocarcinogenesis has been confirmed in a second mouse model, where myr-AKT was co-transfected with the protooncogene c-Met (AKT/c-Met mice). In this model, the co-expression of AKT and c-Met was found to dramatically accelerate HCC development in mice when compared to those transfected with AKT or c-Met alone, with all AKT/c-Met mice being required to be euthanized within 8 weeks post-injection due to high tumor burden (33). Thus, AKT, c-Met, and Cre plasmids were transfected into FASNfl/fl mice, allowing the simultaneous expression of AKT and c-Met oncogenes, while deleting FASN in the same subset of mouse hepatocytes (AKT/c-Met/Cre) (33). Once again, genetic inactivation of FASN completely blunted AKT/c-Met-driven hepatocarcinogenesis in AKT/c-Met/Cre mice, implying that although extremely aggressive, AKT/c-Met tumors fully depend on FASN activity to develop (33). Similar results were obtained more recently by Guri et al. (34). These authors generated a mouse model consisting of lack of *Tsc1* and *Pten* tumor suppressor genes, which inhibit the mTORC1 and mTORC2 pathways, specifically in the liver (termed L-dKO mouse). In these mice, liver-specific activation of the mTOR signaling cascade promoted fatty acid synthesis, liver steatosis, and HCC development. Noticeably, either treatment with the FASN inhibitor Orlistat or Fasn knockdown using adenovirus associated virus suppressed hepatocarcinogenesis in L-dKO mice (34).

Altogether, the present data indicate that FASN and related fatty acid biosynthesis play a critical pathogenetic role in hepatocarcinogenesis.

INHIBITION OF FATTY ACID SYNTHASE IN HUMAN HEPATOCELLULAR CARCINOMA: IS IT A FEASIBLE OPTION?

Based on the body of evidence presented before, it can be envisaged that FASN inhibition might represent a potentially effective therapeutic strategy against human HCC (35). Several FASN inhibitors have been tested against cancer in preclinical studies, including cerulenin, Orlistat, C75, Fasnall, TVB-2640, and others (**Figure 3**). However, only TVB-2640 is currently under evaluation, alone or in combination with other medications, in clinical trials against human cancers, not comprising HCC (**Table 1**).

Pioneering examples of studies investigating the lipogenic dependency of cancer in recent years include those performed with small molecule FASN inhibitors like cerulenin, an antibiotic isolated from fungal extracts. Cerulenin was found to be active against numerous cancer cell lines and xenograft models; however, the highly reactive nature of the cysteine-reactive epoxide group and off-target activities hampered its clinical application (36). In particular, activation of β-oxidation and excessive energy expenditure, leading to weight-loss or anorexia, represented the major factors limiting the application of cerulenin in humans (35). Similar reasons prevented the clinical use of C-75, a synthetic inhibitor of FASN, which was also demonstrated to possess profound antineoplastic effects in experimental models, and to enhance radiation-induced apoptosis in prostate cancer cells, promoting cell cycle arrest in the G2/M phase (37–39).

Orlistat is an anti-obesity drug, which acts by blocking the absorption of free fatty acids from the gastrointestinal tract through the inhibition of pancreatic and gastric lipase that hydrolyze triglycerides (40, 41). Specifically, Orlistat possesses a highly reactive beta-lactone that covalently captures reactive serine residues in the FASN thioesterase domain (42). Despite its potency in restraining the growth of *in vitro* and *in vivo* cancer models (43, 44), the off-target activities together with the poor water solubility and gastrointestinal absorption have hindered the use of Orlistat as anti-tumor agent in patients (45).

C93 is one of the first inhibitors synthesized, which showed antineoplastic activity initially in lung cancer cell lines, and subsequently in trophoblastic neoplasias (46, 47), but no significant further studies were recently performed. Fasnall, a thiophenopyrimidine-based FASN inhibitor with potent and broad antitumor activity against various breast cancer models, might represent a promising alternative. Fasnall inhibits the FASN capacity to facilitate the production of phospholipids with saturated acyl chains, whereas it promotes the uptake of exogenous unsaturated fatty acids, with consequent alterations in signal transduction messages and promotion of apoptosis. Of note, Fasnall have been shown to act synergistically to prolong the survival of mouse models of breast cancer when associated with

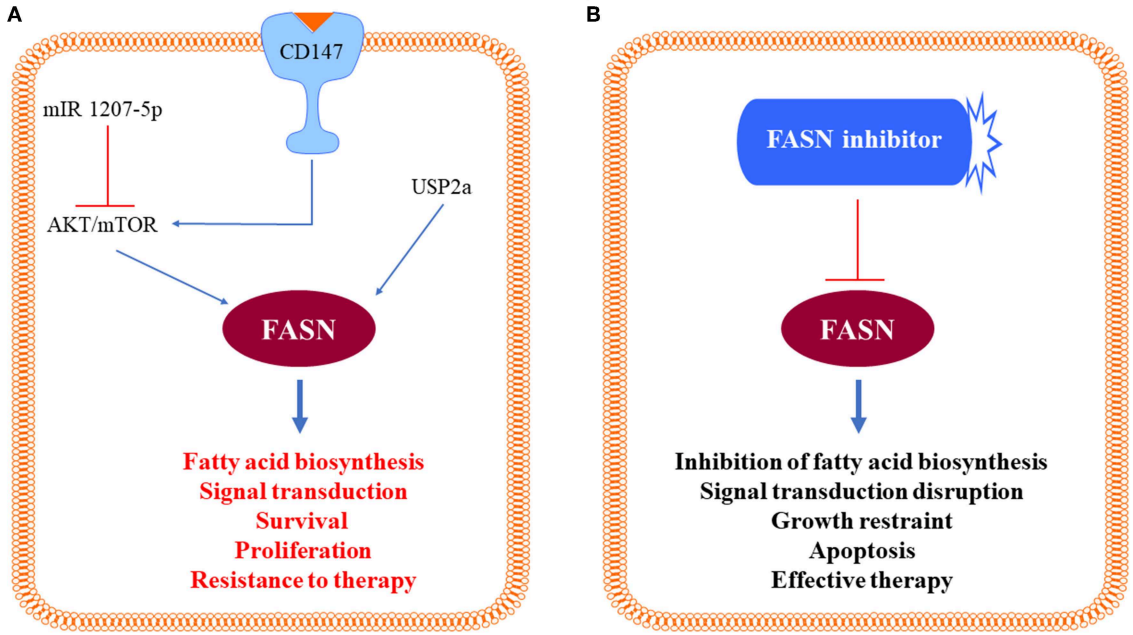

FIGURE 2 | Schematic representation of the identified molecular mechanisms triggering unrestrained fatty acid synthase (FASN) activity in hepatocellular carcinoma cells. **(A)** Positive signals inducing activation of the AKT/mTOR pathway (CD147) and loss of negative stimuli (mIR 1207-5p) toward the same pathway lead to activation of FASN and induction of its multiple, pro-oncogenic biologic effects, which are blunted by FASN inhibitors **(B)**. Further details are reported in the text.

the chemotherapeutic agent carboplatin; in this study Fasnall was well tolerated, with no changes in feeding behavior or weight loss being detected in these mice, further suggesting its possible application in the clinical practice (48).

Other high potential FASN inhibitors have been recently developed. Among them, TVB-3166 is a imidazopyridine-based, orally-available, FASN inhibitor, which suppresses *de novo* palmitate synthesis *in vitro* and *in vivo*, and displays antineoplastic activity in several experimental cancer models (49, 50). The mechanism of action of TVB-3166 on aberrant lipogenesis resides on its property to disrupt the architecture of lipid rafts. Alterations in lipid rafts by TVB-3166 promote the mis-localization of membrane-associated oncoproteins, such as Ras, AKT, and members of the canonical Wnt/β-catenin pathway. As a consequence, TVB-3166 administration leads to the abrogation of several signaling cascades and the induction of tumor cell apoptosis (49). Lu et al. have synthesized several FASN inhibitors recently using a structure-based approach guided by X-ray crystallography approach (51). Among them, compound 34 showed a high FASN inhibitory potential and favorable pharmacological features; in addition, it strongly inhibited cell proliferation in several cancer cell lines including A2780 (ovarian), PC3M (prostate), LNCaP (prostate), OCI LY1 (lymphoma), MV4-11 (leukemia/lymphoma/myeloma), H460 (lung), A549 (lung), and MDA-MB-468 (breast), becoming an interesting candidate for future studies (51).

The synthetic drug IPI-9119, which has been recently developed, strongly inhibits FASN by promoting acylation of the catalytic serine, with high selectivity and negligible off-target activity (52). IPI-9119 was shown able to effectively block cell growth and proliferation in several cell lines, including prostatic cancer cells, reducing the proportion of S-phase cells and increased that of G0/G1 cells, and decreasing expression of cyclin A2 (52). GSK837149A was identified as a reversible low inhibitor of the FASN β-ketoacyl reductase domain, but its poor cell permeability prevented the study of its mechanism in cells (53), while other synthetic inhibitors like GSK2194069 and JNJ- 54302833 remain to be tested in pre-clinical models. In addition, several natural plant-derived polyphenols have been shown to inhibit FASN, including epigallocatechin-3-gallate (EGCG) and the flavonoids luteolin, taxifolin, kaempferol, quercetin, and apigenin (54); EGCG in a recent study reduced the growth of adenocarcinoma human lung cancer xenografts without inducing body weight loss (37). Other natural FASN inhibitors may have similar properties, and merit evaluation in future studies.

Currently, the most promising anti-FASN drug is TVB-2640, an oral, small-molecule possessing *in vitro* and *in vivo* antitumor activity associated with an acceptable non-clinical safety profile. Of note, preclinical and early efficacy data from a dose-escalation trial demonstrated a wide activity of TVB-2640 as a single agent in multiple solid tumors, including cases of stable disease (clinicaltrials.gov/ct2/show/NCT02223247). These encouraging results were achieved with relatively low side effects, which could be eliminated with therapy discontinuation (55). Currently four further clinical trials are testing TVB-2640 alone or in combination with other drugs in NSCLC (NCT03808558),

TABLE 1 | Current evidence on the antineoplastic properties of main FASN inhibitors in cancer.

Name	Molecular formula	Antineoplastic activity and targeted tumor type	Current clinical trials	References
Cerulenin	$C_{12}H_{17}NO_3$	Breast cancer, promyelocytic leukemia and other cells, mouse liver metastases	–	(35, 36)
C75	$C_{14}H_{22}O_4$	Lung cancer cells, radio-sensitization in prostate cancer cells	–	(37–39)
Orlistat	$C_{29}H_{53}O_5$	Prostate, melanoma, breast and other cells, and xenograft tumor models	–	(40–45)
C93	$C_{13}H_{15}NO_5$	Lung, ovarian and trophoblastic neoplasia cells	–	(46, 47)
Fasnall	$C_{19}H_{22}N_4S{\cdot}C_6H_6O_3S$	Breast cancer (combination therapy)	–	(48)
TVB-3166	$C_{24}H_{24}N_4O$	Lung, ovarian, prostate, and pancreatic xenograft tumor models, combination with taxanes	–	(49, 50)
Compound 34	$C_{31}H_{24}F_3N_3O_3$	Ovarian, prostate, prostate, lymphoma, leukemia, myeloma, lung, breast cells		(51)
IPI-9119	$C_{24}H_{19}F_2N_5O_5$	Prostate cancer cells	–	(52)
GSK837149A	$C_{23}H_{22}N_8O_5S_2$	–	–	(53)
GSK2194069	$C_{25}H_{24}N_4O_3$	–	–	(35)
JNJ-54302833	$C_{30}H_{31}N_5O_2$	–	–	(35)
TVB-2640	$C_{27}H_{29}N_5O$	Numerous solid tumors, several combinations with chemotherapeutic agents under evaluation	NCT03808558 NCT02223247 NCT02980029 NCT03179904 NCT03032484	(55)

The code of the clinical trials refers to the ClinicalTrials.gov repository.

colorectal (NCT02980029), breast cancer (NCT03179904), and astrocytomas (NCT03032484).

TVB-2640 combination treatments are based on evidences that FASN inhibitors synergize with multiple chemotherapeutic agents, such as taxanes, vinca alkaloids, 5-fluorouracil, platinum compounds, and anthracyclines. Furthermore, FASN inhibitors have been found to restore the sensitivity to chemotherapeutic drugs, including doxorubicin, and to targeted therapies, such as those including trastuzumab or lapatinib. In addition, FASN suppression might also cooperate in radio-sensitization and with antiangiogenic agents, by triggering strong tumor hypoxia because cancer cells escape antiangiogenic-driven hypoxia by upregulation of FASN-related lipogenesis (38, 56). These evidences strongly suggest that FASN inhibitors will play an important role in future therapeutic attempts against cancer, hopefully also against HCC.

CONCLUSION

HCC is a highly aggressive and frequent tumor worldwide, with its incidence rising also in low-frequency areas. Thus, these data, together with the lack of effective therapies against this tumor type, indicate that HCC represents a major health concern globally. Understanding the intricated molecular pathogenesis of this cancer entity is therefore necessary for the identification of specific targets suitable of therapeutic intervention. Recently, among the potential, novel therapeutic targets identified in HCC is FASN and the related *de novo* lipogenesis pathway. Mounting and solid evidence underscores the fact that aberrant fatty biosynthesis contributes to hepatocellular carcinogenesis in experimental models as well as in humans. Albeit several features of FASN and related lipogenesis remain to be explored, it appears clear from the data summarized in the present review article that anti-FASN-based therapies might be helpful for the treatment of HCC treatment. The use of existing drugs against FASN for the treatment of HCC (and other tumors) has been impeded by the low potency and consistent off-target effects of these molecules. However, the most recent FASN inhibitors (e.g. Fasnall, TVB-3166, and TVB-2640) seem to have overcome most of these limitations (56).

Among the critical questions that still need to be addressed for the clinical practice, is how the HCC patients can be selected for anti-FASN treatments. It is clear from HCC TCGA analysis (https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp) as well as other genomic studies (57) that human HCC is a highly heterogeneous disease. Not all HCCs express FASN and its related lipogenesis genes at high levels. Consequently, some HCCs might not depend on FASN and *de novo* lipogenesis for growth. This possibility was revealed by *in vivo* mouse studies. Indeed, there was no increase in Fasn expression in mouse HCCs induced by c-Met and gain of function mutant of β-Catenin (c-Met/β-Catenin) via hydrodynamic injection. Consistently, ablation of *Fasn* did not affect HCC growth in mice (35). For this purpose, reliable biomarkers able to uncover the patients who would presumably benefit from this therapeutic strategy should be identified.

Furthermore, as *de novo* lipogenesis is an integrated part of a metabolic network, it is conceivable that disruption of fatty acid synthesis may lead to other biochemical events. These feedback

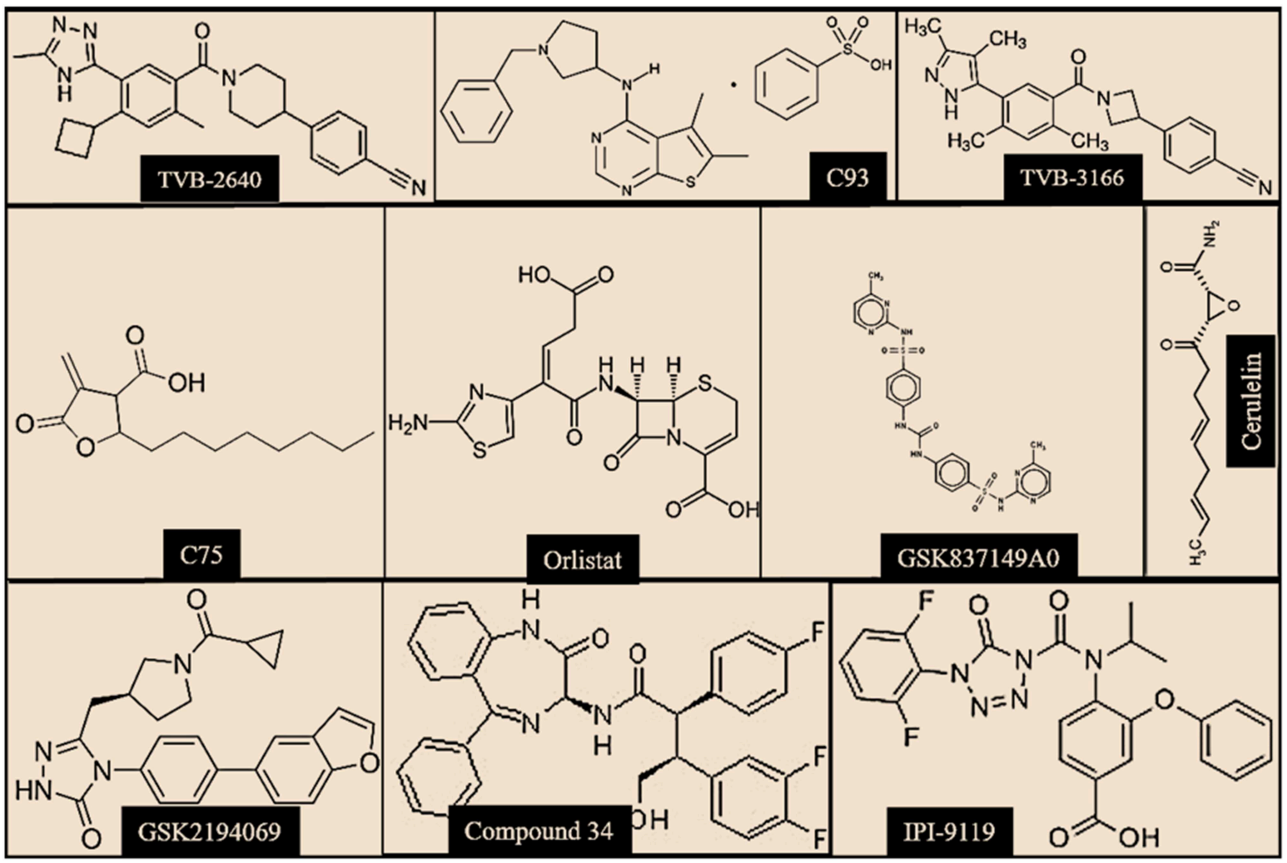

FIGURE 3 | Chemical structures of the main FASN inhibitors tested in preclinical and clinical studies.

biochemical and metabolic events may contribute to HCC development. For instance, in the diethylnitrosamine (DEN) induced mouse HCC model, inhibition of lipogenesis via deletion of Acac1 and Acac2 genes in the liver led to an increased HCC development (58). Mechanistically, this unexpected finding was due to the marked increased in antioxidants, including increased NADPH and reduced glutathione, which protected hepatocytes from oxidant-mediated cell death. In another example, in murine HCCs induced by overexpression of c-Met and loss of Pten (c-Met/sgPten), loss of Fasn significantly repressed HCC formation. However, over long time, HCC lesions could emerge from Fasn null genetic background. Further molecular and metabolomic analysis revealed that there was an increased cholesterol biosynthesis due to increased Srebp2 activity in the mouse liver tissues. This augmented cholesterogenesis eventually compensated for the loss of *de novo* lipogenesis, ultimately leading to HCC formation (59).

It is also important to acknowledge that two major mechanisms whereby cells acquire fatty acids required for cell growth exist: one involves FASN and its mediated *de novo* lipogenesis, while the other consists of the transport of circulating fatty acids via the "lipolytic" pathway (60). This process requires lipoprotein lipase (LPL), which releases fatty acids from lipoproteins, as well as fatty acid transporter proteins for fatty acids uptake (61). The role of exogenous fatty acids during tumor initiation and progression has been studied marginally to date. However, recent reports suggested the key role of this pathway in tumorigenesis. For instance, it was recently found that fatty acids derived from adipocytes could be transferred to melanoma cells through the fatty acid transporter protein SLC27A1. Blocking fatty acid uptake via the fatty acid transport proteins inhibitor Lipofermata significantly reduced melanoma growth and invasion (62). In HCC cells, it has been shown that LPL mediated fatty acid uptake could at least partly compensate the blockade of *de novo* lipogenesis (63). These studies indicate that presumably both *de novo* fatty acid synthesis and exogenous fatty acid uptake should be inhibited to achieve significant anti-cancer effects.

In addition, future studies are required to determine whether anti-FASN drugs can be used in combination with FDA-approved anti-HCC multi-kinase inhibitors (Sorafenib, Regorafenib, Cabozantinib), immune modulators (checkpoint inhibitors), and/or conventional chemotherapeutic drugs for the treatment of HCC. Studies to address this important point should be conducted. An alternative approach to suppress FASN in HCC (and other tumor types) could be the inhibition of FASN

upstream inducers, such as USP2a and CD147. As concerns USP2a, ML364, a small molecule inhibitor of this deubiquitinase has been recently developed. Of note, ML364 administration caused cell cycle arrest in colorectal cancer and lymphoma cell lines, although the specific effect of the drug on FASN levels was not investigated (64). Preliminary results obtained in our laboratory indicate a strong growth restraint as well as downregulation of FASN in HCC cell lines treated with ML364 (Cigliano et al., unpublished observation), suggesting that inhibition of USP2a might be a promising therapy for this deadly disease. Furthermore, targeting CD147 has revealed promising results in the treatment of human HCC patients. Indeed, HCC recurrence rate was found to be significantly decreased, and the survival length of HCC patients subjected to liver transplantation prolonged, following the administration of a monoclonal antibody against CD147, in a randomized controlled trial (65).

AUTHOR CONTRIBUTIONS

DC and XC conceived the work, designed the outline of the review, and supervised all aspects of the manuscript. All authors participated in the literature search, scrutiny, and interpretation, as well as in writing and editing all contents of the manuscript.

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REFERENCES

1. Pascual S, Herrera I, Irurzun J. New advances in hepatocellular carcinoma. *World J Hepatol*. (2016) 8:421–38. doi: 10.4254/wjh.v8.i9.421
2. El-Serag HB. Hepatocellular carcinoma. *N Engl J Med*. (2011) 365:1118–27. doi: 10.1056/NEJMra1001683
3. Tsukuma H, Hiyama T, Tanaka S, Nakao M, Yabuuchi T, Kitamura T, et al. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N Engl J Med*. (1993) 328:1797–801. doi: 10.1056/NEJM199306243282501
4. Knudsen ES, Gopal P, Singal AG. The changing landscape of hepatocellular carcinoma: etiology, genetics, and therapy. *Am J Pathol*. (2014) 184:574–83. doi: 10.1016/j.ajpath.2013.10.028
5. Choo SP, Tan WL, Goh BK, Tai WM, Zhu AX. Comparison of hepatocellular carcinoma in Eastern versus Western populations. *Cancer*. (2016) 122:3430–46. doi: 10.1002/cncr.30237
6. Sanyal AJ, Yoon SK, Lencioni R. The etiology of hepatocellular carcinoma and consequences for treatment. *Oncologist*. (2010) 4:14–22. doi: 10.1634/theoncologist.2010-S4-14
7. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med*. (2008) 359:378–90. doi: 10.1056/NEJMoa0708857
8. Bruix J, Tak WY, Gasbarrini A, Santoro A, Colombo M, Lim HY, et al. Regorafenib as second-line therapy for intermediate or advanced hepatocellular carcinoma: multicentre, open-label, phase II safety study. *Eur J Cancer*. (2013) 49:3412–19. doi: 10.1016/j.ejca.2013.05.028
9. Llovet JM, Bruix J. Molecular targeted therapies in hepatocellular carcinoma. *Hepatology*. (2008) 48:1312–27. doi: 10.1002/hep.22506
10. Menendez JA, Lupu R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat Rev Cancer*. (2007) 7:763–77. doi: 10.1038/nrc2222
11. Currie E, Schulze A, Zechner R, Walther TC, Farese RV Jr. Cellular fatty acid metabolism and cancer. *Cell Metab*. (2013) 18:153–61. doi: 10.1016/j.cmet.2013.05.017
12. Rohrig F, Schulze A. The multifaceted roles of fatty acid synthesis in cancer. *Nat Rev Cancer*. (2016) 16:732–49. doi: 10.1038/nrc.2016.89
13. Warburg, O. On the origin of cancer cells. *Science*. (1956) 123:309–14. doi: 10.1126/science.123.3191.309
14. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
15. Peck B, Schulze A. Lipid desaturation - the next step in targeting lipogenesis in cancer? *FEBS J*. (2016) 283:2767–78. doi: 10.1111/febs.13681
16. Khwairakpam AD, Shyamananda MS, Sailo BL, Rathnakaram SR, Padmavathi G, Kotoky J, et al. ATP citrate lyase (ACLY): a promising target for cancer prevention and treatment. *Curr Drug Targets*. (2015) 16:156–63. doi: 10.2174/1389450115666141224125117
17. Asturias FJ, Chadick JZ, Cheung IK, Stark H, Witkowski A, Joshi AK, et al. Structure and molecular organization of mammalian fatty acid synthase. *Nat Struct Mol Biol*. (2005) 12:225–32. doi: 10.1038/nsmb899
18. Jayakumar A, Tai MH, Huang WY, al-Feel W, Hsu M, Abu-Elheiga L, et al. Human fatty acid synthase: properties and molecular cloning. *Proc Natl Acad Sci USA*. (1995) 92:8695–99. doi: 10.1073/pnas.92.19.8695
19. Vazquez-Martin A, Colomer R, Brunet J, Lupu R, Menendez JA. Overexpression of fatty acid synthase gene activates HER1/HER2 tyrosine kinase receptors in human breast epithelial cells. *Cell Prolif*. (2008) 41:59–85. doi: 10.1111/j.1365-2184.2007.00498.x
20. Migita T, Ruiz S, Fornari A, Fiorentino M, Priolo C, Zadra G, et al. Fatty acid synthase: a metabolic enzyme and candidate oncogene in prostate cancer. *J Natl Cancer Inst*. (2009) 101:519–32. doi: 10.1093/jnci/djp030
21. Yahagi N, Shimano H, Hasegawa K, Ohashi K, Matsuzaka T, Najima Y, et al. Co-ordinate activation of lipogenic enzymes in hepatocellular carcinoma. *Eur J Cancer*. (2005) 41:1316–22. doi: 10.1016/j.ejca.2004.12.037
22. Yamashita T, Honda M, Takatori H, Nishino R, Minato H, Takamura H, et al. Activation of lipogenic pathway correlates with cell proliferation and poor prognosis in hepatocellular carcinoma. *J Hepatol*. (2009) 50:100–10. doi: 10.1016/j.jhep.2008.07.036
23. Gao Y, Lin LP, Zhu CH, Chen Y, Hou YT, Ding J. Growth arrest induced by C75, A fatty acid synthase inhibitor, was partially modulated by p38 MAPK but not by p53 in human hepatocellular carcinoma. *Cancer Biol Ther*. (2006) 5:978–85. doi: 10.4161/cbt.5.8.2883
24. Calvisi DF, Wang C, Ho C, Ladu S, Lee SA, Mattu S, et al. Increased lipogenesis, induced by AKT-mTORC1-RPS6 signaling, promotes development of human hepatocellular carcinoma. *Gastroenterology*. (2011) 140:1071–83. doi: 10.1053/j.gastro.2010.12.006
25. Graner E, Tang D, Rossi S, Baron A, Migita T, Weinstein LJ, et al. The isopeptidase USP2a regulates the stability of fatty acid synthase in prostate cancer. *Cancer Cell*. (2004) 5:253–61. doi: 10.1016/S1535-6108(04)00055-8
26. Lamming DW, Sabatini DM. A central role for mTOR in lipid homeostasis. *Cell Metab*. (2013) 18:465–69. doi: 10.1016/j.cmet.2013.08.002
27. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. (2012) 149:274–93. doi: 10.1016/j.cell.2012.03.017
28. Zhao G, Dong L, Shi H, Li H, Lu X, Guo X, et al. MicroRNA-1207–5p inhibits hepatocellular carcinoma cell growth and invasion through the fatty acid synthase-mediated Akt/mTOR signalling pathway. *Oncol Rep*. (2016) 36:1709–16. doi: 10.3892/or.2016.4952
29. Li J, Huang Q, Long X, Zhang J, Huang X, Aa J, et al. CD147 reprograms fatty acid metabolism in hepatocellular carcinoma cells through Akt/mTOR/SREBP1c and P38/PPARα pathways. *J Hepatol*. (2015) 63:1378–89. doi: 10.1016/j.jhep.2015.07.039
30. Calvisi DF. CD147/Basigin: a Warburg oncogene in hepatocellular carcinoma? *Chin J Cancer Res*. (2016) 28:377–79. doi: 10.21147/j.issn.1000-9604.2016.03.13

31. Chakravarthy MV, Pan Z, Zhu Y, Tordjman K, Schneider JG, Coleman T, et al. "New" hepatic fat activates PPARalpha to maintain glucose, lipid, and cholesterol homeostasis. *Cell Metab*. (2005) 1:309–22. doi: 10.1016/j.cmet.2005.04.002
32. Li L, Pilo GM, Li X, Cigliano A, Latte G, Che L, et al. Inactivation of fatty acid synthase impairs hepatocarcinogenesis driven by AKT in mice and humans. *J Hepatol*. (2016) 64:333–41. doi: 10.1016/j.jhep.2015.10.004
33. Hu J, Che L, Li L, Pilo MG, Cigliano A, Ribback S, et al. Co-activation of AKT and c-Met triggers rapid hepatocellular carcinoma development via the mTORC1/FASN pathway in mice. *Sci Rep*. (2016) 6:20484. doi: 10.1038/srep20484
34. Guri Y, Colombi M, Dazert E, Hindupur SK, Roszik J, Moes S, et al. mTORC2 promotes tumorigenesis via lipid synthesis. *Cancer Cell*. (2017) 32:807–23.e12. doi: 10.1016/j.ccell.2017.11.011
35. Che L, Pilo MG, Cigliano A, Latte G, Simile MM, Ribback S, et al. Oncogene dependent requirement of fatty acid synthase in hepatocellular carcinoma. *Cell Cycle*. (2017) 16:499–507. doi: 10.1080/15384101.2017.1282586
36. Angeles TS, Hudkins RL. Recent advances in targeting the fatty acid biosynthetic pathway using fatty acid synthase inhibitors. *Expert Opin Drug Discov*. (2016) 11:1187–99. doi: 10.1080/17460441.2016.1245286
37. Relat J, Blancafort A, Oliveras G, Cufí S, Haro D, Marrero PF, et al. Different fatty acid metabolism effects of (-)-epigallocatechin-3-gallate and C75 in adenocarcinoma lung cancer. *BMC Cancer*. (2012) 12:280. doi: 10.1186/1471-2407-12-280
38. Rae C, Haberkorn U, Babich JW, Mairs RJ. Inhibition of fatty acid synthase sensitizes prostate cancer cells to radiotherapy. *Radiat Res*. (2015) 184:482–93. doi: 10.1667/RR14173.1
39. Cheng X, Li L, Uttamchandani M, Yao SQ. *In situ* proteome profiling of C75, a covalent bioactive compound with potential anticancer activities. *Org Lett*. (2014) 16:1414–17. doi: 10.1021/ol500206w
40. Kridel SJ, Axelrod F, Rozenkrantz N, Smith JW. Orlistat is a novel inhibitor of fatty acid synthase with antitumor activity. *Cancer Res*. (2004) 64:2070–75. doi: 10.1158/0008-5472.CAN-03-3645
41. Hadváry P, Lengsfeld H, Wolfer H. Inhibition of pancreatic lipase *in vitro* by the covalent inhibitor tetrahydrolipstatin. *Biochem J*. (1988) 256:357–61. doi: 10.1042/bj2560357
42. Pemble CW IV, Johnson LC, Kridel SJ, Lowther WT. Crystal structure of the thioesterase domain of human fatty acid synthase inhibited by Orlistat. *Nat Struct Mol Biol*. (2007) 14:704–9. doi: 10.1038/nsmb1265
43. Buckley D, Duke G, Heuer TS, O'Farrell M, Wagman AS, McCulloch W, et al. Fatty acid synthase - Modern tumor cell biology insights into a classical oncology target. *Pharmacol Ther*. (2017) 177:23–31. doi: 10.1016/j.pharmthera.2017.02.021
44. Liu H, Liu JY, Wu X, Zhang JT. Biochemistry, molecular biology, and pharmacology of fatty acid synthase, an emerging therapeutic target and diagnosis/prognosis marker. *Int J Biochem Mol Biol*. (2010) 1:69–89.
45. Mullen GE, Yet L. Progress in the development of fatty acid synthase inhibitors as anticancer targets. *Bioorg Med Chem Lett*. (2015) 25:4363–69. doi: 10.1016/j.bmcl.2015.08.087
46. Orita H, Coulter J, Lemmon C, Tully E, Vadlamudi A, Medghalchi SM, et al. Selective inhibition of fatty acid synthase for lung cancer treatment. *Clin Cancer Res*. (2007) 13:7139–45. doi: 10.1158/1078-0432.CCR-07-1186
47. Ueda SM, Mao TL, Kuhajda FP, Vasoontara C, Giuntoli RL, Bristow RE, et al. Trophoblastic neoplasms express fatty acid synthase, which may be a therapeutic target via its inhibitor C93. *Am J Pathol*. (2009) 175:2618–24. doi: 10.2353/ajpath.2009.081162
48. Alwarawrah Y, Hughes P, Loiselle D, Carlson DA, Darr DB, Jordan JL, et al. Fasnall, a selective FASN Inhibitor, shows potent anti-tumor activity in the MMTV-Neu model of HER2(+) breast cancer. *Cell Chem Biol*. (2016) 23:678–88. doi: 10.1016/j.chembiol.2016.04.011
49. Ventura R, Mordec K, Waszczuk J, Wang Z, Lai J, Fridlib M, et al. Inhibition of *de novo* palmitate synthesis by fatty acid synthase induces apoptosis in tumor cells by remodeling cell membranes, inhibiting signaling pathways, and reprogramming gene expression. *EBioMedicine*. (2015) 2:808–24. doi: 10.1016/j.ebiom.2015.06.020
50. Heuer TS, Ventura R, Mordec K, Lai J, Fridlib M, Buckley D, et al. FASN inhibition and taxane treatment combine to enhance anti-tumor efficacy in diverse xenograft tumor models through disruption of tubulin palmitoylation and microtubule organization and FASN inhibition-mediated effects on oncogenic signaling and gene expression. *EBioMedicine*. (2017) 16:51–62. doi: 10.1016/j.ebiom.2016.12.012
51. Lu T, Schubert C, Cummings MD, Bignan G, Connolly PJ, Smans K, et al. Design and synthesis of a series of bioavailable fatty acid synthase (FASN) KR domain inhibitors for cancer therapy. *Bioorg Med Chem Lett*. (2018) 28:2159–64. doi: 10.1016/j.bmcl.2018.05.014
52. Zadra G, Ribeiro CF, Chetta P, Ho Y, Cacciatore S, Gao X, et al. Inhibition of *de novo* lipogenesis targets androgen receptor signaling in castration-resistant prostate cancer. *Proc Natl Acad Sci USA*. (2019) 116:631–40. doi: 10.1073/pnas.1808834116
53. Vázquez MJ, Leavens W, Liu R, Rodríguez B, Read M, Richards S, et al. Discovery of GSK837149A, an inhibitor of human fatty acid synthase targeting the β-ketoacyl reductase reaction. *FEBS J*. (2008) 275:1556–67. doi: 10.1111/j.1742-4658.2008.06314.x
54. Flavin R, Peluso S, Nguyen PL, Loda M. Fatty acid synthase as a potential therapeutic target in cancer. *Future Oncol*. (2010) 6:551–62. doi: 10.2217/fon.10.11
55. Jones SF, Infante JR. Molecular pathways: fatty acid synthase. *Clin Cancer Res*. (2015) 21:5434–38. doi: 10.1158/1078-0432.CCR-15-0126
56. Bueno MJ, Sanchez J, Colomer R, Quintela-Fandino M. Antiangiogenics and hypoxic response: role of fatty acid synthase inhibitors. *Curr Drug Targets*. (2016) 17:1735–46. doi: 10.2174/1389450117666160502151857
57. Dhanasekaran R, Nault JC, Roberts LR, Zucman-Rossi J. Genomic medicine and implications for hepatocellular carcinoma prevention and therapy. *Gastroenterology*. (2019) 156:492–509. doi: 10.1053/j.gastro.2018.11.001
58. Nelson ME, Lahiri S, Chow JD, Byrne FL, Hargett SR, Breen DS, et al. Inhibition of hepatic lipogenesis enhances liver tumorigenesis by increasing antioxidant defence and promoting cell survival. *Nat Commun*. (2017) 8:14689. doi: 10.1038/ncomms14689
59. Che L, Chi W, Qiao Y, Zhang J, Song X, Liu Y, et al. Cholesterol biosynthesis supports the growth of hepatocarcinoma lesions depleted of fatty acid synthase in mice and humans. *Gut*. (2019) gutjnl-2018-317581. doi: 10.1136/gutjnl-2018-317581. [Epub ahead of print].
60. Zaidi N, Lupien L, Kuemmerle NB, Kinlaw WB, Swinnen JV, Smans K. Lipogenesis and lipolysis: the pathways exploited by the cancer cells to acquire fatty acids. *Prog Lipid Res*. (2013) 52:585–9. doi: 10.1016/j.plipres.2013.08.005
61. Kinlaw WB, Baures PW, Lupien LE, Davis WL, Kuemmerle NB. Fatty acids and breast cancer: make them on site or have them delivered. *J Cell Physiol*. (2016) 231:2128–41. doi: 10.1002/jcp.25332
62. Zhang M, Di Martino JS, Bowman RL, Campbell NR, Baksh SC, Simon-Vermot T, et al. Adipocyte-derived lipids mediate melanoma progression via FATP proteins. *Cancer Discov*. (2018) 8:1006–25. doi: 10.1158/2159-8290.CD-17-1371
63. Cao D, Song X, Che L, Li X, Pilo MG, Vidili G, et al. Both *de novo* synthetized and exogenous fatty acids support the growth of hepatocellular carcinoma cells. *Liver Int*. (2016) 37:80–9. doi: 10.1111/liv.13183
64. Xu J, Shen ZY, Chen XG, Zhang Q, Bian HJ, Zhu P, et al. A randomized controlled trial of Licartin for hepatic recurrence after liver transplantation. *Hepatology*. (2007) 45:269–76. doi: 10.1002/hep.21465
65. Davis MI, Pragani R, Fox JT, Shen M, Parmar K, Gaudiano EF, et al. Small molecule inhibition of the ubiquitin-specific protease USP2 accelerates cyclin D1 degradation and leads to cell cycle arrest in colorectal cancer and mantle cell lymphoma models. *J Biol Chem*. (2016) 291:24628–40. doi: 10.1074/jbc.M116.738567

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MicroRNAs in Tumor Cell Metabolism: Roles and Therapeutic Opportunities

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Dysregulated metabolism is a common feature of cancer cells and is considered a hallmark of cancer. Altered tumor-metabolism confers an adaptive advantage to cancer cells to fulfill the high energetic requirements for the maintenance of high proliferation rates, similarly, reprogramming metabolism confers the ability to grow at low oxygen concentrations and to use alternative carbon sources. These phenomena result from the dysregulated expression of diverse genes, including those encoding microRNAs (miRNAs) which are involved in several metabolic and tumorigenic pathways through its post-transcriptional-regulatory activity. Further, the identification of key actionable altered miRNA has allowed to propose novel targeted therapies to modulated tumor-metabolism. In this review, we discussed the different roles of miRNAs in cancer cell metabolism and novel miRNA-based strategies designed to target the metabolic machinery in human cancer.

Keywords: microRNAs, reprogramming metabolism, regulation, therapeutic targets, tumor cell metabolism

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INTRODUCTION

Ever since their discovery in 1993 (1), microRNAs emerged as a new class of small RNAs with a critical role in the regulation of gene expression. MicroRNAs (miRNAs or miRs) are endogenous small non-coding RNAs from 18-25 nucleotides in length that regulate gene expression via base complementarity between the seed region of the microRNA and the 3′-untranslated region (UTR) of the target mRNA (1, 2). Depending on the degree of complementarity, miRNAs binding can induce mRNA degradation, translational repression, or both (3–5). For the considerable relevance of miRNAs in gene expression, these tiny RNA molecules have recently been called "master regulators of gene expression" (6).

The biogenesis of miRNAs has been extensively studied (7–9). For instance, genes encoding miRNAs show distinct genomic locations, such as intergenic, intronic, exonic, or mirtronic (a type

of miRNA that is located in the introns of the mRNAs). Genes encoding miRNAs are transcribed in the nucleus in the form of long primary transcripts (pri-miRNAs) typically, although not exclusively, by RNA Pol II (10). Afterward, pri-miRNAs are processed into a small stem-loop transcript of approximately 55-70 nucleotides by the RNA-binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) and Drosha (a ribonuclease III enzyme) (11, 12). This new structure, termed pre-miRNA, is recognized by Exportin 5 (Exp 5) and is exported from the nucleus to the cytoplasm (13). Once in the cytoplasm, pre-miRNA hairpins are cleaved by the Dicer RNase III enzyme and TRBP (TAR RNA-binding protein), resulting in a ∼22 nucleotide mature miRNA-miRNA* duplex (14–16).

Finally, the mature miRNA is loaded onto Argonaute 2 protein (AGO2) and the RNA-induced silencing complex (RISC) to catalyze site-specific cleavage or translational repression of their mRNA targets (17, 18). The post-transcriptional regulation of gene expression by miRNAs is of paramount importance, thus, it is estimated that miRNAs could regulate nearly 60% of all human protein-coding genes (19). miRNAs are involved in several cellular processes, such as proliferation, development, differentiation, apoptosis, carcinogenesis, and energy metabolism (20–26).

During tumorigenesis, dysregulated metabolism represents an adaptive advantage of cancer cell that promote uncontrolled cell division, cell growth, and survival (27, 28). One of the best characterized metabolic disorders during cancer development is the Warburg effect, that increase glucose uptake and lactate production. During the Warburg effect, miRNAs activity contributes to keeping high levels of glycolysis. miRNAs also control other crucial steps of the energy metabolism, including glucose transport, glycolysis, tricarboxylic acid cycle, glutaminolysis, altered lipid metabolism, insulin production, cholesterol, and lipid homeostasis, as well as amino acid and nucleotide biogenesis (29–33).

In this review, we focus on the different roles of miRNAs in cancer metabolism and discuss novel miRNA-based strategies designed to target different processes in human cancer. We also explore the links between microbiota and miRNA networks and cancer, with a particular focus on genotoxicity and tumor-metabolism.

METABOLIC REPROGRAMMING IN CANCER CELLS

Upon cancer onset and progression, cells exhibit various growth, proliferation, and survival phenotypes. These cancer hallmarks are supported by a catabolic and anabolic metabolism reprogramming. Increasing evidence has shown that metabolic changes are the result of complex processes, and several cellular pathways are implicated (34–36). Recent findings have led to a significant shift in our understanding of altered metabolic states, which now are seen as a central transformative force in cancer development (37–39).

The Warburg effect is thought to be an early event in cancer that promotes rapid adaptation to higher bioenergetic demands, such as, excessive proliferation and hypoxic microenvironments. Warburg effect is characterized by: (a) supports the demand for ATP synthesis and promotes its flux into biosynthetic pathways to achieve an uncontrolled proliferation; (b) maintains an acidic microenvironment via the accumulation of lactate; and (c) allows for ROS signaling homeostasis (40–43). Moreover, reprogramming energy metabolism promotes tumor cells to use alternative carbon sources such as glutamine, considered to be the second source of nutrients after glucose. Glutamine is the most abundant amino acid in cells, and its catabolism results in several amounts of cellular precursors, including glutamate, aspartate, pyruvate, lactate, alanine, and citrate (44–46).

For many years, the Warburg effect was considered as a synonym for metabolic reprogramming. However, it is clear that this phenotype alone cannot explain all the metabolic alterations that enhance the formation of primary tumors and their development throughout invasion and metastasis. Recent publications have also reported the metabolic interactions between tumors and the microenvironment involving cancer-associated fibroblasts, immune cells, and microbiota, which allows us to expand our understanding of the metabolic reprogramming and reveals the complex interaction networks required to establish the tumor phenotype (47–49). Most of the aforementioned metabolic features are a consequence of the deregulation of several cell pathways and often involve altered oncogenes, tumor suppressors, and miRNA.

miRNAs REGULATION OF METABOLIC PATHWAYS IN CANCER

In the last decade, a growing volume of evidence has revealed the role of miRNAs in the regulation of energy metabolism, directly, through the regulation of glucose transporters (GLUT family), enzymes (hexokinase 1/2, Aldolase A), and protein kinases (AMPK, PI3K), or indirectly, through inhibition of several transcriptional factors (p53, c-Myc) (50–52). In any case, the role of miRNAs in the regulation of energy metabolism has gained much interest by their nature to modulate cellular metabolism and the possibility to use miRNAs-targets genes circuits as cancer therapies. Therefore, we review the main pathways of energy metabolism, the genes involved in each metabolic signaling and their transcriptional landscapes articulated by the miRNAs in cancer programs.

miRNAs AND GLUCOSE TRANSPORTERS

Glucose represents the main source of cellular energy. In cancer, tumor cells increase their glucose consumption to maintain the high energy requirements. However, due to the hydrophilic composition of glucose, it is not able to cross the plasma membrane by its own. To overcome this situation, tumor cells induce the expression of several members of the glucose transporters family (GLUTs, also named SLC2A proteins). Glucose transporters are membrane-associated carrier proteins responsible for facilitating the transport of glucose across the plasma membrane. In the human genome, 14 GLUT proteins

have been found. Among different members of the GLUTs family, the expression of GLUT1, GLUT2, and GLUT3 has been reported to be upregulated in different types of tumors, whereas GLUT4 and GLUT5 are downregulated (53, 54). miRNAs control glucose uptake by regulating the GLUTs expression; for example, miR-144 and miR-132 are two miRNAs that have been associated with the regulation of GLUT1, one of the most broadly expressed isoforms in various cell types. Lui et al. reported that the downregulation of miR-144 induces an increase in glucose uptake in lung cancer (55). Moreover, Qu et al. demonstrated that the decrease in miR-132 expression altered glucose metabolism in prostate cancer (56). Additionally, miR-150 has been reported as a GLUT1 regulator in CD4+ cells (57). In renal cell carcinoma, miR-138, miR-150, miR-199a-3p, and miR-532-5p overexpression are associated with a decreased expression of GLUT 1, whereas miR-19a, miR-19b, miR-130b, and miR-301a decrease are directly associated with an over-expression of GLUT 1 (58).

GLUT3, another member of the glucose transport proteins family, is also regulated by miRNAs. Fei et al. demonstrated that miR-195-5p directly regulates the expression of GLUT3, and consequently decreases glucose uptake and inhibits cell growth in T24 bladder cancer cells (59). Similar results were reported by Dai DW in U251 and LN229 glioblastoma cells through the activity of miR-106a over GLUT3. Additionally, the authors indicated that miR-106a down-regulation is associated with glioblastoma patients survival (60).

Other examples of miRNAs that regulate glucose uptake are miR-233 and miR-133, which directly regulate the expression of GLUT4 (26, 61). Interestingly, miR-21 and miR-23a indirectly regulate the expression of GLUT4, as a result of their regulation over two GLUT4 translocators: PTEN and SMAD4 (62, 63). An exhaustive work published by Esteves et al., highlight the role of miR-21a-5p, miR-29a-3p, miR-29c-3p, miR-93-5p, miR-106b-5p, miR-133a-3p, miR-133b-3p, miR-222-3p, and miR-223-3p that directly or indirectly regulate the expression of GLUT4 (64). To our knowledge, there are no reports describing other members of the GLUT family regulated by miRNAs, although miRNA target prediction analysis identifies a set of miRNAs capable to silence them; however, further studies are needed to determine their contribution to aberrant tumor cell metabolism.

miRNAs IN GLYCOLYSIS

Unlike tumor cells, normal cells obtain energy in the form of ATP through the glucose-derived pyruvate by the mitochondrial oxidative phosphorylation. Conversely, regardless of oxygen conditions, tumor cells prefer anaerobic glycolysis, a less efficient process for obtaining ATP that produces large amounts of lactate. To compensate for this apparent decrease in energy flow, tumor cells increase glucose uptake and trigger alternative pathways to metabolize alternative carbon sources, such as glutamine, and some amino acids, such as arginine and glycine. This change in the energy metabolism confers several advantages to tumor cells, in addition it also provides necessary biomolecules for the high rates of cell division (65, 66).

During the first step of glycolysis, glucose is transformed into glucose-6 phosphate through the phosphorylation of the 6-hydroxyl group of glucose by the enzyme hexokinase (HK). The hexokinase family of enzymes comprises four isoforms (HK1–HK4) (67–69). Isoform 2 (HK2) has been reported to be upregulated in a wide variety of tumors (70–72).

One of the first works that demonstrated the regulation of miRNAs on the HK2 enzyme was published by Fang et al. Interestingly, they demonstrated that miR-125a and miR-143 regulate HK2, which modifies glucose metabolism and cell proliferation in lung cancer cells (73). This finding was confirmed by Peschiaroli et al. in head and neck squamous cell carcinoma (HNSCC)-derived cell lines (74), and by Gregersen et al. in colon cancer cells (75). Another miRNA, miR-199a-5p, regulates HK2 expression and has been reported to be under-expressed in liver cancer cells. Remarkably, overexpression of HIF1α decreased miR-199a-5p expression, which promotes glycolysis and lactate production (30). In stomach cancer cells, miR-181b directly inhibits the expression of HK2 and causes a decrease in glucose uptake and lactate production (76). In addition, miR-155 has also been reported as a regulator of the expression of HK2. Jiang et al. demonstrated that miR-155 regulates the expression of HK2 by two different mechanisms. First, miR-155 promotes the indirect transcription of HK2 through the activation of STAT3, a transcriptional activator of HK2. Second, miR-155 regulates the expression of C/EBPβ, a transcriptional activator of miR-143, whose overexpression is related to the inhibition of HK2 (77).

A couple of works showed that the enzyme responsible for catalyzing the second reaction of glycolysis, glucose-6-phosphate isomerase (GPI), is regulated by miR-200 in breast cancer cells (78) and by miR-302b and miR-17-5p in chicken primordial germ cells (79). Another glycolytic enzyme regulated by miRNAs is phosphofructokinase 1 (PFK1). PFK1 is the main regulatory enzyme for glycolysis; it catalyzes the phosphorylation reaction of fructose-6-phosphate to convert it into fructose-1,6-bisphosphate. In this sense, Yang et al. demonstrated that miR-135 targets PFK1, inhibits aerobic glycolysis, and suppresses tumor growth (31).

Similarly, Aldolase A, a glycolytic enzyme that catalyzes the conversion of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP), is targeted by several miRNAs. Among the miRNAs that have been reported to regulate Aldolase A expression are the following: miR-122 in liver cells (80), miR-15a and miR-16-1 in leukemia (81), and miR-31 and miR-200a in Y79 retinoblastoma cells (82).

The expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has been widely used for normalizing quantitative gene expression experiments. GAPDH catalyzes the sixth reaction of glycolysis, where a molecule of NADH is released. Like other enzymes in glycolysis, GAPDH is targeted by some miRNAs such as miR-644a (83) and miR-155 (84).

The last reaction of the glycolysis pathway is catalyzed by pyruvate kinase 2 (PKM2) enzyme. PKM2 dephosphorylates phosphoenolpyruvate to produce pyruvate regardless of oxygen concentration. PKM2 has been reported to be over-expressed in many tumors due to the dysregulation of various miRNAs that down-modulate it. Some of the miRNAs reported to directly

regulate the expression of PKM2 are miR-133a, miR-133b, miR-326, and miR-122 (85–87) whereas those that indirectly regulate it are miR-99a, miR-124, miR-137, and miR-340 (88, 89).

miRNAs INVOLVED IN LACTATE METABOLISM

In tumors, after the glycolysis phase, pyruvate is converted into lactate by the lactate dehydrogenase enzyme (LDH). Some works have reported increased levels of LDH and its correlation with tumor aggressiveness (90–92). Interestingly, LDH expression is also regulated by miRNAs. For instance, miR-375 regulates the subunit B of LDH (LDHB) in maxillary sinus and esophageal anaplasias (93). In addition, subunit A of LHA (LDHA) has been reported to be regulated by miR-34a, miR-34c, miR-369-3p, miR-374, miR4524a/b, miR-323a-3p, miR-200c, miR-30d-5p, and miR-30a-5p in breast cancer cells and osteosarcoma tissues, which induces a decrease in glycolysis, lactate production, ATP generation, and cell proliferation (94–99).

Lactate fluxes are mainly maintained by monocarboxylate transporter (MCTs). MCTs are membrane proteins acting as carriers for lactate, pyruvate, and ketone bodies. Up to now, four MCT isoforms (MCT1, MCT2, MCT2, and MCT4) have been described in humans, and each of them exhibits a distinct cellular distribution (100). In the same way as LDH enzymes, lactate carriers (MCT proteins) are regulated by diverse miRNAs. For example, MCT1 is targeted by miR-29a, miR-29b, miR-124, and miR-495 in pancreatic β cells (101, 102). Another MCT1-regulatory miRNA is miR-342-3p, which promotes alterations in lactate and glucose flows. In addition, miR-342-3p over-expression significantly decreased cell proliferation, viability, and migration in breast cancer cell lines (103). MCT4, another member of the family of lactate transporters, is regulated by miR-145, which causes the accumulation of lactate within tumor cells in hepatocellular carcinoma cells (HCC) (104).

miRNAs INVOLVED IN GLUTAMINE METABOLISM

Glutamine metabolism (glutaminolysis) represents the second source of nutrients in cancer cells. Actually, high rates of glutaminolysis are necessary for metabolic reprogramming as it provides substrates for increased lipogenesis and nucleic acid biosynthesis that are critical to preserve the high proliferation rates of tumor cells (105, 106). Glutaminolysis converts glutamine into TCA cycle metabolites through the activity of multiple enzymes. First, glutamine is transported into the cells by solute transporters SLC1A5 and SLC7A5. Once inside the cell, glutamine is converted into glutamate and later into alpha-ketoglutarate (α-KG) by glutaminase (GLS), glutamate dehydrogenase (GDH, and other enzymes, such as glutamate pyruvate transaminase (GPT) for alanine production and glutamate oxaloacetate transaminase (GOT) for aspartate production. In addition, glutaminolysis produces considerable amounts of succinate, fumarate, malate, NADH, and ATP molecules. The transport of glutamine into the cell is strictly regulated by the membrane protein SLC1A5 (also called ASCT2 protein). SLC1A5 and other members of the ASC solute transporters family have been reported to be overexpressed in a wide variety of tumors. Dong J et al. showed that the exogenous expression of miR-137 and miR-122 markedly inhibited the SLC1A5 expression in a dose-dependent manner therefore altering tumor glutamine metabolism (107).

In a well-conducted work, Gao et al. demonstrated that the repression of miR-23a and miR-24b by the oncogenic transcription factor c-Myc resulted in a greater expression of GLS proteins and led to the upregulation of glutamine catabolism (108). Another miRNA reported to regulate GLS protein expression is miR-203, which additionally sensitizes malignant melanoma cells to temozolomide chemotherapy (109). Expression of glutamate cysteine ligase, the rate limiting enzyme of glutathione (GSH) synthesis, is attenuated by miR-18a in liver cancer (110) and by miRNA-153 in glioblastoma (111). Additionally, miR-450a limits the metastatic potential of ovarian cancer cells by targeting a set of mitochondrial mRNAs to reduce glycolysis and glutaminolysis (112).

miRNAs REGULATION OF OXPHOS

Oxidative phosphorylation (OXPHOS) is a metabolic pathway combining two cellular processes to generate energy in the form of ATP. First, in an oxidative stage, the electron donors such as NADH and FADH2 are oxidized by the electron transport chain that turns the released energy into a proton gradient across the mitochondrial inner membrane. In the second stage, phosphorylation, ATP synthase uses the proton gradient to phosphorylate ADP to ATP. OXPHOS involves a system of protein complexes with oxidoreductase functions (complex I–IV) and ATP synthase (complex V). Even though OXPHOS is the most efficient way to produce cellular energy, tumor cells prefer to metabolize glucose via aerobic glycolysis. Several studies have recently indicated that, contrary to what is generally accepted, tumor cells could alternate between these two processes, OXPHOS and aerobic glycolysis, depending on the tumor microenvironment (113–115).

Interestingly, it has been proposed that several miRNAs regulate OXPHOS by inducing the inhibition of many components of the electron transport chain. For instance, miR-210 regulates the activity of the mitochondrial complex I (NADH: ubiquinone oxidoreductase) via the iron-sulfur cluster assembly enzyme (ISCU) by reducing the availability of iron and sulfur ions (116). Another study published by Muralimanoharan et al. revealed that miR-210 overexpression significantly reduces the complex III expression of the electron transport chain (ubiquinone:cytochrome c oxidoreductase) (117). Cytochrome c oxidase (complex IV), another enzyme of the electron transport chain, is also regulated by miRNAs. The following miRNAs have been reported to regulate cytochrome c oxidase: miR-181c (118), miR-338 (119), and miR-210 (117).

Finally, ATP synthase (complex V), a transmembrane enzyme that catalyzes ATP synthesis from an ADP molecule, is also regulated by miRNAs. Willers et al. reported that miR-127-5p

reduces the expression of the catalytic subunit of ATP synthase (β-F1 subunit) in BT-549 cells in breast cancer (120). Another miRNA, miR-141, reduces the activity of ATP synthase by reducing SLC25A3 proteins (121).

miRNAs REGULATION OF TRANSCRIPTION FACTORS AND SIGNALING PATHWAYS

miRNAs are also capable of modulating metabolic reprogramming through regulating various transcription factors relevant in metabolic pathways (122). The metabolic shift of tumor cells may be a potential strategy to evade programmed cell death and triggers cell survival and growth by activating oncogenes, such as RAS, MYC, and p53 (51, 123–126). Tumor metabolic reprogramming seems to be influenced by oncogenes and tumor suppressor networks. For example, phosphatidylinositol 3-kinase (PI3K), a lipid kinase that regulates the levels of phosphorylated phosphatidylinositol at the plasma membrane and enhances glucose uptake and glycolysis in cancer cell metabolism, is targeted by miR-320, miR-123a, miR-422, miR-506, and miR-136 (127). Catanzaro et al. showed evidence that downregulation of miR-139-5p in pediatric low-grade gliomas drives cell proliferation by regulating PI3K/AKT signaling (128). Furthermore, miR-33a/b, targets metabolic enzymes involved in fatty acid metabolism and the AMPK pathway, whereas miR-29b targets amino acid catabolism, which regulates cancer cell metabolism and biogenesis to support tumor growth and proliferation (61, 129–131). Like PI3K, AKT, and mTORC1, the MYC transcription factor has important metabolic roles beyond enhancing glycolysis. MYC promotes mitochondrial gene expression and mitochondrial biogenesis. MYC mainly depends on glutamine as a carbon source for mitochondrial metabolism (132). The oncogene MYC can bind to the promoter region of other oncogenes such as some miRNAs; for example, miR-9 is frequently upregulated in glioma specimens and cells, and it could significantly enhance proliferation, migration, and invasion of glioma cells (133).

On the other hand, miRNAs regulate important signaling pathways in mitochondria by triggering adaptive mechanisms to optimize the oxidative phosphorylation concerning the substrate supply and energy demands. For example, exogenous exosomes carrying miRNAs can induce metabolic reprogramming by restoring respiration in cancer cells and thus suppressing tumor growth. The exosomal-miRNAs involved in the modulation of cancer metabolism may be used for better diagnoses and therapies (134, 135).

Hypoxia-inducible factor 1 (HIF-1), another pathway related to tumor metabolism, is also regulated by miRNAs. HIF-1 activation can stablished oncogenic signaling by promoting glycolysis of cancer cells; but also, an alternative mechanisms over the glucose carbon mitochondrial metabolism confers HIF-1 a tumor suppressor role in some types of cancer (136, 137). In this way, miR-125-5p, miR-33-5p, and miR-190-5p are known to target the master regulator of oxygen deprivation response, HIF-1 (138). On the other hand, HIF-1 is a key molecule in adapting cancer cells to the reduced oxygen availability in the microenvironment (139–141). HIF-1 induces metabolic reprogramming as it upregulates genes such as HK1, HK2, LDHA, PDK1, GLUT1, and GLUT3, which enhance lactate production through the glycolytic pathway (142, 143). HIF1 also influences the activity of the pentose phosphate pathway, nucleotide biogenesis, angiogenesis, and suppresses the mitochondrial function (144, 145).

Finally, the oncogene c-MYC regulates HIF1 expression regardless of oxygen levels, and both act in concert to "fine-tune" adaptive responses during tumor growth (146–149). Moreover, it has been reported that more than 50% of tumors have mutations in the tumor suppressor p53, which leads to metabolic changes and contributes to the Warburg effect through the upregulation of c-MYC, HIF-1, and a broad range of genes involved in other aspects of cancer biology, including tumor cell survival and proliferation, migration, drug resistance, and immune evasion (51, 150, 151). The advance in molecular biology techniques has allowed us to detect how a diversity of miRNAs regulate tumors metabolism, as we show in **Table 1** and **Figure 1**.

DRUGGABLE miRNA-METABOLIC NETWORKS WITH POTENTIAL VALUE FOR CANCER THERAPY

The unveiled connection between cancer profiles and metabolic reprogramming shed light on the reassessment of metabolism-targeting pharmacologic therapies as potential opportunities in cancer. Alterations in key miRNA regulatory networks contribute to the oncogenic transformation of cancer cells through genes involved in the metabolic switch (163). New insights into the altered tumor metabolism have provided novel therapeutic strategies that are being evaluated in preclinical models or clinical trials as effective therapies for many human cancers (164). Pharmacological targeting of altered miRNAs may have therapeutic effects by suppressing relevant cancer signaling pathways without affecting normal cells (165). Furthermore, pleuritic effects of metabolic drugs include miRNAs modulation that impairs signaling pathways and regulates cell energy production, which reveal miRNAs as potential drug targets.

Numerous studies now suggest that drug repurposing, which is the discovery of new therapeutic indications for known drugs, represents an attractive route in drug harnessing in cancer. Unlike the development of new molecules, drug repurposing identifies new uses for existing drugs that already have clinical and safety descriptions (166). Repurposing drugs with an oncological and non-oncological primary purpose, such as metabolic-based drugs, might be an attractive strategy to offer more effective treatment options to cancer patients and faster translate the research knowledge into the clinics (167). Interestingly, a growing body of evidence has shown that many of the antineoplastic effects and improved responses to these metabolic-based drugs may be mediated through induction of tumor suppressor miRNAs and suppression of oncogenic miRNAs.

TABLE 1 | Main miRNAs that regulate cellular metabolism in different types of cancer.

miRNA	Location	Cancer type	Target gene/pathway	References
miR-125a	19q13.41	Hepatocellular carcinoma	HK2	(152, 153)
miR-192/215-5p	11q13.1, 1q41	Colorectal cancer	ZEB1 and ZEB2, Type I collagens	(104)
miR-140-3p	16q22.1	Chronic myeloid leukemia	SIX	(154)
miR-140-3p	16q22.1	Spindle cell oncocytomas	TCA, carbohydrate, lipid metabolism	(155)
miR-940	16p13.3	Glioma	MTHFD2	(156)
miR-139-5p	11q13.4	Pediatric low-grade gliomas	PI3K/AKT signaling	(128)
miR-151a-5p	8q24.3	Malignant pleural mesothelioma	FASN, OXSM, ACACB	(157)
miR-361-5p	Xq21.2	Prostate cancer	Sp1/PKM2 axis	(158)
miR-7, let-7a, miR-34a and miR-143	9q21.32, 9q22.32, 1p36.22; 1p36.22, 5q3	Glioblastoma	Critical regulators of aerobic glycolysis	(159)
miR-125	19q13.41	Hepatocellular carcinoma	HK2	(160)
miR-122	18q21.31	Hepatocellular carcinoma	PKM2 and represses glycolytic metabolism	(161)
miR-126	9q34.3	Mesothelioma, hepatocellular, pancreatic and breast cancer	Insulin receptor substrate-1 (IRS1)	(134)
miR-195-5p	17p13.1	Bladder cancer	GLUT-3	(59)
miR-155	21q21.3	Breast cancer	miR-143	(77)
miR-378	5q32	Breast cancer	ERRγ and GABPA	(162)

In this section, we describe existing evidence of molecules with biochemical mechanisms impairing tumor metabolism. These molecules appear as the most promising repurposing and *de-novo* pharmacological interventions as shown by preclinical and clinical studies. Particular emphasis was put on chemo-resistance, which is recognized as a critical cause of treatment failure. It is reported that dysregulations of miRNAs contribute to therapy resistance via drug efflux mechanisms, alterations in drug targets, energy metabolism, DNA repair pathways, evasion of apoptosis, cell cycle control, among others (6, 168, 169). We briefly described below some pharmacologic therapies employed in different metabolic-related diseases and how they could selectively target metabolic pathways in cancer cells and modulate miRNAs networks, we will also comment some of the most relevant evidence of each of the metabolic therapeutically intervention and its anti-carcinogenic properties via miRNA activity. A more extensive over-view of miRNA expression portraits modulated by pharmacological treatment, as well as cooperative or resistance phenotypes toward drug activity is listed in **Table 2** and **Figure 2**.

TARGETING GLUCOSE METABOLISM: METFORMIN

Metformin, a commonly prescribed drug for treating type 2 diabetes, inhibits the mitochondrial complex I that impairs respiration, which results in a systemic impede of glucose uptake and neoglucogenesis (217–220) that reduces blood glycemia and insulinemia in hyperglycemic/diabetic patients. The tumor-suppressing effect of metformin has been reported in epidemiological studies describing a statistical association between metformin use and improved clinical outcomes in cancer (221–224). One striking example of this onco-suppressive feature is the cooperative effect of metformin and neoadjuvant chemotherapy to achieve complete tumor regression in some breast cancer patients (225). Although the precise anti-tumorigenic mechanism of action is not well-described, recent studies have shown that metformin can partially direct mitochondrial complex I inhibition, reduce NADH oxidation, and increase AMP/ATP ratio in tumors, with the consequent inhibition of mTOR signaling and decrease of fatty acid and cholesterol synthesis (218, 220, 226). Thus, metformin favors a catabolic process over an anabolic one in tumor cells. Overall, this metabolic pressure causes proliferation decline and triggers apoptosis in cancer cell lines [(227); **Table 2** and **Figure 2**].

A variety of evidence, both *in-vitro* and *in-vivo* along with epidemiological studies, supported the protective effect of metformin against cancer development (228–231). Even more, the role of metformin on cancer not only fall in limiting its incidence, but also as a novel therapeutically intervention as shown by the 335 registered clinical trials that have evaluated patients benefit of incorporate Metformin in their treatment. The

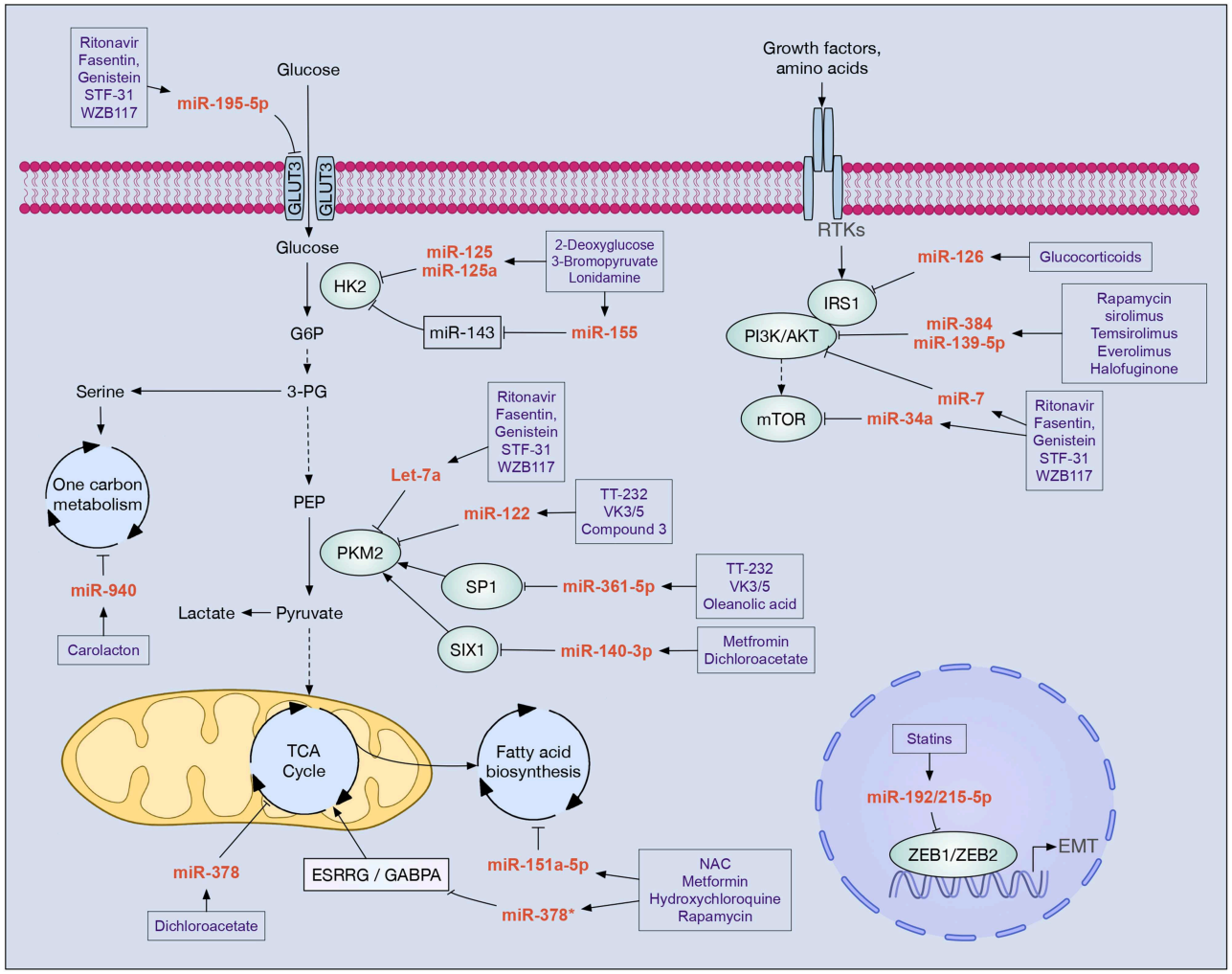

FIGURE 1 | Drugs with clinical potential in cancer that modulate miRNAs implicated in cell metabolism. In boxes are shown drugs that potentially modulate the main miRNAs involved in the metabolic reprogramming of tumor cells. Increased glycolysis flow, alteration of the PI3K/AKT/mTOR pathway, and epithelial-mesenchymal transition (EMT) are key processes that allow tumor cells to reprogram their metabolism in order to survive, proliferate, migrate, and evade new niches. Different miRNAs participate in these processes inhibiting the expression of enzymes (e.g., HK2, PKM2, IRS1, PI3K, AKT, mTOR), transcription factors (e.g., SP1, SIX1, ZEB1, ZEB2, GABPA), and cellular receptors (e.g., GLUT3, ESRRG).

underlying mechanism of the anticancer activity of Metformin can be partially explained through its ability to modulate miRNA expression, activity and biogenesis in a variety of tumor types (**Table 2** and **Figure 2**). For instance, overexpression of the tumor suppressors let-7, miR-26, and miR-200 family members has been reported in the literature as a pleuritic effect of Metformin molecular activity in breast, colorectal, pancreatic, oral and renal cancer. Briefly, Metformin up-modulates let-7a, that epigenetically inhibits the oncomiR miRNA-181a, which actively participated in the epithelial-to-mesenchymal transition, thus, abrogating this aggressive phenotype in BRCA (170). In CRC, the metabolic drug overexpress let-7, miR-200b/c, and miR-26a that limit the stem-like phenotype, which has been linked to poor clinical outcomes (171). Consistently, in pancreatic tumors Metformin induces the expression of miR-26a and let-7c miRNAs reducing cell proliferation, invasion, and migration. Particularly, miR-26a down-regulates the oncogene HMGA1 contributing to the observed phenotype (172). Studies in oral cancer cell models reveal that Metformin significantly increases miR-26a levels which directly decreases Mcl-1 expression that enhances apoptotic rates and reduces tumor-cell viability (173). Finally, in renal carcinoma Metformin treatment limits cell proliferation by miR-26a up-modulation that in turn down-regulates Bcl-2, cyclin D1 and upregulates the tumor suppressor PTEN, which all together influence cell cycle and cell death (174).

TARGETING AEROBIC GLYCOLYSIS: PDK INHIBITORS

Dichloroacetate (DCA, PDK inhibitor) is a small molecule that inhibits the pyruvate dehydrogenase kinase (PDK) and

TABLE 2 | miRNAs target by metabolic-drugs or miRNAs related to therapy resistance.

Drug	Druggable miRNA/Therapy-resistance miRNA*	Cancer	References
Targeting glucose metabolism			
Metformin	↑let-7a, let-7b, miR-26a, 101, 192, 200b and 200c. Over-expression of miR-26a decrease cancer stem-cells markers, an enhanced apoptosis rate. Let-7b re-expression blocks stem cells features	PC BRCA Oral Renal	(170–174)
	↑miR-34a in obese mice reducing its putative targets (Notch, Slug, Snail) ↑miR-34a which in turn restrict Sirt1/Pgc1α/Nrf2 signaling pathway and decrease proliferation rates	PC	(175) (176, 177)
	↓miR-27a which AMPKα and ↑miR-193 family that increased AMPKα and decrease FASN levels, resulting in limiting mammospheres phenotype	BRCA	(178, 179)
	Combined treatment of metformin + FuOx ↓miR-21 and ↑miR-145, that suppress β-catenin and c-Myc signaling expression colon cancer cells	CRC	(180)
	↑miR-141, 200a, 205 and 429 inhibiting EMT, thus, modulating metastatic traits	GC	(181)
	↑mir-124, 182, 27b, let7b and ↓miR-221 and 181a; inhibiting cell proliferation	CLC	(182)
	↑miR-192-5p, 584-3p, and 1246; suppressing cell motility and cell cycle	M	(183)
	↑DROSHA, modulate the miRNA biogenesis, to affect these miRNAs expression	CLC	(182)
	↓miR-222 resulting in enhance abundance of p27, p57, and PTEN ↓miR-222 resulting in enhance abundance of p27, p57, and PTEN	Lung	(184)
	↑DICER expression and miR-33a that targets c-MYC	BRCA	(185)
	↓miR-146a, 100, 425, 193a-3p and 106b involved in cell migration, invasion and proliferation	PCA	(186)
	↑miR-192-5p, miR-584-3p, and miR-1246 enhance EFEMP1 and SCAMP3 downmodulation favoring the suppression of cancer cell motility and growth through G2/M cell cycle arrest and cell apoptosis	M	(183)
	RS:↑miR-21 ↓miR-21 and ↑miR-145 over combined treatment with 5-fluorouracil and oxaliplatin, that suppress β-catenin and c-Myc expression, and consequently reduce cell growth and sphere formation ↓miR-21-5p in cell lines model, xenograft murine model and in tissue from human patients. Since also the pre-miRNA sequence is down-modulated the modulation seems to be at the transcriptional level. Functional reduction of miR-21-5p allow the expression of upstream activators of the AMPK, CAB39L and SESN1	CRC BRCA	(187) (180) (188)
Dichloroacetate (DCA)	Promising therapeutic agents to ↓miR-210	Cancer	(189)
	↑miR-375 resulting in anti-proliferating effects	PCA	(190)
CPI-613	May improve miR-497-5p,−449a,−25-3p,−6838-5p,−520d-3p that down-modulates the expression of Cyclin D3, E1, E2, F, A2, B1 and CDK2 genes of BxPC-3	Cancer	(189)
Targeting FA metabolism			
Statins	Lovastatin upregulated miR-33b expression, reduced cell proliferation and impaired c-Myc expression	MB	(191)
	Simvastatin: inhibits the growth of human CRPC cells by suppressing NF-κB and LIN28B and ↑let-7 miRNA family	PCA	(192)
	Simvastatin: ↓miR-34a, which regulates the NAD+-dependent histone deacetylase SIRT1. ↑miR-612, which is known to reduce stemness	BRCA, PCA, OsC	(193)
	Simvastatin is an activator of miR-192 which subsequently led to suppressed proliferation, migration and invasion	CRC	(194)
	Atorvastatin: ↑miR-182 that targets the anti-apoptotic Bcl-2 and p21	PCA	(195)
	↑miR-140-5p activating the transcription factor NRF1 that reduced cell proliferation and induced apoptosis	BRCA	(196)
	Fluvastatin: ↓miR-140-3p-1 and its downstream pathway such as cell growth	BRCA	(197)
	Statin: ↑miR-33a promoting a proliferation inhibitory effect	PCA	(198)
	lovastatin: ↓miR-133a promoting GCH1 important for endothelial nitric oxide synthase	Cancer	(199)
Rapamycin	Rapamycin-dependent miRNA: ↑miR-29b, 21, 24, 221, 106a, and 199a	Renal	(200)
	↑let-7, miR-125a,−125b,−21, and−26a. Rapamycin is mediated by let-7 family with anti-proliferative effects	Renal	(201)
	*RS: miR-21 supports mitochondrial function and adaptation to rapamycin	Renal	(200)
	Long-term rapamycin treatment RS: ↑MYC that results in ↑miR-17–92	Brain	(201)

(Continued)

TABLE 2 | Continued

Drug	Druggable miRNA/Therapy-resistance miRNA*	Cancer	References
Aspirin and non-steroidal anti-inflammatory agents	↑miR-98 that targets WNT1, suppressing cell proliferation	Lung	(202)
	Sulindac drug: ↓miR-9,−10b,−17, and−21 by suppressing NF-κB-mediated transcription of miRNAs	BRCA CRC	(203)
	↓miR-21 decreasing cell proliferation and invasion upon inactivation of β-catenin/TCF4 signaling	CRC	(204)
	↑let-7 by decreasing the miRNA-sponge H19, resulting in the down-modulation Hypoxia-inducible factor 1α reducing I PDK1, attenuating glycolysis	BRCA	(195)
	Celecoxib: ↑miR-29c supress the oncogen MCL-1 reducing apoptosis	GC	(205)
TVB-2640	miR-15 and miR-16-6: Inhibition of FASN: Agonist effect	BRCA	(206)
Targeting lactate metabolism: LDHA inhibitors			
AZD3965	miR-342-3p: Inhibition of the monocarboxylate transporter MCT1: Agonist effect	BRCA	(103)
Antimetabolite chemotherapeutic agents			
Methotrexate (MTX)	*RS: ↑*miR-24 SNP*results		(207)
	*RS: ↑miR-140	OsC, CRC	(208)
	*RS: ↑miR-215 modulated DTL, a cell cycle-regulated gene	OsC, CRC	(209)
Capecitabine	↑miR-125b-5p ↑miR-137	Cancer	(189)
5-Fluorouracil	↓Relevant oncogenes such as miR-210	HCC CRC OsC	(208, 210, 211)
	↑Relevant tumor suppressor miRs: let-7 family, miR-15b,−16,−23a,−23b, and−200c	BRCA	(189)
	*ES: ↑miR122 through the inhibition of M2 splice isoform of pyruvate kinase (PKM2) *in vitro* and *in vivo*	CRC	(212)
	*RS: ↑miR-21 and−221	BRCA	(213)
	*RS: ↑miR-21,−34,−140	HCC CRC OsC	(212)
Gemcitabine	May impact the expression of 56 relevant miRNAs such as miR-200,−205,−27a,−27b, and let 7 family	Cancer	(214, 215)
	*ES: ↑microRNA-218 by inhibiting the secretion of HMGB1 by PANC-1 cells and the PI3K/Akt pathway	PC	(212)
	*RS: ↑miR-21,−34,−140	PC	(214, 215)
Targeting glutamine metabolism			
Pegylated arginine deiminase (ADI-PEG)	Bioengineered pre-miR-1291 processed to high levels of mature miR-1291. *ER: ↑miR-1291 increases sensitivity to ADI-PEG (trough modulation of ASS1 and GLUT1)	PC	(216)

**Therapy-resistance miRNA. ↑, over-expression; ↓, down-regulation. Therapy-resistance miRNA: RS, reduce sensitivity; ES, enhanced sensitivity. Cancer: BRCA, breast cancer; CRC, colorectal cancer; PCA, prostate cancer; PC, pancreatic cancer; HCC hepatacarcinoma; CLC, cholangiocarcinoma; MB, medulloblastoma; OsC, osteosarcoma; GC, Gastric; M,Melanoma.*

regulates mitochondrial pyruvate dehydrogenase complex that catalyzes the irreversible decarboxylation of pyruvate into acetyl-CoA (232). PDK is overexpressed in several tumors and favors pyruvate conversion into lactate (233). Inhibition of PDK by DCA in cancer cells prompts glucose oxidation, reverses mitochondrial apoptosis, and suppresses tumor growth (234). CPI-613 is a novel anticancer agent (lipoic acid analog) that inhibits PDK through targeting lipoyl-binding pockets and selectively target the altered mitochondrial energy metabolism in tumor cells and produces changes in mitochondrial and redox status, which leads to tumor cells death (232, 235, 236). One of the main clinical challenges in colorectal cancer management is the development of chemoresistance. Interestingly, DCA treatment improve chemosensitivity to 5-fluorouracil. The evidence pointed out that the DCA over-express miR-149-3p which consequently enhanced 5-FU-induced apoptosis. Importantly, miR-149-3p is a post-transcriptional regulator of PDK2 transcript. Thus, DCA treatment overcome chemoresistant phenotype by modulating miR-149-3p/PDK2 axis (237).

TARGETING FA METABOLISM

Several pieces of evidence propose that targeting *de novo* fatty acid synthesis might be effective in the treatment of some cancers. For example, statins, cholesterol-lowering drugs, have been recently related to antitumor, cytostatic, and cytotoxic activity in diverse clinical trials of advanced malignancies (238); however, the studies are still inconclusive. Epidemiological studies have shown that statins lower the risk of presenting lung, breast, bowel, and prostate cancer (239, 240). Furthermore, different preclinical *in-vitro* studies show that statins may produce a variety of antineoplastic responses in cancer cells, including a cytostatic

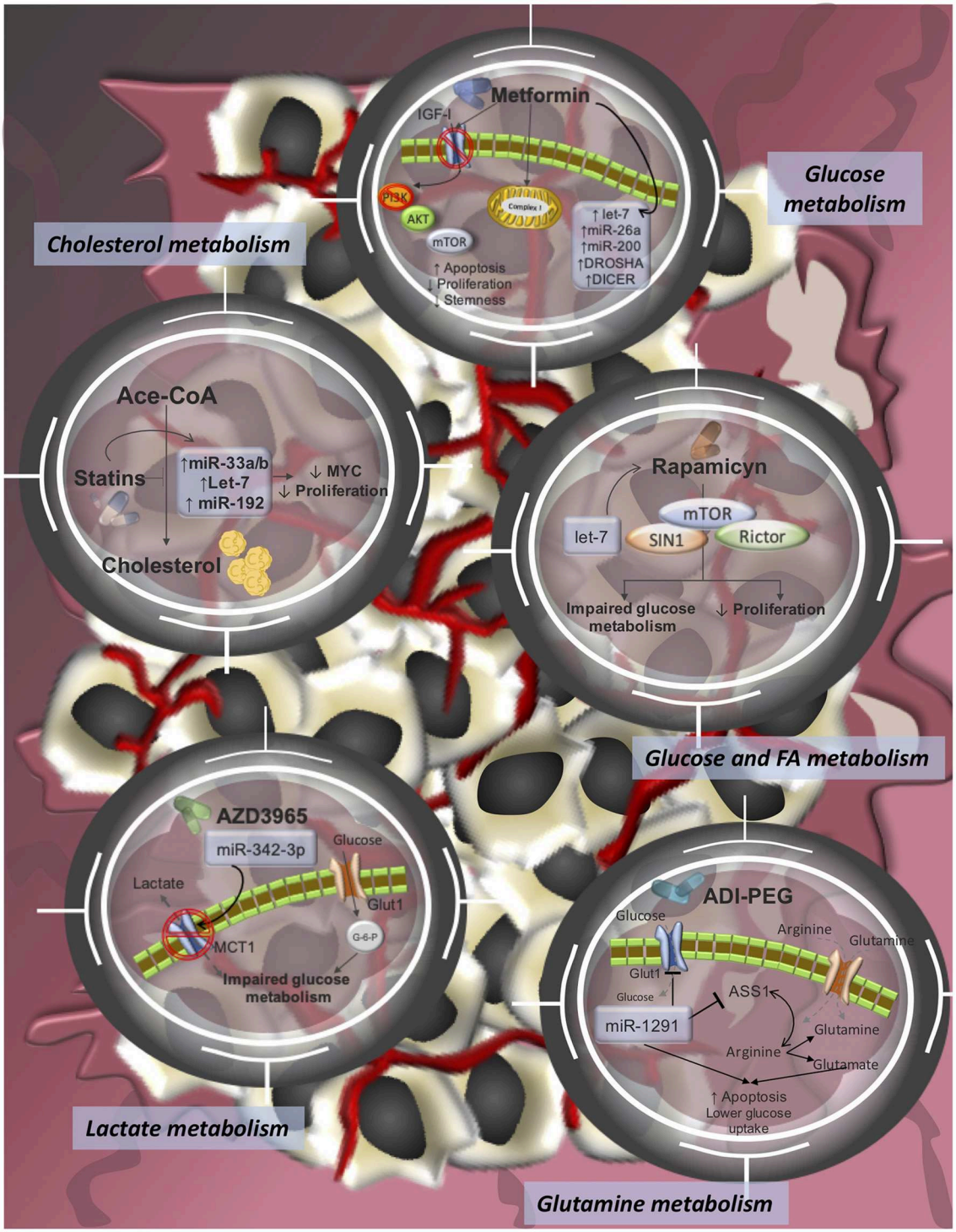

FIGURE 2 | Pharmacological-targeting of tumor metabolism and miRNA-modulating networks of drugs tested in clinical trials or already approved FDA drugs for cancer treatments. It is reported that dysregulations of miRNAs contribute to therapy resistance via drug efflux mechanisms, alterations in drug targets, energy metabolism, Glutamine metabolism, lactate metabolism, cholesterol metabolism, among others.

effect (cell cycle G1/S phase arrest), pro-apoptotic activity by downmodulating BCL-2 (241, 242), anti-metastatic properties through NF-kB and matrix metalloproteinase inactivation (243, 244) and anti-angiogenic properties. Different studies have provided novel evidence of the pleiotropic effects of statins independent to its cholesterol signaling modulation in cancer.

For instance, *in-vitro* assays have shown that more than 400 miRNAs are altered by statins interventions. Including, some well-known tumor suppressor miRNAs such as miR-612, which is up-modulated after statins treatment promoting cancer cell differentiation and enhancing cancer cells response to chemotherapy (193). In another study, miR33a (198), and miR-33b (191) resulted up-modulated and participates in the anti-oncogenic properties of statins by promoting proliferation inhibitory effects and down-regulating the oncogene c-Myc. Another statin-regulated miRNA is miR-182, which down-regulates the antiapoptotic Bcl-2 transcript and consequently favors cell apoptosis (195). In a more complex regulatory circuit, simvastatin reduces NF-κB and LIN28B expression and subsequently increased let-7 levels, that in summary significantly inhibited cell viability and clonal proliferation [(192); **Table 2** and **Figure 2**].

In a different fashion, rapalogs that inhibit mTOR (e.g., rapamycin and its derivatives, everolimus, and temsirolimus) exhibit anti-tumor effects by targeting PI3K/Akt/mTOR axis and cell proliferation. A wide spectrum of tumors is being evaluated in monotherapy or in combination. Temsirolimus and everolimus have been recently approved for the treatment of patients with advanced renal cell carcinoma (245, 246). Since mTOR is also involved in glucose metabolism by stimulating GLUT1, it is reasonable to propose a combinatory therapy with metformin to synergistically kill tumor cells [(247); **Table 2**]. Once again, let-7 family is one of the most reported miRNAs related with Rapamycin mechanism, playing a dual role. In one hand, in short-term treatments the inhibitory effect of rapamycin over cancer cells is mediated by increased expression of let-7 members that regulates c-MYC post-transcriptionally regulates c-MYC. On the other hand, re-expression of let-7 restore rapamycin sensitivity in resistant tumor cells (201). Long-term rapamycin treatment up-modulates miR-17–92 cluster that is related to rapamycin resistance, probably by its positive regulation over c-Myc (201). From a combinatory point of view rapamycin and metformin are able to synergize their activities against cancer cells, since this last one inhibits miR-21-5p which induces signaling of mTOR, a rapamycin-target (188).

Finally, TVB- 2640 compound is one of the first bioavailable fatty acid synthase (FASN) inhibitor to enter clinical trials for breast, colon, and astrocytic tumors, in combination with chemotherapy with the aim of enhancing clinical responses and prolonging stable disease times (NIH). Its antineoplastic activity leads to reduced cell signaling, induces tumor cell apoptosis, and inhibits cell proliferation in tumor cells by restricting lipid signaling, mainly fatty acid production, which is necessary to satisfy tumors metabolic needs [(248–250); **Table 2** and **Figure 2**].

ASPIRIN: ANTI-INFLAMMATORY AND METABOLIC DRUG IN CANCER CELLS

Aspirin, a non-steroidal anti-inflammatory drug (NSAIDs), has shown metabolic and antitumor properties (251). Aspirin may impair tumor cell migration and metastasis through preventing platelet clot formation (252). Aspirin also activates AMPK and inhibits mTOR and FA synthesis in cancer cell lines (253). Recently, aspirin has been demonstrated to have effective anti-tumor effects against RAS/RAF-mutant cells in colorectal cancer by simultaneously affecting BRAF/CRAF dimerization and hyper-activating the AMPK and ERK pathway [(254); **Table 2** and **Figure 2**]. Besides the well-described cardioprotective effects of NSAIDs, there are substantial preclinical, clinical, and observational data that supports its activity in preventing cancer, with strong evidence in colorectal (255), lung (256), and ovarian cancer (257, 258). In preclinical studies NSAIDs administration confer a chemopreventive effect in different cancer cell models and *in-vivo* assays, probably via miRNA modulation. Recently, a novel mechanism of action of aspirin has been reported, in which the drug induces the expression of well-known tumor suppressors miRNAs, such as miR-98 that in turn suppress WNT1 and consequently limits cell proliferation in lung cancer (202). Moreover, NSAIDs favor let-7 expression by decreasing the abundance of one of its ncRNA-sponge, attenuating in this way glycolysis in breast cancer [(195); **Table 2**]. Anti-inflammatory drugs are also able to abrogate the oncogene miR-21, that results in low cell proliferation and invasion rates in BRCA and CRC (203, 204).

TARGETING LACTATE METABOLISM: LDHA INHIBITORS

Several clinical trials evaluating LDHA inhibitors in different solid cancers are currently underway. One mechanism of action of LDHA inhibitors is to limit lactate export from cancer cells into the extracellular space. Accumulating intracellular lactate moves LDHA catalyzed-reaction to produce pyruvate, which prevents NAD+ regeneration and affects the energy source that established a fine competition between cancer cells that resulted in cell death. AZD3965, a drug affecting lactate metabolism, inhibits lactate transporter MCT1, which is overexpressed in several tumors and is associated with poor outcomes (259–262). MCT1 inhibitors probably synergize with the exogenous restauration of miR-342-3p that should provide a more effective inhibition of lactate transportation, which result in loss of cancer cell metabolism homeostasis [(103); **Table 2** and **Figure 2**].

ANTI-TUMORAL THERAPY WITH ANTIMETABOLITE CHEMOTHERAPEUTIC AGENTS

Antimetabolites as chemotherapeutic agents (e.g., methotrexate, capecitabine, 5-fluorouracil, and gemcitabine) are small molecules that resemble nucleotide metabolites; they inhibit the activity of enzymes involved in nucleotide synthesis by preventing cell division and triggering cell death. They are widely used in clinics to treat cancer since neoplastic cells have an increased metabolic demand that requires a huge nucleotide biosynthesis and DNA replication (263). More in detail, methotrexate is a folate analog that inhibits carbon transfer reactions required for *de novo* nucleotide synthesis.

Fluorouracil (5-FU) is a synthetic analog of uracil that inhibits thymidylate synthase by limiting the availability of thymidine nucleotides for DNA synthesis (264) and has been reported that enhances the expression of relevant tumor suppressors such as let-7 family, miR-15b,−16,−23a,−23b, and−200c, some of them well-describe metabolic modulators (189). Moreover, 5-FU represses miR-210 (208, 210, 211), that down-modulates GPD1L, a negative regulator of HIF, restricting HIF-1α stability [(265, 266); **Table 2**]. Similarly, capecitabine is widely used in chemotherapies for gastrointestinal cancers. It halts tumor cells by inhibiting DNA and RNA synthesis and limiting the precursor of thymidine triphosphate (267, 268). Gemcitabine, another nucleoside analog, is intercalated into the DNA molecule and blocks DNA polymerases (269). Notably, the literature reports its effect over several miRNAs such as miR-200,−205,−27a,−27b, and let 7 family [(215, 269); **Table 2**]. All these agents can achieve important clinical responses and lead to complete remission in many cases.

In recent years, there has been substantial attention to the role of miRNAs in regulating metabolic reprogramming. Researchers have tried to reveal the mechanisms that regulate metabolic alterations in tumor cells and identify the interactions (miRNA-mRNA, miRNA-transcription factor, and miRNA-metabolic pathway) that are susceptible of being therapeutically actionable. Although studies are still incipient, robust data have been generated, describing how miRNAs directly or indirectly regulate the dysregulated metabolism of tumor cells. Based on the evidence described in this work, it is appropriate to hypothesize that there are miRNA interactions susceptible of being modulated by therapeutic interventions to reverse the metabolic alterations that allow tumor cells to uncontrollably proliferate. In addition, it is necessary to emphasize the usefulness of miRNAs-based gene therapies to enhance the regulatory activity of those identified miRNAs. However, more studies need to be conducted in a broader spectrum of components of the energetic metabolism of tumor cells, such as enzymes, transcription factors, positive regulators, and enhancers to provide more evidence on the impact of regulation mediated by miRNAs and their signaling networks on oncogenic processes.

NEW DRUGGABLE TARGETS WITH HUGE IMPACT IN CANCER METABOLISM: THE EMERGENCE OF miRNA-BASED THERAPIES

In the last section we discussed how metabolic-target drugs and chemotherapy can modulate miRNA signaling programs as a beneficial pleiotropic effect. But it is also necessary to emphasize the usefulness of miRNAs-based therapies to improve or moderate their regulatory activity. Recent advances have permitted to study the effects of directly manipulating cellular miRNA levels by suppressing the expression of oncomiRs, that somehow enhance cancer metabolism, and which are frequently overexpressed in human cancers. Or on the contrary, by reestablishing the expression of tumor suppressor miRNAs that in many cases collaborate to restrict cancer energetics programs (270). Evidences obtained from these studies have prompted the designing and refinement of dedicated technology aimed at, either, inhibiting miRNAs (i.e., antisense oligonucleotides, locked nucleic acid, antagomiRs, miRNA sponges, and small molecule inhibitors that inactivate mature miRNA sequence in the RISC complex) (271–273) or restoring their levels by mimic sequences that can be recognized by Dicer and Ago2 proteins to be functional. Notable, miRNA delivery systems have been improved during the last years, resulting in robust and more specific devices such as liposomes, adenovirus, adeno-associated virus, EDV nanocell, and nano-particles accompanied with conjugate antibodies (274–276).

Below we briefly describe some examples of clinical trials that have been evaluated the therapeutic impact of targeting miRNAs involved in the regulation of emerging hallmarks of cancer like tumor metabolism, already described in previous sections. For instance, MRX34 was the first miRNA-based therapy undergoing in a clinical trial for cancer treatment, its aim was to re-express miR-34, that regulates LDHA, by introducing a mimic sequence through the lipid carrier NOV40 to treat patients with lymphoma, melanoma, multiple myeloma, liver, small cell lung, and renal carcinoma. Unfortunately, although promising results were observed, the trial was terminated due to severe immune-related reactions developed by some patients (277).

The first completed phase 1 trial evaluated the TargomiR technology, intended for delivering miRNA mimic sequences in vehicles containing bacterially derived minicells and a targeting moiety antibody against EGFR to treat non-small cell lung cancer. A similar example is the MesomiR-1 drug, which reintroduces miR-16, a miRNA that regulates Aldolase A in glycolysis process (278, 279). Another, drug delivery system being evaluated in stage 1 clinical trial is the locked oligonucleotide acid-modified inhibitor for miR-155 (MRG-106), as part of the clinical intervention for cutaneous T-cell lymphoma patients (280, 281). This therapeutic intervention re-expresses miR-155 targets such as miR-143, that negatively regulates HK2 and consequently limits the active glycolytic phenotype (77). Other examples include the new miRNA delivery system from Regulus company named RGLS5579, an anti-miRNA against miR-10b, for patients diagnosed with glioblastoma multiforme (282). Interestingly, under hypoxic conditions, HIF1 upregulates the transcription factor TWIST that results in the induction of the oncomiR miR-10b (283).

A further candidate of miRNA-based therapeutic currently under evaluation by Regulus and Sanofi companies, although not for cancer patient's treatment, is RG-012 which silences miR-21 in patients with Alport syndrome (284). Along the text we widely discussed miR-21 activity as a promoter of the tumoral-metabolism and its role in resistance against metabolic-based drugs. Miragen, another company, maintains also an active phase 1 study for miR-29 mimic (MRG-201) to treat keloid, fibrosis and scar tissue formation (ClinicalTrials: NCT03601052). Importantly, miR-29 is frequently lost in cancer and has been reported to negatively regulates MCT1, a lactate transporter (101, 102).

Lastly, combinatorial therapy strategies have provided successful results to treat cancer since this approach can target several tumor cell survival pathways and establish molecular landscapes to overcome resistance, offering a holistic way

to reduce tumor development and evolution (285). Taking advantage of the technological advances, chemotherapeutic agents can be coordinately administrated with miRNA-based therapeutics to provide synergistic effects and enhance patient response. Since, these examples represent the first generation of miRNA-based therapeutics, there are some challenges and limitations. As an illustration, preclinical experiments in *in-vivo* models have shown low RNA stability, numerous mRNAs targets can be regulated by a single miRNA and different biological effects can be achieved by a miRNA in different tissues (286). Thus, it is important to guarantee tumor-specific delivery and local retention of miRNAs, for example by nanoparticle which facilitates target-specific shipment of miRNAs (286, 287).

PERSPECTIVES: HOW TO TAKE ADVANTAGE OF THE LOCAL AND SYSTEMIC METABOLIC CONTEXT AND ITS CONNECTION TO microRNA REGULATORY CIRCUITS IN CANCER?

In addition to tumoral-intracellular metabolic reprogramming, tumor cells encounter a variety of systemic factors that can influence tumorigenesis and cell metabolism (27, 34, 38, 41, 164, 288). For instance, obesity is a metabolic disorder that promote tumor growth and a connection between obesity and certain cancers, including colorectal, renal, breast cancer, esophageal, adenocarcinoma, thyroid, endometrial, prostate, and leukemia, have been reported in numerous cohort studies (289–292). In recent years, there has been substantial attention to miRNA roles in obesity-linked cancer (293). miRNA regulation programs can modulate adipogenic differentiation by controlling signaling pathways related to its biogenesis, additionally, several miRNAs associated with obesity also have well-described roles in carcinogenesis, thus, their deregulated expression portrait may act as a functional link between obesity and cancer (294–296).

Furthermore, over the last decade, a huge advent of next-generation sequencing occurred, allowing to deeply characterize the diversity of microorganisms that colonize human epitheliums, known as microbiota. Human microbiome produces small molecules and metabolites through a complex community network with relevant biological effects both at local and systemic levels and its dysregulation contributes to cancer establishment, progression and therapy response (297–300). Carcinogenesis is a complex process on which exogenous, as well as, endogenous factors could impact in different ways on malignant transformation. Among endogenous factors, metabolites generated as byproducts of metabolic activity can either act as carcinogen compounds (i.e., nitrosamines, conversion of alcohol to acetaldehyde, and tumor-promoting secondary bile acids) or as anticarcinogens (i.e., activation of dietary phytochemicals and inactivation of hormones that stimulate tumor cells growth). Even more, metabolism of different substances within the body can be affected by different health conditions like diabetes or obesity, which is characterized by chronic inflammation. In this context, bacterial metagenome has revealed to be an important player in fine-tuning tumor metabolic function, as is enriched in genes that participates in nutrients, bile acid and xenobiotic metabolism, as well as biosynthesis of vitamins and isoprenoids, therefore has become an emergent factor that affects tumor development (301–303). Based on these novel data, the gut microbiome is increasingly being recognized as a dynamic ecosystem influenced by environmental conditions such as diet and drug therapy with relevant effects on tumoral biology and metabolism (304, 305). As an open system, gut microbes elicit their effects on cancer cells via their capacity to induce pro-inflammatory responses (306–308) or more indirectly by the production of secondary metabolites (309–311). Recent evidence showed that short chain fatty acids (SCFAs), hydrogen sulfide (H2S), bile acids, and some other metabolites are produced by gut microbiota and impact the genome and epigenome of cancer cells, including miRNAs. Thus, the gut microbiome is an important regulator of host transcriptional dynamics in part through the establishment of inter-communications via miRNA signaling (312).

Host microbiome has pointed out as a potential modulator of cancer metabolism and could be a future target for precision medicine. While there is less evidence of how microbiota affects most of the miRNA landscapes in human tumors, there are growing data that explain how the microbiota confers some

TABLE 3 | miRNA portrait and gut microbiota in cancer.

miRNA	Activity	Cancer	References
miR-182, miR-503, and mir-17∼92 cluster	Differentially expression of these oncogenic miRNAs was correlated with the relative abundances of: *Firmicutes, Bacteroidetes,* and *Proteobacteria*. Possible role of these miRNAs in driven glycan production in tumor location through the recruitment of pathogenic microbial taxa and thus impact tumor development	CCR	(319)
Upregulation of miR-21	*Fusobacterium nucleatum* induces CRC cell proliferation by up-modulating the oncogenic miR-21 via TLR4 signaling	CRC	(320)
Upregulation of miR-20a-5p	The colibactin genotoxin produced by *Escherichia coli* promotes cellular senescence by the upregulation of miR-20a-5p, which in turn downregulates SENP1, resulting in the proliferation of uninfected cells and, subsequently, tumor growth. The over-expression of miR-20a-5p also alters p53 SUMOylation, which has been shown to promote tumor growth and metastasis	CRC	(321–323)

effect on cancer pathways in colorectal cancer (CRC). Under physiological conditions, the microbiota promotes a metabolic niche that produces a huge amount of the energy required by the intestinal epithelial cells (313) through the production of butyrate, a SCFAs, as a result of complex carbohydrates fermentation. CRC cells preferentially use glucose over butyrate

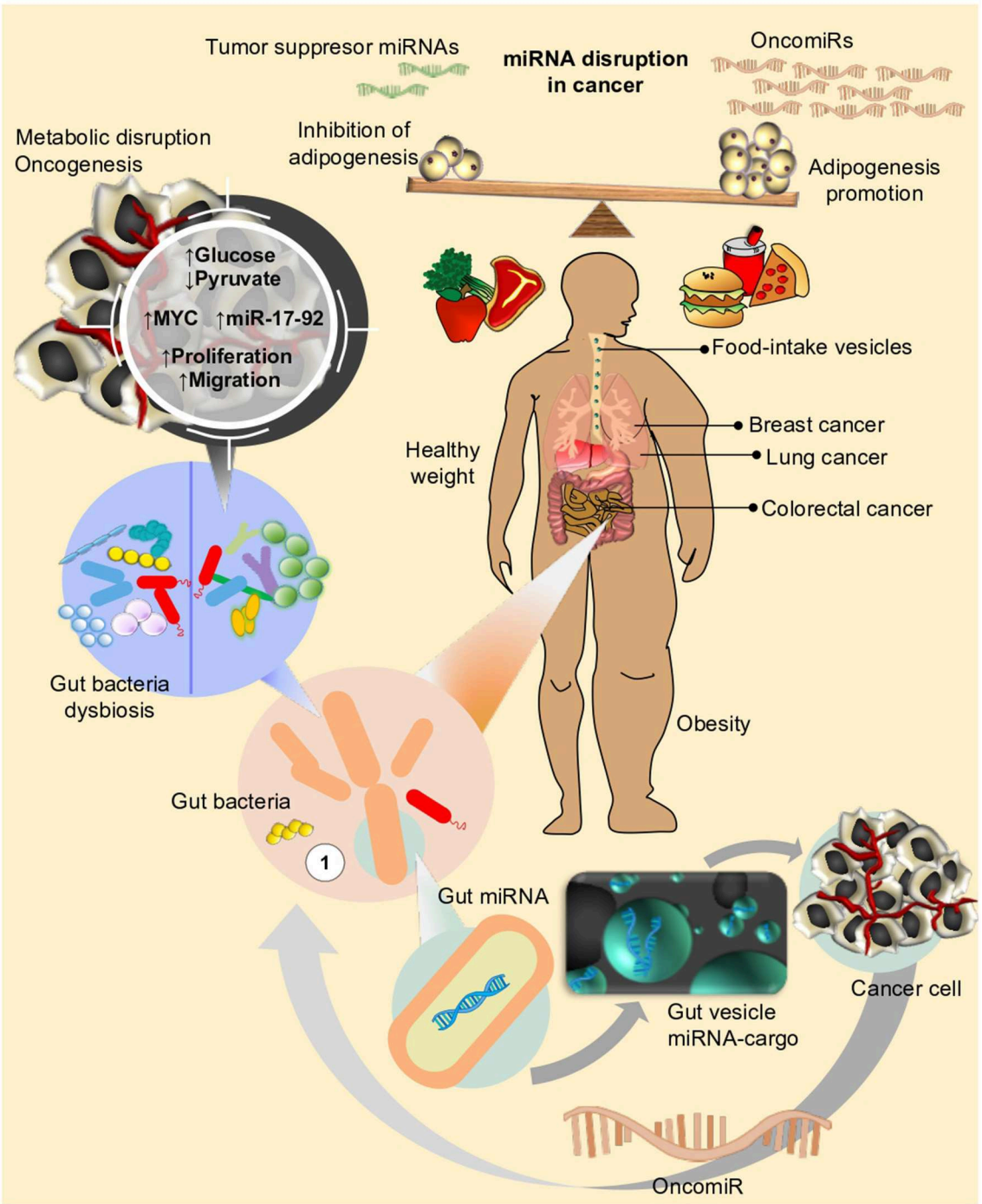

FIGURE 3 | Life style and diet has an impact on different metabolic mechanisms in human cells. Disruption of metabolic fluxes, might particularly affect expression of genes and miRNAs related to control of cell proliferation, cell cycle, and adhesion, eventually leading or favoring neoplastic processes to take place in different organs (i.e., Breast, Prostate, Lung, Colon, etc.). Microbiota, on the other side, the new star player in the complex interaction between environment and human organism, can also influence the effect of nutrients or drug intake within host. In an unhealthy weight scenario (i.e., obesity), disequilibrium in adipogenesis leads to chronic inflammation and triggering of signals for over-expression of oncomiRs. Under this condition, dysbiosis (e.g., loss of balance in gut bacteria composition) could further concur to sustain or even enhance the metabolic perturbations favoring neoplastic transformations.

as the major source of energy, resulting in a gut microbiome related dysbiosis (314). In the tumoral context, low butyrate concentrations enhance MYC expression, which in turn up-modulates the levels of the oncogenic miR-17-92 cluster (315). The overexpression of miR-17-92a cluster has been shown to enhance cell proliferation, metastasis, and angiogenesis (316–318). This data demonstrates an antitumor mechanism of butyrate over the MYC /miR-17-92a axis in CRC cells. As exemplified, miRNAs activity is a relevant feature in mediating metabolic changes and modulating the interaction of host transcriptional portrait and microbiota. Some other evidences are described in **Table 3**. Results from numerous studies now suggest an additionally level in the complex interplay between miRNAs and gut microbiome, including data describing the influence of miRNAs in controlling gut composition and growth rates by improving selectively pressure on the surrounding microenvironment (**Table 3**).

Furthermore, results from numerous studies suggest that intestinal miRNAs come from two main sources: host and food (55, 324). The intestinal epithelial cells are the main contributors of host-derived miRNAs, but miRNAs contained in food can as well be absorbed by the host and regulate gene expression in a cross-species regulation manner (325, 326). Recently it has been showed that Ginger derived exosome-like vesicles, containing RNA, are taken up by the gut microbiota and can alter microbiome composition and host physiology. Briefly, the exosomal particles are preferentially engulfed by *Lactobacillus rhamnosus* and the exosomal microRNAs-cargo target various genes in the bacteria, such as Ginger miR7267-3p that mediate the production of IL-22, favoring an improvement in the colitis via IL-22-dependent mechanisms (324). These findings reveal how plant products and their effects on the microbiome may be exploited to specially target host processes to modify tumor growth through specific diet interventions (**Figure 3**).

Although studies are still incipient, robust data have been generated, describing how microRNAs serve as important communication factors between the gut microbiome and the host. On the basis of these evidences, it's appropriate to hypothesize there is an open bi-directional communication between host cells and microbes, potentially mediated through miRNA activity. However, more studies are required to be conducted in a broader spectra of cancers, to provide more evidence on the impact of gut microbiota and their miRNAs signaling networks on oncogenic and metabolic processes (300), which finally will allow us take advantage of these changes and devise new strategies to translate the modulation of metabolic alterations into patient management.

AUTHOR CONTRIBUTIONS

AP-T, GH, SR-C, MJ-G, IS-G, RR-B, and SM conceived, designed, and wrote most of the manuscript. RM-M, CA-C, VF-O, RÁ-G, and LH contributed to the writing as well as gathered information for the manuscript. SR-C, MJ-G, IS-G, and RR-B assembled figures and tables.

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REFERENCES

1. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*. (1993) 75:843–54. doi: 10.1016/0092-8674(93)90529-y
2. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell*. (1993) 75:855–62. doi: 10.1016/0092-8674(93)90530-4
3. Behm-Ansmant I, Rehwinkel J, Doerks T, Stark A, Bork P, Izaurralde E. mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes Dev*. (2006) 20:1885–98. doi: 10.1101/gad.1424106
4. Behm-Ansmant I, Rehwinkel J, Izaurralde E. MicroRNAs silence gene expression by repressing protein expression and/or by promoting mRNA decay. *Cold Spring Harb Symp Quant Biol*. (2006) 71:523–30. doi: 10.1101/sqb.2006.71.013
5. Vella MC, Choi E-Y, Lin S-Y, Reinert K, Slack FJ. The *C. elegans* microRNA let-7 binds to imperfect let-7 complementary sites from the lin-41 3'UTR. *Genes Dev*. (2004) 18:132–7. doi: 10.1101/gad.1165404
6. Garofalo M, Croce CM. microRNAs: master regulators as potential therapeutics in cancer. *Annu Rev Pharmacol Toxicol*. (2011) 51:25–43. doi: 10.1146/annurev-pharmtox-010510-100517
7. Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol*. (2009) 10:126–39. doi: 10.1038/nrm2632
8. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. (2004) 116:281–97. doi: 10.1016/s0092-8674(04)00045-5
9. Ha M, Kim VN. Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol*. (2014) 15:509–24. doi: 10.1038/nrm3838
10. Lee Y, Kim M, Han J, Yeom K-H, Lee S, Baek SH, et al. MicroRNA genes are transcribed by RNA polymerase II. *EMBO J*. (2004) 23:4051–60. doi: 10.1038/sj.emboj.7600385
11. Han J, Lee Y, Yeom K-H, Kim Y-K, Jin H, Kim VN. The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev*. (2004) 18:3016–27. doi: 10.1101/gad.1262504
12. Wang Y, Medvid R, Melton C, Jaenisch R, Blelloch R. DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nat Genet*. (2007) 39:380–5. doi: 10.1038/ng1969
13. Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev*. (2003) 17:3011–6. doi: 10.1101/gad.1158803

14. Zhang H, Kolb FA, Jaskiewicz L, Westhof E, Filipowicz W. Single processing center models for human Dicer and bacterial RNase III. *Cell*. (2004) 118:57–68. doi: 10.1016/j.cell.2004.06.017
15. Koscianska E, Starega-Roslan J, Krzyzosiak WJ. The role of Dicer protein partners in the processing of microRNA precursors. *PLoS ONE*. (2011) 6:e28548. doi: 10.1371/journal.pone.0028548
16. Fareh M, Yeom K-H, Haagsma AC, Chauhan S, Heo I, Joo C. TRBP ensures efficient Dicer processing of precursor microRNA in RNA-crowded environments. *Nat Commun*. (2016) 7:13694. doi: 10.1038/ncomms13694
17. Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K, et al. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature*. (2005) 436:740–4. doi: 10.1038/nature03868
18. Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell*. (2004) 15:185–97. doi: 10.1016/j.molcel.2004.07.007
19. Friedman RC, Farh KK-H, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*. (2009) 19:92–105. doi: 10.1101/gr.082701.108
20. Roccaro AM, Sacco A, Thompson B, Leleu X, Azab AK, Azab F, et al. MicroRNAs 15a and 16 regulate tumor proliferation in multiple myeloma. *Blood*. (2009) 113:6669–80. doi: 10.1182/blood-2009-01-198408
21. Chen J-F, Tao Y, Li J, Deng Z, Yan Z, Xiao X, et al. microRNA-1 and microRNA-206 regulate skeletal muscle satellite cell proliferation and differentiation by repressing Pax7. *J Cell Biol*. (2010) 190:867–79. doi: 10.1083/jcb.200911036
22. Yamakuchi M, Ferlito M, Lowenstein CJ. miR-34a repression of SIRT1 regulates apoptosis. *Proc Natl Acad Sci USA*. (2008) 105:13421–6. doi: 10.1073/pnas.0801613105
23. Schickel R, Park S-M, Murmann AE, Peter ME. miR-200c regulates induction of apoptosis through CD95 by targeting FAP-1. *Mol Cell*. (2010) 38:908–15. doi: 10.1016/j.molcel.2010.05.018
24. Otsubo T, Akiyama Y, Hashimoto Y, Shimada S, Goto K, Yuasa Y. MicroRNA-126 inhibits SOX2 expression and contributes to gastric carcinogenesis. *PLoS ONE*. (2011) 6:e16617. doi: 10.1371/journal.pone.0016617
25. Hu Q, Du K, Mao X, Ning S. miR-197 is downregulated in cervical carcinogenesis and suppresses cell proliferation and invasion through targeting forkhead box M1. *Oncol Lett*. (2018) 15:10063–9. doi: 10.3892/ol.2018.8565
26. Lu H, Buchan RJ, Cook SA. MicroRNA-223 regulates Glut4 expression and cardiomyocyte glucose metabolism. *Cardiovasc Res*. (2010) 86:410–20. doi: 10.1093/cvr/cvq010
27. Pavlova NN, Thompson CB. The emerging hallmarks of cancer metabolism. *Cell Metab*. (2016) 23:27–47. doi: 10.1016/j.cmet.2015.12.006
28. Oermann EK, Wu J, Guan K-L, Xiong Y. Alterations of metabolic genes and metabolites in cancer. *Semin Cell Dev Biol*. (2012) 23:370–80. doi: 10.1016/j.semcdb.2012.01.013
29. Chen D, Wang H, Chen J, Li Z, Li S, Hu Z, et al. MicroRNA-129-5p Regulates glycolysis and cell proliferation by targeting the glucose transporter SLC2A3 in gastric cancer cells. *Front Pharmacol*. (2018) 9:502. doi: 10.3389/fphar.2018.00502
30. Guo W, Qiu Z, Wang Z, Wang Q, Tan N, Chen T, et al. MiR-199a-5p is negatively associated with malignancies and regulates glycolysis and lactate production by targeting hexokinase 2 in liver cancer. *Hepatol Baltim Md*. (2015) 62:1132–44. doi: 10.1002/hep.27929
31. Yang Y, Ishak Gabra MB, Hanse EA, Lowman XH, Tran TQ, Li H, et al. MiR-135 suppresses glycolysis and promotes pancreatic cancer cell adaptation to metabolic stress by targeting phosphofructokinase-1. *Nat Commun*. (2019) 10:809. doi: 10.1038/s41467-019-08759-0
32. Zhang X, Li Z, Xuan Z, Xu P, Wang W, Chen Z, et al. Novel role of miR-133a-3p in repressing gastric cancer growth and metastasis via blocking autophagy-mediated glutaminolysis. *J Exp Clin Cancer Res CR*. (2018) 37:320. doi: 10.1186/s13046-018-0993-y
33. Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, et al. miR-122 regulation of lipid metabolism revealed by *in vivo* antisense targeting. *Cell Metab*. (2006) 3:87–98. doi: 10.1016/j.cmet.2006.01.005
34. DeBerardinis RJ, Chandel NS. Fundamentals of cancer metabolism. *Sci Adv*. (2016) 2:e1600200. doi: 10.1126/sciadv.1600200
35. Dang CV. Links between metabolism and cancer. *Genes Dev*. (2012) 26:877–90. doi: 10.1101/gad.189365.112
36. Levine AJ, Puzio-Kuter AM. The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. *Science*. (2010) 330:1340–4. doi: 10.1126/science.1193494
37. Vaupel P, Schmidberger H, Mayer A. The Warburg effect: essential part of metabolic reprogramming and central contributor to cancer progression. *Int J Radiat Biol*. (2019) 95:912–9. doi: 10.1080/09553002.2019.1589653
38. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*. (2009) 324:1029–33. doi: 10.1126/science.1160809
39. Lunt SY, Vander Heiden MG. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu Rev Cell Dev Biol*. (2011) 27:441–64. doi: 10.1146/annurev-cellbio-092910-154237
40. Lebelo MT, Joubert AM, Visagie MH. Warburg effect and its role in tumourigenesis. *Arch Pharm Res*. (2019) 42:833–47. doi: 10.1007/s12272-019-01185-2
41. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. *Nat Rev Cancer*. (2011) 11:85–95. doi: 10.1038/nrc2981
42. Wu W, Zhao S. Metabolic changes in cancer: beyond the Warburg effect. *Acta Biochim Biophys Sin*. (2013) 45:18–26. doi: 10.1093/abbs/gms104
43. Soga T. Cancer metabolism: key players in metabolic reprogramming. *Cancer Sci*. (2013) 104:275–81. doi: 10.1111/cas.12085
44. Kato Y, Maeda T, Suzuki A, Baba Y. Cancer metabolism: new insights into classic characteristics. *Jpn Dent Sci Rev*. (2018) 54:8–21. doi: 10.1016/j.jdsr.2017.08.003
45. Li Z, Zhang H. Reprogramming of glucose, fatty acid and amino acid metabolism for cancer progression. *Cell Mol Life Sci CMLS*. (2016) 73:377–92. doi: 10.1007/s00018-015-2070-4
46. Li C, Zhang G, Zhao L, Ma Z, Chen H. Metabolic reprogramming in cancer cells: glycolysis, glutaminolysis, and Bcl-2 proteins as novel therapeutic targets for cancer. *World J Surg Oncol*. (2016) 14:15. doi: 10.1186/s12957-016-0769-9
47. Schwörer S, Vardhana SA, Thompson CB. Cancer metabolism drives a stromal regenerative response. *Cell Metab*. (2019) 29:576–91. doi: 10.1016/j.cmet.2019.01.015
48. Ippolito L, Morandi A, Taddei ML, Parri M, Comito G, Iscaro A, et al. Cancer-associated fibroblasts promote prostate cancer malignancy via metabolic rewiring and mitochondrial transfer. *Oncogene*. (2019) 38:5339–55. doi: 10.1038/s41388-019-0805-7
49. Netea-Maier RT, Smit JWA, Netea MG. Metabolic changes in tumor cells and tumor-associated macrophages: a mutual relationship. *Cancer Lett*. (2018) 413:102–9. doi: 10.1016/j.canlet.2017.10.037
50. Papa S, Choy PM, Bubici C. The ERK and JNK pathways in the regulation of metabolic reprogramming. *Oncogene*. (2019) 38:2223–40. doi: 10.1038/s41388-018-0582-8
51. Yeung SJ, Pan J, Lee M-H. Roles of p53, MYC and HIF-1 in regulating glycolysis - the seventh hallmark of cancer. *Cell Mol Life Sci CMLS*. (2008) 65:3981–99. doi: 10.1007/s00018-008-8224-x
52. Zwaans BMM, Lombard DB. Interplay between sirtuins, MYC and hypoxia-inducible factor in cancer-associated metabolic reprogramming. *Dis Model Mech*. (2014) 7:1023–32. doi: 10.1242/dmm.016287
53. Mueckler M, Thorens B. The SLC2 (GLUT) family of membrane transporters. *Mol Aspects Med*. (2013) 34:121–38. doi: 10.1016/j.mam.2012.07.001
54. Thorens B, Mueckler M. Glucose transporters in the 21st century. *Am J Physiol Endocrinol Metab*. (2010) 298:E141–5. doi: 10.1152/ajpendo.00712.2009
55. Liu M, Gao J, Huang Q, Jin Y, Wei Z. Downregulating microRNA-144 mediates a metabolic shift in lung cancer cells by regulating GLUT1 expression. *Oncol Lett*. (2016) 11:3772–6. doi: 10.3892/ol.2016.4468
56. Qu W, Ding S-M, Cao G, Wang S-J, Zheng X-H, Li G-H. miR-132 mediates a metabolic shift in prostate cancer cells by targeting Glut1. *FEBS Open Bio*. (2016) 6:735–41. doi: 10.1002/2211-5463.12086
57. King BC, Esguerra JLS, Golec E, Eliasson L, Kemper C, Blom AM. CD46 activation regulates miR-150–mediated control of GLUT1 expression and

cytokine secretion in human CD4 $^{+}$ T Cells. *J Immunol*. (2016) 196:1636–45. doi: 10.4049/jimmunol.1500516
58. Chow T-FF, Mankaruos M, Scorilas A, Youssef Y, Girgis A, Mossad S, et al. The miR-17-92 cluster is over expressed in and has an oncogenic effect on renal cell carcinoma. *J Urol*. (2010) 183:743–51. doi: 10.1016/j.juro.2009.09.086
59. Fei X, Qi M, Wu B, Song Y, Wang Y, Li T. MicroRNA-195-5p suppresses glucose uptake and proliferation of human bladder cancer T24 cells by regulating GLUT3 expression. *FEBS Lett*. (2012) 586:392–7. doi: 10.1016/j.febslet.2012.01.006
60. Dai D-W, Lu Q, Wang L-X, Zhao W-Y, Cao Y-Q, Li Y-N, et al. Decreased miR-106a inhibits glioma cell glucose uptake and proliferation by targeting SLC2A3 in GBM. *BMC Cancer*. (2013) 13:478. doi: 10.1186/1471-2407-13-478
61. Horie T, Ono K, Nishi H, Iwanaga Y, Nagao K, Kinoshita M, et al. MicroRNA-133 regulates the expression of GLUT4 by targeting KLF15 and is involved in metabolic control in cardiac myocytes. *Biochem Biophys Res Commun*. (2009) 389:315–20. doi: 10.1016/j.bbrc.2009.08.136
62. Ling H-Y, Hu B, Hu X-B, Zhong J, Feng S-D, Qin L, et al. MiRNA-21 reverses high glucose and high insulin induced insulin resistance in 3T3-L1 adipocytes through targeting phosphatase and tensin homologue. *Exp Clin Endocrinol Diabetes Off J Ger Soc Endocrinol Ger Diabetes Assoc*. (2012) 120:553–9. doi: 10.1055/s-0032-1311644
63. Raychaudhuri S. MicroRNAs overexpressed in growth-restricted rat skeletal muscles regulate the glucose transport in cell culture targeting central TGF-β factor SMAD4. *PLoS ONE*. (2012) 7:e34596. doi: 10.1371/journal.pone.0034596
64. Esteves JV, Enguita FJ, Machado UF. MicroRNAs-mediated regulation of skeletal muscle GLUT4 expression and translocation in insulin resistance. *J Diabetes Res*. (2017) 2017:7267910. doi: 10.1155/2017/7267910
65. Yin C, Qie S, Sang N. Carbon source metabolism and its regulation in cancer cells. *Crit Rev Eukaryot Gene Expr*. (2012) 22:17–35. doi: 10.1615/critreveukargeneexpr.v22.i1.20
66. Dang CV. Glutaminolysis: supplying carbon or nitrogen or both for cancer cells? *Cell Cycle Georget Tex*. (2010) 9:3884–6. doi: 10.4161/cc.9.19.13302
67. John S, Weiss JN, Ribalet B. Subcellular localization of hexokinases I and II directs the metabolic fate of glucose. *PLoS ONE*. (2011) 6:e17674. doi: 10.1371/journal.pone.0017674
68. Mandarino LJ, Printz RL, Cusi KA, Kinchington P, O'Doherty RM, Osawa H, et al. Regulation of hexokinase II and glycogen synthase mRNA, protein, and activity in human muscle. *Am J Physiol*. (1995) 269:E701–8. doi: 10.1152/ajpendo.1995.269.4.E701
69. Wyatt E, Wu R, Rabeh W, Park H-W, Ghanefar M, Ardehali H. Regulation and cytoprotective role of hexokinase III. *PLoS ONE*. (2010) 5:e13823. doi: 10.1371/journal.pone.0013823
70. Yasuda S, Arii S, Mori A, Isobe N, Yang W, Oe H, et al. Hexokinase II and VEGF expression in liver tumors: correlation with hypoxia-inducible factor-1α and its significance. *J Hepatol*. (2004) 40:117–23. doi: 10.1016/S0168-8278(03)00503-8
71. Jiao L, Zhang H-L, Li D-D, Yang K-L, Tang J, Li X, et al. Regulation of glycolytic metabolism by autophagy in liver cancer involves selective autophagic degradation of HK2 (hexokinase 2). *Autophagy*. (2018) 14:671–84. doi: 10.1080/15548627.2017.1381804
72. Palmieri D, Fitzgerald D, Shreeve SM, Hua E, Bronder JL, Weil RJ, et al. Analyses of resected human brain metastases of breast cancer reveal the association between up-regulation of hexokinase 2 and poor prognosis. *Mol Cancer Res*. (2009) 7:1438–45. doi: 10.1158/1541-7786.MCR-09-0234
73. Fang R, Xiao T, Fang Z, Sun Y, Li F, Gao Y, et al. MicroRNA-143 (miR-143) regulates cancer glycolysis via targeting hexokinase 2 gene. *J Biol Chem*. (2012) 287:23227–35. doi: 10.1074/jbc.M112.373084
74. Peschiaroli A, Giacobbe A, Formosa A, Markert EK, Bongiorno-Borbone L, Levine AJ, et al. miR-143 regulates hexokinase 2 expression in cancer cells. *Oncogene*. (2013) 32:797–802. doi: 10.1038/onc.2012.100
75. Gregersen LH, Jacobsen A, Frankel LB, Wen J, Krogh A, Lund AH. MicroRNA-143 down-regulates Hexokinase 2 in colon cancer cells. *BMC Cancer*. (2012) 12:232. doi: 10.1186/1471-2407-12-232
76. Li L-Q, Yang Y, Chen H, Zhang L, Pan D, Xie W-J. MicroRNA-181b inhibits glycolysis in gastric cancer cells via targeting hexokinase 2 gene. *Cancer Biomark Sect Dis Markers*. (2016) 17:75–81. doi: 10.3233/CBM-160619
77. Jiang S, Zhang L-F, Zhang H-W, Hu S, Lu M-H, Liang S, et al. A novel miR-155/miR-143 cascade controls glycolysis by regulating hexokinase 2 in breast cancer cells. *EMBO J*. (2012) 31:1985–98. doi: 10.1038/emboj.2012.45
78. Ahmad A, Aboukameel A, Kong D, Wang Z, Sethi S, Chen W, et al. Phosphoglucose isomerase/autocrine motility factor mediates epithelial-mesenchymal transition regulated by miR-200 in breast cancer cells. *Cancer Res*. (2011) 71:3400–9. doi: 10.1158/0008-5472.CAN-10-0965
79. Rengaraj D, Park TS, Lee SI, Lee BR, Han BK, Song G, et al. Regulation of glucose phosphate isomerase by the 3'UTR-specific miRNAs miR-302b and miR-17-5p in chicken primordial germ cells. *Biol Reprod*. (2013) 89:33. doi: 10.1095/biolreprod.112.105692
80. Fabani MM, Gait MJ. miR-122 targeting with LNA/2'-O-methyl oligonucleotide mixmers, peptide nucleic acids (PNA), and PNA-peptide conjugates. *RNA NYN*. (2008) 14:336–46. doi: 10.1261/rna.844108
81. Calin GA, Cimmino A, Fabbri M, Ferracin M, Wojcik SE, Shimizu M, et al. MiR-15a and miR-16-1 cluster functions in human leukemia. *Proc Natl Acad Sci USA*. (2008) 105:5166–71. doi: 10.1073/pnas.0800121105
82. Montoya V, Fan H, Bryar PJ, Weinstein JL, Mets MB, Feng G, et al. Novel miRNA-31 and miRNA-200a-mediated regulation of retinoblastoma proliferation. *PLoS ONE*. (2015) 10:e0138366. doi: 10.1371/journal.pone.0138366
83. Sikand K, Singh J, Ebron JS, Shukla GC. Housekeeping gene selection advisory: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin are targets of miR-644a. *PLoS ONE*. (2012) 7:e47510. doi: 10.1371/journal.pone.0047510
84. Khanna M, Saini S, Shariff M, Ronsard L, Singh JK, Kumar H. Data highlighting miR-155 and GAPDH correlation. *Data Brief*. (2019) 24:103945. doi: 10.1016/j.dib.2019.103945
85. Wong T-S, Liu X-B, Chung-Wai Ho A, Po-Wing Yuen A, Wai-Man Ng R, Ignace Wei W. Identification of pyruvate kinase type M2 as potential oncoprotein in squamous cell carcinoma of tongue through microRNA profiling. *Int J Cancer*. (2008) 123:251–7. doi: 10.1002/ijc.23583
86. Kefas B, Comeau L, Erdle N, Montgomery E, Amos S, Purow B. Pyruvate kinase M2 is a target of the tumor-suppressive microRNA-326 and regulates the survival of glioma cells. *Neuro-Oncol*. (2010) 12:1102–12. doi: 10.1093/neuonc/noq080
87. Liu AM, Xu Z, Shek FH, Wong K-F, Lee NP, Poon RT, et al. miR-122 targets pyruvate kinase M2 and affects metabolism of hepatocellular carcinoma. *PLoS ONE*. (2014) 9:e86872. doi: 10.1371/journal.pone.0086872
88. Sun Y, Zhao X, Zhou Y, Hu Y. miR-124, miR-137 and miR-340 regulate colorectal cancer growth via inhibition of the Warburg effect. *Oncol Rep*. (2012) 28:1346–52. doi: 10.3892/or.2012.1958
89. Li W, Wang J, Chen Q-D, Qian X, Li Q, Yin Y, et al. Insulin promotes glucose consumption via regulation of miR-99a/mTOR/PKM2 pathway. *PLoS ONE*. (2013) 8:e64924. doi: 10.1371/journal.pone.0064924
90. Feng Y, Xiong Y, Qiao T, Li X, Jia L, Han Y. Lactate dehydrogenase A: a key player in carcinogenesis and potential target in cancer therapy. *Cancer Med*. (2018) 7:6124–36. doi: 10.1002/cam4.1820
91. Li J, Zhu S, Tong J, Hao H, Yang J, Liu Z, et al. Suppression of lactate dehydrogenase A compromises tumor progression by downregulation of the Warburg effect in glioblastoma. *Neuroreport*. (2016) 27:110–5. doi: 10.1097/WNR.0000000000000506
92. Dong T, Liu Z, Xuan Q, Wang Z, Ma W, Zhang Q. Tumor LDH-A expression and serum LDH status are two metabolic predictors for triple negative breast cancer brain metastasis. *Sci Rep*. (2017) 7:6069. doi: 10.1038/s41598-017-06378-7
93. Kinoshita T, Nohata N, Yoshino H, Hanazawa T, Kikkawa N, Fujimura L, et al. Tumor suppressive microRNA-375 regulates lactate dehydrogenase B in maxillary sinus squamous cell carcinoma. *Int J Oncol*. (2012) 40:185–93. doi: 10.3892/ijo.2011.1196
94. Xiao X, Huang X, Ye F, Chen B, Song C, Wen J, et al. The miR-34a-LDHA axis regulates glucose metabolism and tumor growth in breast cancer. *Sci Rep*. (2016) 6:21735. doi: 10.1038/srep21735

95. Chen H, Gao S, Cheng C. MiR-323a-3p suppressed the glycolysis of osteosarcoma via targeting LDHA. *Hum Cell*. (2018) 31:300–9. doi: 10.1007/s13577-018-0215-0
96. Li L, Kang L, Zhao W, Feng Y, Liu W, Wang T, et al. miR-30a-5p suppresses breast tumor growth and metastasis through inhibition of LDHA-mediated Warburg effect. *Cancer Lett*. (2017) 400:89–98. doi: 10.1016/j.canlet.2017.04.034
97. Wang J, Wang H, Liu A, Fang C, Hao J, Wang Z. Lactate dehydrogenase A negatively regulated by miRNAs promotes aerobic glycolysis and is increased in colorectal cancer. *Oncotarget*. (2015) 6:19456–468. doi: 10.18632/oncotarget.3318
98. Yuan D, Zheng S, Wang L, Li J, Yang J, Wang B, et al. MiR-200c inhibits bladder cancer progression by targeting lactate dehydrogenase A. *Oncotarget*. (2017) 8:67663–9. doi: 10.18632/oncotarget.18801
99. He Y, Chen X, Yu Y, Li J, Hu Q, Xue C, et al. LDHA is a direct target of miR-30d-5p and contributes to aggressive progression of gallbladder carcinoma. *Mol Carcinog*. (2018) 57:772–83. doi: 10.1002/mc.22799
100. Halestrap AP. The SLC16 gene family - structure, role and regulation in health and disease. *Mol Aspects Med*. (2013) 34:337–49. doi: 10.1016/j.mam.2012.05.003
101. Pullen TJ, da Silva Xavier G, Kelsey G, Rutter GA. miR-29a and miR-29b contribute to pancreatic beta-cell-specific silencing of monocarboxylate transporter 1 (Mct1). *Mol Cell Biol*. (2011) 31:3182–94. doi: 10.1128/MCB.01433-10
102. Liang D, Zhang Y, Han J, Wang W, Liu Y, Li J, et al. Embryonic stem cell-derived pancreatic endoderm transplant with MCT1-suppressing miR-495 attenuates type II diabetes in mice. *Endocr J*. (2015) 62:907–20. doi: 10.1507/endocrj.EJ15-0186
103. Romero-Cordoba SL, Rodriguez-Cuevas S, Bautista-Pina V, Maffuz-Aziz A, D'Ippolito E, Cosentino G, et al. Loss of function of miR-342-3p results in MCT1 over-expression and contributes to oncogenic metabolic reprogramming in triple negative breast cancer. *Sci Rep*. (2018) 8:12252. doi: 10.1038/s41598-018-29708-9
104. Zhao Y, Li W, Li M, Hu Y, Zhang H, Song G, et al. Targeted inhibition of MCT4 disrupts intracellular pH homeostasis and confers self-regulated apoptosis on hepatocellular carcinoma. *Exp Cell Res*. (2019)111591. doi: 10.1016/j.yexcr.2019.111591
105. Hensley CT, Wasti AT, DeBerardinis RJ. Glutamine and cancer: cell biology, physiology, and clinical opportunities. *J Clin Invest*. (2013) 123:3678–84. doi: 10.1172/JCI69600
106. Daye D, Wellen KE. Metabolic reprogramming in cancer: unraveling the role of glutamine in tumorigenesis. *Semin Cell Dev Biol*. (2012) 23:362–9. doi: 10.1016/j.semcdb.2012.02.002
107. Dong J, Xiao D, Zhao Z, Ren P, Li C, Hu Y, et al. Epigenetic silencing of microRNA-137 enhances ASCT2 expression and tumor glutamine metabolism. *Oncogenesis*. (2017) 6:e356. doi: 10.1038/oncsis.2017.59
108. Gao P, Tchernyshyov I, Chang T-C, Lee Y-S, Kita K, Ochi T, et al. c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature*. (2009) 458:762–5. doi: 10.1038/nature07823
109. Chang X, Zhu W, Zhang H, Lian S. Sensitization of melanoma cells to temozolomide by overexpression of microRNA 203 through direct targeting of glutaminase-mediated glutamine metabolism. *Clin Exp Dermatol*. (2017) 42:614–21. doi: 10.1111/ced.13119
110. Anderton B, Camarda R, Balakrishnan S, Balakrishnan A, Kohnz RA, Lim L, et al. MYC-driven inhibition of the glutamate-cysteine ligase promotes glutathione depletion in liver cancer. *EMBO Rep*. (2017) 18:569–85. doi: 10.15252/embr.201643068
111. Liu Z, Wang J, Li Y, Fan J, Chen L, Xu R. MicroRNA-153 regulates glutamine metabolism in glioblastoma through targeting glutaminase. *Tumour Biol J Int Soc Oncodevelopmental Biol Med*. (2017) 39:1010428317691429. doi: 10.1177/1010428317691429
112. Muys BR, Sousa JF, Plaça JR, de Araújo LF, Sarshad AA, Anastasakis DG, et al. miR-450a acts as a tumor suppressor in ovarian cancer by regulating energy metabolism. *Cancer Res*. (2019) 79:3294–305. doi: 10.1158/0008-5472.CAN-19-0490
113. Zacksenhaus E, Shrestha M, Liu JC, Vorobieva I, Chung PED, Ju Y, et al. Mitochondrial OXPHOS induced by RB1 deficiency in breast cancer: implications for anabolic metabolism, stemness, and metastasis. *Trends Cancer*. (2017) 3:768–79. doi: 10.1016/j.trecan.2017.09.002
114. Marchiq I, Le Floch R, Roux D, Simon M-P, Pouyssegur J. Genetic disruption of lactate/H+ symporters (MCTs) and their subunit CD147/BASIGIN sensitizes glycolytic tumor cells to phenformin. *Cancer Res*. (2015) 75:171–80. doi: 10.1158/0008-5472.CAN-14-2260
115. Ippolito L, Marini A, Cavallini L, Morandi A, Pietrovito L, Pintus G, et al. Metabolic shift toward oxidative phosphorylation in docetaxel resistant prostate cancer cells. *Oncotarget*. (2016) 7:61890–904. doi: 10.18632/oncotarget.11301
116. Patel B, Gove CD, Hutchinson WL, Mowbray J. Substantial quantities of the high energy derivative oligophosphoglyceroyl-ATP are located in mitochondria in rat liver. *Biochim Biophys Acta*. (1991) 1074:178–81. doi: 10.1016/0304-4165(91)90058-o
117. Muralimanoharan S, Maloyan A, Mele J, Guo C, Myatt LG, Myatt L. MIR-210 modulates mitochondrial respiration in placenta with preeclampsia. *Placenta*. (2012) 33:816–23. doi: 10.1016/j.placenta.2012.07.002
118. Das S, Ferlito M, Kent OA, Fox-Talbot K, Wang R, Liu D, et al. Nuclear miRNA regulates the mitochondrial genome in the heart. *Circ Res*. (2012) 110:1596–603. doi: 10.1161/CIRCRESAHA.112.267732
119. Aschrafi A, Schwechter AD, Mameza MG, Natera-Naranjo O, Gioio AE, Kaplan BB. MicroRNA-338 regulates local cytochrome c oxidase IV mRNA levels and oxidative phosphorylation in the axons of sympathetic neurons. *J Neurosci*. (2008) 28:12581–90. doi: 10.1523/JNEUROSCI.3338-08.2008
120. Willers IM, Martínez-Reyes I, Martínez-Diez M, Cuezva JM. miR-127-5p targets the 3'UTR of human β-F1-ATPase mRNA and inhibits its translation. *Biochim Biophys Acta*. (2012) 1817:838–48. doi: 10.1016/j.bbabio.2012.03.005
121. Baseler WA, Thapa D, Jagannathan R, Dabkowski ER, Croston TL, Hollander JM. miR-141 as a regulator of the mitochondrial phosphate carrier (Slc25a3) in the type 1 diabetic heart. *Am J Physiol Cell Physiol*. (2012) 303:C1244–51. doi: 10.1152/ajpcell.00137.2012
122. Pucci S, Mazzarelli P. MicroRNA Dysregulation in colon cancer microenvironment interactions: the importance of small things in metastases. *Cancer Microenviron*. (2011) 4:155–62. doi: 10.1007/s12307-011-0062-y
123. Morton MJ, Pitel ML. Reflex sympathetic dystrophy syndrome complicating the management of TMJ symptoms. A case report. *Cranio J Craniomandib Pract*. (1989) 7:239–42.
124. Wise DR, DeBerardinis RJ, Mancuso A, Sayed N, Zhang X-Y, Pfeiffer HK, et al. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc Natl Acad Sci USA*. (2008) 105:18782–7. doi: 10.1073/pnas.0810199105
125. Kimmelman AC. Metabolic dependencies in RAS-driven cancers. *Clin Cancer Res Off J Am Assoc Cancer Res*. (2015) 21:1828–34. doi: 10.1158/1078-0432.CCR-14-2425
126. Simabuco FM, Morale MG, Pavan ICB, Morelli AP, Silva FR, Tamura RE. p53 and metabolism: from mechanism to therapeutics. *Oncotarget*. (2018) 9:23780–823. doi: 10.18632/oncotarget.25267
127. Jones RG, Thompson CB. Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes Dev*. (2009) 23:537–48. doi: 10.1101/gad.1756509
128. Catanzaro G, Besharat ZM, Miele E, Chiacchiarini M, Po A, Carai A, et al. The miR-139-5p regulates proliferation of supratentorial paediatric low-grade gliomas by targeting the PI3K/AKT/mTORC1 signalling. *Neuropathol Appl Neurobiol*. (2018) 44:687–706. doi: 10.1111/nan.12479
129. Koh H-J, Toyoda T, Fujii N, Jung MM, Rathod A, Middelbeek RJ-W, et al. Sucrose nonfermenting AMPK-related kinase (SNARK) mediates contraction-stimulated glucose transport in mouse skeletal muscle. *Proc Natl Acad Sci USA*. (2010) 107:15541–6. doi: 10.1073/pnas.1008131107
130. Dávalos A, Goedeke L, Smibert P, Ramírez CM, Warrier NP, Andreo U, et al. miR-33a/b contribute to the regulation of fatty acid metabolism and insulin signaling. *Proc Natl Acad Sci USA*. (2011) 108:9232–7. doi: 10.1073/pnas.1102281108
131. Mersey BD, Jin P, Danner DJ. Human microRNA (miR29b) expression controls the amount of branched chain alpha-ketoacid dehydrogenase complex in a cell. *Hum Mol Genet*. (2005) 14:3371–7. doi: 10.1093/hmg/ddi368

132. Ward PS, Thompson CB. Metabolic reprogramming: a cancer hallmark even Warburg did not anticipate. *Cancer Cell*. (2012) 21:297–308. doi: 10.1016/j.ccr.2012.02.014
133. Chen X, Yang F, Zhang T, Wang W, Xi W, Li Y, et al. MiR-9 promotes tumorigenesis and angiogenesis and is activated by MYC and OCT4 in human glioma. *J Exp Clin Cancer Res CR*. (2019) 38:99. doi: 10.1186/s13046-019-1078-2
134. Tomasetti M, Lee W, Santarelli L, Neuzil J. Exosome-derived microRNAs in cancer metabolism: possible implications in cancer diagnostics and therapy. *Exp Mol Med*. (2017) 49:e285. doi: 10.1038/emm.2016.153
135. Tomasetti M, Santarelli L, Neuzil J, Dong L. MicroRNA regulation of cancer metabolism: role in tumour suppression. *Mitochondrion*. (2014) 19(Pt A):29–38. doi: 10.1016/j.mito.2014.06.004
136. Shen G, Li X, Jia Y, Piazza GA, Xi Y. Hypoxia-regulated microRNAs in human cancer. *Acta Pharmacol Sin*. (2013) 34:336–41. doi: 10.1038/aps.2012.195
137. Al Tameemi W, Dale TP, Al-Jumaily RMK, Forsyth NR. Hypoxia-modified cancer cell metabolism. *Front Cell Dev Biol*. (2019) 7:4. doi: 10.3389/fcell.2019.00004
138. English SG, Hadj-Moussa H, Storey KB. MicroRNAs regulate survival in oxygen-deprived environments. *J Exp Biol*. (2018) 221(Pt 23):jeb190579. doi: 10.1242/jeb.190579
139. Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell Metab*. (2006) 3:187–97. doi: 10.1016/j.cmet.2006.01.012
140. Kim J, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab*. (2006) 3:177–85. doi: 10.1016/j.cmet.2006.02.002
141. Hagen T. Oxygen versus reactive oxygen in the regulation of HIF-1α: the balance tips. *Biochem Res Int*. (2012) 2012:436981. doi: 10.1155/2012/436981
142. Marín-Hernández A, Gallardo-Pérez JC, Ralph SJ, Rodríguez-Enríquez S, Moreno-Sánchez R. HIF-1alpha modulates energy metabolism in cancer cells by inducing over-expression of specific glycolytic isoforms. *Mini Rev Med Chem*. (2009) 9:1084–101. doi: 10.2174/138955709788922610
143. Nagao A, Kobayashi M, Koyasu S, Chow CCT, Harada H. HIF-1-Dependent reprogramming of glucose metabolic pathway of cancer cells and its therapeutic significance. *Int J Mol Sci*. (2019) 20:238. doi: 10.3390/ijms20020238
144. Singh D, Arora R, Kaur P, Singh B, Mannan R, Arora S. Overexpression of hypoxia-inducible factor and metabolic pathways: possible targets of cancer. *Cell Biosci*. (2017) 7:62. doi: 10.1186/s13578-017-0190-2
145. Tokuda K, Baron B, Yamashiro C, Kuramitsu Y, Kitagawa T, Kobayashi M, et al. Up-regulation of the pentose phosphate pathway and HIF-1α expression during neural progenitor cell induction following glutamate treatment in rat *ex vivo* retina. *Cell Biol Int*. (2019) doi: 10.1002/cbin.11212. [Epub ahead of print].
146. Gordan JD, Thompson CB, Simon MC. HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation. *Cancer Cell*. (2007) 12:108–13. doi: 10.1016/j.ccr.2007.07.006
147. Podar K, Anderson KC. A therapeutic role for targeting c-Myc/Hif-1-dependent signaling pathways. *Cell Cycle Georget Tex*. (2010) 9:1722–8. doi: 10.4161/cc.9.9.11358
148. Zhang H, Gao P, Fukuda R, Kumar G, Krishnamachary B, Zeller KI, et al. HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity. *Cancer Cell*. (2007) 11:407–20. doi: 10.1016/j.ccr.2007.04.001
149. Kim J, Gao P, Liu Y-C, Semenza GL, Dang CV. Hypoxia-inducible factor 1 and dysregulated c-Myc cooperatively induce vascular endothelial growth factor and metabolic switches hexokinase 2 and pyruvate dehydrogenase kinase 1. *Mol Cell Biol*. (2007) 27:7381–93. doi: 10.1128/MCB.00440-07
150. Amelio I, Mancini M, Petrova V, Cairns RA, Vikhreva P, Nicolai S, et al. p53 mutants cooperate with HIF-1 in transcriptional regulation of extracellular matrix components to promote tumor progression. *Proc Natl Acad Sci USA*. (2018) 115:E10869–78. doi: 10.1073/pnas.1808314115
151. Obacz J, Pastorekova S, Vojtesek B, Hrstka R. Cross-talk between HIF and p53 as mediators of molecular responses to physiological and genotoxic stresses. *Mol Cancer*. (2013) 12:93. doi: 10.1186/1476-4598-12-93
152. Jin F, Wang Y, Zhu Y, Li S, Liu Y, Chen C, et al. The miR-125a/HK2 axis regulates cancer cell energy metabolism reprogramming in hepatocellular carcinoma. *Sci Rep*. (2017) 7:3089. doi: 10.1038/s41598-017-03407-3
153. Lin YX, An FM, Zhan Q. [Research progress of microRNAs in regulation of lipid metabolism-related proteins for influencing the pathogenesis of hepatocellular carcinoma]. *Zhonghua Gan Zang Bing Za Zhi Zhonghua Ganzangbing Zazhi Chin J Hepatol*. (2019) 27:219–22. doi: 10.3760/cma.j.issn.1007-3418.2019.03.011
154. Nie Z-Y, Liu X-J, Zhan Y, Liu M-H, Zhang X-Y, Li Z-Y, et al. miR-140-5p induces cell apoptosis and decreases Warburg effect in chronic myeloid leukemia by targeting SIX1. *Biosci Rep*. (2019) 39:BSR20190150. doi: 10.1042/BSR20190150
155. Krokker L, Nyíro G, Reiniger L, Darvasi O, Szücs N, Czirják S, et al. Differentially expressed miRNAs influence metabolic processes in pituitary oncocytoma. *Neurochem Res*. (2019) 44:2360–71. doi: 10.1007/s11064-019-02789-2
156. Xu T, Zhang K, Shi J, Huang B, Wang X, Qian K, et al. MicroRNA-940 inhibits glioma progression by blocking mitochondrial folate metabolism through targeting of MTHFD2. *Am J Cancer Res*. (2019) 9:250–69.
157. De Santi C, Melaiu O, Bonotti A, Cascione L, Di Leva G, Foddis R, et al. Deregulation of miRNAs in malignant pleural mesothelioma is associated with prognosis and suggests an alteration of cell metabolism. *Sci Rep*. (2017) 7:3140. doi: 10.1038/s41598-017-02694-0
158. Ling Z, Liu D, Zhang G, Liang Q, Xiang P, Xu Y, et al. miR-361-5p modulates metabolism and autophagy via the Sp1-mediated regulation of PKM2 in prostate cancer. *Oncol Rep*. (2017) 38:1621–8. doi: 10.3892/or.2017.5852
159. Alfardus H, McIntyre A, Smith S. MicroRNA regulation of glycolytic metabolism in glioblastoma. *BioMed Res Int*. (2017) 2017:9157370. doi: 10.1155/2017/9157370
160. Guo H, Nan Y, Zhen Y, Zhang Y, Guo L, Yu K, et al. miRNA-451 inhibits glioma cell proliferation and invasion by downregulating glucose transporter 1. *Tumour Biol*. (2016) 37:13751–61. doi: 10.1007/s13277-016-5219-3
161. Fong MY, Zhou W, Liu L, Alontaga AY, Chandra M, Ashby J, et al. Breast-cancer-secreted miR-122 reprograms glucose metabolism in premetastatic niche to promote metastasis. *Nat Cell Biol*. (2015) 17:183–94. doi: 10.1038/ncb3094
162. Eichner LJ, Perry M-C, Dufour CR, Bertos N, Park M, St-Pierre J, et al. miR-378(∗) mediates metabolic shift in breast cancer cells via the PGC-1β/ERRγ transcriptional pathway. *Cell Metab*. (2010) 12:352–61. doi: 10.1016/j.cmet.2010.09.002
163. Hatziapostolou M, Polytarchou C, Iliopoulos D. miRNAs link metabolic reprogramming to oncogenesis. *Trends Endocrinol Metab*. (2013) 24:361–73. doi: 10.1016/j.tem.2013.03.002
164. Vernieri C, Casola S, Foiani M, Pietrantonio F, de Braud F, Longo V. Targeting cancer metabolism: dietary and pharmacologic interventions. *Cancer Discov*. (2016) 6:1315–33. doi: 10.1158/2159-8290.CD-16-0615
165. Drakaki A, Hatziapostolou M, Iliopoulos D. Therapeutically targeting microRNAs in liver cancer. *Curr Pharm Des*. (2013) 19:1180–91. doi: 10.2174/138161213804805658
166. Nosengo N. Can you teach old drugs new tricks? *Nature*. (2016) 534:314–6. doi: 10.1038/534314a
167. Pantziarka P, Bouche G, Meheus L, Sukhatme V, Sukhatme VP, Vikas P. The repurposing drugs in oncology (ReDO) project. *Ecancermedicalscience*. (2014) 8:442. doi: 10.3332/ecancer.2014.442
168. Naidu S, Garofalo M. microRNAs: an emerging paradigm in lung cancer chemoresistance. *Front Med*. (2015) 2:77. doi: 10.3389/fmed.2015.00077
169. Ayers D, Vandesompele J. Influence of microRNAs and long non-coding RNAs in cancer chemoresistance. *Genes*. (2017) 8:95. doi: 10.3390/genes8030095
170. Oliveras-Ferraros C, Cufí S, Vazquez-Martin A, Torres-Garcia VZ, Del Barco S, Martin-Castillo B, et al. Micro(mi)RNA expression profile of breast cancer epithelial cells treated with the anti-diabetic drug metformin: induction of the tumor suppressor miRNA let-7a and suppression of

the TGFβ-induced oncomiR miRNA-181a. *Cell Cycle*. (2011) 10:1144–51. doi: 10.4161/cc.10.7.15210
171. Bao B, Wang Z, Ali S, Ahmad A, Azmi AS, Sarkar SH, et al. Metformin inhibits cell proliferation, migration and invasion by attenuating CSC function mediated by deregulating miRNAs in pancreatic cancer cells. *Cancer Prev Res.* (2012) 5:355–64. doi: 10.1158/1940-6207.CAPR-11-0299
172. Li W, Yuan Y, Huang L, Qiao M, Zhang Y. Metformin alters the expression profiles of microRNAs in human pancreatic cancer cells. *Diabetes Res Clin Pract*. (2012) 96:187–95. doi: 10.1016/j.diabres.2011.12.028
173. Wang F, Xu J, Liu H, Liu Z, Xia F. Metformin induces apoptosis by microRNA-26a-mediated downregulation of myeloid cell leukaemia-1 in human oral cancer cells. *Mol Med Rep*. (2016) 13:4671–6. doi: 10.3892/mmr.2016.5143
174. Yang F-Q, Wang J-J, Yan J-S, Huang J-H, Li W, Che J-P, et al. Metformin inhibits cell growth by upregulating microRNA-26a in renal cancer cells. *Int J Clin Exp Med*. (2014) 7:3289–96.
175. Cifarelli V, Lashinger LM, Devlin KL, Dunlap SM, Huang J, Kaaks R, et al. Metformin and rapamycin reduce pancreatic cancer growth in obese prediabetic mice by distinct microRNA-regulated mechanisms. *Diabetes*. (2015) 64:1632–42. doi: 10.2337/db14-1132
176. Do MT, Kim HG, Choi JH, Jeong HG. Metformin induces microRNA-34a to downregulate the Sirt1/Pgc-1α/Nrf2 pathway, leading to increased susceptibility of wild-type p53 cancer cells to oxidative stress and therapeutic agents. *Free Radic Biol Med*. (2014) 74:21–34. doi: 10.1016/j.freeradbiomed.2014.06.010
177. Xie W, Wang L, Sheng H, Qiu J, Zhang D, Zhang L, et al. Metformin induces growth inhibition and cell cycle arrest by upregulating microRNA34a in renal cancer cells. *Med Sci Monit*. (2017) 23:29–37. doi: 10.12659/msm.898710
178. Wahdan-Alaswad RS, Cochrane DR, Spoelstra NS, Howe EN, Edgerton SM, Anderson SM, et al. Metformin-induced killing of triple-negative breast cancer cells is mediated by reduction in fatty acid synthase via miRNA-193b. *Horm Cancer*. (2014) 5:374–89. doi: 10.1007/s12672-014-0188-8
179. Zhao W, Zhang X, Liu J, Sun B, Tang H, Zhang H. miR-27a-mediated antiproliferative effects of metformin on the breast cancer cell line MCF-7. *Oncol Rep*. (2016) 36:3691–9. doi: 10.3892/or.2016.5199
180. Nangia-Makker P, Yu Y, Vasudevan A, Farhana L, Rajendra SG, Levi E, et al. Metformin: a potential therapeutic agent for recurrent colon cancer. *PLoS ONE*. (2014) 9:e84369. doi: 10.1371/journal.pone.0084369
181. Cufí S, Vazquez-Martin A, Oliveras-Ferraros C, Quirantes R, Segura-Carretero A, Micol V, et al. Metformin lowers the threshold for stress-induced senescence: a role for the microRNA-200 family and miR-205. *Cell Cycle*. (2012) 11:1235–46. doi: 10.4161/cc.11.6.19665
182. Jiang X, Ma N, Wang D, Li F, He R, Li D, et al. Metformin inhibits tumor growth by regulating multiple miRNAs in human cholangiocarcinoma. *Oncotarget*. (2015) 6:3178–94. doi: 10.18632/oncotarget.3063
183. Tseng H-W, Li S-C, Tsai K-W. Metformin treatment suppresses melanoma cell growth and motility through modulation of microRNA expression. *Cancers*. (2019) 11:209. doi: 10.3390/cancers11020209
184. Wang Y, Dai W, Chu X, Yang B, Zhao M, Sun Y. Metformin inhibits lung cancer cells proliferation through repressing microRNA-222. *Biotechnol Lett*. (2013) 35:2013–9. doi: 10.1007/s10529-013-1309-0
185. Blandino G, Valerio M, Cioce M, Mori F, Casadei L, Pulito C, et al. Metformin elicits anticancer effects through the sequential modulation of DICER and c-MYC. *Nat Commun*. (2012) 3:865. doi: 10.1038/ncomms1859
186. Avci CB, Harman E, Dodurga Y, Susluer SY, Gunduz C. Therapeutic potential of an anti-diabetic drug, metformin: alteration of miRNA expression in prostate cancer cells. *Asian Pac J Cancer Prev*. (2013) 14:765–8. doi: 10.7314/APJCP.2013.14.2.765
187. Tsao C-J. microRNA-21-mediated regulation of Sprouty2 protein expression enhances the cytotoxic effect of 5-fluorouracil and metformin in colon cancer cells. *Int J Mol Med*. (2012) 29:920–6. doi: 10.3892/ijmm.2012.910
188. Pulito C, Mori F, Sacconi A, Goeman F, Ferraiuolo M, Pasanisi P, et al. Metformin-induced ablation of microRNA 21-5p releases Sestrin-1 and CAB39L antitumoral activities. *Cell Discov*. (2017) 3:17022. doi: 10.1038/celldisc.2017.22
189. Devlin C, Greco S, Martelli F, Ivan M. miR-210: more than a silent player in hypoxia. *IUBMB Life*. (2011) 63:94–100. doi: 10.1002/iub.427
190. Harting T, Stubbendorff M, Willenbrock S, Wagner S, Schadzek P, Ngezahayo A, et al. The effect of dichloroacetate in canine prostate adenocarcinomas and transitional cell carcinomas *in vitro*. *Int J Oncol*. (2016) 49:2341–50. doi: 10.3892/ijo.2016.3720
191. Takwi AAL, Li Y, Becker Buscaglia LE, Zhang J, Choudhury S, Park AK, et al. A statin-regulated microRNA represses human c-Myc expression and function. *EMBO Mol Med*. (2012) 4:896–909. doi: 10.1002/emmm.201101045
192. Kang M, Lee K-H, Lee HS, Jeong CW, Ku JH, Kim HH, et al. Concurrent treatment with simvastatin and NF-κB inhibitor in human castration-resistant prostate cancer cells exerts synergistic anti-cancer effects via control of the NF-κB/LIN28/let-7 miRNA signaling pathway. *PLoS ONE*. (2017) 12:e0184644. doi: 10.1371/journal.pone.0184644
193. Karlic H, Thaler R, Gerner C, Grunt T, Proestling K, Haider F, et al. Inhibition of the mevalonate pathway affects epigenetic regulation in cancer cells. *Cancer Genet*. (2015) 208:241–52. doi: 10.1016/j.cancergen.2015.03.008
194. Zheng X, Liu K, Wang X, Zhang R, Li X. MicroRNA-192 acts as a tumor suppressor in colon cancer and simvastatin activates miR-192 to inhibit cancer cell growth. *Mol Med Rep*. (2019) 19:1753–60. doi: 10.3892/mmr.2019.9808
195. Peng F, Wang J-H, Fan W-J, Meng Y-T, Li M-M, Li T-T, et al. Glycolysis gatekeeper PDK1 reprograms breast cancer stem cells under hypoxia. *Oncogene*. (2018) 37:1062–74. doi: 10.1038/onc.2017.368
196. Bai F, Yu Z, Gao X, Gong J, Fan L, Liu F. Simvastatin induces breast cancer cell death through oxidative stress up-regulating miR-140-5p. *Aging*. (2019) 11:3198–219. doi: 10.18632/aging.101974
197. Bhardwaj A, Singh H, Trinidad CM, Albarracin CT, Hunt KK, Bedrosian I. The isomiR-140-3p-regulated mevalonic acid pathway as a potential target for prevention of triple negative breast cancer. *Breast Cancer Res*. (2018) 20:150. doi: 10.1186/s13058-018-1074-z
198. Karataş ÖF, Ittmann M. MiR-33a and statins collaboratively reduce the proliferative capacity of prostate cancer cells. *Eur Res J*. (2018) 4:266–74. doi: 10.18621/eurj.380619
199. Li P, Yin Y-L, Guo T, Sun X-Y, Ma H, Zhu M-L, et al. Inhibition of aberrant microRNA-133a expression in endothelial cells by statin prevents endothelial dysfunction by targeting GTP cyclohydrolase 1 *in vivo*. *Circulation*. (2016) 134:1752–65. doi: 10.1161/CIRCULATIONAHA.116.017949
200. Lam HC, Liu H-J, Baglini CV, Filippakis H, Alesi N, Nijmeh J, et al. Rapamycin-induced miR-21 promotes mitochondrial homeostasis and adaptation in mTORC1 activated cells. *Oncotarget*. (2017) 8:64714–27. doi: 10.18632/oncotarget.19947
201. Totary-Jain H, Sanoudou D, Ben-Dov IZ, Dautriche CN, Guarnieri P, Marx SO, et al. Reprogramming of the MicroRNA transcriptome mediates resistance to rapamycin. *J Biol Chem*. (2013) 288:6034–44. doi: 10.1074/jbc.M112.416446
202. Gan H, Lin L, Hu N, Yang Y, Gao Y, Pei Y, et al. Aspirin ameliorates lung cancer by targeting the miR-98/WNT1 axis. *Thorac Cancer*. (2019) 10:744–50. doi: 10.1111/1759-7714.12992
203. Li X, Gao L, Cui Q, Gary BD, Dyess DL, Taylor W, et al. Sulindac inhibits tumor cell invasion by suppressing NF-κB-mediated transcription of microRNAs. *Oncogene*. (2012) 31:4979–86. doi: 10.1038/onc.2011.655
204. Kang C. Genome-wide identification of TCF7L2/TCF4 target miRNAs reveals a role for miR-21 in Wnt-driven epithelial cancer. *Int J Oncol*. (2011) 40:519–26. doi: 10.3892/ijo.2011.1215
205. Saito Y, Suzuki H, Imaeda H, Matsuzaki J, Hirata K, Tsugawa H, et al. The tumor suppressor *microRNA-29c* is downregulated and restored by celecoxib in human gastric cancer cells. *Int J Cancer*. (2013) 132:1751–60. doi: 10.1002/ijc.27862
206. Wang J, Zhang X, Shi J, Cao P, Wan M, Zhang Q, et al. Fatty acid synthase is a primary target of MiR-15a and MiR-16-1 in breast cancer. *Oncotarget*. (2016) 7:78566–76. doi: 10.18632/oncotarget.12479
207. Mishra PJ, Humeniuk R, Mishra PJ, Longo-Sorbello GSA, Banerjee D, Bertino JR. A miR-24 microRNA binding-site polymorphism in dihydrofolate reductase gene leads to methotrexate resistance. *Proc Natl Acad Sci USA*. (2007) 104:13513–8. doi: 10.1073/pnas.0706217104

208. Song B, Wang Y, Xi Y, Kudo K, Bruheim S, Botchkina GI, et al. Mechanism of chemoresistance mediated by miR-140 in human osteosarcoma and colon cancer cells. *Oncogene*. (2009) 28:4065–74. doi: 10.1038/onc.2009.274
209. Song B, Wang Y, Titmus MA, Botchkina G, Formentini A, Kornmann M, et al. Molecular mechanism of chemoresistance by miR-215 in osteosarcoma and colon cancer cells. *Mol Cancer*. (2010) 9:96. doi: 10.1186/1476-4598-9-96
210. Valeri N, Gasparini P, Braconi C, Paone A, Lovat F, Fabbri M, et al. MicroRNA-21 induces resistance to 5-fluorouracil by down-regulating human DNA MutS homolog 2. (hMSH2). *Proc Natl Acad Sci USA*. (2010) 107:21098–103. doi: 10.1073/pnas.1015541107
211. Akao Y, Noguchi S, Iio A, Kojima K, Takagi T, Naoe T. Dysregulation of microRNA-34a expression causes drug-resistance to 5-FU in human colon cancer DLD-1 cells. *Cancer Lett*. (2011) 300:197–204. doi: 10.1016/j.canlet.2010.10.006
212. He J, Xie G, Tong J, Peng Y, Huang H, Li J, et al. Overexpression of microRNA-122 re-sensitizes 5-FU-resistant colon cancer cells to 5-FU through the inhibition of PKM2 *in vitro* and *in vivo*. *Cell Biochem Biophys*. (2014) 70:1343–50. doi: 10.1007/s12013-014-0062-x
213. Shah MY, Pan X, Fix LN, Farwell MA, Zhang B. 5-fluorouracil drug alters the microrna expression profiles in MCF-7 breast cancer cells. *J Cell Physiol*. (2011) 226:1868–78. doi: 10.1002/jcp.22517
214. Park J-K, Lee EJ, Esau C, Schmittgen TD. Antisense Inhibition of microRNA-21 or−221 arrests cell cycle, induces apoptosis, and sensitizes the effects of gemcitabine in pancreatic adenocarcinoma: *Pancreas*. (2009) 38:e190–9. doi: 10.1097/MPA.0b013e3181ba82e1
215. Almeida MI, Reis RM, Calin GA. Decoy activity through microRNAs: the therapeutic implications. *Expert Opin Biol Ther*. (2012) 12:1153–9. doi: 10.1517/14712598.2012.693470
216. Kim EJ, Tu M-J, Duan Z, Gonzalez FJ, Bold RJ, Yu A. MicroRNA-1291 effects on pancreatic cancer (PC) cells sensitivity to arginine deprivation and chemotherapy through modulation of ASS1 and GLUT1. *J Clin Oncol*. (2018) 36:334. doi: 10.1200/JCO.2018.36.4_suppl.334
217. El-Mir M-Y, Nogueira V, Fontaine E, Avéret N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem*. (2000) 275:223–8. doi: 10.1074/jbc.275.1.223
218. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest*. (2001) 108:1167–74. doi: 10.1172/JCI13505
219. Bridges HR, Jones AJY, Pollak MN, Hirst J. Effects of metformin and other biguanides on oxidative phosphorylation in mitochondria. *Biochem J*. (2014) 462:475–87. doi: 10.1042/BJ20140620
220. Wheaton WW, Weinberg SE, Hamanaka RB, Soberanes S, Sullivan LB, Anso E, et al. Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. *Elife*. (2014) 3:e02242. doi: 10.7554/eLife.02242
221. Evans JMM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. *BMJ*. (2005) 330:1304–5. doi: 10.1136/bmj.38415.708634.F7
222. Lee JH, Kim TI, Jeon SM, Hong SP, Cheon JH, Kim WH. The effects of metformin on the survival of colorectal cancer patients with diabetes mellitus. *Int J Cancer*. (2012) 131:752–9. doi: 10.1002/ijc.26421
223. Noto H, Goto A, Tsujimoto T, Noda M. Cancer risk in diabetic patients treated with metformin: a systematic review and meta-analysis. *PLoS ONE*. (2012) 7:e33411. doi: 10.1371/journal.pone.0033411
224. Gandini S, Puntoni M, Heckman-Stoddard BM, Dunn BK, Ford L, DeCensi A, et al. Metformin and cancer risk and mortality: a systematic review and meta-analysis taking into account biases and confounders. *Cancer Prev Res*. (2014) 7:867–85. doi: 10.1158/1940-6207.CAPR-13-0424
225. Jiralerspong S, Palla SL, Giordano SH, Meric-Bernstam F, Liedtke C, Barnett CM, et al. Metformin and pathologic complete responses to neoadjuvant chemotherapy in diabetic patients with breast cancer. *J Clin Oncol*. (2009) 27:3297–302. doi: 10.1200/JCO.2009.19.6410
226. Gui DY, Sullivan LB, Luengo A, Hosios AM, Bush LN, Gitego N, et al. Environment dictates dependence on mitochondrial complex I for NAD+ and aspartate production and determines cancer cell sensitivity to metformin. *Cell Metab*. (2016) 24:716–27. doi: 10.1016/j.cmet.2016.09.006
227. Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, et al. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc Natl Acad Sci USA*. (2004) 101:3329–35. doi: 10.1073/pnas.0308061100
228. Kim HJ, Lee S, Chun KH, Jeon JY, Han SJ, Kim DJ, et al. Metformin reduces the risk of cancer in patients with type 2 diabetes: an analysis based on the Korean national diabetes program cohort. *Medicine*. (2018) 97:e0036. doi: 10.1097/MD.0000000000010036
229. Lee M-Y, Lin K-D, Hsiao P-J, Shin S-J. The association of diabetes mellitus with liver, colon, lung, and prostate cancer is independent of hypertension, hyperlipidemia, and gout in Taiwanese patients. *Metabolism*. (2012) 61:242–9. doi: 10.1016/j.metabol.2011.06.020
230. He K, Hu H, Ye S, Wang H, Cui R, Yi L. The effect of metformin therapy on incidence and prognosis in prostate cancer: a systematic review and meta-analysis. *Sci Rep*. (2019) 9:2218. doi: 10.1038/s41598-018-38285-w
231. Libby G, Donnelly LA, Donnan PT, Alessi DR, Morris AD, Evans JMM. New users of metformin are at low risk of incident cancer: a cohort study among people with type 2 diabetes. *Diabetes Care*. (2009) 32:1620–5. doi: 10.2337/dc08-2175
232. Saunier E, Benelli C, Bortoli S. The pyruvate dehydrogenase complex in cancer: an old metabolic gatekeeper regulated by new pathways and pharmacological agents: pyruvate dehydrogenase complex in cancer. *Int J Cancer*. (2016) 138:809–17. doi: 10.1002/ijc.29564
233. Kim K, Kim JK, Han SH, Lim J-S, Kim KI, Cho DH, et al. Adiponectin is a negative regulator of NK cell cytotoxicity. *J Immunol*. (2006) 176:5958–64. doi: 10.4049/jimmunol.176.10.5958
234. Michelakis ED, Webster L, Mackey JR. Dichloroacetate (DCA) as a potential metabolic-targeting therapy for cancer. *Br J Cancer*. (2008) 99:989–94. doi: 10.1038/sj.bjc.6604554
235. Wen S, Zhu D, Huang P. Targeting cancer cell mitochondria as a therapeutic approach. *Future Med Chem*. (2013) 5:53–67. doi: 10.4155/fmc.12.190
236. Bingham PM, Stuart SD, Zachar Z. Lipoic acid and lipoic acid analogs in cancer metabolism and chemotherapy. *Expert Rev Clin Pharmacol*. (2014) 7:837–46. doi: 10.1586/17512433.2014.966816
237. Liang Y, Hou L, Li L, Li L, Zhu L, Wang Y, et al. Dichloroacetate restores colorectal cancer chemosensitivity through the p53/miR-149-3p/PDK2-mediated glucose metabolic pathway. *Oncogene*. (2019) doi: 10.1038/s41388-019-1035-8
238. Bjarnadottir O, Romero Q, Bendahl P-O, Jirström K, Rydén L, Loman N, et al. Targeting HMG-CoA reductase with statins in a window-of-opportunity breast cancer trial. *Breast Cancer Res Treat*. (2013) 138:499–508. doi: 10.1007/s10549-013-2473-6
239. Lin JJ, Ezer N, Sigel K, Mhango G, Wisnivesky JP. The effect of statins on survival in patients with stage IV lung cancer. *Lung Cancer*. (2016) 99:137–42. doi: 10.1016/j.lungcan.2016.07.006
240. Hung M-S, Chen I-C, Lee C-P, Huang R-J, Chen P-C, Tsai Y-H, et al. Statin improves survival in patients with EGFR-TKI lung cancer: a nationwide population-based study. *PLoS ONE*. (2017) 12:e0171137. doi: 10.1371/journal.pone.0171137
241. Newman A, Clutterbuck RD, Powles RL, Catovsky D, Millar JL. A comparison of the effect of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors simvastatin, lovastatin and pravastatin on leukaemic and normal bone marrow progenitors. *Leuk Lymphoma*. (1997) 24:533–7. doi: 10.3109/10428199709055590
242. Dimitroulakos J, Thai S, Wasfy GH, Hedley DW, Minden MD, Penn LZ. Lovastatin induces a pronounced differentiation response in acute myeloid leukemias. *Leuk Lymphoma*. (2000) 40:167–78. doi: 10.3109/10428190009054894
243. Denoyelle C. Cerivastatin, an inhibitor of HMG-CoA reductase, inhibits the signaling pathways involved in the invasiveness and metastatic properties of highly invasive breast cancer cell lines: an *in vitro* study. *Carcinogenesis*. (2001) 22:1139–48. doi: 10.1093/carcin/22.8.1139
244. Mannello F, Tonti GA. Statins and breast cancer: may matrix metalloproteinase be the missing link. *Cancer Invest*. (2009) 27:466–70. doi: 10.1080/07357900802491444
245. Chiarini F, Evangelisti C, McCubrey JA, Martelli AM. Current treatment strategies for inhibiting mTOR in cancer. *Trends Pharmacol Sci*. (2015) 36:124–35. doi: 10.1016/j.tips.2014.11.004

246. Tolcher AW, Bendell JC, Papadopoulos KP, Burris HA, Patnaik A, Jones SF, et al. A phase IB trial of the oral MEK inhibitor trametinib (GSK1120212) in combination with everolimus in patients with advanced solid tumors. *Ann Oncol*. (2015) 26:58–64. doi: 10.1093/annonc/mdu482
247. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. (2012) 149:274–93. doi: 10.1016/j.cell.2012.03.017
248. Zaytseva YY, Rychahou PG, Gao T, Lee EY, Weiss HL, Heuer TS, et al. Abstract 1010: evaluation of small-molecule FASN inhibitors in preclinical models of colorectal cancer. *Cancer Res.* (2016) 76:1010. doi: 10.1158/1538-7445.AM2016-1010
249. Brenner A, Falchook G, Patel M, Infante J, Arkenau H-T, Dean E, et al. Abstract P6-11-09: heavily pre-treated breast cancer patients show promising responses in the first in human study of the first-In-class fatty acid synthase (FASN) inhibitor, TVB-2640 in combination with paclitaxel. *Cancer Res.* (2017) 77(Suppl. 4). doi: 10.1158/1538-7445.SABCS16-P6-11-09
250. Zaytseva YY, Rychahou PG, Le A-T, Scott TL, Flight RM, Kim JT, et al. Preclinical evaluation of novel fatty acid synthase inhibitors in primary colorectal cancer cells and a patient-derived xenograft model of colorectal cancer. *Oncotarget*. (2018) 9: 24787–800. doi: 10.18632/oncotarget.25361
251. Hundal RS, Petersen KF, Mayerson AB, Randhawa PS, Inzucchi S, Shoelson SE, et al. Mechanism by which high-dose aspirin improves glucose metabolism in type 2 diabetes. *J Clin Invest*. (2002) 109:1321–6. doi: 10.1172/JCI0214955
252. Paez Espinosa EV, Murad JP, Khasawneh FT. Aspirin: pharmacology and clinical applications. *Thrombosis*. (2012) 2012:1–15. doi: 10.1155/2012/173124
253. Hawley SA, Fullerton MD, Ross FA, Schertzer JD, Chevtzoff C, Walker KJ, et al. The ancient drug salicylate directly activates AMP-activated protein kinase. *Science*. (2012) 336:918–22. doi: 10.1126/science.1215327
254. Hammerlindl H, Ravindran Menon D, Hammerlindl S, Emran AA, Torrano J, Sproesser K, et al. Acetylsalicylic acid governs the effect of sorafenib in *RAS* -mutant cancers. *Clin Cancer Res*. (2018) 24:1090–102. doi: 10.1158/1078-0432.CCR-16-2118
255. Drew DA, Cao Y, Chan AT. Aspirin and colorectal cancer: the promise of precision chemoprevention. *Nat Rev Cancer*. (2016) 16:173–86. doi: 10.1038/nrc.2016.4
256. Rothwell PM, Fowkes FGR, Belch JFF, Ogawa H, Warlow CP, Meade TW. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. *Lancet Lond Engl*. (2011) 377:31–41. doi: 10.1016/S0140-6736(10)62110-1
257. Simon TG, Ma Y, Ludvigsson JF, Chong DQ, Giovannucci EL, Fuchs CS, et al. Association between aspirin use and risk of hepatocellular carcinoma. *JAMA Oncol*. (2018) 4:1683–90. doi: 10.1001/jamaoncol.2018.4154
258. Barnard ME, Poole EM, Curhan GC, Eliassen AH, Rosner BA, Terry KL, et al. Association of analgesic use with risk of ovarian cancer in the nurses' health studies. *JAMA Oncol*. (2018) 4:1675–82. doi: 10.1001/jamaoncol.2018.4149
259. Pinheiro C, Longatto-Filho A, Scapulatempo C, Ferreira L, Martins S, Pellerin L, et al. Increased expression of monocarboxylate transporters 1, 2, and 4 in colorectal carcinomas. *Virchows Arch*. (2008) 452:139–46. doi: 10.1007/s00428-007-0558-5
260. Pinheiro C, Albergaria A, Paredes J, Sousa B, Dufloth R, Vieira D, et al. Monocarboxylate transporter 1 is up-regulated in basal-like breast carcinoma: MCT1 in breast cancer. *Histopathology*. (2010) 56:860–7. doi: 10.1111/j.1365-2559.2010.03560.x
261. Bola BM, Chadwick AL, Michopoulos F, Blount KG, Telfer BA, Williams KJ, et al. Inhibition of monocarboxylate transporter-1 (MCT1) by AZD3965 enhances radiosensitivity by reducing lactate transport. *Mol Cancer Ther*. (2014) 13:2805–16. doi: 10.1158/1535-7163.MCT-13-1091
262. Halford SER, Jones P, Wedge S, Hirschberg S, Katugampola S, Veal G, et al. A first-in-human first-in-class (FIC) trial of the monocarboxylate transporter 1 (MCT1) inhibitor AZD3965 in patients with advanced solid tumours. *J Clin Oncol*. (2017) 35:2516. doi: 10.1200/JCO.2017.35.15_suppl.2516
263. Mehrmohamadi M, Jeong SH, Locasale JW. Molecular features that predict the response to antimetabolite chemotherapies. *Cancer Metab*. (2017) 5:8. doi: 10.1186/s40170-017-0170-3
264. Showalter SL, Showalter TN, Witkiewicz A, Havens R, Kennedy EP, Hucl T, et al. Evaluating the drug-target relationship between thymidylate synthase expression and tumor response to 5-fluorouracil: Is it time to move forward? *Cancer Biol Ther*. (2008) 7:986–94. doi: 10.4161/cbt.7.7.6181
265. Costales MG, Haga CL, Velagapudi SP, Childs-Disney JL, Phinney DG, Disney MD. Small molecule inhibition of microRNA-210 reprograms an oncogenic hypoxic circuit. *J Am Chem Soc*. (2017) 139:3446–55. doi: 10.1021/jacs.6b11273
266. Kelly TJ, Souza AL, Clish CB, Puigserver P. A hypoxia-induced positive feedback loop promotes hypoxia-inducible factor 1alpha stability through miR-210 suppression of glycerol-3-phosphate dehydrogenase 1-like. *Mol Cell Biol*. (2011) 31:2696–706. doi: 10.1128/MCB.01242-10
267. Heidelberger C, Chaudhuri NK, Danneberg P, Mooren D, Griesbach L, Duschinsky R, et al. Fluorinated pyrimidines, a new class of tumour-inhibitory compounds. *Nature*. (1957) 179:663–6. doi: 10.1038/179663a0
268. Wagner AD, Grothe W, Haerting J, Kleber G, Grothey A, Fleig WE. Chemotherapy in advanced gastric cancer: a systematic review and meta-analysis based on aggregate data. *J Clin Oncol*. (2006) 24:2903–9. doi: 10.1200/JCO.2005.05.0245
269. Parker WB. Enzymology of purine and pyrimidine antimetabolites used in the treatment of cancer. *Chem Rev*. (2009) 109:2880–93. doi: 10.1021/cr900028p
270. Hanna J, Hossain GS, Kocerha J. The potential for microRNA therapeutics and clinical research. *Front Genet*. (2019) 10:478. doi: 10.3389/fgene.2019.00478
271. Garzon R, Marcucci G, Croce CM. Targeting microRNAs in cancer: rationale, strategies and challenges. *Nat Rev Drug Discov*. (2010) 9:775–89. doi: 10.1038/nrd3179
272. van Rooij E, Kauppinen S. Development of microRNA therapeutics is coming of age. *EMBO Mol Med*. (2014) 6:851–64. doi: 10.15252/emmm.201100899
273. Shah MY, Ferrajoli A, Sood AK, Lopez-Berestein G, Calin GA. microRNA therapeutics in cancer - an emerging concept. *EBioMedicine*. (2016) 12:34–42. doi: 10.1016/j.ebiom.2016.09.017
274. Yingchoncharoen P, Kalinowski DS, Richardson DR. Lipid-based drug delivery systems in cancer therapy: what is available and what is yet to come. *Pharmacol Rev*. (2016) 68:701–87. doi: 10.1124/pr.115.012070
275. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang H-W, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell*. (2009) 137:1005–17. doi: 10.1016/j.cell.2009.04.021
276. MacDiarmid JA, Mugridge NB, Weiss JC, Phillips L, Burn AL, Paulin RP, et al. Bacterially derived 400 nm particles for encapsulation and cancer cell targeting of chemotherapeutics. *Cancer Cell*. (2007) 11:431–45. doi: 10.1016/j.ccr.2007.03.012
277. Beg MS, Brenner AJ, Sachdev J, Borad M, Kang Y-K, Stoudemire J, et al. Phase I study of MRX34, a liposomal miR-34a mimic, administered twice weekly in patients with advanced solid tumors. *Invest New Drugs*. (2017) 35:180–8. doi: 10.1007/s10637-016-0407-y
278. Reid G, Kao SC, Pavlakis N, Brahmbhatt H, MacDiarmid J, Clarke S, et al. Clinical development of TargomiRs, a miRNA mimic-based treatment for patients with recurrent thoracic cancer. *Epigenomics*. (2016) 8:1079–85. doi: 10.2217/epi-2016-0035
279. van Zandwijk N, Pavlakis N, Kao SC, Linton A, Boyer MJ, Clarke S, et al. Safety and activity of microRNA-loaded minicells in patients with recurrent malignant pleural mesothelioma: a first-in-man, phase 1, open-label, dose-escalation study. *Lancet Oncol*. (2017) 18:1386–96. doi: 10.1016/S1470-2045(17)30621-6
280. Seto AG, Beatty X, Lynch JM, Hermreck M, Tetzlaff M, Duvic M, et al. Cobomarsen, an oligonucleotide inhibitor of miR-155, co-ordinately regulates multiple survival pathways to reduce cellular proliferation and survival in cutaneous T-cell lymphoma. *Br J Haematol*. (2018) 183:428–44. doi: 10.1111/bjh.15547
281. Querfeld C, Pacheco T, Foss FM, Halwani AS, Porcu P, Seto AG, et al. Preliminary results of a phase 1 trial evaluating MRG-106, a synthetic microRNA antagonist (LNA antimiR) of microRNA-155, in patients with CTCL. *Blood*. (2016) 128:1829. doi: 10.1182/blood.V128.22.1829.1829

282. Ghosh D, Nandi S, Bhattacharjee S. Combination therapy to checkmate Glioblastoma: clinical challenges and advances. *Clin Transl Med*. (2018) 7:33. doi: 10.1186/s40169-018-0211-8
283. Haque I, Banerjee S, Mehta S, De A, Majumder M, Mayo MS, et al. Cysteine-rich 61-connective tissue growth factor-nephroblastoma-overexpressed 5 (CCN5)/Wnt-1-induced signaling protein-2 (WISP-2) regulates microRNA-10b via hypoxia-inducible factor-1α-TWIST signaling networks in human breast cancer cells. *J Biol Chem*. (2011) 286:43475–85. doi: 10.1074/jbc.M111.284158
284. Gomez IG, MacKenna DA, Johnson BG, Kaimal V, Roach AM, Ren S, et al. Anti-microRNA-21 oligonucleotides prevent Alport nephropathy progression by stimulating metabolic pathways. *J Clin Invest*. (2015) 125:141–56. doi: 10.1172/JCI75852
285. Steeg PS, Theodorescu D. Metastasis: a therapeutic target for cancer. *Nat Clin Pract Oncol*. (2008) 5:206–19. doi: 10.1038/ncponc1066
286. Chakraborty C, Wen Z-H, Agoramoorthy G, Lin C-S. Therapeutic microRNA delivery strategies with special emphasis on cancer therapy and tumorigenesis: current trends and future challenges. *Curr Drug Metab*. (2016) 17:469–77. doi: 10.2174/1389200217666160126142408
287. Brannon-Peppas L, Blanchette JO. Nanoparticle and targeted systems for cancer therapy. *Adv Drug Deliv Rev*. (2004) 56:1649–59. doi: 10.1016/j.addr.2004.02.014
288. Vander Heiden MG, DeBerardinis RJ. Understanding the intersections between metabolism and cancer biology. *Cell*. (2017) 168:657–69. doi: 10.1016/j.cell.2016.12.039
289. Larsson SC, Wolk A. Overweight and obesity and incidence of leukemia: a meta-analysis of cohort studies: overweight and obesity and incidence of leukemia. *Int J Cancer*. (2008) 122:1418–21. doi: 10.1002/ijc.23176
290. Roberts DL, Dive C, Renehan AG. Biological mechanisms linking obesity and cancer risk: new perspectives. *Annu Rev Med*. (2010) 61:301–16. doi: 10.1146/annurev.med.080708.082713
291. Castillo JJ, Reagan JL, Ingham RR, Furman M, Dalia S, Merhi B, et al. Obesity but not overweight increases the incidence and mortality of leukemia in adults: a meta-analysis of prospective cohort studies. *Leuk Res*. (2012) 36:868–75. doi: 10.1016/j.leukres.2011.12.020
292. Lehr S, Hartwig S, Sell H. Adipokines: a treasure trove for the discovery of biomarkers for metabolic disorders. *Proteomics Clin Appl*. (2012) 6:91–101. doi: 10.1002/prca.201100052
293. Arner P, Kulyté A. MicroRNA regulatory networks in human adipose tissue and obesity. *Nat Rev Endocrinol*. (2015) 11:276–88. doi: 10.1038/nrendo.2015.25
294. Iacomino G, Siani A. Role of microRNAs in obesity and obesity-related diseases. *Genes Nutr*. (2017) 12:23. doi: 10.1186/s12263-017-0577-z
295. Kasiappan R, Rajarajan D. Role of microRNA regulation in obesity-associated breast cancer: nutritional perspectives. *Adv Nutr Int Rev J*. (2017) 8:868–88. doi: 10.3945/an.117.015800
296. Ayers D, Boughanem H, Macías-González M. Epigenetic influences in the obesity/colorectal cancer axis: a novel theragnostic avenue. *J Oncol*. (2019) 2019:7406078. doi: 10.1155/2019/7406078
297. Garrett WS. Cancer and the microbiota. *Science*. (2015) 348:80–6. doi: 10.1126/science.aaa4972
298. Tsilimigras MCB, Fodor A, Jobin C. Carcinogenesis and therapeutics: the microbiota perspective. *Nat Microbiol*. (2017) 2:17008. doi: 10.1038/nmicrobiol.2017.8
299. Elinav E, Garrett WS, Trinchieri G, Wargo J. The cancer microbiome. *Nat Rev Cancer*. (2019) 19:371–6. doi: 10.1038/s41568-019-0155-3
300. Helmink BA, Khan MAW, Hermann A, Gopalakrishnan V, Wargo JA. The microbiome, cancer, and cancer therapy. *Nat Med*. (2019) 25:377–88. doi: 10.1038/s41591-019-0377-7
301. Gill SR, Pop M, DeBoy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. *Science*. (2006) 312:1355–9. doi: 10.1126/science.1124234
302. Philipp B. Bacterial degradation of bile salts. *Appl Microbiol Biotechnol*. (2011) 89:903–15. doi: 10.1007/s00253-010-2998-0
303. Schwabe RF, Jobin C. The microbiome and cancer. *Nat Rev Cancer*. (2013) 13:800–12. doi: 10.1038/nrc3610
304. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. *Nature*. (2012) 486:207–14. doi: 10.1038/nature11234
305. McDonald D, Hyde E, Debelius JW, Morton JT, Gonzalez A, Ackermann G, et al. American gut: an open platform for citizen science microbiome research. *mSystems*. (2018) 3:e00031-18. doi: 10.1128/mSystems.00031-18
306. Francescone R, Hou V, Grivennikov SI. Microbiome, inflammation, and cancer. *Cancer J*. (2014) 20:181–9. doi: 10.1097/PPO.0000000000000048
307. Irrazábal T, Belcheva A, Girardin SE, Martin A, Philpott DJ. The multifaceted role of the intestinal microbiota in colon cancer. *Mol Cell*. (2014) 54:309–20. doi: 10.1016/j.molcel.2014.03.039
308. Chen J, Pitmon E, Wang K. Microbiome, inflammation and colorectal cancer. *Semin Immunol*. (2017) 32:43–53. doi: 10.1016/j.smim.2017.09.006
309. Mischke M, Plösch T. The gut microbiota and their metabolites: potential implications for the host epigenome. In: A. Schwiertz, editor. *Microbiota of the Human Body*. Cham: Springer International Publishing (2016). p. 33–44. doi: 10.1007/978-3-319-31248-4_3
310. Bhat MI, Kapila R. Dietary metabolites derived from gut microbiota: critical modulators of epigenetic changes in mammals. *Nutr Rev*. (2017) 75:374–89. doi: 10.1093/nutrit/nux001
311. Ye J, Wu W, Li Y, Li L. Influences of the gut microbiota on DNA methylation and histone modification. *Dig Dis Sci*. (2017) 62:1155–64. doi: 10.1007/s10620-017-4538-6
312. Williams MR, Stedtfeld RD, Tiedje JM, Hashsham SA. MicroRNAs-based inter-domain communication between the host and members of the gut microbiome. *Front Microbiol*. (2017) 8:1896. doi: 10.3389/fmicb.2017.01896
313. Yuan C, Subramanian S. microRNA-mediated tumor–microbiota metabolic interactions in colorectal cancer. *DNA Cell Biol*. (2019) 38:281–5. doi: 10.1089/dna.2018.4579
314. Warburg O. On the Origin of Cancer Cells. *Science*. (1956) 123:309–14. doi: 10.1126/science.123.3191.309
315. Hu S, Liu L, Chang EB, Wang J-Y, Raufman J-P. Butyrate inhibits pro-proliferative miR-92a by diminishing c-Myc-induced miR-17-92a cluster transcription in human colon cancer cells. *Mol Cancer*. (2015) 14:180. doi: 10.1186/s12943-015-0450-x
316. Dews M, Fox JL, Hultine S, Sundaram P, Wang W, Liu YY, et al. The Myc-miR-17 92 axis blunts TGF signaling and production of multiple TGF -dependent antiangiogenic factors. *Cancer Res*. (2010) 70:8233–46. doi: 10.1158/0008-5472.CAN-10-2412
317. Zhang G, Zhou H, Xiao H, Liu Z, Tian H, Zhou T. MicroRNA-92a functions as an oncogene in colorectal cancer by targeting PTEN. *Dig Dis Sci*. (2014) 59:98–107. doi: 10.1007/s10620-013-2858-8
318. Ke T-W, Wei P-L, Yeh K-T, Chen WT-L, Cheng Y-W. MiR-92a promotes cell metastasis of colorectal cancer through PTEN-mediated PI3K/AKT pathway. *Ann Surg Oncol*. (2015) 22:2649–55. doi: 10.1245/s10434-014-4305-2
319. Yuan C, Burns MB, Subramanian S, Blekhman R. Interaction between host microRNAs and the gut microbiota in colorectal cancer. *mSystems*. (2018) 3:e00205-17. doi: 10.1128/mSystems.00205-17
320. Yang Y, Weng W, Peng J, Hong L, Yang L, Toiyama Y, et al. *Fusobacterium nucleatum* increases proliferation of colorectal cancer cells and tumor development in mice by activating toll-like receptor 4 signaling to nuclear factor–κB, and up-regulating expression of microRNA-21. *Gastroenterology*. (2017) 152:851–66.e24. doi: 10.1053/j.gastro.2016.11.018
321. Cougnoux A, Dalmasso G, Martinez R, Buc E, Delmas J, Gibold L, et al. Bacterial genotoxin colibactin promotes colon tumour growth by inducing a senescence-associated secretory phenotype. *Gut*. (2014) 63:1932–42. doi: 10.1136/gutjnl-2013-305257
322. Dalmasso G, Cougnoux A, Delmas J, Darfeuille-Michaud A, Bonnet R. The bacterial genotoxin colibactin promotes colon tumor growth by modifying the tumor microenvironment. *Gut Microbes*. (2014) 5:675–80. doi: 10.4161/19490976.2014.969989

323. Baek SH. A novel link between SUMO modification and cancer metastasis. *Cell Cycle*. (2006) 5:1492–5. doi: 10.4161/cc.5.14.3008
324. Teng Y, Ren Y, Sayed M, Hu X, Lei C, Kumar A, et al. Plant-derived exosomal microRNAs shape the gut microbiota. *Cell Host Microbe*. (2018) 24:637–52.e8. doi: 10.1016/j.chom.2018.10.001
325. Zhang L, Hou D, Chen X, Li D, Zhu L, Zhang Y, et al. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. *Cell Res*. (2012) 22:107–26. doi: 10.1038/cr.2011.158
326. Izumi H, Tsuda M, Sato Y, Kosaka N, Ochiya T, Iwamoto H, et al. Bovine milk exosomes contain microRNA and mRNA and are taken up by human macrophages. *J Dairy Sci*. (2015) 98:2920–33. doi: 10.3168/jds.2014-9076

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REVIEW
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Predisposition to Apoptosis in Hepatocellular Carcinoma: From Mechanistic Insights to Therapeutic Strategies

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Hepatocellular carcinoma (HCC) ranks among the most rapidly evolving cancers in the Western world. The majority of HCCs develop on the basis of a chronic inflammatory liver damage that predisposes liver cancer development and leads to deregulation of multiple cellular signaling pathways. The resulting dysbalance between uncontrolled proliferation and impaired predisposition to cell death with consecutive failure to clear inflammatory damage is a key driver of malignant transformation. Therefore, resistance to death signaling accompanied by metabolic changes as well as failed immunological clearance of damaged pre-neoplastic hepatocytes are considered hallmarks of hepatocarcinogenesis. Hereby, the underlying liver disease, the type of liver damage and individual predisposition to apoptosis determines the natural course of the disease as well as the therapeutic response. Here, we will review common and individual aspects of cell death pathways in hepatocarcinogenesis with a particular emphasis on regulatory networks and key molecular alterations. We will further delineate the potential of targeting cell death-related signaling as a viable therapeutic strategy to improve the outcome of HCC patients.

Keywords: hepatocellular carcinoma, cell death, inflammation, BCL-2 family, BH3, primed to death, BH3 profiling, mitochondrial apoptosis

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INTRODUCTION

The common hallmark of the vast majority of Hepatocellular carcinomas (HCC) is a chronic inflammatory liver damage induced by a diverse spectrum of etiological risk factors (1). Depending on the type of liver injury and persistence of the underlying inflammatory stimulus, HCCs are particularly characterized by a significant phenotypic and molecular heterogeneity. Therefore, HCCs are oncogenic paradigms for inflammation-induced cancers (2). Herein, the underlying causes of the chronic liver disease range from chronic hepatitis B (HBV) and C viruses (HCV) infections over excessive alcohol abuse to metabolic liver diseases. Importantly, the obesity-associated alterations of the hepatic microenvironment resembling non-alcoholic fatty liver disease and, more importantly, steatohepatitis (NAFLD/NASH) are now among the most prominent etiological risk factors for HCC in several Western countries (3). The particular type of inflammatory liver damage induced by NASH is also responsible for a high number of

HCCs without underlying cirrhosis (4, 5). Given the rising incidence of the metabolic syndrome worldwide, it is not surprising that HCC currently ranks among the most rapidly evolving and deadliest cancers in the Western world. Further, the impaired liver function and observed molecular heterogeneity renders effective treatments of HCCs particularly challenging (6, 7).

In the context of HCC development and progression, special importance can be assigned to the type of liver damage and associated changes to the hepatic micromilieu that create a pro-oncogenic field effect and precede malignant transformation of hepatocytes (8, 9).

Various types of liver injury and associated chronic cell death responses have been identified to trigger inflammatory liver diseases, fibrosis development and, ultimately, hepatocarcinogenesis (10, 11). Accordingly, major cell death processes as well as signaling pathways are associated with liver cancer development and mainly involve apoptosis and necrosis. However, other forms of cell death, such as autophagy, necroptosis, pyroptosis, ferroptosis, or combinations of these death programs, have also been linked to HCC development and progression (11). Damaged hepatocytes induce activation and cross-talk of other non-parenchymal, immune and stromal cells with subsequent release of cytokine that fuel inflammation-induced damage and prone cancer development (12). Abnormalities in glucose and lipid metabolisms as well as microbiota composition further aggravate the oncogenic process. While the mentioned cell death mechanisms are relevant for hepatocarcinogenesis, regardless of the underlying etiological risk factors, oxidative stress and consecutive impairment of mitochondrial function seem to particularly induce hepatocyte death during metabolic liver damage and lead to signaling through B-cell lymphoma-2 (BCL-2) family proteins and activation of caspases and c-Jun N-terminal kinase during NASH-induced HCC (13). Besides prominent roles of cell death pathways in HCC development, cell death regulation and associated changes are also important for diagnosis and therapy. Several surrogate methods to assess and quantify liver injury, predominant mode of cell death and activation of inflammatory processes have been successfully evaluated in the context of acute and chronic liver diseases (14, 15). However, reliable and non-invasive cell death markers are not available in clinical routine. Cell death and inflammatory markers have also been assessed as prognostic markers or to facilitate monitoring of therapy response in the context of liver cancer (16, 17). In addition, inhibitors of apoptosis, particularly inhibitors of BCL-2 family members or caspases, have recently been introduced to target several chronic inflammatory diseases including NASH. These inhibitors might not only prevent malignant transformation and, thus, be effective as preventive compounds, but also be viable therapeutic strategies for HCC. Together, inflammatory cell death is particular relevant for mechanistic and clinical applications in liver cancer.

The here presented review aims to summarize key cellular and molecular mechanisms involved in liver cell death during hepatocarcinogenesis with a main focus on apoptosis. We will also delineate the importance of predisposition to apoptosis as a key factor for malignant transformation and specify factors that affect differential predisposition to apoptotic stimuli during liver cancer development and therapy. Finally, the impact for personalized medicine and precision oncology will be discussed.

MECHANISMS OF CELL DEATH IN HEPATOCARCINOGENESIS

Cell death is intrinsically associated with chronic inflammation in various organs including the liver (10). Herein, infectious and metabolic changes induced by the underlying etiological agent prone hepatocytes for further damage. Liver fibrogenesis and carcinogenesis are significantly accelerated by oxidative stress, cell death and inflammation. Thus, HCC is the final and most deadly consequence of all major chronic liver diseases (2). Consistently, continuous inflammatory cell death is one of the hallmarks of hepatocarcinogenesis. Almost all HCC patients show signs of cell death in sera and tissue and their emergence is indicative of adverse biological traits (18).

The apoptosis program governs the cell-autonomous removal of superfluous, infected, or damaged cells (19, 20) and thus constitutes the most prominent defense mechanism against hepatocarcinogenesis. During chronic damage, apoptosis is regulated on the outer mitochondrial membrane (OMM) by BCL-2 proteins. The pro-apoptotic BCL-2 proteins, BCL-2-associated X protein (BAX) and BCL-2 antagonist killer 1 (BAK) permeabilize the OMM and release intermembrane space proteins, such as cytochrome *c*, into the cytoplasm in order to activate the caspase cascade (21). Therefore, BAX and BAK can commit the cell to apoptosis. The cell is protected from BAX and BAK activity by functionally redundant pro-survival BCL-2 proteins. Although, BAX/BAK activation is usually followed by irreversible cellular commitment to apoptosis, cell survival is possible after limited OMM permeabilization (22). Even cells with the capacity to undergo death receptor-dependent apoptosis without mitochondrial apoptosis signaling enhance their apoptotic response by BAX/BAK activation (23). Therefore, therapeutic success of anti-tumor strategies, including targeted strategies, immune therapies as well as chemotoxic stress, rely on efficient BAX/BAK engagement in targeted cells. Several molecular alterations could be associated with induction or imbalance of pro- and anti-apoptotic BCL-2 proteins in liver cancer. They play an essential role in maintaining genomic integrity of hepatocytes. Disruption of the apoptotic program is frequently observed already during chronic liver diseases (12). Activation of BCL-x_L is further observed at high frequencies in human HCC, whereas concomitant downregulation of BAX is a common feature of HCC with p53 alterations and observed at progressed stages of the disease (24). Moreover, inhibition of caspases e.g., by XIAP is also common in human HCC and associated with TGFβ signaling and subsequent acquisition of pro-metastatic properties. In addition to the inhibition of pro-apoptotic proteins or caspases, activation of pro-survival genes as well as pathways contributes to liver cancer development and progression (25).

A prominent molecular alteration detected in a sizable number of HCC patients is NF-kB pathway that is also particularly important in metabolic liver diseases and NASH-induced HCC (26–28). The pathway controls diverse functions in a cell type and context-dependent manner and activity is observed during chronic inflammation, fibrogenesis as well as development and progression of HCC (29, 30). In hepatocytes, NF-κB mainly mediates survival during chronic damage in response to e.g., oxidative stress while suppression contributes to malignant transformation. However, NF-κB activation in non-parenchymal and immune cells can aggravate inflammation and fibrogenesis (31). Tumor necrosis factor-α and interleukin-6 are among the major inflammatory cytokines that induce this pathway. NF-κB downstream signaling resembling c-Jun N-terminal kinase (JNK), and signal transducer and activator of transcription 3 play a major role in inflammation-associated HCC (32). NF-κB activation can also be critically linked to several anti-apoptotic molecules including (cIAP1, cIAP2), XIAP, the BCL-2 family members A1 and BCL- x_L, cFLIP, TRAF1, TRAF2, and GADD45β (33). Besides JNK, NF-κB also activates other pro-survival and pro-proliferative pathways, resembling p38 MAPK (mitogen-activated protein kinase) kinase (34, 35). In this context, upstream regulators, such as the NF-κB essential modulator (NEMO), the IKK kinase complex as well as death-domain kinase receptor-interacting protein kinase 1 (RIPK1) are of particular importance. The central regulators of cell death resembling TAK1 and RIPK1 are, consequently, other common findings mechanistically linked to malignant transformation in the liver. TAK1 (MAP3-kinase TGF-β-activated kinase 1) is critically involved in the modulation of innate and adaptive immune responses. Activation of TAK1 in parenchymal cells significantly inhibits apoptosis and demonstrated anti-tumorigenic effects mediated by NF-κB activation via TNF (36). Conversely, deficiency of TAK1 impaired NF-κB activity and induced hepatocyte apoptosis, inflammation as well as HCC development in a NEMO-dependent manner (36). Consistently, alteration of the immune cell composition and impairment of immune-mediated clearance of damaged hepatocytes is an important driver of liver cancer. It has recently been shown that dysregulation of lipid metabolism in NAFLD induces selective ablation of intrahepatic CD4+ cells, which impairs mitochondrial function and generates high levels of oxidative damage, thus, corroborating lipid dysregulation with impaired anti-tumor immune-surveillance (37). Accordingly, impaired senescence surveillance by myeloid cells also induced failure in immune-mediated clearance of damaged hepatocytes and accelerated hepatocarcinogenesis. ER stress induced by metabolic liver damage following a high fat diet further enhanced resulting liver damage, increased immune infiltration, and lipogenesis and, ultimately, led to HCC development (28).

Another form of cell death recently linked to HCC development is necroptosis. Again, the mentioned TAK1 model with liver-specific ablation was employed to clarify the relative contribution of necroptosis during hepatocarcinogenesis. While response to apoptosis in the model promoted inflammation and tumorigenesis, necroptotic response had opposing effects and conferred anti-inflammatory and tumor-suppressive functions. These results indicate the diverse molecular functions of key cell death pathways in mediating apoptotic, necroptotic or other forms of cell death. Detailed dissection of the relative contribution and mechanistic hallmarks are urgently needed (12).

An improved biological understanding of the exact mechanisms driving hepatocyte cell death and, ultimately, cancer growth are not only of particular scientific interest, but also directly imply translational applications. Besides identification of patients at risk for cancer development, biomarkers of cell death might also be instrumental to delineate the biological trait, i.e., prognosis, of a tumor but also for prediction and monitoring of treatment response. Nevertheless, excessive cell death was successfully identified predict the development as well as progression of liver cancer. Furthermore, expression of key markers in cell death and surrogate characteristics were associated with clinical outcome. As such, the new checkpoint molecules RIPK1 and TRAF2 were recently confirmed as independent prognostic markers in liver cancer (38). Furthermore, the serum cell death parameter M65, which detects cleaved and uncleaved CK-18 fragments, was also demonstrated to possess clinical utility as a non-invasive marker for tumor initiation as well as prognosis, corroborating the potential as a new diagnostic tool for HCC (16). Finally, it is well-established that transcriptome profiles conferring to cell death resistance are significantly enriched in HCCs with low differentiation, high invasion and a particularly poor outcome (39). In summary, imbalance of a broad range of molecules with critical function of cell death, including dysregulation of cytokines and inflammatory as well as survival pathways during chronic liver disease, possess high relevance for clinical application and harbor potential as translational biomarkers of malignant transformation as well as progression.

ANALYSIS OF BCL-2 PROTEINS TO PREDICT TUMOR CELL APOPTOSIS

While liver tumors possess molecular characteristics that set them apart from other types of tumors, general mechanisms of apoptosis regulation apply as they have been shown in many different cell types. The discovery of opposing BCL-2 protein activities led to the rheostat model to describe regulatory interactions in mitochondrial apoptosis signaling (40, 41). The model postulates that pro-survival BCL-2 proteins act anti-apoptotic by binding to BAX and BAK. Therefore, mitochondrial apoptosis would largely dependent on different protein expression and degradation rates. In fact, platelets contain a molecular timer that commits them to apoptosis when BAK levels exceed the levels of the predominant pro-survival BCL-2 (42). The rheostat model sparked a body of work suggesting prediction of therapeutic success based on measuring BAX levels (**Figure 1**). The refined version of this approach investigated the BAX/BCL-2 ratio. However, subsequent research expanded our knowledge on protein localizations and interactions, revealing the absence of the prerequisite of the rheostat model: stable protein

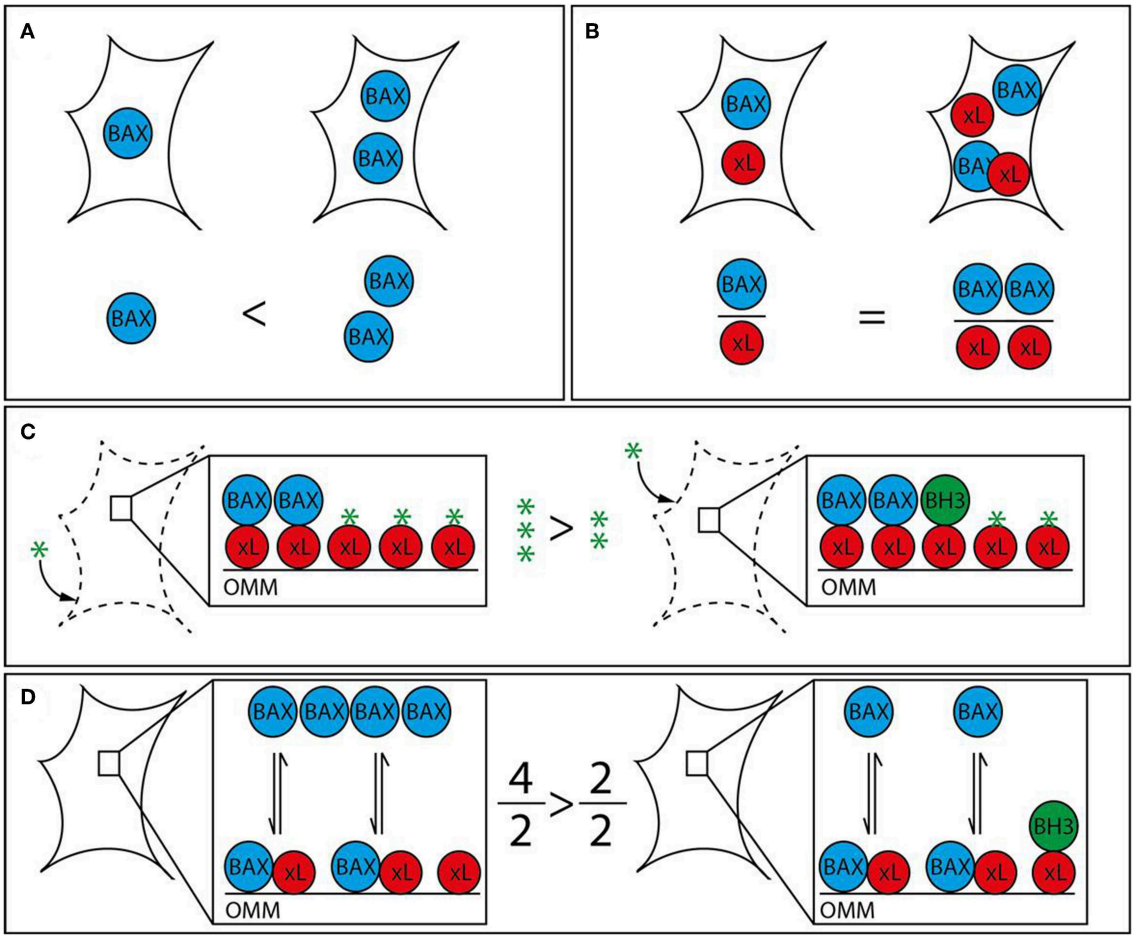

FIGURE 1 | Strategies to analyze apoptotic predisposition based on BCL-2 proteins. **(A)** BAX level. The right cell contains more BAX (blue) than the left and is therefore considered to have a higher apoptosis predisposition. Other relevant factors are not measured. **(B)** BAX vs. BCL-xL level. The ratio between BAX and a single pro-survival BCL-2 protein (BCL-xL, red) is similar in both cells. Therefore, both cells would be judged to have the same tendency to initiate apoptosis. The redundancy of the BCL-2 family would require this analysis to be expanded to all BCL-2 proteins in order to be insightful. **(C)** BH3 profiling. Permeabilized and cultured cells are incubated with BH3 peptides (green star) in order to titrate the amount of free BH3 binding sites on the outer mitochondrial membrane (OMM). BH3-only proteins (green) associated with the OMM following prior cell stress reduce the amount of free BH3 binding sites and thus increase the sensitivity toward BH3 mimetics. The increased capacity of the left cell would translate into a reduced sensitivity toward BH3 mimetics. **(D)** Relative BAX localization. Determination of the cytosolic and mitochondrial BAX pools in intact cells describes the position of the BAX localization equilibrium and thus the cellular predisposition to apoptosis. While single contributing factors cannot be dissected, all contributing factors, e.g., BCL-2 protein interactions with BH3 motifs and other segments, interacting proteins outside the BCL-2 family, are included. BH3-only proteins reduce the shuttling rate and thus the cytosolic BAX pool. The larger cytosolic pool of the left cell shows reduced predisposition to apoptosis. The analysis can be supplemented with measuring the functionally redundant and similarly regulated BAK, which is usually shifted toward the mitochondria but shows a similar range of localizations in human samples.

complexes. Prediction of apoptotic outcome based on protein levels encountered another major problem with the discovery of new members of the BCL-2 family. Their functional redundancies forsake all educated guesses, whether pro-survival BCL-2 proteins outnumber pro-apoptotic BCL-2 proteins. Therefore, apoptosis predictions based on protein levels, although occasionally attempted, are unrewarding.

A group of proteins that has influenced revised paradigms for apoptosis signaling and predictions of apoptotic outcome contains BH3-only proteins (**Figure 2**). BH3-only proteins are defined by harboring a single BH3 motif, while the remaining protein structures diverge as much as the type of stress signaled to the OMM, including DNA damage, ER stress, death receptor signaling and other types of stress (43). BH3-only proteins are thought to either inhibit pro-survival BCL-2 proteins and/or directly activate BAX and BAK (44). Inhibition of pro-survival BCL-2 proteins by BH3-only proteins is structurally well-characterized and has led to the development of low molecular weight inhibitors. These targeted anti-cancer small molecule inhibitors called BH3 mimetics bind to and inhibit pro-survival BCL-2 proteins in a manner similar to BH3-only proteins.

The concept that BH3-only proteins loaded on the OMM could determine the cellular response to apoptosis has led to the strategy to profile BH3-only proteins. "Mitochondrial

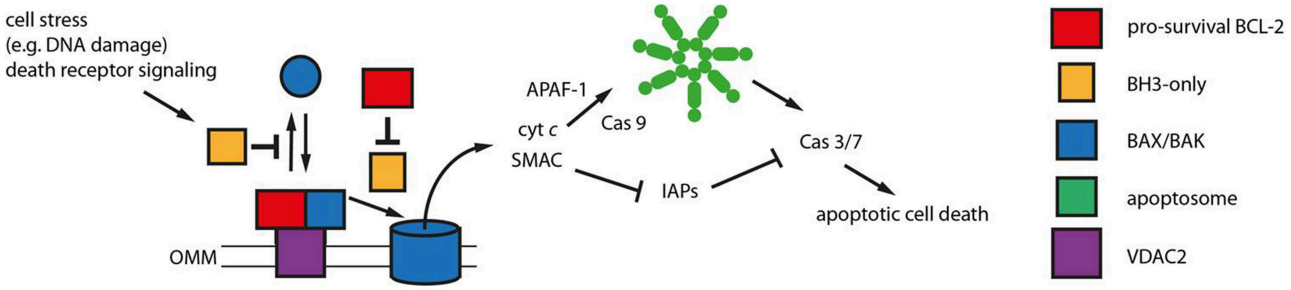

FIGURE 2 | Mitochondrial apoptosis signaling. Mitochondrial apoptosis is regulated by members of the BCL-2 protein family on the outer mitochondrial membrane (OMM). The pro-apoptotic BCL-2 proteins BAX and BAK (blue) constantly translocate to the OMM undergoing a conformational change. The porin voltage-dependent anion channel 2 (VDAC 2, purple) acts as mitochondrial BAX/BAK receptor and as platform for the retrotranslocation of BAX and BAK back into the cytosol dependent on the activities of pro-survival BCL-2 proteins (red). The equilibrium between BAX/BAK translocation and retrotranslocation determines the cellular predisposition to apoptosis. Intrinsic stress as well as death receptor signaling is mediated by BH3-only proteins (yellow) that inhibit BAX/BAK retrotranslocation shifting BAX and BAK toward the mitochondria. The BH3-only proteins tBID, BIM and PUMA are also thought to directly activate BAX and BAK initiating OMM permeabilization and the release of cytochrome *c* (cyt *c*) and SMAC into the cytosol. This function can be inhibited by pro-survival BCL-2 proteins. Cytosolic cyt *c* initiates in turn the formation of the apoptosome (green), an APAF-1 complex activating Caspase 9 (Cas 9). Subsequently, Caspases 3 and 7 are activated that can be inhibited by IAPs in the absence of SMAC in the cytosol. Caspase 3/7 activation leads to the efficient dismantling of the cell into apoptotic bodies that are later phagocytosed.

priming" in this context is the resulting stress capacity of cells dependent on the presence of pro-survival BCL-2 proteins, OMM-accumulated BH3-only proteins and BAX/BAK (**Figure 1**). Actually, BH3 profiling is again based on the rheostat model. It expands the model by emphasizing the potential role of BH3-only proteins, but does not take into account the transient nature of BCL-2 protein interactions and interactions among BCL-2 proteins other than through the BH3 motif. Extensive work shows the feasibility of "BH3 profiling" in different cellular settings (45–49). The analysis involves the short culturing of cells, limited cell lysis, incubation with peptides corresponding to BH3 domains and the analysis of OMM permeabilization through a membrane potential-sensitive dye. Cell culturing is prone to changes the apoptotic predisposition of a given tumor clone despite relative genetic stability. In addition, recent research has provided evidence of several secondary binding sites in BCL-2 protein interactions that BH3 profiling cannot account for (50–53). Therefore, BH3 profiling can particularly identify the contribution of pro-survival BCL-2 activities to the survival and therefore support selection of the potentially most effective BH3 mimetic. On the other hand, the appropriate BH3 mimetics could be tested directly, as procedure and readout would be similar.

APOPTOSIS PREDISPOSITION BY THE POSITION OF THE BAX/BAK LOCALIZATION EQUILIBRIUM

Prior stress and stress response influence the apoptotic predisposition but are also reflected in the cellular localization of the pro-apoptotic BCL-2 proteins. Despite their functional redundancy, BAK is found largely on the OMM in many cell types, while BAX resides primarily in the cytoplasm (54, 55). This apparent difference is important, since the sizes of the mitochondrial protein pools prior apoptotic stress determines apoptotic response (56). The mitochondrial BAX pool as much as the corresponding BAK pool is variable because both proteins are inhibited by a dynamic shuttling equilibrium between cytosol and mitochondria (57). Pro-survival BCL-2 proteins constantly retrotranslocate BAX and BAK from the mitochondria and cell stress mediated by BH3-only proteins shifts both pro-apoptotic BCL-2 proteins back onto the mitochondria. The importance of mitochondrial BAX for apoptosis induction implies that (i) the total cell protein population is not critical for apoptosis induction and (ii) accurately measuring mitochondrial BAX (or BAK) fractions or shuttling rates could predict apoptotic outcome in response to stress (**Figure 1**). Experimental observations have shown that the ratio between cytosolic and mitochondrial BAX/BAK is the best available representation of the average localization dynamics of BAX/BAK molecules (58). The paradigm that relevant protein pool and total protein level are not necessarily connected is true for BAX, BAK, pro-survival proteins, like BCL-2 and BCL-x_L, and BH3-only proteins, like BID (53, 59, 60). Relative BAX/BAK localization reflects the combined contributions of all players, known and unknown, to the cellular predisposition to apoptosis. Similar differences in the cellular BAX localization could also be present in HCC and could be associated with distinct molecular and clinical characteristics of the tumors.

TARGETING OF CELL DEATH AS A THERAPEUTIC STRATEGY FOR HCC

Hepatocyte damage and consecutive activation of cell death signaling plays a pivotal role for liver cancer initiation, but is also of particular importance for modulating treatment effects during established therapies. Herein, cell death can be induced by chemotherapeutic as well as targeted approaches (61). Sorafenib and lenvatinib are the only approved first line therapies for advanced stages in liver cancer (62). Both

TABLE 1 | Selected targets of cell death in liver diseases and cancer.

Drug	Target, function, pathway	Target population	Phase clinical development
PRIMA-1	Restoration of p53 function	Pre-clinical	N/A
Emricasan/IDN-6556 GS9450	Pan-Caspase Inhibitor	NASH, liver cirrhosis	Phase II (e.g., NCT02960204, NCT02686762, NCT03205345)
Venetoclax/ABT-199	BH3 mimetic	Pre-clinical	N/A
GSK2982772	RIPK1/RIPK3 Inhibitor	Pre-clinical	N/A
Etanercept	TNF Inhibitor	Alcoholic hepatitis, chronic viral hepatitis, NAFLD/NASH, AIH, PBC	Phase I-II
AEG35156	XIAP Antisense	HCC	Phase I-II (e.g., NCT00882869)
Curcumin	NF-kB, RIPK Inhibitor	HCC, NAFLD/NASH	Phase I-II (e.g., NCT03864783)

compounds are multi-tyrosine kinase inhibitors with anti-angiogenic properties. It is well-known, that sorafenib is a strong inducer of apoptosis and exposure to hepatoma cells leads to BAX/BAK activation, at least in part through the BH3-only protein PUMA (63). Furthermore, high numbers of objective response rates observed in HCC patients further indicate that considerable cell death follows lenvatinib treatment (64). However, several recent reports suggest that in addition to induction of cell death the treatment effect is also significantly induced by immunomodulation through targeted therapies (65). In consequence, several combination therapies with PD1/PD-L1 therapies are currently under clinical evaluation (62).

Direct targeting of cell death pathways and modulation of the apoptotic response might be a viable preventive strategy in chronic liver diseases but also exert direct anti-tumorigenic properties in HCC (**Table 1**). Given central role of p53 as a regulator of cell death, restoration of its function was attempted to induce anti-tumor activity in several studies. While adenoviral delivery of recombinant p53 did not reveal promising results, modulation of p53 activity by e.g., ubiquitination through inhibition of COP1, was recently explored (66). Blockade of COP1 by systemic delivery of RNAi decreased *in vivo* cancer growth and significantly induced apoptosis in several HCC cell lines. Furthermore, several compounds were identified to restore p53 functions. Prominently, p53 reactivation and induction of massive apoptosis (PRIMA-1) and PRIMA-1Met are currently evaluated in several clinical trials (67). In the liver, application of the compounds is currently restricted to preclinical data and shows promising anti-tumor effects when mutant p53 is silenced by siRNA. Other therapeutic strategies aimed to directly target proteins involved in apoptosis to enhance the apoptotic response of cancer therapies. Interestingly, XIAP antisense therapy in combination with sorafenib showed synergistic anti-tumor effects in a recent phase II clinical trial (68). Results showed a moderate increase in progression-free survival (4.0 months vs. 2.6 months), overall survival (6.5 months vs. 5.4 months), and objective response rates (16.1% vs. 9.7%) compared with Sorafenib monotherapy. Notably, drug-related adverse events were moderate.

Pro-survival BCL-2 proteins are also under intensive preclinical and clinical evaluation as cancer therapy targets. The use of the BH3 mimetic venetoclax or ABT-199 in chronic lymphocytic leukemia (CLL) has shown the potential of this strategy, as response rates of about 80% can be achieved with single-agent venetoclax even in a relapsed/refractory setting (69). Current efforts explore the combination of venetoclax with rituximab, obinutuzumab or ibrutinib in order to suppress acquired resistance observed during monotherapy (70, 71). Venetoclax in combination with hypomethylating agents (HMAs) has also received special attention for the treatment of acute myeloid leukemia (AML) (72, 73). The combination has been shown to target leukemia stem cells (74). In the liver, recent evidence suggest that BH3-only protein BID significantly contributes to the development of liver cancer (75). Loss of BID was shown to delay hepatocarcinogenesis by reducing cell death, liver inflammation, and compensatory proliferation (76). Thus, modulation of the BCL-2 protein interplay might be a promising therapeutic strategie for liver cancer.

In addition to the therapeutic targeting of critical apoptosis regulators, pan-caspase inhibitors, e.g., Emricasan/IDN-6556, or selective caspase-1,8,9 inhibitors, e.g., GS9450, have been explored in preclinical models as well as clinical trials, mainly in the context of chronic liver diseases (77). While the majority of these trials showed improved liver enzymes as a surrogate for hepatocyte protection, effect on degree of hepatitis and fibrogenesis is still unclear and is currently under evaluation in large phase III trials for the treatment NASH with and without liver cirrhosis (NCT02960204, NCT02686762, NCT03205345). Importantly, caspase inhibition might induce necroptosis or other complications and, thus, require further investigations addressing the safety of long-term administration (12).

Although no clinical trials have yet been initiated to test the clinical efficacy of necroptosis inhibition in liver disease, preclinical studies and early phase clinical trials in inflammatory (auto-immune) disease indicate that inhibition of RIPK1 kinase activity might also be a promising therapeutic strategy and prevent apoptosis in chronic liver diseases (78). However, while the importance of several key proteins including RIPK1, TAK1, and NEMO has been shown, the therapeutic potential for HCC remains to be demonstrated. Based on the regulatory functions of RIPK1, inhibition might even cause paradox reactions depending on the context of inhibition and affected cell type (30). Finally, given the redundancy in the different pathways, combination of

different anti-tumor therapies with one or several modulators of cell death pathways might be of particular therapeutic potential.

SUMMARY AND CONCLUSIONS

Hepatocyte death is a key driver of chronic inflammatory liver diseases and hepatocarcinogenesis. Several lines of evidence suggest that apoptosis and other types of cell death are critically linked to initiation and progression of liver cancer. They participate in shaping the biological trait of the tumor, thus, ultimately determining patient prognosis. Herein, existence and degree of cell death infers several mechanistic and translational implications. While detection of the apoptotic predisposition might be a powerful diagnostic tool, direct targeting of mitochondrial apoptosis might complement the limited therapeutic strategies for HCC. In light of recent advances in immune-oncological approaches, targeting of cell death might also exert synergistic immunomodulatory properties that could be explored in combination treatment strategies. However, our understanding of the detailed mechanisms and triggers of activation underlying the diverse mechanisms of cell death remains limited. Thus, definition of the actual state of cell death signaling effect in the distinct parenchymal and non-parenchymal cell types within the liver is urgently needed. Furthermore, the relative importance for distinct disease stages, i.e., chronic liver disease, tumor initiation as well as progression should be conclusively dissected to advance the field and before application of specific modulators of cell death in human is warranted. Furthermore, intensive translational research is needed to characterize the molecular hallmarks that operate on the intersection between cell death and inflammation. In this context, individual predisposition to apoptosis of cancer cells or cells within the hepatic microenvironment might be of particular relevance and might require distinct therapeutic strategy, thus, precision oncological approaches. Nevertheless, targeting of apoptosis is a promising avenue of HCC treatment that might yield to novel treatment strategies for this deadly inflammatory-driven cancer.

AUTHOR CONTRIBUTIONS

JM and FE conceptualized, wrote, and edited the review.

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REFERENCES

1. Yang JD, Hainaut P, Gores GJ, Amadou A, Plymoth A, Roberts LR. A global view of hepatocellular carcinoma: trends, risk, prevention and management. *Nat Rev Gastroenterol Hepatol*. (2019) 16:589–60. doi: 10.1038/s41575-019-0186-y
2. Marquardt JU, Andersen JB, Thorgeirsson SS. Functional and genetic deconstruction of the cellular origin in liver cancer. Nature reviews. *Cancer*. (2015) 15:653–67. doi: 10.1038/nrc4017
3. Xiao J, Wang F, Wong NK, He J, Zhang R, Sun R, et al. Global liver disease burdens and research trends: Analysis from a Chinese perspective. *J Hepatol*. (2019) 71:212–21. doi: 10.1016/j.jhep.2019.03.004
4. Marquardt JU, Galle PR, Teufel A. Molecular diagnosis and therapy of hepatocellular carcinoma (HCC): an emerging field for advanced technologies. *J Hepatol*. (2012) 56:267–75. doi: 10.1016/j.jhep.2011.07.007
5. Liu Z, Jiang Y, Yuan H, Fang Q, Cai N, Suo C, et al. The trends in incidence of primary liver cancer caused by specific etiologies: results from the Global Burden of Disease Study 2016 and implications for liver cancer prevention. *J Hepatol*. (2019) 70:674–83. doi: 10.1016/j.jhep.2018.12.001
6. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Cancer J Clin*. (2018) 68:394–424. doi: 10.3322/caac.21492
7. El-Serag HB. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology*. (2012) 142:1264–73 e1. doi: 10.1053/j.gastro.2011.12.061
8. Hernandez-Gea V, Toffanin S, Friedman SL, Llovet JM. Role of the microenvironment in the pathogenesis and treatment of hepatocellular carcinoma. *Gastroenterology*. (2013) 144:512–27. doi: 10.1053/j.gastro.2013.01.002
9. Todoric J, Karin M. The fire within: cell-autonomous mechanisms in inflammation-driven cancer. *Cancer Cell*. (2019) 35:714–20. doi: 10.1016/j.ccell.2019.04.001
10. Kondylis V, Pasparakis M. RIP Kinases in Liver Cell Death, Inflammation and Cancer. Trends Mol Med. (2019) 25:47–63. doi: 10.1016/j.molmed.2018.10.007
11. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P, et al. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ*. (2018) 25:486–541. doi: 10.1038/s41418-017-0012-4
12. Schwabe RF, Luedde T. Apoptosis and necroptosis in the liver: a matter of life and death. *Nat Rev*. (2018) 15:738–52. doi: 10.1038/s41575-018-0065-y
13. Garcia-Ruiz C, Fernandez-Checa JC. Mitochondrial oxidative stress and antioxidants balance in fatty liver disease. *Hepatol Commun*. (2018) 2:1425–39. doi: 10.1002/hep4.1271
14. Ku NO, Strnad P, Bantel H, Omary MB. Keratins: biomarkers and modulators of apoptotic and necrotic cell death in the liver. *Hepatology*. (2016) 64:966–76. doi: 10.1002/hep.28493
15. Mazzolini G, Sowa JP, Canbay A. Cell death mechanisms in human chronic liver diseases: a far cry from clinical applicability. *Clin Sci*. (2016) 130:2121–38. doi: 10.1042/CS20160035
16. Waidmann O, Koberle V, Bettinger D, Trojan J, Zeuzem S, Schultheiss M, et al. Diagnostic and prognostic significance of cell death and macrophage activation markers in patients with hepatocellular carcinoma. *J Hepatol*. (2013) 59:769–79. doi: 10.1016/j.jhep.2013.06.008
17. Bock B, Hasdemir D, Wandrer F, Rodt T, Manns MP, Schulze-Osthoff K, et al. Serum cell death biomarker mirrors liver cancer regression after transarterial chemoembolisation. *Aliment Pharmacol Ther*. (2016) 44:747–54. doi: 10.1111/apt.13758
18. Castven D, Fischer M, Becker D, Heinrich S, Andersen JB, Strand D, et al. Adverse genomic alterations and stemness features are induced by field cancerization in the microenvironment of hepatocellular carcinomas. *Oncotarget*. (2017) 8:48688–700. doi: 10.18632/oncotarget.16231

19. Claveria C, Giovinazzo G, Sierra R, Torres M. Myc-driven endogenous cell competition in the early mammalian embryo. *Nature*. (2013) 500:39–44. doi: 10.1038/nature12389
20. Sulston JE, Horvitz HR. Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev Biol*. (1977) 56:110–56. doi: 10.1016/0012-1606(77)90158-0
21. Eskes R, Antonsson B, Osen-Sand A, Montessuit S, Richter C, Sadoul R, et al. Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg2+ ions. *J Cell Biol*. (1998) 143:217–24. doi: 10.1083/jcb.143.1.217
22. Ichim G, Lopez J, Ahmed SU, Muthalagu N, Giampazolias E, Delgado ME, et al. Limited mitochondrial permeabilization causes DNA damage and genomic instability in the absence of cell death. *Mol Cell*. (2015) 57:860–72. doi: 10.1016/j.molcel.2015.01.018
23. Hotchkiss RS, Strasser A, McDunn JE, Swanson PE. Cell death. *N Eng J Med*. (2009) 361:1570–83. doi: 10.1056/NEJMra0901217
24. Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. Nature reviews. *Cancer*. (2006) 6:674–87. doi: 10.1038/nrc1934
25. Moreno-Caceres J, Fabregat I. Apoptosis in liver carcinogenesis and chemotherapy. *Hepatol Oncol*. (2015) 2:381–397. doi: 10.2217/hep.15.27
26. Locatelli I, Sutti S, Vacchiano M, Bozzola C, Albano E. NF-kappaB1 deficiency stimulates the progression of non-alcoholic steatohepatitis (NASH) in mice by promoting NKT-cell-mediated responses. *Clin Sci*. (2013) 124:279–87. doi: 10.1042/CS20120289
27. Grohmann M, Wiede F, Dodd GT, Gurzov EN, Ooi GJ, Butt T, et al. Obesity drives STAT-1-dependent NASH and STAT-3-dependent HCC. *Cell*. (2018) 175:1289–306 e20. doi: 10.1016/j.cell.2018.09.053
28. Nakagawa H, Umemura A, Taniguchi K, Font-Burgada J, Dhar D, Ogata H, et al. ER stress cooperates with hypernutrition to trigger TNF-dependent spontaneous HCC development. *Cancer Cell*. (2014) 26:331–43. doi: 10.1016/j.ccr.2014.07.001
29. Elsharkawy AM, Mann DA. Nuclear factor-kappaB and the hepatic inflammation-fibrosis-cancer axis. *Hepatology*. (2007) 46:590–7. doi: 10.1002/hep.21802
30. Czauderna C, Castven D, Mahn FL, Marquardt JU. Context-dependent role of NF-kappaB signaling in primary liver cancer-from tumor development to therapeutic implications. *Cancers*. (2019) 11:E1053. doi: 10.3390/cancers11081053
31. Luedde T, Schwabe RF. NF-kappaB in the liver–linking injury, fibrosis and hepatocellular carcinoma. *Nat Rev*. (2011) 8:108–18. doi: 10.1038/nrgastro.2010.213
32. Yang YM, Kim SY, Seki E. Inflammation and liver cancer: molecular mechanisms and therapeutic targets. *Semin Liver Dis*. (2019) 39:26–42. doi: 10.1055/s-0038-1676806
33. Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene*. (1999) 18:6853–66. doi: 10.1038/sj.onc.1203239
34. Heinrichsdorff J, Luedde T, Perdiguero E, Nebreda AR, Pasparakis M. p38 alpha MAPK inhibits JNK activation and collaborates with IkappaB kinase 2 to prevent endotoxin-induced liver failure. *EMBO Rep*. (2008) 9:1048–54. doi: 10.1038/embor.2008.149
35. Tang G, Minemoto Y, Dibling B, Purcell NH, Li Z, Karin M, et al. Inhibition of JNK activation through NF-kappaB target genes. *Nature*. (2001) 414:313–7. doi: 10.1038/35104568
36. Bettermann K, Vucur M, Haybaeck J, Koppe C, Janssen J, Heymann F, et al. TAK1 suppresses a NEMO-dependent but NF-kappaB-independent pathway to liver cancer. *Cancer Cell*. (2010) 17:481–96. doi: 10.1016/j.ccr.2010.03.021
37. Ma C, Kesarwala AH, Eggert T, Medina-Echeverz J, Kleiner DE, Jin P, et al. NAFLD causes selective CD4(+) T lymphocyte loss and promotes hepatocarcinogenesis. *Nature*. (2016) 531:253–7. doi: 10.1038/nature16969
38. Schneider AT, Gautheron J, Feoktistova M, Roderburg C, Loosen SH, Roy S, et al. RIPK1 suppresses a TRAF2-dependent pathway to liver cancer. *Cancer Cell*. (2017) 31:94–109. doi: 10.1016/j.ccell.2016.11.009
39. Lee JS, Chu IS, Heo J, Calvisi DF, Sun ZT, Roskams T, et al. Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. *Hepatology*. (2004) 40:667–76. doi: 10.1002/hep.20375
40. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*. (1993) 74:609–19. doi: 10.1016/0092-8674(93)90509-O
41. Sedlak TW, Oltvai ZN, Yang E, Wang K, Boise LH, Thompson CB, et al. Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. *Proc Natl Acad Sciences USA*. (1995) 92:7834–8. doi: 10.1073/pnas.92.17.7834
42. Mason KD, Carpinelli MR, Fletcher JI, Collinge JE, Hilton AA, Ellis S, et al. Programmed anuclear cell death delimits platelet life span. *Cell*. (2007) 128:1173–86. doi: 10.1016/j.cell.2007.01.037
43. Hinds MG, Smits C, Fredericks-Short R, Risk JM, Bailey M, Huang DC, et al. Bim, Bad and Bmf: intrinsically unstructured BH3-only proteins that undergo a localized conformational change upon binding to prosurvival Bcl-2 targets. *Cell Death Differ*. (2007) 14:128–36. doi: 10.1038/sj.cdd.4401934
44. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol*. (2008) 9:47–59. doi: 10.1038/nrm2308
45. Brunelle JK, Ryan J, Yecies D, Opferman JT, Letai A. MCL-1-dependent leukemia cells are more sensitive to chemotherapy than BCL-2-dependent counterparts. *J Cell Biol*. (2009) 187:429–42. doi: 10.1083/jcb.200904049
46. Certo M, Del Gaizo Moore V, Nishino M, Wei G, Korsmeyer S, Armstrong SA, V. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell*. (2006) 9:351–65. doi: 10.1016/j.ccr.2006.03.027
47. Ni Chonghaile T, Sarosiek KA, Vo TT, Ryan JA, Tammareddi A, Moore Vdel G, V. Pretreatment mitochondrial priming correlates with clinical response to cytotoxic chemotherapy. *Science*. (2011) 334:1129–33. doi: 10.1126/science.1206727
48. Ryan JA, Brunelle JK, Letai A. Heightened mitochondrial priming is the basis for apoptotic hypersensitivity of CD4+ CD8+ thymocytes. *Proc Natl Acad Sci USA*. (2010) 107:12895–900. doi: 10.1073/pnas.0914878107
49. Vo TT, Ryan J, Carrasco R, Neuberg D, Rossi DJ, Stone RM, et al. Relative mitochondrial priming of myeloblasts and normal HSCs determines chemotherapeutic success in AML. *Cell*. (2012) 151:344–55. doi: 10.1016/j.cell.2012.08.038
50. Andreu-Fernandez V, Sancho M, Genoves A, Lucendo E, Todt F, Lauterwasser J, et al. Bax transmembrane domain interacts with prosurvival Bcl-2 proteins in biological membranes. *Proc Natl Acad Sci USA*. (2017) 114:310–15. doi: 10.1073/pnas.1612322114
51. Barclay LA, Wales TE, Garner TP, Wachter F, Lee S, Guerra RM, et al. Inhibition of pro-apoptotic BAX by a noncanonical interaction mechanism. *Mol Cell*. (2015) 57:873–86. doi: 10.1016/j.molcel.2015.01.014
52. Gavathiotis E, Suzuki M, Davis ML, Pitter K, Bird GH, Katz SG, et al. BAX activation is initiated at a novel interaction site. *Nature*. (2008) 455:1076–81. doi: 10.1038/nature07396
53. Liu Q, Osterlund EJ, Chi X, Pogmore J, Leber B, Andrews DW. Bim escapes displacement by BH3-mimetic anti-cancer drugs by double-bolt locking both Bcl-XL and Bcl-2. *Elife*. (2019) 8:e37689. doi: 10.7554/eLife.37689
54. Griffiths GJ, Dubrez L, Morgan CP, Jones NA, Whitehouse J, Corfe BM, et al. Cell damage-induced conformational changes of the pro-apoptotic protein Bak *in vivo* precede the onset of apoptosis. *J Cell Biol*. (1999) 144:903–14. doi: 10.1083/jcb.144.5.903
55. Hsu YT, Youle RJ. Bax in murine thymus is a soluble monomeric protein that displays differential detergent-induced conformations. *J Biol Chem*. (1998) 273:10777–83. doi: 10.1074/jbc.273.17.10777
56. Todt F, Cakir Z, Reichenbach F, Youle RJ, Edlich F. The C-terminal helix of Bcl-x(L) mediates Bax retrotranslocation from the mitochondria. Cell Death Differ. (2013) 20:333–42. doi: 10.1038/cdd.2012.131
57. Edlich F, Banerjee S, Suzuki M, Cleland MM, Arnoult D, Wang C, Neutzner A, Tjandra N, Youle RJ, Bcl-x(L) retrotranslocates Bax from the mitochondria into the cytosol. *Cell*. (2011) 145:104–16. doi: 10.1016/j.cell.2011.02.034
58. Reichenbach F, Wiedenmann C, Schalk E, Becker D, Funk K, Scholz-Kreisel P, et al. Mitochondrial BAX determines the predisposition to apoptosis in human AML. *Clin Cancer Res*. (2017) 23:4805–16. doi: 10.1158/1078-0432.CCR-16-1941
59. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell*. (1998) 94:491–501. doi: 10.1016/S0092-8674(00)81590-1

60. Todt F, Cakir Z, Reichenbach F, Emschermann F, Lauterwasser J, Kaiser A, et al. Differential retrotranslocation of mitochondrial Bax and Bak. *EMBO J*. (2015) 34:67–80. doi: 10.15252/embj.201488806
61. Pinyol R, Nault JC, Quetglas IM, Zucman-Rossi J, Llovet JM. Molecular profiling of liver tumors: classification and clinical translation for decision making. *Semin Liver Dis*. (2014) 34:363–75. doi: 10.1055/s-0034-1394137
62. Marquardt JU, Saborowski A, Czauderna C, Vogel A. The changing landscape of systemic treatment of advanced hepatocellular carcinoma: new targeted agents and immunotherapies. *Target Oncol*. (2019) 14:115–23. doi: 10.1007/s11523-019-00624-w
63. Fernando J, Sancho P, Fernandez-Rodriguez CM, Lledo JL, Caja L, Campbell JS, et al. Sorafenib sensitizes hepatocellular carcinoma cells to physiological apoptotic stimuli. *J Cell Physiol*. (2012) 227:1319–25. doi: 10.1002/jcp.22843
64. Kudo M, Finn RS, Qin S, Han KH, Ikeda K, Piscaglia F, et al. Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. *Lancet*. (2018) 391:1163–73. doi: 10.1016/S0140-6736(18)30207-1
65. Kimura T, Kato Y, Ozawa Y, Kodama K, Ito J, Ichikawa K, et al. Immunomodulatory activity of lenvatinib contributes to antitumor activity in the Hepa1-6 hepatocellular carcinoma model. *Cancer Sci*. (2018) 109:3993–4002. doi: 10.1111/cas.13806
66. Lee YH, Andersen JB, Song HT, Judge AD, Seo D, Ishikawa T, et al. Definition of ubiquitination modulator COP1 as a novel therapeutic target in human hepatocellular carcinoma. *Cancer Res*. (2010) 70:8264–9. doi: 10.1158/0008-5472.CAN-10-0749
67. Perdrix A, Najem A, Saussez S, Awada A, Journe F, Ghanem G, et al. PRIMA-1 and PRIMA-1(Met) (APR-246): from mutant/wild type p53 reactivation to unexpected mechanisms underlying their potent anti-tumor effect in combinatorial therapies. *Cancers*. (2017) 9:E172. doi: 10.3390/cancers9120172
68. Lee FA, Zee BC, Cheung FY, Kwong P, Chiang CL, Leung KC, et al. Randomized phase II study of the X-linked Inhibitor of Apoptosis (XIAP) antisense AEG35156 in combination with sorafenib in patients with advanced Hepatocellular Carcinoma (HCC). *Am J Clin Oncol*. (2016) 39:609–13. doi: 10.1097/COC.0000000000000099
69. Stilgenbauer S, Eichhorst B, Schetelig J, Hillmen P, Seymour JF, Coutre S, et al. Venetoclax for patients with chronic lymphocytic leukemia with 17p deletion: results from the full population of a phase II pivotal trial. *J Clin Oncol*. (2018) 36:1973–80. doi: 10.1200/JCO.2017.76.6840
70. Kater AP, Seymour JF, Hillmen P, Eichhorst B, Langerak AW, Owen C, et al. Fixed duration of venetoclax-rituximab in relapsed/refractory chronic lymphocyticleukemia eradicates minimal residual disease and prolongs survival: post-treatment follow-up of the MURANO phase III study. *J Clin Oncol*. (2019) 37:269–77. doi: 10.1200/JCO.18.01580
71. Seymour JF, Kipps TJ, Eichhorst B, Hillmen P, D'Rozario J, Assouline S, et al. Venetoclax-rituximab in relapsed or refractory chronic lymphocytic leukemia. *N Eng J Med*. (2018) 378:1107–20. doi: 10.1056/NEJMoa1713976
72. DiNardo CD, Pratz K, Pullarkat V, Jonas BA, Arellano M, Becker PS, et al. Venetoclax combined with decitabine or azacitidine in treatment-naive, elderly patients with acute myeloid leukemia. *Blood*. (2019) 133:7–17. doi: 10.1182/blood-2018-08-868752
73. Pollyea DA, Jordan CT. Why are hypomethylating agents or low-dose cytarabine and venetoclax so effective? *Curr Opin Hematol*. (2019) 26:71–6. doi: 10.1097/MOH.0000000000000485
74. Pollyea DA, Stevens BM, Jones CL, Winters A, Pei S, Minhajuddin M, et al. Venetoclax with azacitidine disrupts energy metabolism and targets leukemia stem cells in patients with acute myeloid leukemia. *Nat Med*. (2018) 24:1859–66. doi: 10.1038/s41591-018-0233-1
75. Orlik J, Schungel S, Buitrago-Molina LE, Marhenke S, Geffers R, Endig J, et al. The BH3-only protein BID impairs the p38-mediated stress response and promotes hepatocarcinogenesis during chronic liver injury in mice. *Hepatology*. (2015) 62:816–28. doi: 10.1002/hep.27888
76. Wree A, Johnson CD, Font-Burgada J, Eguchi A, Povero D, Karin M, et al. Hepatocyte-specific Bid depletion reduces tumor development by suppressing inflammation-related compensatory proliferation. *Cell Death Differ*. (2015) 22:1985–94. doi: 10.1038/cdd.2015.46
77. Linton SD, Aja T, Armstrong RA, Bai X, Chen LS, Chen N, et al. First-in-class pan caspase inhibitor developed for the treatment of liver disease. *J Med Chem*. (2005) 48:6779–82. doi: 10.1021/jm050307e
78. Kopalli SR, Kang TB, Koppula S. Necroptosis inhibitors as therapeutic targets in inflammation mediated disorders - a review of the current literature and patents. *Expert Opin Ther Pat*. (2016) 26:1239–56. doi: 10.1080/13543776.2016.1230201

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REVIEW
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mTORC1 as a Regulator of Mitochondrial Functions and a Therapeutic Target in Cancer

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Continuous proliferation of tumor cells requires constant adaptations of energy metabolism to rapidly fuel cell growth and division. This energetic adaptation often comprises deregulated glucose uptake and lactate production in the presence of oxygen, a process known as the "Warburg effect." For many years it was thought that the Warburg effect was a result of mitochondrial damage, however, unlike this proposal tumor cell mitochondria maintain their functionality, and is essential for integrating a variety of signals and adapting the metabolic activity of the tumor cell. The mammalian/mechanistic target of rapamycin complex 1 (mTORC1) is a master regulator of numerous cellular processes implicated in proliferation, metabolism, and cell growth. mTORC1 controls cellular metabolism mainly by regulating the translation and transcription of metabolic genes, such as peroxisome proliferator activated receptor γ coactivator-1 α (PGC-1α), sterol regulatory element-binding protein 1/2 (SREBP1/2), and hypoxia inducible factor-1 α (HIF-1α). Interestingly it has been shown that mTORC1 regulates mitochondrial metabolism, thus representing an important regulator in mitochondrial function. Here we present an overview on the role of mTORC1 in the regulation of mitochondrial functions in cancer, considering new evidences showing that mTORC1 regulates the translation of nucleus-encoded mitochondrial mRNAs that result in an increased ATP mitochondrial production. Moreover, we discuss the relationship between mTORC1 and glutaminolysis, as well as mitochondrial metabolites. In addition, mitochondrial fission processes regulated by mTORC1 and its impact on cancer are discussed. Finally, we also review the therapeutic efficacy of mTORC1 inhibitors in cancer treatments, considering its use in combination with other drugs, with particular focus on cellular metabolism inhibitors, that could help improve their anti neoplastic effect and eliminate cancer cells in patients.

Keywords: mTORC1, mitochondria, mitochondrial functions, cancer, therapy

INTRODUCTION

Cellular metabolism involves a set of highly coordinated activities in which numerous enzymes collaborate to convert nutrients into building blocks toward generation of macromolecules, energy, and cellular biomass. In cancer, genetic, and epigenetic changes can disturb key enzymes or rewire oncogenic pathways, resulting in cell metabolism alterations (1). In 1924 Otto Warburg observed that tumor cells prefer aerobic glycolysis to generate ATP and lactate even in presence of oxygen, process known as the "Warburg effect" (2). For a long time it was believed that this preference for the Warburg effect was due to a failure in the mitochondrial function. Nevertheless, in recent years, there were significant progresses in our understanding of metabolic regulation in cancer and contrariwise, it was demonstrated that cancer cells have a functional mitochondrion. Furthermore, it was shown that oxidative phosphorylation (OXPHOS) is crucial for ATP production and tumor progression (3). However, mitochondria perform many functions beyond energetic production, including generation of redox molecules and the regulation of cell signaling, cell death, biosynthetic metabolism, and generation of reactive oxygen species (ROS) (4).

Mitochondrial ROS are the byproducts of metabolic processes during which electrons escape from the mitochondrial electron transport chain and then are captured by molecular oxygen to generate superoxide anions (O_2^-) (5). Mitochondrial ROS exhibit both, a tumor promoting or tumor suppressing roles, depending on their levels and their oxidative potential. ROS are highly reactive species that produce oxidized proteins, lipids and nucleic acids, either behaving as damaging or as signaling species in cell metabolism. For instance, low levels of ROS have a pronounced proliferative effect but high levels induce tissue damage and consequently cell death (6). Despite the potential damaging roles of high ROS, cancer cells posses ROS-scavenging systems aimed to maintain ROS homeostasis, being the two major players Glutathione (GSH) and Thioredoxin (Txn) (7). Mitochondrial functions confer high levels of cellular plasticity, which permits a fast adaptation to challenging microenvironments conditions, such as hypoxia and nutrient deficiency, two very common consequences in tumors (8). On the other hand, accumulation of damaged mitochondria can be dangerous to cells; mitochondrial quality and quantity are processes severely monitored to ensure balance in cell physiology (9). Damaged or unwanted mitochondria can be selectively removed by mitophagy, a lysosome-dependent catabolic degradation process (10). Mitochondrial functions are matched by their morphological and structural changes, during the lifetime of a cell, the mitochondrial homeostasis network is constantly shaped by fission and fusion events (11).

In the process of tumor initiation and progression, cancer cells are exposed to harsh condition such as hypoxia or nutrient depletion in the tumor microenvironment. To survive in this severe environment, cancer cells must sense, and respond to the status of nutrient availability in the extracellular environment. The cell has several nutrients sensors responsible for maintaining cell homeostasis with the extracellular environment, such as the mammalian/mechanistic target of rapamycin complex 1 (mTORC1) that drives ATP-consuming cellular processes (anabolic) necessaries for cellular proliferation and growth (12). Another important sensor is the serine/threonine kinase AMP-activated protein kinase (AMPK), which, as its name implies, senses cellular AMP levels and coordinate a metabolic switch from anabolism toward catabolism under energy deprivation conditions such as hypoxia and hypoglycemia (13). AMPK has a wide variety of cell targets, one of which is mTORC1. AMPK activation suppresses mTORC1 signaling, thus regulating energy metabolism by stimulating the activity of several transcriptional controllers of metabolic enzymes such as peroxisome proliferator activated receptor γ coactivator-1 α (PGC-1α), sterol regulatory element-binding protein 1/2 (SREBP1/2), and hypoxia inducible factor-1 α (HIF-1α) (14). Interestingly, has been shown that mTORC1 also regulates mitochondrial oxidative metabolism (15–17). Moreover, mitochondrial oxidative metabolism is a very important mechanism for cancer development, acquired resistance against chemotherapy, and increased hypoxia tolerance in tumor microenvironment.

In this review we explain the participation of mTORC1 in the regulation of mitochondrial ATP producing capacity and we discuss how this process affects tumor cells. On the other hand, the mitochondrial function is directly associated with mitochondrial morphology regulated through fusion and fission processes thus, we review the current knowledge about the relationship of mitochondrial morphology highlighting mTORC1 participation in cancer. On the other hand it is known that glutamine, the most abundant free amino acid in blood, is uptaked by tumor cells and converted into α-ketoglutarate (α-KG) that fuels the tricarboxilic acid (TCA) cycle and OXPHOS in tumor mitochondrial. Therefore, we discuss how glutamine and mTORC1 participate in tumor development. Additionally, it was shown that mutations in nuclear and mitochondrial DNA lead to deregulation of important metabolic enzymes promoting the accumulation of intermediary metabolites, known as oncometabolites which in turn support cancer development. In this review, we depict the role of mTORC1 in the regulation of oncometabolites, as well as the therapeutic efficacy of mTORC1 inhibitors in cancer treatment.

STRUCTURE AND FUNCTIONS OF mTORC1

The protein serine threonine kinase TOR (target of rapamycin) was initially identified in *Saccharomyces cerevisiae* as a target of the macrolide fungicide rapamycin and later, the mammalian counterpart was identified and named mammalian/mechanistic target of rapamycin (mTOR), also known as FRAP (FKBP12-rapamycin-associated protein), RAFT (rapamycin and FKB12 target), and RAPT1 (rapamycin target 1) (18, 19). TOR is a large (∼280 kDa) serine/threonine protein kinase that belongs to the family of phosphoinositide 3-kinase (PI3K)-related kinase (20). The mTOR protein interacts with other proteins and form two distinct multiprotein complexes: mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2), either one with a different sensitivities to rapamycin (21). mTORC1 is inhibited

by rapamycin, while mTORC2 is resistant to acute rapamycin treatment, however in some types of cells (HeLa and PC3) this mTORC2 complex can be inhibited by longer rapamycin treatments (over 24 h) (22).

mTORC1 is composed by the regulatory-associated protein of mTOR (Raptor), a scaffolding protein important for mTORC1 assembly, stability, substrate specificity and regulation (23), and by the proline-rich AKT substrate 40 kDa factor protein (PRAS40) (24), that associates with Raptor and inhibits mTORC1 activity. mTORC2 complex is composed by the rapamycin-insensitive companion (Rictor) (25), a component essential for both, complex formation, and their biological function, the mammalian stress-activated map kinase-interacting protein 1 (mSin1) an essential component required for complex formation and kinase activity (26), and by Protor 1 (Protein observed with Rictor 1), required to allow efficient regulation of mTORC2 targets (27). Both mTORC1 and mTORC2 are composed by mTOR protein, a mammalian lethal with sec13 protein 8 (mLST8, also known as GβL), DEP domain-containing mTOR interacting protein (DEPTOR), and Tel two interacting protein 1 (TTI1/TEL2) complex. mLST8 is associated with the catalytic domain of mTOR and may stabilize the kinase activation loop, DEPTOR on the contrary inhibits mTOR activity, TTI1/TEL2 is a mTOR interacting protein important for mTOR stability and assembly of the mTOR complex and maintain their activities (28) (**Figure 1**).

mTORC1 is activated via growth factors stimulation [epidermal growth factor (EGF), insulin-like growth factor (IGF)], increase in amino acid levels such as leucin and arginine and cellular energy status (29–31), promoting protein and lipid synthesis, as well as ribosome biogenesis impacting on cell proliferation and growth, autophagy and metabolic processes are also stimulated by mTORC1 action (32). Moreover, it was demonstrated that mTORC1 signaling is strongly implicated in the aging process of diverse organisms, including yeast, worms flies, and mammals (33).

On the other hand, mTORC2 is activated by growth factors but unlike mTORC1 its activity is not regulated by amino acids. mTORC2 phosphorylates PKC-α, AKT/PKB (Ser473) and paxillin (focal adhesion-associated adaptor protein) (Tyr118), to regulate the activity of the small GTPases Rac and Rho, controlling cell survival and cytoskeletal organization and cell migration (34).

REGULATION OF mTORC1 SIGNALING IN CANCER

The mTORC1 is often deregulated in numerous cancer types, such as breast, cervical cancer, esophageal squamous cell carcinoma, lung and hepatic cancers (35–39). mTORC1 is often activated by mutations in its upstream regulators. These include gain-of-function mutation of PI3K and loss-of-function mutation of the tumor suppressor PTEN (40). In a number of *in vitro* cell-lines and *in vivo* murine xenograft models, it has been demonstrated that aberrant mTORC1 contributes to tumor growth, angiogenesis, invasion and metastasis (41). Given its key role in the regulation of process associated with cell growth and metabolism in cancer, specifically with the mitochondrial functions, we focus on mTORC1.

It has been shown that mTORC1 is regulated by growth factors through the phosphoinositide 3-kinase/protein kinase B, also known as Akt (PI3K/PKB or Akt) pathway and by Ras/mitogen-activated protein kinase (MAPK) pathway (42). Binding of insulin or insulin-like growth factor (IGF) to their receptor lead to recruitment and phosphorylation of the insulin receptor substrate and subsequent recruitment of PI3K. PI3K phosphorylates the inositol ring of the membrane phospholipid, phosphatidylinositol-4,5-biphosphate (PI-4,5-P_2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP_3) at the cytoplasmic side of the cellular membrane (43). PIP_3 recruits a subset of pleckstrin homology (PH) domain-containing proteins, such as the same protein kinase Akt and constitutively active phosphoinositide-dependent kinase 1 (PDK1) (44, 45). In turn PDK1 phosphorylates Akt in T308 (46), however the maximal activation of Akt requires its additional phosphorylation on S473 located at the carboxyl-terminus site, mediated by mTORC2 (47). Akt inhibits the tuberous sclerosis complex (TSC) that limit the mTORC1 signaling, TSC complex is composed by three subunits: TSC1 (Harmatin), TSC2 (Tuberin), and TBC1D7 (48, 49). Akt phosphorylate TSC2 on five residues (S939, S981, S1130, S1132, and T1462) leading to its inactivation (50, 51). The TSC complex is a negative regulator of the small GTPase Rheb (Ras homolog enriched in brain) (52), via stimulation of GTP hydrolysis. On the other hand Rheb-GTP is translocated to the lysosomal membrane, where directly interacts with the catalytic domain of mTOR promoting its activation (53). Once mTORC1 is activated, positively controls cell growth through stimulation of protein synthesis by induction the phosphorylation of its two main targets, the eukaryotic initiation factor 4E binding protein 1 (4E-BP1), and the ribosomal protein S6 kinase (S6K). Raptor-mTOR binds to S6K and 4E-BP1 through their respective TOR signaling (TOS) motifs (54, 55) enhancing translation of proteins involved in the control of cell growth/size and cell cycle progression.

The 4E-BPs are small (∼15–20 kDa) proteins that interact with eukaryotic translation initiation factor 4E (eIF4E) inhibiting translation initiation, this being a very important regulation point in protein translation. Although there are three 4E-BPs known isoforms in mammals (4E-BP 1, 2, and 3), most studies had focus on 4E-BP1. mTORC1 phosphorylates 4E-BP1 in Thr37/Thr46, followed by Thr70, and finally Ser65 (56). Phosphorylation of Thr70 and Ser65 are part of the response to extracellular signals such as serum stimulation. Phosphorylation of all of these sites inhibits 4E-BPs' binding to eIF4E. The 4E-BPs prevents the formation of the translation initiation complex (eIF4F) by competing with eIF4G for binding to the dorsal side of eIF4E and reduces cap-dependent translation initiation (57). On the other hand the ribosomal protein S6 kinase (rpS6) known as S6K was first identified in unfertilized *Xenopus laevis* eggs as a 90 kDa polypeptide, termed p90 or rpS6 kinase (RSK, also known as p90RSK) (58). Later a protein with a molecular weight of 65–70 kDa was purified from chicken embryos and 3T3 cells, and referred to as S6K (59). Mammalian cells express both S6K1 and

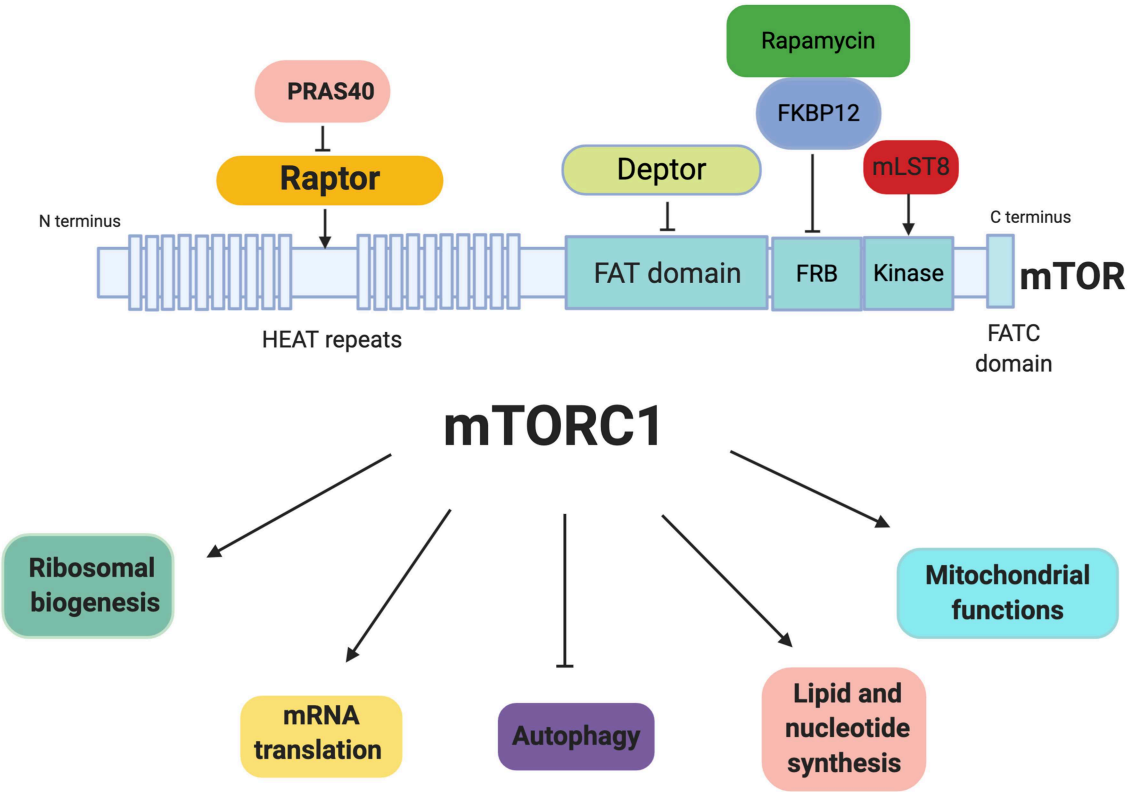

FIGURE 1 | Mechanistic target of rapamycin complex 1 (mTORC1) and regulation of cellular processes. mTORC1 is a complex with DEPTOR and PRAS40 as negative regulators and RAPTOR and mSLT8 as positive regulators. Rapamycin-FKBP12 inhibits the mTOR kinase by directly blocking substrates recruitment and by further restricting active-site access. mTORC1 regulates different cellular processes such as ribosomal biogenesis, mRNA translation, autophagy, lipid and nucleotide synthesis, and mitochondrial functions.

S6K2 also known as S6Kα and S6Kβ, respectively, which are encoded by two different genes and share a very high level of overall sequence homology (60). S6K1 has cytosolic and nuclear isoforms (p70 S6K1 and p85 S6K1, respectively) (61), whereas both S6K2 isoforms (p54 S6K2 and p56 S6K2) are primarily nuclear. S6K was identified as the main kinase responsible for ribosomal protein S6 phosphorylation (60), S6K regulates the mRNA biogenesis, translation initiation, and elongation.

MITOCHONDRIA AND CANCER

In addition to genetic aberrations, tumor cells rewiring their metabolism, such as aerobic glycolysis, glutamine uptake, accumulation of intermediates of glycolysis, and upregulation of lipid and amino acid synthesis, and OXPHOS, enable support their high demands on nutrients for building blocks and energy production (62). In cancer development tumor cells reprogram their metabolism to guarantee survival and proliferation in an often nutrient-scare and stressful microenvironment. (40). Moreover, several findings demonstrate that mutations in oncogenes and /or tumor suppressor genes can mediate metabolic rewiring in cancer cells to support the high demands for building blocks and energy production (63). Tumor cells acquire a metabolic plasticity that allows alternate between aerobic glycolysis and OXPHOS in order to maintain malignant phenotypes, such as a chemotherapy resistance, tumor invasion, and metastasis, and mitochondria play a central role in this dynamic (64).Changes in mitochondrial respiration rates are accompanied by changes in mitochondrial mass, the rate of fission, fusion, mitochondria biogenesis and mitophagy as well as mtDNA copy number, transcription and translation (64). In recent years, several evidences have established the role of mTORC1 as a central regulatory node in such events, which coordinates energy consumption by the translation apparatus and ATP production in mitochondria (65) (**Figure 2**).

It has been demonstrated that the role of mitochondria in cancer can vary depending of input genetic, environmental, and tissue-of-origin between tumors (4). The mitochondrion, contains its own DNA (mDNA) which is replicated independently of the host genome, mDNA comprises a circular genome of 16, 569 base pairs and encodes 37 genes, including 13 subunits of the electron transport chain (ETC), 2 ribosomal RNAs and 22 tRNAs, the remaining mitochondrial proteins are encoded by the nuclear genome and are imported into the mitochondria (66). Higher mtDNA copy number and

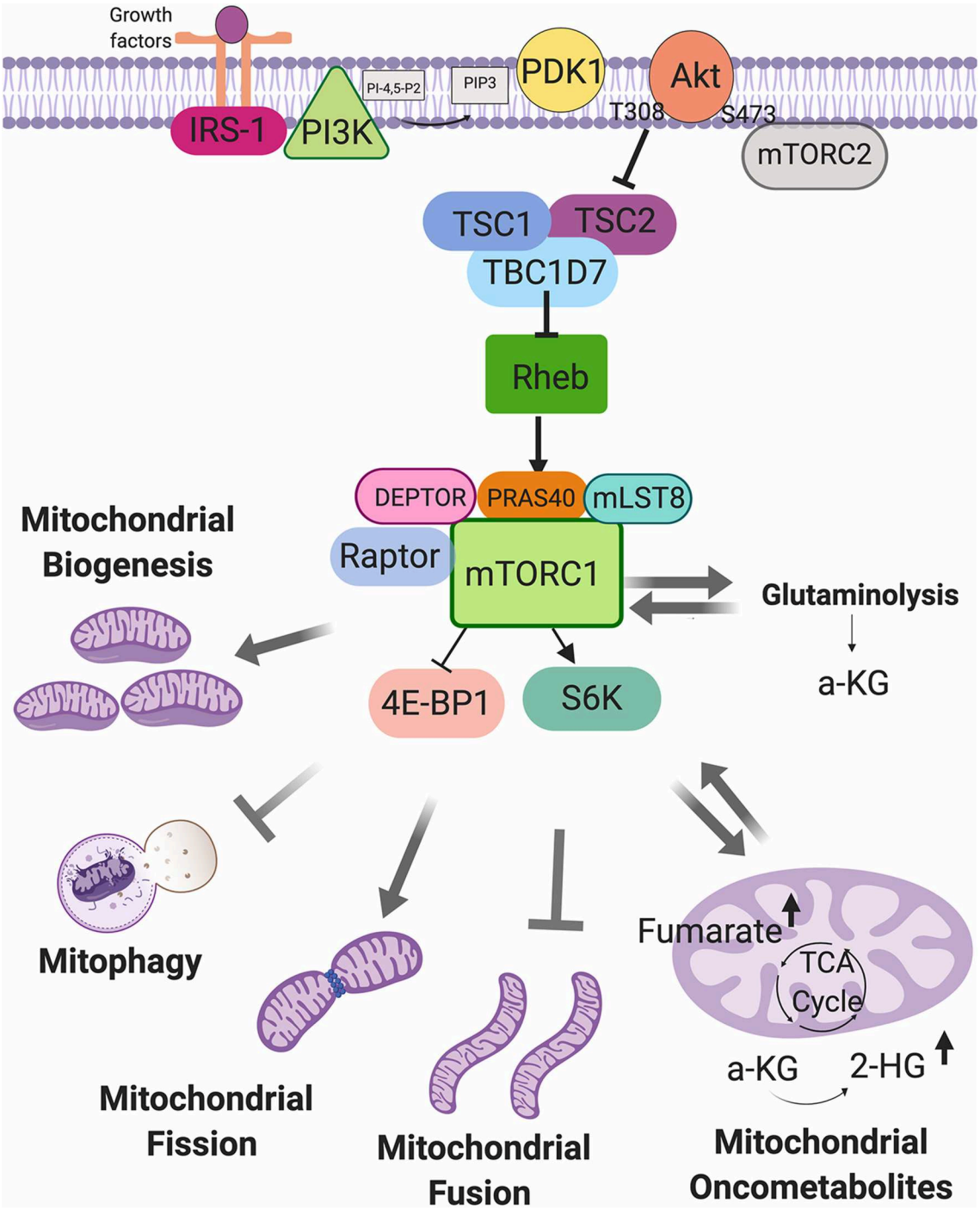

FIGURE 2 | Mechanistic target of rapamycin complex 1 (mTORC1) as a regulator of mitochondrial functions. mTORC1 can be activated by growth factor, and can regulate the mitochondrial biogenesis, mitophagy, fission and fusion processes, glutaminolysis, and mitochondrial oncometabolites generation.

mitochondrial function may confer an invasive advantage to human colorectal cancer (67).

Respiratory chain protein complexes (complexes I-IV) are placed into the inner membrane of mitochondria together with adenosine triphosphate (ATP) synthase, protein import machinery and transport proteins regulating metabolites passage through the matrix. The generation of ATP in mitochondria is coupled to the oxidation of NADH and $FADH_2$, and reduction of oxygen to water (68). Abnormalities in mitochondrial complex I activity increase the aggressiveness of human breast cancer

cells (69). The complex I, II and IV have all been shown to be hyperactive in human breast cancer cells; compared to tumor stromal cells and normal epithelial ductal cells (70). Interestingly it was shown that COX7RP is overexpressed in breast and endometrial cancer cells and promotes *in vitro* and *in vivo* growth by stabilizing mitochondrial supercomplex assembly even in hypoxic states, and increases hypoxia tolerance (71). Recently it was shown that OXPHOS is regulated by fascin, an actin-bundling protein that promotes lung cancer metastatic colonization by augmenting metabolic stress resistance by remodeling mitochondrial actin filaments (72).

The wide regulation of the mitochondria in cancer is of great importance and is a promising target in the development of cancer therapy (73), a number of therapeutic strategies have been based on targeting tumor mitochondrial proteins and their functions, such as metformin that has had currently a lot of impact on cancer therapy (74). Metformin induce the inhibition of OXPHOS due to reduced function of mitochondrial complex I underlies cellular and whole organism actions (75), this topic will be reviewed later in this review.

mTORC1 AND MITOCHONDRIAL REGULATION BY miRNAs IN CANCER

The expression of a large number of oncogenes and tumor suppressor genes is regulated by miRNAs, which altered expression, is currently though as a hallmark of cancer. miRNAs or microRNAs are small non-coding RNAs (21–25 nt), that regulate gene expression by targeting mRNAs for degradation or suppressing translation (76). In cancer, miRNAs are divided into two categories, oncogenic miRNAs and tumor suppressor miRNAs, which are up regulated and down regulated during tumorigenesis (77). According to its role as a master regulator of cell growth, mTORC1 activity is modulated by different extracellular signals and intracellular mechanisms, interestingly it has been shown that some miRNAs can also regulate the mTORC1 activity directly or through targeting upstream regulators such as PI3K/Akt pathway. For instance, miR-451 is upregulated in glioma compared with control brain tissue; furthermore decreased miR-451 expression was associated to a suppressed tumor cell proliferation via CAB39/AMPK/mTOR pathway in two glioma cell lines (78). Furthermore, over expression of miR-405 promoted caspase-3/-9 and Bax protein expression, and suppressed cyclin D1 protein expression and the PI3K/Akt/mTOR pathway inhibiting cell proliferation and promoting cell apoptosis in gastric cancer-derived cells (78). On the other hand evidence shown that mTORC1 regulates miRNAs biogenesis and given the broad function of miRNAs in cancer development, it is possible that a significant portion of mTORC1 function, may be through its ability to control miRNA biogenesis. It was shown that chronic treatment with rapamycin leads to significant alterations in miRNA profiles and these changes correlate with resistance to rapamycin. The miRNAs associated to rapamycin resistance were miR-370, miR-17-92 and its related miR-106a-92, and miR-106b-25 clusters, which have been shown to have oncogenic properties in several types of cancer (79). Ye and collaborators (2015) report that mTORC1 activation downregulates miRNA biogenesis through upregulation of Mdm2, which is a necessary and sufficient E3 ligase for ubiquitinylation of Drosha an essential RNase dedicated to processing pri-miRNA in response to the cellular environment (80). On the other hand it was shown that mTORC1 in TSC2 deficient cells, promotes the miRNA biogenesis through of GSK3β regulation. mTORC1 induces the activity of the microprocessor, a nuclear complex that includes the nuclease Drosha and its partner DGCR8, this complex cleaves the stem loop of pri-miRNA to form premiRNA via the nuclease activity of Drosha (81).

On the other hand it was reported that several miRNAs targeting several mRNAs of nuclear-encoded mitochondrial proteins, integrating miRNAs into the landscape of translational regulation of mitochondrial functions such as TCA cycle, production of ROS and glutamine metabolism and mitochondrial fission process (82). miR-125a is frequently downregulated in several human cancer such as ovarian, non small-cell lung and gastric cancer and colorectal cancer (83–85) Moreover low expression of miR-125a is associated with increased tumor diameter, high Ki67 expression and poor overall survival of patients with gastric carcinoma (86) Additionally miR-125a deficiency enhances agiogenic processes through metabolic reprogramming of endothelial cells (87). Interestingly it was demonstrated that miR-125a is decreased in pancreatic cancer cells (PANC-1), accompanied by an increase in the contents of mitofusin 2 (MFN2) an important regulator of mitochondrial fission. Interestingly reintroduction of miR-125a triggered mitochondrial fission via downregulation of MFN2. Excessive mitochondrial fission contributes to activation of mitochondria-dependent apoptosis and impairs cellular migration via induction of F-actin degradation (88).

miRNAs are encoded in the nuclear genome and exported to the cytosol where they perform most of their functions, however, the expression of miRNAs within the mitochondrion has been recently demonstrated, which can be either mitochondrial encoded or transcribed within the nucleus and subsequently localized to mitochondria, this miRNAs are termed as mitomiRs (89). MitomiRs are likely to contribute to some post-transcriptional regulation of gene expression related to the mitochondrial functions (90). Interestingly mitomiRs have been shown to play a very important role in chemotherapy resistance through the regulation of metabolic changes. For instance, it was demonstrated that mito-miR-2392 regulates the cisplatin resistance by reprogramming the balance between OXPHOS and glycolysis in tongue squamous cell carcinoma (TSCC) cells. Furthermore, in a retrospective analysis of TSCC patient tumor revealed a significant association of miR2392 and the expression of mitochondrial gene with chemosensitivity and overall survival (91).

Although several cancer processes are regulated by miRNAs, there is a lacking of investigation aimed to determine the role of the mitomiRs and mTORC1 regulation either, in metabolic responses to therapy as well as mitochondrial functions, representing an open opportunity for future research.

mTORC1 REGULATES TRANSLATION OF MITOCHONDRIAL PROTEINS ENCODED IN THE NUCLEI

Protein synthesis or mRNA translation, is the major energy-consuming process in the cell (92, 93). It is well-established that deregulation of mRNA translation is a prominent characteristic of cancer cells (94). Protein translation can be simplified into four stages: initiation, elongation, termination, and ribosomes recycling, however the critical regulation point occurs in the step of mRNA translation initiation, this step is regulated by several key signaling pathways, including PI3K/Akt/mTORC1 that in fact are over expressed in several neoplasms (95). The mitochondrial translation comprises the same four stages, although mitochondria have their own translation machinery with distinct mitochondrial ribosomes (mitoribosome), tRNAs and translation factors than the cytosolic counterparts. Yet the majority of the mitochondrial proteins, including all factors required for mtDNA maintenance and expression, and some components of the ETC complexes are encoded in the nuclear genome (96) and are translated in cytosolic ribosomes, and transported into mitochondria via peptides that function as import signals, this mitochondrial proteins are widely regulated via mTORC1 (97). Since mTORC1 regulates the cellular most energy consuming process, it is reasonable that mTORC1 responds to bioenergetics variation, a process controlled by mitochondria. Additionally, it was shown that mTORC1 regulates the capacity of the mitochondria to produce ATP as well as cell cycle progression in cancer cells (98).

Larsson et al. (99) evaluated the impact of different mTORC1 inhibitors in the global regulation of protein translation in MCF7 cells, interestingly, the authors found several mRNAs involved in mitochondrial functions (99). In another study, it was demonstrated that mTORC1 regulates the translation of the ATP synthase components, included ATP synthase subunit delta (ATP5D), and the transcription factor A, mitochondrial (TFAM), which promotes mitochondrial DNA replication and transcription through 4E-BPs, moreover, this was related with a higher mitochondrial activity (100). In conclusion, there is a feed-forward mechanism in the cells whereby translation of nucleus-encoded mitochondria-related mRNAs is regulated via mTORC1/4E-BP pathway to induce mitochondrial ATP production capacity and thus provide sufficient energy for protein synthesis (100). In support with this, using nano-cap analysis, which allows determination of transcription start sites on a genome-wide scale, a large number of non-TOP mRNAs were found to be mTOR sensitive (101). Among these non-TOP mRNAs, mRNAs with short 5′ UTRs were in fact mRNAs encoding for protein involved in mitochondrial functions, including components of the respiratory chain complexes (ATP50, ATP5D, UQCC2) (101).

This demonstrates that mTORC1 drives cell proliferation and neoplastic growth not only by inducing the translation of genes involved in cell growth but also by promoting the translation of mitochondrial proteins involved in cellular energy production, proteins implicated in mitochondrial DNA replication and mitochondrial repair, transcription, and translation.

MITOCHONDRIAL LOCALIZATION OF mTORC1: REGULATION OF THE MITOCHONDRIAL OXIDATIVE METABOLISM

As described previously, mTORC1 regulates the translation of mitochondrial proteins encoded in the nucleus, however it is not the only function by which this important metabolic regulator acts. Interestingly, it has been shown that mTORC1 is found in mitochondrial fractions suggesting a regulatory ATP producing capacity.

Desai et al. (102) described the first association between mTOR and mitochondria through subcellular fractionation of human T cells. They identified that mTOR co-interact with purified mitochondria elements, and specifically mTOR is associated with the outer mitochondrial membrane. In addition, they demonstrated that when treating with mitochondrial inhibitors, the activity of mTORC1 was decreased (102). In support of these data, another study showed that mTOR-raptor complex is also present in the mitochondrial fraction of Jurkat T cells; this complex was tightly correlated with mitochondrial activity, specifically with high consumption of oxygen and mitochondrial membrane potential as well as with a higher capacity for ATP production. Moreover, disruption of the mTOR-raptor complexes with rapamycin or with RNAi resulted in a decreased mitochondrial metabolism (103).

The voltage-dependent anion channels (VDACs) are pore forming proteins found in the outer mitochondrial membrane of all eukaryotes, and are the binding sites for several cytosolic enzymes, including the isoforms of hexokinase and glycerol kinase, allowing a preferential access to mitochondrial ATP (104). This mitochondrial protein is often overexpressed in several cancers, and it has been shown that VDAC1 depletion leads to a rewiring of cancer cell metabolism in breast cancer, lung cancer and glioblastoma, resulting in cell growth arrest, and tumor growth inhibition (105). Ramanathan et al. (106) showed that leukemic cells treated with rapamycin, showed a decreased mitochondrial activity. Interestingly, they found that mTOR coimmunoprecipitates with the VDAC1 and with the anti-apoptotic protein B-cell lymphoma-extra-large (Bcl-xl). They also demonstrated that mTOR phosphorylates Bcl-xl in serine 62 and increases its activity. Since Bcl-xl is a key mediator of mitochondrial function and cellular apoptosis that has been shown to bind to VDAC1 and increase the substrate permeability, its suggested that mTOR could control mitochondrial metabolism in a Bcl-xl-VDAC1 dependent manner (106). On the other hand, it was demonstrated that under radiation stress, mTOR relocates to mitochondria in MCF7, HCT116, and U87 cells, where it interacts with hexokinase II, an enzyme that phosphorylates glucose during glycolysis switching bioenergetics from aerobic glycolysis to OXPHOS which is related to an increased tumor resistance to radiation treatment (107), this interaction was also observed in another study in neonatal rat ventricular myocytes under glucose starvation (108). In another study, it was demonstrated that mTOR/Akt pathway regulates the mitochondrial respiratory activities and the expression of complex I, II and IV of the electron

transport chain trough 4E-BP1 (109). Furthermore, another study suggested that mTOR-raptor may acts as a metabolic checkpoint in G1 phase of cell cycle by regulating mitochondrial function (110).

Triple-negative breast cancer cells possess special metabolic characteristics compared to estrogen receptor (ER) positive cells, manifested by high glucose uptake, increased lactate production, and low mitochondrial respiration which is correlated with attenuation of mTOR pathway and decreased expression of p70S6K. Re-expression of p70S6K reverses their glycolytic phenotype to OXPHOS state, while knockdown of p70S6K in ER positive cells leads to suppression of mitochondrial OXPHOS (111). It was demonstrated that global targeting of mTOR caused both anti-survival and pro-survival mitochondrial response, which were differentially exhibited in diverse cancer cells according to their intrinsic sensitivity to mTOR inhibition and hyperactive PI3K/AKT/mTOR activity status and/or growth factor-dependence (112).

mTORC1 AND MITOCHONDRIAL DYNAMIC IN CANCER

The mitochondrial dynamic is a balance between fission and fusion processes (113). Mitochondria fusion is the union of two mitochondria resulting in one mitochondrion; organelle movement along cellular tracks that permit the encounter between two different mitochondria facilitating the fusion process (114). Fusion helps cells to mitigate stress by sharing multiple elements, which sustain mitochondrial biology as a form of complementation. Mitochondrial fusion involves two sequential steps: first, the outer membranes (OMs) of two mitochondria fuse; second, the inner membranes (IMs) fuse. OM fusion is mediated by mitofusin 1 (MFN1) (115) and MNF2 (116), which are dynamin-related GTPases at the OM (117). IM fusion is mediated by the dynamin-related protein optic atrophy 1 (OPA1) (118).

On the other hand, the mitochondrial fission is characterized by the division of one mitochondrion in two daughters, this process is required for segregation of damaged mitochondria for mitophagy, mtDNA replication, and mitochondria redistribution and motility during cell division (113). The fusion process requires the recruitment of dynamin-related protein 1 (DRP1) (119) from the cytosol to the mitochondrial OM. Assembly of DRP1 on the mitochondrial surface causes constraint of the mitochondria and leads to division of the organelle (120). In mammals exist four DRP1 receptors: mitochondrial fission 1 (FIS1) (121), mitochondrial fission factor (MFF) (122), Mitochondrial dynamics proteins of 49 kDa (MID49), and MID51 that are located on the mitochondrial OM (123).

It has been established that the alteration of mitochondrial dynamics impact tumor development broadly. Alterations to the mitochondrial dynamic network also result in specific therapeutic susceptibilities, in particular, tumors with increased mitochondrial fragmentation or connectivity are hypersensitive to SMAC mimetics and induce apoptosis by blocking the action of inhibitor of apoptosis proteins (IAPs) (124). On the other hand, it was demonstrated that Drp1 expression was strongly increased in distant metastasis of hepatocellular carcinoma (HCC) compared to primary tumors. In contrast, Mfn1 showed an opposite trend (125). Moreover, *in vitro* experiments with HCC cells, demonstrated that mitochondrial fission significantly promoted the reprogramming of focal adhesion dynamics and lamellipodia formation mainly, by activating the CA^{2+}/CaMKII/ERK/FAK pathway, which was associated with a greater capacity for migration and invasion (123, 125).

A very important protein in mitochondrial fission is the mitochondrial fission process protein 1 (MTFP1), also called mitochondrial fission process 1,18 kDa (MTFP18), an integral pro-fission protein located at the mitochondrial inner membrane whose loss results in a hyperfused mitochondrial reticulum, whereas its overexpression produces mitochondrial fragmentation (126). As mentioned earlier, mTORC1 promotes the translation of mitochondrial proteins encoded in nuclei, interestingly, using a genome-wide polysome profiling and translatome, it was demonstrated the treatment with rapamycin, PP242 and metformin (mTORC1 inhibitors) suppressed the translation of MTFP1 (99). Morita and collaborators recently demonstrated that mTORC1 is a regulator of mitochondrial dynamics and cell survival via MTFP1 translation. Using mouse embryonic fibroblasts (MEFs) and human malignant melanoma cells treated with active-site mTOR inhibitor (asTORi), was demonstrate that mTORC1 stimulates the translation of MTFP1 mediated by 4E-BP, and therefore the mTOR inhibition induces the phosphorylation of the DRP1 at Ser 637, this phosphorylation prevents it translocation to mitochondria, conversely, the pro-fission phosphorylation site of DRP1 at Ser 616 was decreased in asTORi treated cells. This process was associated with a high mitochondrial elongation, branching, and circularization (127).

In support with these results it has been shown that cellular starvation inhibits mTORC1 pathway, interestingly, it was shown that the cells show a mitochondrial elongation phenotype under starvation (128, 129) similar to that observed in asTORi treatment. Combination between mTOR inhibitors and an increase of mitochondrial fission activates cell apoptosis, converting the mTOR inhibitors action of cytostatic to cytotoxic (127). In other study, it was shown that S6K1 contributes to mitochondrial dynamics, homeostasis and function, since MEFs-lacking S6K1 exhibited more fragmented mitochondria and a higher level of Drp1 with greater phosphorylation levels in Ser 616 (130). The depletion of S6K1 induced mitochondrial fission but not mitophagy. These changes in mitochondrial morphology alter its function disrupting the balance of OXPHOS, ATP production and changing cellular energy metabolism (130).

MITOCHONDRIAL BIOGENESIS AND MITOPHAGY: mTORC1 IN CANCER

Mitochondrial mass is regulated by two opposite pathways, mitochondrial biogenesis and mitophagy, both processes emerging as dual regulators of tumorigenesis (4). Mitochondrial

biogenesis is the growth and division of pre-existing mitochondria, whereas mitophagy is a form of autophagy that selectively degrades damaged mitochondria (131).

Mitochondrial biogenesis is widely regulated at transcriptional, translational and post-translational levels. Peroxisome proliferator-activated receptor γ co-activator 1α (PGC1-α) and related transcription co-activator are the master transcriptional regulators of mitochondrial biogenesis (132). PCG1-α binds to various transcription factors and nuclear receptors that recognize specific sequences in their target genes and promotes the mitochondrial biogenesis and oxidative phosphorylation in cancer cells and also promotes tumor metastasis (133) and drug resistance in colorectal cancer cells by regulating endoplasmic reticulum stress (134). The targets of PGC1-α include enzymes of energy metabolism as well as essential factors for the replication and transcription of mtDNA. PGC1-α is a transcription factor for mitochondrial genes, which action depends on its association with other transcription factors such as yin-yang (YY1), nuclear respiratory factor 1(NRF1) and 2 (NRF2), estrogen-related receptor α (ERRα) (132, 135). YY1 is a zinc finger protein and a member of the GLI-Kruppel family that can activate or inactivate gene expression depending on its interacting partners (136), YY1 is overexpressed in multiple cancer types and correlates with poor clinical outcomes (137, 138). However, other papers report that YY1 inhibits the cell growth in different tumor cell types *in vitro*, including human breast carcinoma cells and glioblastoma cells (139).

Using skeletal muscle cells was showed that rapamycin decreased the expression of the PGC1-α, RREα, and NRF1 in correlation with decreased oxygen consumption. Moreover, it was identified that mTOR-raptor complex interacts with YY1, and in association with PCG1-α, regulates the mitochondrial gene expression (ATP5G1, Cox5A, cytochrome c, NDUF88, and UCP2) (140). In support with this results, it was demonstrated that mTOR induces the phosphorylation of YY1 (T30 and S356) consequently favoring the interaction with PGC1-α and increased mitochondrial morphology and bioenergetics state, in skeletal muscle (141). These results demonstrate that mTORC1 regulates mitochondrial biogenesis by promoting the transcription of mitochondrial genes. On the other hand, mTORC1 controls mitochondrial activity and biogenesis by selectively promoting translation of nucleus-encoded mitochondria related mRNAs via inhibition 4E-BPs. Moreover, the stimulation of the translation increases ATP production capacity, a required energy source for translation in MCF7 cells (100). In addition to stimulation of mitochondrial biogenesis by antagonizing 4E-BP1 dependent translation repression of mitochondria related mRNAs, mTORC1 inhibits mitochondrial degradation by suppressing autophagy (100).

PGC-1β is also an important mitochondrial biogenesis regulator, through regulation of the expression of NRF1 (142). It was shown that the levels of PGC-1β and mTOR correlated with overall mitochondrial activity in breast cancer samples. Moreover, the knockdown of endogenous PGC-1β, leads to a decreased expression of mTOR pathway related genes and induces apoptosis in MDA-MB-231 cells (143). Interestingly, it was demonstrated that the branched chain amino acid transaminase 1 (BCAT1) actives mTORC1 and in consequence promotes the mitochondrial biogenesis, ATP production and defense of oxidative stress (143). The inhibition of mTORC1 with rapamycin, neutralized the roles of BCAT1 in mitochondrial function and breast cancer cell growth (143). Recently, it was shown that rapamycin, enhanced the processes of apoptosis and initiation of autophagy in LKB1 deficient urothelial carcinoma of the bladder both *in vitro* and *in vivo*, which was associated with deregulated mitochondrial biogenesis and AMPK activation (144). These results are relevant because AMPK is an important regulator of mitochondrial biogenesis via PGC1-α (145), which also inhibits the mTORC1 pathway.

Mitophagic status was assessed in a panel of human cytoplasmic hybrid (cybrid) cell lines carrying a variety of pathogenic mtDNA mutations. It was found that both genetic and chemically induced loss of mitochondrial transmembrane potentially caused recruitment of the pro-mitophagic factor Parkin to mitochondria but it was insufficient to prompt mitophagy. They found that mitophagy could be induced following treatment with the mTORC1 inhibitor rapamycin (146).

These findings suggest that, mTORC1 is an important regulator of mitochondrial biogenesis, by regulating the expression of important factors in the regulation of mitochondrial biogenesis, both at the transcriptional level and at the translation level (**Figure 3**).

GLUTAMINOLYSIS AND mTORC1 IN CANCER

Glutaminolysis is a set of reactions that occurs in mitochondrial matrix and cytosol in proliferating cells. In such reactions, the amino acid glutamine is degraded to glutamate, ammonium, aspartate and pyruvate, among others. Glutamine, glutamate as well as aspartate, are used for nucleic acid synthesis, other important function of glutamine is replenishing the TCA cycle intermediate α-KG.

It has been reported that glutamine is the amino acid most frequently found in plasma and muscle (147), glutamine concentration ranges from 450 to 800 μM in human plasma (148). Glutamine has been defined as a non-essential amino acid; nevertheless, evidence has showed that glutamine becomes essential in stressful conditions (149). As an example, when cells are under hypoxic stress, glutamine-derived α-KG is used to stimulate lipids synthesis (150). Carbon and nitrogen from the glutamine present in blood are used for biosynthesis and also for providing energy to the cell (151). Specifically, glutamine is the leading donor of nitrogen for purine and pyrimidine nucleotide synthesis, as well as a supplier for amino groups for non-essential amino acids synthesis, such as aspartate, alanine, glycine and serine, moreover, nitrogen from glutamine participates in nucleic acid and *de novo* protein synthesis (149, 152). Finally, the glutamine-derived carbon is source for fatty acid and amino acid synthesis as well (151).

Glutamine enters to the cells via SLC (solute carrier)-type transporters. Fourteen of these transporters are known for transporting glutamine to the plasma membrane which are

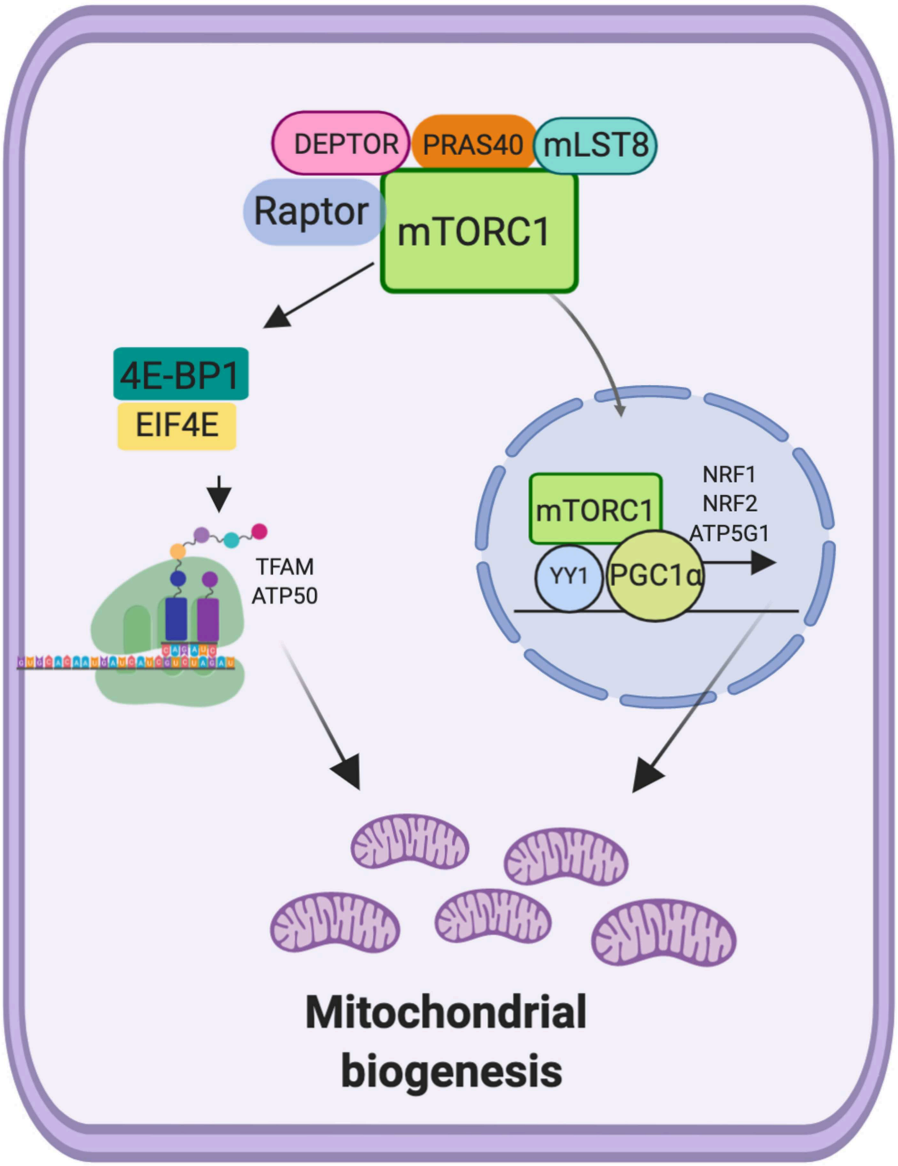

FIGURE 3 | Mechanistic target of rapamycin complex 1 (mTORC1) and mitochondrial biogenesis. mTORC1 promotes mitochondrial biogenesis via upregulation of translation genes and moreover via transcriptional regulation of TFAM, ATP50, NRF1, NRF2 genes.

classified into four families: SLC1, SLC6, SLC7, and SLC38 (153). Glutamine is metabolized within the mitochondrion via two deamination steps. The first one produces glutamate through an irreversible reaction catalyzed by glutaminase (GLS1 and GLS2 in mammals); in the following deamination reaction, α-KG is produced by the enzyme glutamate dehydrogenase (GDH) (154). The α-KG generated by glutaminolysis is a major anaplerotic source in the TCA cycle.

Importantly, it was demonstrated that glutamine could be useful for cancer cells to drive tumor growth due to is used for energy generation as well as for biomass accumulation being a source of carbon and nitrogen as mentioned before (152), moreover glutamine can be consumed by proliferating cells more rapidly than needed to satisfy nitrogen requirements (155). As a result of glutamine depletion, most cancer patient's loss body weight due to muscle mass consumption provoking weakness, all these symptoms are known as cachexia (155, 156). It is important to notice that, when cancer cells are deprived of glutamine, undergo cell cycle arrest due to nitrogen deficiency since nitrogen is necessary for nucleotides synthesis (157). In 1978, Lawrence et al. observed that glutamine is the major energy source in HeLa cell line (158). Additionally, evidence supports that glutaminolysis provides metabolites, such as glutamate to promote tumor growth, as observed by Dornier

et al. (159). The group investigated the participation of glutamine metabolism in invasive processes so that, they showed that mammary epithelial cells from normal tissue uptake glutamine, yet glutamate secretion was not observed. Extracellular glutamate is needed at low concentrations for mammary epithelial phenotype maintenance, but higher concentrations promote key characteristics of the invasive phenotype, moreover, in primary cultures of invasive breast cancer cells it was observed a high conversion glutamine to glutamate (159).

Autophagy and cell growth are found to be under control of mTORC1; those two cellular processes are regulated by glutaminolysis, so that mTOR activity is tightly controlled to prevent inappropriate cell growth (**Figure 4**). In fact, it has been found an upregulation of mTORC1 in several cancers and such activation is required for cell growth and protein synthesis. Further, glutamine metabolism is found disrupted in several cancer types, including papillary thyroid cancer where using cell lines was demonstrated that such cells are dependent on glutamine and glutaminolysis-associated proteins.

Through different experimental approaches, an aberrant overexpression of GLS was showed in cancer; moreover, pharmacological inhibition (by using inhibitors BPTES and CB-8939 that target both isoforms of GLS) and genetic knockdown of GLS repressed glutaminolysis and diminished mitochondrial respiration. Additionally, using tissues and cells from patients with papillary thyroid cancer, an altered overexpression of glutaminase was observed. When GLS was inhibited using a siRNA, mTORC1-signaling pathway was deactivated leading to an increase of autophagy and apoptosis (160).

It has been demonstrated that arginine and leucine prompt mTORC1 by activating RAS-related GTPase (RAG) complex; as a result, mTORC1 is recruited and triggers lysosome activity. Studies have demonstrated that glutamine positively regulates the mTORC1 pathway when promoting leucine uptake (161) and as well-boosting mTORC1 assembly as well as its localization into the lysosome; indeed, the presence of α-KG is considered to be enough to promote mTORC1 localization into the lysosome (162) (**Figure 3**).

The mentioned RAG-dependent regulation of mTOR could rely on glutamine, arginine and leucine transporter SLC38A9 (163–165). Although the mechanism is not well-understood, it has been hypothesized that α-KG could be able to regulate RAGB activity as well as mTOR activation at a downstream glutamine metabolism level (151). On the other hand, Jewell and her group reported in 2015 that mTORC1 activation could be independent of the Rag GTPases and supported the fact that mTORC1 is differentially regulated by the amino acids leucine and glutamine. Using mouse embryonic fibroblasts RagA and RagB knockout cells, they demonstrated that leucine stimulates mTORC1 by Rag GTPase-dependent mechanism meanwhile glutamine stimulates mTORC1 through a mechanism that is carried out by Rag in an GTPase-independent mechanism in order to translocate mTORC1 to the lysosome (166).

In 2013, Csibi and collaborators reported that mTORC1 pathway regulates glutamine uptake and metabolism. The results showed that the mTORC1 pathway negatively controls SIRT4, an ADP-ribosyltransferase that is found in the mitochondria and inhibits glutamine dehydrogenase (GDH), through stimulation of proteasome-mediated degradation of cAMP-responsive element-binding (CREB) 2. In fact, it has also been reported that SIRT4 expression is decreased in several human cancers (167).

The same research group postulated a previous model in which they concluded that cells are addicted to glutamine as a result of mTORC1 activation (168). It was shown that α-KG could be exported to the cytosol by the mitochondrial carrier protein α-KG/malate named SLC25A11(154). At high glutaminolyc rate, cytosolic α-KG activates the enzymes that function as oxygen and nutrient of the cell sensors EGLNs (prolyl hydroxylase enzymes PHD) such enzymes are required for mTORC1 activation-dependent of amino acids in a HIF-1α independent manner to promote cell growth and anabolism and inhibit autophagy (154, 162).

An elevated glutaminolysis is related to the promotion of cancer progression at early stages by stimulating cell growth through the mTORC1 pathway and diminishing elimination of altered proteins and organelles by inhibiting autophagy (154, 162). In another case, glutamine dependence was evaluated in six different cell lines from squamous cell carcinoma and it was found that five out of six cell lines were glutamine-dependent, also, glutamine depletion, using GLS1- inhibitors BPTES and compound 968, decreased cell proliferation in those five cell lines, meanwhile inhibition of cell proliferation in QG56 glutamine-independent cell line was not reported as significant. Further, it was observed that the inhibition of glutaminolysis suppressed mTORC1 activity, by evaluating pS6 levels in the glutamine-dependent RERF-LC-AI cell line but the activity of mTORC1 was not affected in the QG56 glutamine-independent cell line. Finally, inhibition of glutaminolysis induced autophagy in RERF-LC-AI cell line (169). Furthermore, the activation of mTORC1 inhibits the family of enzymes that catalyze phosphorylation of phosphatidyl inositol, one of the main phospholipids present in the cell, specifically at the d-3 position of the inositol ring, to generate PtdIns (3)P complex I and unc-51 like autophagy activating kinase complex (ULK), both proteins participate in the initiation step of autophagy and mTORC1 activation limits the initiation steps of autophagy. On the other hand, glutaminolysis products GSH, NADPH, and α-KG limit ROS production to prevent autophagy induction (154).

It has been observed that a reactivation of mTORC1 by glutaminolysis is also required for lysosome regeneration and autophagy termination (154). In the specific case of autophagy, it has been reported that autophagy has a dual role in cancer, acting as tumor suppressor in some cases. For instance, metabolic stress causes the expression of p62, a sustained autophagy substrate protein, resulting in autophagy defects and an altered expression of NF-kB, finally promoting tumorigenesis, this information indicates that autophagy suppresses tumorigenesis by limiting p62 accumulation (151, 170). On the contrary, autophagy seems to support cancer cells survival by facilitating nutrients and suppressing stress pathways. For instance, expression of H-ras and K-ras oncogenes in immortal non-tumorigenic baby mouse kidney epithelial cells upregulated basal autophagy promoting tumor cell survival (151, 171). Another interesting relation between mTOR pathway and autophagy is the association to

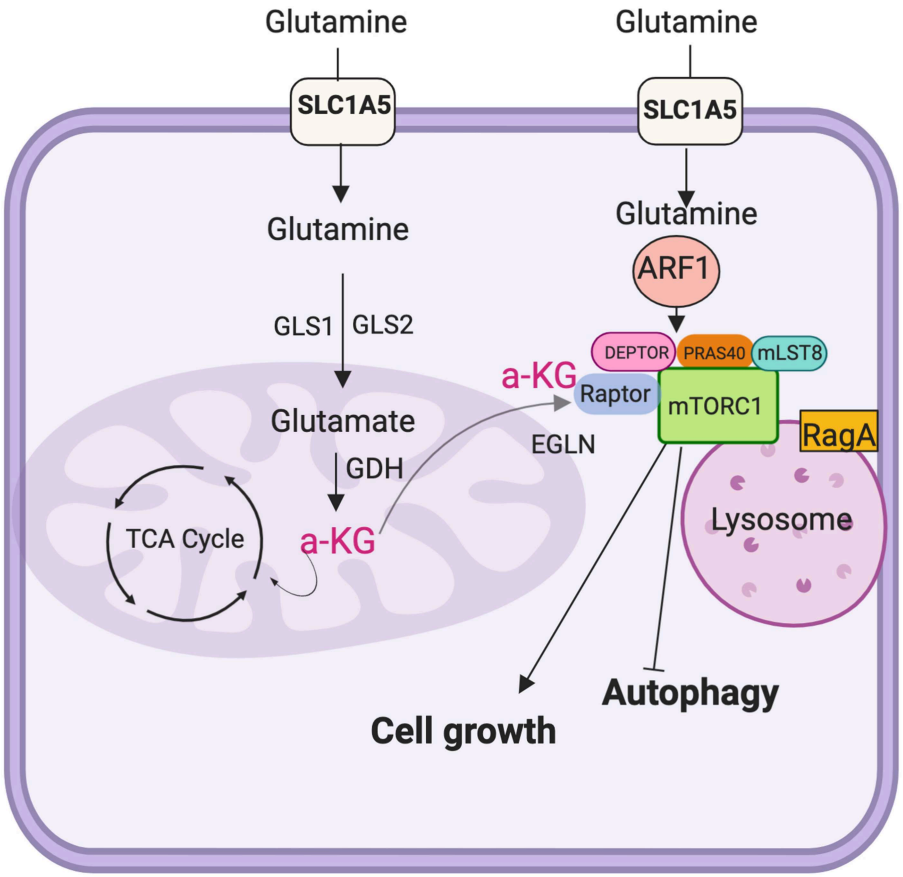

FIGURE 4 | Glutaminolysis and Mechanistic target of rapamycin complex 1 (mTORC1) The α-ketoglutarate (α-KG) produced by glutaminolysis is used for tricarboxilic acid (TCA) cycle intermediates replenish, a process known as anaplerosis. Once α-KG is exported from the mitochondria to the cytosol activates EGLNs, which in turn triggers mTORC1 activity promoting cell growth and inhibits autophagy.

lifespan and aging; mainly because it has been observed that inhibition of mTOR could bring as a consequence delay of aging due to autophagy stimulation resulting in a mitophagy increase (172). In fact, it is well-documented that inhibition of key components of mTOR and its counterpart in invertebrates TOR pathways, results in an extension of life span in part by the influence of mTOR on the called "hallmarks of aging," an interesting an extensive review about this subject is broadly reviewed in Papadopoli publication (173).

The regulation of both, mTORC1 and glutaminolysis suggests that mTORC1 and glutaminolysis act in both directions hence they are found to regulate each other for promoting cell growth and cancer progression; mTORC1 also induces glutaminolysis by activating c-MYC-GLS and because c-MYC is GLS and GLUD1 transcription factor, glutamine metabolism is favored; additionally, the glutaminolysis-mediated activation of mTORC1 participates in autophagy inhibition and the DNA double-strand breaks sensor serine/threonine protein kinase ATM which participates in cell cycle delay after DNA damage. The mTORC1 pathway suppresses ATM via S6K1/2 signaling pathways and by upregulating mircroRNA-18a and microRNA-421 that target ATM (154, 174). An increase in glutamine synthetase abolishes the production of α-KG from glutaminolysis, as a result, an inhibition of mTORC1 is observed as well as an enhancement of autophagy, which is imperative for cancer cell survival (154, 175). There is an increasing interest in inhibiting simultaneously both, glutaminolysis and autophagy in order to trigger a synergistic effect that may be useful for patient outcome improvement and also to diminish toxicity.

A very interesting publication of 2016 shows that autophagy could be a survival mechanism upon rapamycin treatment. Interestingly, in conditions of nutrient restrictions, mTORC1 is activated by glutaminolysis during nutritional restrictions and autophagy is inhibited, so then apoptosis is induced, via upregulation of p62 in U-2 OS cells (176).

MITOCHONDRIAL ONCOMETABOLITES AND mTORC1

Dominant mutations in mitochondrial enzymes led to identification of mitochondrial derived signaling molecules,

called oncometabolites. The term of oncometabolites refers to intermediates of metabolism that abnormally accumulate in cancer cells upstream or downstream of metabolic defects, often through loss-of-function or gain-of function mutations, respectively, in genes encoding the corresponding enzymes (177). This oncometabolites are 2-hydroxyglutarate (2HG), succinate and fumarate which have been demonstrated to contribute to the development and progression of cancer (178). The oncometabolites are produced by mutations in the nuclear-encoded TCA enzymes, isocitrate dehydrogenase 1 and 2 (IDH1/2), succinate dehydrogenase (SDH), and fumarate hydratase (FH) (177). Chin and co-workers discovered that metabolite α-KG increases the lifespan of adult *C. elegans* by inhibiting the highly conserved ATP synthase and mTORC1, mimicking dietary restriction in longevity (179). Interestingly, it has been shown that mTORC1 promotes the generation of oncometabolites in addition it was also shown that these oncometabolites regulate mTORC1, as a feedback regulation.

2HG and mTORC1

Isocitrate dehydrogenases 1 and 2 (IDH1, and IDH2) are key TCA cycle enzymes that are nicotinamide adenine dinucleotide phosphate ($NADP^+$) dependent. IDH1 and 2 catalyze the oxidative decarboxylation of isocitrate to α-KG with production of reduced nicotinamide adenine dinucleotide phosphate (NADPH) (180). Mutations in IDH1 and IDH2 genes are mostly missense variants leading to a single amino-acid substitution of arginine residues at codon 132 in exon 4 of the IDH gene or codons 140 or 172 of the IDH2 gene. Mutant of IDH1 and IDH2 enzymes have a gain the function of catalyzing the reduction of α-KG to its (R)-enantiomer of 2-hydroxyglutarate (2HG), which accumulates to exceedingly high levels in patients with glioma, acute myeloid leukemia, esophageal squamous cell carcinoma (180–183) thus, 2HG levels being used as a biomarker for IDH mutation in these cancers (184). 2HG is an oncometabolite impairing epigenetic and hypoxic regulation through its binding to α-KG-dependent dioxygenases.

Recently, it was shown that 2HG induces angiogenic activity, because it induces the levels of secreted vascular endothelial growth factor (VEGF) in breast cancer cells, and finally enhance the endothelial cell proliferation and migration cell inducing MMP2 activity (185).

It was shown that both (R)-2HG and (S)-2HG bind and inhibit ATP synthase and mTOR signaling. Consistently, this inhibition is sufficient for growth arrest and tumor cell killing under conditions of glucose limitation in glioblastoma cells (186). Contrary to these results, it was demonstrated that mutations $IDH1^{R132H}$ or $IDH2^{R172K}$ in MEF and HeLa cells induce an increase in 2HG levels that stimulate both mTORC1 and mTORC2 signaling as highlighted by enhanced phosphorylation of p70S6K, pS6 and Rictor, and Akt, respectively. They also showed that 2HG inhibits the α-KG-dependent enzyme KDM4A and consequently, this affects the stability of DEPTOR a negative regulator of mTORC1 and mTORC2, leading to mTOR activation independently of the PI3K/Akt/TSC1-2 pathway (187).

In other study it was shown that rapamycin reduced 2-HG levels derived of lactate, in IDH1 mutant fibrosarcoma cell line (HT-1080 cells). Furthermore, they shown that rapamycin inhibit the growth in HT-1080 xenografts *in vivo* and 2HG production (188). In support with this, using two mutant cell lines for IDH and orthotopic mutant IDH tumor model, showed that the treatment with dual PI3K/mTOR inhibitor (XL765), induced a significant reduction in 2HG levels, and enhanced the survival (189).

Fumarate and mTORC1

In the TCA cycle the reversible hydration of fumarate to malate is catalyzed by FH. The oncogenic properties of FH loss have been mostly associated with a high intracellular accumulation of fumarate. This oncometabolite shares structural similarity with another TCA cycle intermediate α-ketoglutarate, also referred to as 2-oxoglutarate (2-OG). 2-OG is a cofactor for a family of enzymes called 2-OG-dependent dioxygenases that catalyze the hydroxylation of a wide range of targets (190). The enzymes that belong to this family are the prolyl hydroxylases and the Jumonji C containing family of histone lysine demethylases and TET (ten-eleven translocases) enzymes (190). It was shown that high levels of fumarate inhibit the HIF-1α prolyl hydroxylases, leading to HIF-1α stabilization (191). HIF-1α is inactivated in normoxia by prolyl hydroxylase enzymes (PHD 1-3) using oxygen as a substrate. HIF-1α hydroxilated is associated to E3 ubiquitin ligase Von Hippel Lindau protein (VHLp) for its degradation, whereas in hypoxia condition stabilization and nuclear translocation occur, leading to oncogenes activation (192). HIF-1α is a transcription factor for metabolic genes such as hexokinase (HK), lactate deshydrogenase (LDHA) and glucose transporter (GLUT1) promoting tumor glycolysis (193). In other study it was demonstrated that fumarate accumulation promotes HIF-1α mRNA and protein accumulation independent of the VHL pathway but through an NF-kB dependent mechanism. Fumarate promotes p65 phosphorylation and p65 accumulation at the HIF-1α promoter through non-canonical signaling via the upstream Tank biding kinase (TBK1) promoting cell invasion of renal cancer cells (194). In accordance with the role of the fumarate accumulation with cytotoxicity and oncogenic capacity, it was demonstrated that cells exposed to high levels of fumarate and succinate lead to extensive DNA fragmentation and altering the global DNA methylation patterns via DNA hypermethylation in human hepatocellular carcinoma (195).

Interestingly it was shown that mTORC1 upregulation leads to accumulation of fumarate, and contributes to tumor transformation. Using a mouse model harboring the kidney specific inactivation of TSC1 that developed progressive renal lesions that eventually resulted in cortical renal papillary carcinoma, it was shown that TSC1 inactivation results in the accumulation of fumarate due to mTOR-dependent downregulation of the FH. The re-expression of FH rescued renal epithelial transformation (196). In support with these results, using primary samples from clear cell renal cell carcinoma (ccRCC) a total of 15 of 23 cancer samples displayed increased positive staining for pS6 protein (∼65%), confirming mTORC1 upregulation in a large proportion of ccRCC cases. Among the 23 samples analyzed, 16 samples showed downregulation of FH mRNA levels compared with relative healthy tissue (196).

mTORC1 AS A THERAPEUTIC TARGET

Chemotherapy and radiotherapy represent the leading option for cancer treatment and although responses are observed, relapses in several cancer types are common so then, effective therapeutic options for recurrent disease are lacking. There is a link among mTORC1 signaling upregulation and tumor growth, which establish that tumors could be responsive to mTORC1 inhibitors. The correlation between tumor growth and hyperactive mTORC1 signaling suggests that tumors may be sensitive to mTORC1 inhibitors. mTOR inhibitors are known primarily as cytostatic, so inhibiting cell growth could induce cell death when mTOR inhibitors are administrated alone or combined with different therapeutic drugs. Such inhibitors are a promising therapeutic strategy for treating several cancer types (197).

Rapamycin is the first known allosteric mTORC1 inhibitor studied, however, its poor water solubility and chemical stability have led to implement instead the use of semi-synthetic rapamycin analogs (or rapalogs) that show improved pharmacokinetic properties, solubility and reduced immunosuppressive effects (159, 160). To date, three rapalogs are being tested in clinical trials, CCI-779 (temsirolimus), AP23573 or MK-8669 (ridaforolimus), and RAD001 (everolimus) (198). Temsirolimus is an ester derivative drug, approved for renal-cell carcinoma patients since 2007, and is administrated to patients via intravenous or orally. Ridaforolimus was designed to improve aqueous solubility and is administered orally. And finally, everolimus is a hydroxyethyl ether derivative that is administrated to patients via oral (199). In addition, the prototype rapamycin (sirolimus) is also being tested in kidney transplant recipients, for preventing the occurrence of secondary skin cancers, which are common in these patients (200).

These drugs induce apoptosis inhibition by forming a complex with the intracellular immunophilin FKBP12 thus inhibiting the phosphorylation of the mTOR targets, S6K1 and 4E-BP1, as a result, the activation of cyclin-dependent kinases (CDK) is prevented, specifically, the expression of cyclin D1 is found to be decreased meanwhile p27 increases and consequently, cells arrested at G1/S phase die either by autophagy or apoptosis (197, 198).

In the specific case of everolimus, it is known that this drug inhibits the aberrant activity of mTOR by inducing arrest at G_1-phase and sensitizing endothelial and tumoral cells to cisplatin and radiotherapy effects through apoptosis enhancement (197). Such effect occurs due to everolimus ability to block p53-induced p21 expression (201). Everolimus has also been tested in cervical cancer cell lines with a remarkable ability to inactivate efficiently the HPV16 E7 oncoprotein inhibiting cell proliferation (202). The capacity of everolimus-based combinations to inhibit cell proliferation from several cancer types has been reported for breast cancer (203, 204), renal cell carcinoma (205, 206), and thyroid cancer (207) in clinical trials.

In addition to everolimus and temsirolimus, three natural compounds that have been reported as mTOR inhibitors including curcumin, resveratrol and epigallocatechin gallate (EGCG)(208).These compounds proved to be able to induce cytotoxicity in the HeLa cell line when administrated along with radiation. Nevertheless, it is worth to notice that neither everolimus nor temsirolimus seem to be selective for all cancer cell lines as EGCG, resveratrol or curcumin (209). The pro-apoptotic effect of everolimus combined with paclitaxel has been successfully shown for HeLa and SiHa cell lines. In addition, it has been demonstrated that both compounds inhibit the PI3K/AKT/mTOR pathway (210).

Recently, the combination of a daily everolimus dose administrated with standard chemotherapy for newly diagnosed patients with glioblastoma was evaluated in order to determine its efficacy. Even though everolimus has proved to be effective in several published data, it was evident that its efficacy in clinical trials is not as equal than in *in vitro* models. The administrated treatment was not efficient for improving clinical outcomes yet lead to increased toxicity. Moreover, it was suggested that one of the reasons for such lacking of efficacy could be the activation of the Akt pathway due to S6 feedback loop driven by mTORC2 so, it has been proposed that a dual inhibition of mTORC1 and mTORC2 could prevent such Akt activation (211).

An mTOR inhibitor derived from an active fraction of the ethyl acetate extract of *Streptomyces* sp OA293 was reported in 2018. Although it was fully corroborated that such extract lacks any known natural inhibitor of mTOR to date, the metabolite or metabolites present in such active fraction completely inhibited mTORC1 and controlled Akt activation by blocking mTORC2 phosphorylation at Ser2481. Also, this fraction suppressed the activation of 4E-BP1 and P70S6k in cervical cancer cell lines and, induced autophagy and Bax mediated apoptosis. Such extract may represent a better option for improving clinical outcomes in patients once its proved to perform as well as in cell lines (212).

Other rapalogs have been evaluated in clinical trials showing discouraging results in some cases. In 2013, was reported the use of temsirolimus in a phase II study using a dose of 25 mg once a week 4 times. Of 38 patients with cervical cancer enrolled in the study, one of them experienced partial response and 19 had stable disease rendering the effectiveness of temsirolimus alone as questionable (213). According to previous reports performed with cervical cancer cell lines, it was suggested that using mTOR inhibitors could be more efficient when the inhibitors are administrated in combination with other drugs. Three years later, in 2016, Ferreira and colleagues evaluated the maximum-tolerated dose (MTD) of everolimus combined with cisplatin and pelvic radiotherapy, as well as safety and toxicity in 15 patients with advanced stage of cervical cancer in a phase I study. The results showed that although the acceptable dose of everolimus was 5 mg/day, all patients had at least 1 adverse event. Concerning its efficacy, from 12 patients evaluated, 11 showed a complete response, suggesting that 5 mg everolimus together with cisplatin and chemotherapy is a feasible therapy for cervical cancer treatment (207).

Another promising combination using everolimus has been reported in cancer cell lines using metformin, a drug commonly used for diabetes treatment. Metformin induces the inhibition of OXPHOS due to reduced function of respiratory complex I and AMPK activation, which in turn promotes tumor growth reduction through mTOR inhibition, cell cycle arrest and

activation of autophagy; therefore, a combination of both drugs could be more successful for cancer treatment. This synergistic effect was evaluated in breast cancer cell lines (MCF-7, MDA-MB-231, and T47D), cultured with a physiological concentration of glucose under hypoxic or normoxic conditions. The obtained results showed that everolimus and metformin cooperate to inhibit mTOR activity, tumor cell growth and colony formation, independently of glucose and O_2 concentrations (214). A year later, the synergic effect of metformin and rapamycin was evaluated in a pancreatic cancer cell line (SW1990) where a reduced cell proliferation was observed, moreover, cell viability was also reduced when cells were treated with both rapamycin and metformin, importantly, an evaluation of phosphorylated mTOR showed that only a combination of the two drugs was capable to suppress the mTOR pathway. Finally, using a xenograft tumor model, the capacity of metformin and rapamycin to inhibit tumor growth was confirmed (215).

As mentioned before, the use of mTORC1 inhibitors in clinical trials has not been as successfully demonstrated as it has been in cancer cell lines. A possible explanation to this phenomenon could be that upon mTORC1 inhibition, PI3K-AKT cell signaling is stimulated and, consequently it may increase the survival of cancer cells (199). All this because rapamycin and its rapalogs selectively target only mTORC1 without affecting mTORC2, such selective inhibition could prompt feedback loops resulting in AKT activation at ser473 (216). However, it is important to highlight once more that there is plenty of information, which suggests that the use of such inhibitors in combination with other drugs could improve clinical outcome; what is more, inhibiting both mTORC1 and mTORC2 could improve the poor response of other inhibitors observed in clinical trials.

Besides mTORC1 rapalogs, there is another group of mTOR inhibitors known as ATP analogs; such drugs inhibit mTOR kinase activity trough competing with ATP in order to bind to the mTOR kinase domain. ATP donates the phosphate group by which mTOR phosphorylates its target proteins. The ATP analogs inhibit both mTORC1 and mTORC2, interestingly, and because of the resemblance of the kinase domains of mTOR and PI3Ks, this analogs are able to inhibit also PI3K (199).

Inhibition of both PI3K and mTOR ought be effective in eliminating cancer cells. A recent publication tested a low-dose triple drug combination that inhibits the pathways PI3K, Akt and mTOR in seven cell lines derived from ovarian clear cell carcinoma (OCCC). The use of the drugs AZD8055, GDC0941, and selumetinib decreased cell proliferation and significantly reduced tumor growth in two OCCC patient-derived xenograft mice models. The results and lack of adverse effects in the mice show that the combination of these three drugs could validate future clinical tests (217).

CC-223 is a competitive inhibitor of the mTOR that targets mTORC1 and mTORC2, preventing up regulation of Akt phosphorylation, a great advantage, if comparing to the rapalogs. In a phase I Dose-Escalation study, CC-22 was evaluated in twenty-eight patients with advanced cancer. Safety, tolerability, non-tolerated dosage, maximum tolerated dosage (MTD), and preliminary pharmacokinetic profile were evaluated; the reported adverse effects were hyperglycemia, rash, fatigue and mucositis, 45 mg/d was determined as the MTD and an inhibition of phosphorylation of mTORC1/mTORC2 pathway biomarkers present in blood was observed. Taken together these results suggest that CC-223 was tolerable, with manageable toxicities representing a promising antitumor activity compound (218).

Sapanisertib (TAK-228) is a potent and highly selective mTORC1/mTORC2 inhibitor that has been tested in non-hematological malignancies. In this study, sixty-one patients with advanced solid tumors were given daily or a weekly dose of TAK-228 alone or in combination with paclitaxcel. The results showed that just one patient that received TAK-228 plus paclitaxel showed a complete response, moreover, three patients that took TAK-228 plus paclitaxel and two patients with a daily dose of TAK-228 showed a partial response. Additionally, safety analyses showed that fatigue was the main adverse effect, followed bypruritus, lack of appetite and diarrhea, among others but any severe effect related to the treatment was reported. Contrary to everolimus and temsirolimus treatment, anemia and thrombocytopaenia were not reported as adverse effects by consuming TAK-228. Even though the authors emphasize a positive response to TAK-228 alone or in combination with paclitaxel, which could guarantee further investigations, it is only highlighted a positive response for some solid tumors (219).

Recently, specific mTORC1/mTORC2 inhibitors, torin2, INK-128, and NVP-Bez235 (which also inhibits PI2K), were tested on LNT-229 human glioblastoma cells. INK-228 and NVP-Bez235 inhibited the phosphorylation of mTOR targets S6RP and NDRG1, and together with torin2 showed a better capacity of inhibiting mTOR pathway when compared to rapamycin due to a more effective inhibition of 4EBP phosphorylation. The main contribution of this paper was that they highlight the metabolic effects of partial mTOR pathway inhibition by rapamycin and rapalogs to economize resources when cells are exposed to nutrient deficiency and hypoxic conditions, which could promote survival of tumor cells hence, highlighting the use of dual mTORC1/mTORC2 inhibition because such inhibitors are able to target dividing cells more efficiently (220).

Another mTORC1/mTORC2 inhibitor, CC-223, was evaluated in a phase II study including 47 patients with non-pancreatic neuroendocrine tumors. Tolerability, preliminary efficacy and pharmacokinetic of CC-223 was evaluated in a daily dose. The results were consistent with those presented in cell lines; anti-tumor activity was assessed, and the data obtained indicated that the drug was safe for patients (221). Additionally, other mTORC1/mTORC2 known as vistusertib was evaluated in a phase II study for patients with relapsed or refractory diffuse large B cell lymphoma, in this specific case, the dual inhibitor vistusertib did not show any advantage over mTORC1 inhibitors in the group of patients evaluated (222).

The combination of mTOR inhibitors with other drugs or treatments is thought to be more effective than just one treatment alone. Recently, the oral administration of PQR309, a dual PI3K and mTORC1/mTORC2 inhibitor, was evaluated in a phase I trial of patients with advanced solid tumors. The patients presented several adverse effects as fatigue, rash and loss of appetite and partial response was reported (223).

In sum, rapamicyn and rapalogs inhibit mTORC1 as demonstrated in several *in vitro* experiments (160), though incomplete mTOR signaling occurs due to these drugs incapacity of inhibit mTORC2 too, and in consequence, it has been suggested that cancer cells could survive because of Akt activation, for this reason and aiming to replicate the successful results observed in cell lines to patients, it is imperative to evaluate the synergic effect of mTOR inhibitors with other drugs or treatments that have shown promising results in patients and also lead the inhibition of mTOR signaling by drugs to perform a complete inhibition of mTORC1 and mTORC2 in order to guarantee clinical outcome.

CONCLUDING REMARKS

mTORC1 is widely described as an important regulator of cell growth, acting on the regulation of anabolic processes such as the synthesis of proteins, lipids, and the inhibition of autophagy. Importantly, mTORC1 is also involved in the regulation of mitochondrial metabolism and mitochondrial functions. In tumor exists a continuous two-way communication between mitochondria and the nucelus that orchestrates production of the mitochondrial encoded proteins and the nuclear-encoded mitochondria proteins to meet the cells continually changing energy and biosynthetic requirements. mTORC1 plays the major role in the regulation of the mitochondrial protein translation, moreover mTOR is an important regulator of mitochondrial turnover by regulating mitochondrial fusion and fission processes mainly deregulated in cancer and that are associated with chemotherapy resistance.

However, it is necessary to intensify research to clarify the participation of mTORC1 in the regulation of these mitochondrial functions and their impact on the aggressiveness of tumors. The fact that mitochondria promotes metabolic plasticity associated with resistance to therapy and the existence of several drug able regulators, proposes this events as promising therapeutic targets in cancer. In addition to the regulatory actions performed by mTOR in mitochondrial functions it represents an opportunity to deeply study for therapy, developing treatment plans with synergy, mainly using mTOR inhibitors, and mitochondrial inhibitors. In this manner, the use of metformin is an attractive therapeutic option with probed efficacy in clinical trials.

AUTHOR CONTRIBUTIONS

KC and AG: conception and design. KC, MT, ES, and AG: wrote and critically review the manuscript. KC, MT, and AG: figure design and elaboration. AG: directed manuscript.

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REFERENCES

1. Li H, Ning S, Ghandi M, Kryukov GV, Gopal S, Deik A, et al. The landscape of cancer cell line metabolism. *Nat Med*. (2019) 25:850–60. doi: 10.1038/s41591-019-0404-8
2. Warburg O. On the origin of cancer cells. *Science*. (1956) 123:309–14. doi: 10.1126/science.123.3191.309
3. Spinelli JB, Haigis MC. The multifaceted contributions of mitochondria to cellular metabolism. *Nat Cell Biol*. (2018) 20:745–54. doi: 10.1038/s41556-018-0124-1
4. Vyas S, Zaganjor E, Haigis MC. Mitochondria and cancer. *Cell*. (2016) 166:555–66. doi: 10.1016/j.cell.2016.07.002
5. Wang K, Jiang J, Lei Y, Zhou S, Wei Y, Huang C. Targeting metabolic-redox circuits for cancer therapy. *Trends Biochem Sci*. (2019) 44:401–14. doi: 10.1016/j.tibs.2019.01.001
6. Payen VL, Zampieri LX, Porporato PE, Sonveaux P. Pro- and antitumor effects of mitochondrial reactive oxygen species. *Cancer Metast Rev*. (2019) 38:189–203. doi: 10.1007/s10555-019-09789-2
7. Weinberg F, Ramnath N, Nagrath D. Reactive oxygen species in the tumor microenvironment: an overview. *Cancers*. (2019) 11:1191. doi: 10.3390/cancers11081191
8. Hensley CT, Faubert B, Yuan Q, Lev-Cohain N, Jin E, Kim J, et al. Metabolic heterogeneity in human lung tumors. *Cell*. (2016) 164:681–94. doi: 10.1016/j.cell.2015.12.034
9. National SP, President V, Secretary J, Members E, Secretary O, Secretary F. Mitochondria in health and disease. *Mitochondrion*. (2012) 2014:814042. doi: 10.1016/j.mito.2018.06.006
10. Ebrahimi-fakhari D, Saffari A, Wahlster L, Han M, Ebrahimi-fakhari D, Saffari A, et al. Impaired mitochondrial dynamics and mitophagy in neuronal models of tuberous sclerosis complex article impaired mitochondrial dynamics and mitophagy in neuronal models of tuberous sclerosis complex. *Cell Rep*. (2016) 17:1053–70. doi: 10.1016/j.celrep.2016.09.054
11. Friedman JR, Nunnari J. Mitochondrial form and function. *Nature*. (2014) 505:335–43. doi: 10.1038/nature12985
12. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell*. (2006) 124:471–84. doi: 10.1016/j.cell.2006.01.016
13. Lin S-C, Hardie DG. AMPK: sensing glucose as well as cellular energy status. *Cell Metab*. (2018) 27:299–313. doi: 10.1016/j.cmet.2017.10.009
14. Dodd KM, Yang J, Shen MH, Sampson JR, Tee AR. mTORC1 drives HIF-1α and VEGF-A signalling via multiple mechanisms involving 4E-BP1, S6K1 and STAT3. *Oncogene*. (2015) 34:2239–50. doi: 10.1038/onc.2014.164
15. Toschi A, Lee E, Gadir N, Ohh M, Foster DA. Differential dependence of hypoxia-inducible factors 1α and 2α on mTORC1. *J Biol Chem*. (2008) 283:34495–9. doi: 10.1074/jbc.C800170200
16. Hardwick JS, Kuruvilla FG, Tong JK, Shamji AF, Schreiber SL. Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins. *Proc Natl Acad Sci USA*. (1999) 96:14866–70. doi: 10.1073/pnas.96.26.14866
17. Arsham AM, Howell JJ, Simon MC. A novel hypoxia-inducible factor-independent hypoxic response regulating mammalian target of rapamycin and its targets. *J Biol Chem*. (2003) 278:29655–60. doi: 10.1074/jbc.M212770200
18. Sabatini DM, Erdjument-bromage H, Lui M, Tempst P, Snyder SH. RAFTI : a mammalian protein that binds to FKBP12 in a rapamycin-dependent

fashion and is homologous to yeast TORs. *Cell.* (1994) 78:35–43. doi: 10.1016/0092-8674(94)90570-3
19. Brown EJ, Albers MW, Shin TB, Ichikawa K, Keith CT, Lane WS, et al. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature*. (1994) 369:756–8.
20. Schreiber L. The PIK-related pathways Finny G Kuruvilla and Stuart intercept L Schreiber conventional signaling. *Chem Biol*. (1975) 6:R129–36.
21. Edinger AL, Linardic CM, Chiang GG, Thompson CB, Abraham RT. Differential effects of rapamycin on mammalian target of rapamycin signaling functions in mammalian cells. *Cancer Res.* (2003) 8451–60.
22. Sarbassov DD, Ali SM, Sengupta S, Sheen J, Hsu PP, Bagley AF, et al. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell.* (2006) 22:159–68. doi: 10.1016/j.molcel.2006.03.029
23. Kim D, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-bromage H, et al. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell.* (2002) 110:163–75. doi: 10.1016/S0092-8674(02)00808-5
24. Wang L, Harris TE, Roth RA, Lawrence JC. PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding*. *J Biol Chem.* (2007) 282:20036–44. doi: 10.1074/jbc.M702376200
25. Sarbassov DD. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science.* (2012) 307:1098–101. doi: 10.1126/science.1106148
26. Yang Q, Inoki K, Ikenoue T, Guan K. Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. *Genes Dev.* (2006) 20:2820–32. doi: 10.1101/gad.1461206
27. Ibrahim AFM, Gourlay R, Magnuson MA, Alessi DR. Identification of protor as a novel rictor-binding component of mTOR complex-2. *Biochem J.* (2007) 522:513–22. doi: 10.1042/BJ20070540
28. Kaizuka T, Hara T, Oshiro N, Kikkawa U, Yonezawa K, Takehana K, et al. Tti1 and Tel2 are critical factors in mammalian target of rapamycin complex assembly. *J Biol Chem*. (2010) 285:20109–16. doi: 10.1074/jbc.M110.121699
29. Chantranupong L, Wolfson RL, Orozco JM, Saxton RA, Scaria SM, Bar-Peled L, et al. The sestrins interact with gator2 to negatively regulate the amino-acid-sensing pathway upstream of mTORC1. *Cell Rep*. (2014) 9:1–8. doi: 10.1016/j.celrep.2014.09.014
30. Jung JW, Macalino SJY, Cui M, Kim S, Choi S, Jung JW, et al. Transmembrane 4 L six family member 5 senses arginine for mTORC1 signaling article transmembrane 4 L six family member 5 senses arginine for mTORC1 signaling. *Cell Metab*. (2019) 29:1306–19.e7. doi: 10.1016/j.cmet.2019.03.005
31. Chantranupong L, Scaria SM, Saxton RA, Gygi MP, Shen K, Wyant GA, et al. The CASTOR proteins are arginine sensors for the mTORC1 pathway. *Cell*. (2016) 165:153–64. doi: 10.1016/j.cell.2016.02.035
32. Sabatini DM. Twenty-five years of mTOR: uncovering the link from nutrients to growth. *Proc Natl Acad Sci USA*. (2017) 114:11818–25. doi: 10.1073/pnas.1716173114
33. Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. *Cell*. (2017) 169:361–71. doi: 10.1016/j.cell.2017.03.035
34. Jacinto E, Loewith R, Schmidt A, Lin S, Rüegg MA, Hall A, et al. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol.* (2004) 6:1122–8. doi: 10.1038/ncb1183
35. Krencz I, Sebestyen A, Papay J, Jeney A, Hujber Z, Burger CD, et al. *In situ* analysis of mTORC1/2 and cellular metabolism – related proteins in human. *Hum Pathol*. (2018) 79:199–207. doi: 10.1016/j.humpath.2018.05.018
36. Cotler SJ, Hay N, Xie H, Ling M, Pei C, Xu Z, et al. Immunohistochemical expression of components of the Akt- mTORC1 pathway is associated with hepatocellular carcinoma in patients with chronic liver disease. *Dig Dis Sci*. (2008) 53:844–9. doi: 10.1007/s10620-007-9934-x
37. Faried LS, Faried A, Kanuma T, Aoki H, Sano T, Nakazato T. Expression of an activated mammalian target of rapamycin in adenocarcinoma of the cervix : a potential biomarker and molecular target therapy. *Mol Carcinog.* (2008) 457:446–57. doi: 10.1002/mc.20402
38. Lee H. Phosphorylated mTOR expression profiles in human normal and carcinoma tissues. *Dis Markers*. (2017) 2017:1–8. doi: 10.1155/2017/1397063
39. Jiang W-J, Feng R-X, Liu J-T, Fan L-L, Wang H, Sun G-P. RICTOR expression in esophageal squamous cell carcinoma and its clinical significance. *Med Oncol*. (2017) 34:32. doi: 10.1007/s12032-017-0894-5
40. Mossmann D, Park S, Hall MN. mTOR signalling and cellular metabolism are mutual determinants in cancer. *Nat Rev Cancer*. (2018) 18:744–57. doi: 10.1038/s41568-018-0074-8
41. Pópulo H, Lopes JM, Soares P. The mTOR signalling pathway in human cancer. *Int J Mol Sci*. (2012) 13:1886–918. doi: 10.3390/ijms13021886
42. Dibble CC, Cantley LC. Regulation of mTORC1 by PI3K signaling. *Trends Cell Biol*. (2015) 25:545–55. doi: 10.1016/j.tcb.2015.06.002
43. Rathinaswamy MK, Burke JE. Class I phosphoinositide 3-kinase. (PI3K) regulatory subunits and their roles in signaling and disease. *Adv Biol Regul*. (2019) 2019:100657. doi: 10.1016/j.jbior.2019.100657
44. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, et al. Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J*. (1996) 15:6541–51. doi: 10.1002/j.1460-2075.1996.tb01045.x
45. James SR, Peter downes C, Gigg R, Grove SJA, Holmes AB, Alessi DR. Specific binding of the Akt-1 protein kinase to phosphatidylinositol 3,4,5-trisphosphate without subsequent activation. *Biochem J*. (1996) 315:709–13. doi: 10.1042/bj3150709
46. Ding Z, Liang J, Li J, Lu Y, Ariyaratna V, Lu Z, et al. Physical association of PDK1 with AKT1 is sufficient for pathway activation independent of membrane localization and phosphatidylinositol 3 kinase. *PLoS ONE*. (2010) 5:e9910. doi: 10.1371/journal.pone.0009910
47. Ikenoue T, Inoki K, Yang Q, Zhou X, Guan K. Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. *EMBO J.* (2008) 27:1919–31. doi: 10.1038/emboj.2008.119
48. Dibble CC, Elis W, Menon S, Qin W, Klekota J, Asara JM, et al. TBC1D7 is a third subunit of the TSC1-TSC2 complex upstream of mTORC1. *Mol Cell*. (2012) 47:535–46. doi: 10.1016/j.molcel.2012.06.009
49. Van Slegtenhorst M, Nellist M, Nagelkerken B, Cheadle J, Snell R, Van Den Ouweland A, et al. Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products. *Hum Mol Genet*. (1998) 7:1053–7. doi: 10.1093/hmg/7.6.1053
50. Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol*. (2002) 4:648–57. doi: 10.1038/ncb839
51. Manning BD, Tee AR, Logsdon MN, Blenis J, Cantley LC. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/Akt pathway. *Mol Cell*. (2002) 10:151–62. doi: 10.1016/S1097-2765(02)00568-3
52. Takahashi M, Nokihara K, Mihara H. Construction of a protein-detection system using a loop peptide library with a fluorescence label *Chem Biol*. (2003) 10:53–60. doi: 10.1016/S1074-5521(02)00308-3
53. Kim J, Guan K. mTOR as a central hub of nutrient signalling and cell growth. *Nat Cell Biol*. (2019) 21:63–71. doi: 10.1038/s41556-018-0205-1
54. Hidayat S, Yoshino K, Hara K, Tanaka N, Avruch J, Yonezawa K. Rapamycin. (mTOR) partner, substrates p70 S6 kinase and 4E-BP1 through their TOR signaling. (TOS) Motif *. *J Biol Chem.* (2003) 278:15461–4. doi: 10.1074/jbc.C200665200
55. Schalm SS, Fingar DC, Sabatini DM, Blenis J. TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function. *Curr Biol*. (2003) 13:797–806. doi: 10.1016/S0960-9822(03)00329-4
56. Gingras A, Gygi SP, Raught B, Polakiewicz RD, Abraham RT, Hoekstra MF, et al. Regulation of 4E-BP1 phosphorylation : a novel two-step mechanism. *Genes Dev.* (1999) 13:1422–37. doi: 10.1101/gad.13.11.1422
57. Haghighat A, Mader S, Pause A, Sonenberg N. Repression of cap-dependent translation by 4E-binding protein 1 : competition with p220 for binding to eukaryotic initiation factor-4E. *EMBO J.* (1995) 14:5701–9. doi: 10.1002/j.1460-2075.1995.tb00257.x
58. Erikson E, Maller JL. A protein kinase from Xenopus eggs specific for ribosomal protein S6. *Proc Natl Acad Sci USA*. (1985) 82:742–46. doi: 10.1073/pnas.82.3.742
59. Jeno P, Ballou LM, Novak-hofer I, Thomast G. Identification and characterization of a mitogen-activated S6 kinase. *Proc Natl Acad Sci USA*. (1988) 85:406–410. doi: 10.1073/pnas.85.2.406
60. Reinhard C, Thomas G, Kozma SC. A single gene encodes two isoforms of the p70 S6 kinase: activation upon mitogenic stimulation. (gene and mRNA analyses/immunoprecipitation/serum-stimulated 3T3 cells). *Proc Natl Acad Sci USA*. (1992). 89:4052–6. doi: 10.1073/pnas.89.9.4052

61. Reinhard C, Fernandez A, Lambl NJC, Thomas G. Nuclear localization of p85 entry into S phase functional requirement for. *EMBO J.* (1994) 13:1557–65. doi: 10.1002/j.1460-2075.1994.tb06418.x
62. Crispo F, Condelli V, Lepore S, Notarangelo T, Sgambato A, Esposito F, et al. Metabolic dysregulations and epigenetics: a bidirectional interplay that drives tumor progression. *Cells*. (2019) 8:798. doi: 10.3390/cells8080798
63. Min H-Y, Lee H-Y. Oncogene-driven metabolic alterations in cancer. *Biomol Ther*. (2018) 26:45–56. doi: 10.4062/biomolther.2017.211
64. Herst PM, Grasso C, Berridge MV. Metabolic reprogramming of mitochondrial respiration in metastatic cancer. *Cancer Metast Rev*. (2018) 37:643–53. doi: 10.1007/s10555-018-9769-2
65. Leibovitch M, Topisirovic I. Dysregulation of mRNA translation and energy metabolism in cancer. *Adv Biol Regul*. (2018) 67:30–9. doi: 10.1016/j.jbior.2017.11.001
66. Sazanov LA. A giant molecular proton pump: structure and mechanism of respiratory complex I. *Nat Rev Mol Cell Biol*. (2015) 16:375–88. doi: 10.1038/nrm3997
67. Lin CS, Liu LT, Ou LH, Pan SC, Lin CI, Wei YH. Role of mitochondrial function in the invasiveness of human colon cancer cells. *Oncol Rep*. (2018) 39:316–30. doi: 10.3892/or.2017.6087
68. van der Bliek AM, Sedensky MM, Morgan PG. Cell biology of the mitochondrion. *Genetics*. (2017) 207:843–71. doi: 10.1534/genetics.117.300262
69. Santidrian AF, Matsuno-yagi A, Ritland M, Seo BB, Leboeuf SE, Gay LJ, et al. Mitochondrial complex I activity and NAD + / NADH balance regulate breast cancer progression. *J Clin Invest*. (2013) 123:1068–81. doi: 10.1172/JCI64264
70. Whitaker-Menezes D, Martinez-Outschoorn UE, Flomenberg N, Birbe RC, Witkiewicz AK, Howell A, et al. Hyperactivation of oxidative mitochondrial metabolism in epithelial cancer cells in situ: visualizing the therapeutic effects of metformin in tumor tissue. *Cell Cycle*. (2011) 10:4047–64. doi: 10.4161/cc.10.23.18151
71. Ikeda K, Horie-Inoue K, Suzuki T, Hobo R, Nakasato N, Takeda S, et al. Mitochondrial supercomplex assembly promotes breast and endometrial tumorigenesis by metabolic alterations and enhanced hypoxia tolerance. *Nat Commun*. (2019) 10:4108. doi: 10.1038/s41467-019-12124-6
72. Lin S, Huang C, Gunda V, Sun J, Chellappan SP, Li Z, et al. Fascin controls metastatic colonization and mitochondrial oxidative phosphorylation by remodeling mitochondrial actin filaments. *Cell Rep*. (2019) 28:2824–36.e8. doi: 10.1016/j.celrep.2019.08.011
73. Pustylnikov S, Costabile F, Beghi S, Facciabene A. Targeting mitochondria in cancer: current concepts and immunotherapy approaches. *Transl Res*. (2018) 202:35–51. doi: 10.1016/j.trsl.2018.07.013
74. Saraei P, Asadi I, Kakar MA, Moradi-Kor N. The beneficial effects of metformin on cancer prevention and therapy: a comprehensive review of recent advances. *Cancer Manag Res*. (2019) 11:3295–13. doi: 10.2147/CMAR.S200059
75. Avéret N, Rigoulet M, Fontaine E, El-Mir M-Y, Nogueira V, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem*. (2002) 275:223–8. doi: 10.1074/jbc.275.1.223
76. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov*. (2017) 16:203–22. doi: 10.1038/nrd.2016.246
77. Fasoulakis Z, Daskalakis G, Michail D, Papapanagiotou I, Theodora M, Bourazan A, et al. MicroRNAs determining carcinogenesis by regulating oncogenes and tumor suppressor genes during cell cycle. *MicroRNA*. (2019) 8:1. doi: 10.2174/2211536608666190919161849
78. Zhao K, Wang L, Li T, Zhu M, Zhang C, Chen L, et al. The role of miR-451 in the switching between proliferation and migration in malignant glioma cells: AMPK signaling, mTOR modulation and Rac1 activation required. *Int J Oncol*. (2017) 50:1989–9. doi: 10.3892/ijo.2017.3973
79. Totary-Jain H, Sanoudou D, Ben-Dov IZ, Dautriche CN, Guarnieri P, Marx SO, et al. Reprogramming of the microRNA transcriptome mediates resistance to rapamycin. *J Biol Chem*. (2013) 288:6034–44. doi: 10.1074/jbc.M112.416446
80. Ye P, Liu Y, Chen C, Tang F, Wu Q, Wang X, et al. An mTORC1-Mdm2-Drosha axis for miRNA biogenesis in response to glucose- and amino acid-deprivation. *Mol Cell*. (2015) 57:708–20. doi: 10.1016/j.molcel.2014.12.034
81. Ogórek B, Lam HC, Khabibullin D, Liu H-J, Nijmeh J, Triboulet R, et al. TSC2 regulates microRNA biogenesis via mTORC1 and GSK3β. *Hum Mol Genet*. (2018) 27:1654–63. doi: 10.1093/hmg/ddy073
82. Geiger J, Dalgaard LT. Interplay of mitochondrial metabolism and microRNAs. *Cell Mol Life Sci*. (2017) 74:631–46. doi: 10.1007/s00018-016-2342-7
83. Liu H, Ma Y, Liu C, Li P, Yu T. Reduced miR-125a-5p level in non-small-cell lung cancer is associated with tumour progression. *Open Biol*. (2018) 8:180118. doi: 10.1098/rsob.180118
84. Ye H, Zhu W, Mei L, Lu Z. Prognostic and clinicopathologic significance of MicroRNA-125a-5p in cancers. *Medicine*. (2019) 98:e16685. doi: 10.1097/MD.0000000000016685
85. Zhang Y, Zhou Z, Wang L, Zhang P, Wang M, Cui C, et al. Clinicopathological significance of microRNA-21 and miR-125 expression in colorectal cancer. *Zhonghua Wei Chang Wai Ke Za Zhi*. (2009) 12:623–6. doi: 10.3760/cma.j.issn.1671-0274.2009.06.029
86. Li G, Ao S, Hou J, Lyu G. Low expression of miR-125a-5p is associated with poor prognosis in patients with gastric cancer. *Oncol Lett*. (2019) 18:1483–90. doi: 10.3892/ol.2019.10423
87. Wade SM, Ohnesorge N, McLoughlin H, Biniecka M, Carter SP, Trenkman M, et al. Dysregulated miR-125a promotes angiogenesis through enhanced glycolysis. *EBioMed*. (2019) 47:402–13. doi: 10.1016/j.ebiom.2019.08.043
88. Pan L, Zhou L, Yin W, Bai J, Liu R. miR-125a induces apoptosis, metabolism disorder and migrationimpairment in pancreatic cancer cells by targeting Mfn2-related mitochondrial fission. *Int J Oncol*. (2018) 53:124–36. doi: 10.3892/ijo.2018.4380
89. Sharma P, Bharat, Dogra N, Singh S. Small regulatory molecules acting big in cancer: potential role of mito-miRs in cancer. *Curr Mol Med*. (2019) 19:621–31. doi: 10.2174/1566524019666190723165357
90. Tomasetti M, Neuzil J, Dong L. MicroRNAs as regulators of mitochondrial function: role in cancer suppression. *Biochim Biophys Acta*. (2014) 1840:1441–53. doi: 10.1016/j.bbagen.2013.09.002
91. Fan S, Tian T, Chen W, Lv X, Lei X, Zhang H, et al. Mitochondrial miRNA determines chemoresistance by reprogramming metabolism and regulating mitochondrial transcription. *Cancer Res*. (2019) 79:1069–84. doi: 10.1158/0008-5472.CAN-18-2505
92. Topisirovic I, Sonenberg N. mRNA translation and energy metabolism in cancer: the role of the MAPK and mtorc1 Pathways. *Cold Spring Harb Symp Quant Biol*. (2011) 76:355–67. doi: 10.1101/sqb.2011.76.010785
93. Buttgereit F, Brandt MD. A hierarchy of ATP-consuming. *Control*. (1995) 167:163–7. doi: 10.1042/bj3120163
94. Bhat M, Robichaud N, Hulea L, Sonenberg N, Pelletier J, Topisirovic I. Targeting the translation machinery in cancer. *Nat Rev Drug Discov*. (2015) 14:261–78. doi: 10.1038/nrd4505
95. Mamane Y, Petroulakis E, LeBacquer O, Sonenberg N. mTOR, translation initiation and cancer. *Oncogene*. (2006) 25:6416–22. doi: 10.1038/sj.onc.1209888
96. Yan C, Duanmu X, Zeng L, Liu B, Song Z. Mitochondrial DNA: distribution, mutations, and elimination. *Cells*. (2019) 8:E379. doi: 10.3390/cells8040379
97. Priesnitz C, Becker T. Pathways to balance mitochondrial translation and protein import. *Genes Dev*. (2018) 32:1315–20. doi: 10.1101/gad.316547.118
98. Dowling RJO, Topisirovic I, Alain T, Bidinosti M, Fonseca BD, Petroulakis E, et al. mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs. *Science*. (2010) 328:1172–7. doi: 10.1126/science.1187532
99. Larsson O, Morita M, Topisirovic I, Alain T, Blouin M-J, Pollak M, et al. Distinct perturbation of the translatome by the antidiabetic drug metformin. *Proc Natl Acad Sci USA*. (2012) 109:8977–82. doi: 10.1073/pnas.1201689109
100. Morita M, Gravel S-P, Chénard V, Sikström K, Zheng L, Alain T, et al. mTORC1 controls mitochondrial activity and biogenesis through 4E-BP-dependent translational regulation. *Cell Metab*. (2013) 18:698–711. doi: 10.1016/j.cmet.2013.10.001
101. Gandin V, Masvidal L, Hulea L, Gravel S-P, Cargnello M, McLaughlan S, et al. nanoCAGE reveals 5′ UTR features that define specific modes of translation of functionally related MTOR-sensitive mRNAs. *Genome Res*. (2016) 26:636–48. doi: 10.1101/gr.197566.115

102. Desai BN, Myers BR, Schreiber SL. FKBP12-rapamycin-associated protein associates with mitochondria and senses osmotic stress via mitochondrial dysfunction. *Proc Natl Acad Sci USA*. (2002) 99:4319–24. doi: 10.1073/pnas.261702698
103. Schieke SM, Phillips D, Philip J, Aponte AM, Balaban RS, Finkel T. The mammalian target of rapamycin. (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity *. *J Biol Chem*. (2006) 281:27643–52. doi: 10.1074/jbc.M603536200
104. Blachly-Dyson E, Zambronicz EB, Yu WH, Adams V, McCabe ER, Adelman J, et al. Cloning and functional expression in yeast of two human isoforms of the outer mitochondrial membrane channel, the voltage-dependent anion channel. *J Biol Chem*. (1993) 268:1835–41.
105. Arif T, Paul A, Krelin Y, Shteinfer-Kuzmine A, Shoshan-Barmatz V. Mitochondrial VDAC1 silencing leads to metabolic rewiring and the reprogramming of tumour cells into advanced differentiated states. *Cancers*. (2018) 10:499. doi: 10.3390/cancers10120499
106. Ramanathan A, Schreiber SL. Direct control of mitochondrial function by mTOR. *Proc Natl Acad Sci USA*. (2009) 106:22229–32. doi: 10.1073/pnas.0912074106
107. Lu CL, Qin L, Liu HC, Candas D, Fan M, Li JJ. Tumor cells switch to mitochondrial oxidative phosphorylation under radiation via mTOR-mediated hexokinase II inhibition - A Warburg-reversing effect. *PLoS ONE*. (2015) 10:e0121046. doi: 10.1371/journal.pone.0121046
108. Roberts DJ, Tan-Sah VP, Ding EY, Smith JM, Miyamoto S. Hexokinase-II positively regulates glucose starvation-induced autophagy through TORC1 inhibition. *Mol Cell*. (2014) 53:521–33. doi: 10.1016/j.molcel.2013.12.019
109. Goo CK, Lim HY, Ho QS, Too H, Clement M. PTEN/Akt signaling controls mitochondrial respiratory capacity through 4E-BP1. (2012) *PLoS ONE*.7:e45806. doi: 10.1371/journal.pone.0045806
110. Schieke SM, McCoy JP, Finkel T. Coordination of mitochondrial bioenergetics with G_1 phase cell cycle progression. *Cell Cycle*. (2008) 7:1782–87. doi: 10.4161/cc.7.12.6067
111. Zhang W, Pelicano H, Hammoudi N, Liu J, Xu R, Huang P, et al. Mitochondrial dysfunction in some triple-negative breast cancer cell lines: role of mTOR pathway and therapeutic potential. *Breast Cancer Res*. (2014) 16:1–16. doi: 10.1186/s13058-014-0434-6
112. Kim JE, He Q, Chen Y, Shi C, Yu K. MTOR-targeted therapy: differential perturbation to mitochondrial membrane potential and permeability transition pore plays a role in therapeutic response. *Biochem Biophys Res Commun*. (2014) 447:184–91. doi: 10.1016/j.bbrc.2014.03.124
113. Wai T, Langer T. Mitochondrial dynamics and metabolic regulation. *Trends Endocrinol Metab*. (2016) 27:105–17. doi: 10.1016/j.tem.2015.12.001
114. Trotta AP, Chipuk JE. Mitochondrial dynamics as regulators of cancer biology. *Cell Mol Life Sci*. (2017) 74:1999–2017. doi: 10.1007/s00018-016-2451-3
115. Santel A, Frank S, Gaume B, Herrler M, Youle RJ, Fuller MT. Mitofusin-1 protein is a generally expressed mediator of mitochondrial fusion in mammalian cells. *J Cell Sci*. (2003) 116:2763–74. doi: 10.1242/jcs.00479
116. Eura Y, Ishihara N, Yokota S, Mihara K. Two mitofusin proteins, mammalian homologues of FZO, with distinct functions are both required for mitochondrial fusion. *J Biochem*. (2003) 134:333–44. doi: 10.1093/jb/mvg150
117. Santel A, Fuller MT. Control of mitochondrial morphology by a human mitofusin. *J Cell Sci*. (2001) 114:867–74.
118. Delettre C, Lenaers G, Griffoin JM, Gigarel N, Lorenzo C, Belenguer P, et al. Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. *Nat Genet*. (2000) 26:207–10. doi: 10.1038/79936
119. Kamerkar SC, Kraus F, Sharpe AJ, Pucadyil TJ, Ryan MT. Dynamin-related protein 1 has membrane constricting and severing abilities sufficient for mitochondrial and peroxisomal fission. *Nat Commun*. (2018) 9:5239. doi: 10.1038/s41467-018-07543-w
120. Osellame LD, Singh AP, Stroud DA, Palmer CS, Stojanovski D, Ramachandran R, et al. Cooperative and independent roles of the Drp1 adaptors Mff, MiD49 and MiD51 in mitochondrial fission. *J Cell Sci*. (2016) 129:2170–81. doi: 10.1242/jcs.185165
121. Onoue K, Jofuku A, Ban-Ishihara R, Ishihara T, Maeda M, Koshiba T, et al. Fis1 acts as a mitochondrial recruitment factor for TBC1D15 that is involved in regulation of mitochondrial morphology. *J Cell Sci*. (2013) 126:176–85. doi: 10.1242/jcs.111211
122. Otera H, Wang C, Cleland MM, Setoguchi K, Yokota S, Youle RJ, et al. Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. *J Cell Biol*. (2010) 191:1141–58. doi: 10.1083/jcb.201007152
123. Palmer CS, Osellame LD, Laine D, Koutsopoulos OS, Frazier AE, Ryan MT. MiD49 and MiD51, new components of the mitochondrial fission machinery. *EMBO Rep*. (2011) 12:565–73. doi: 10.1038/embor.2011.54
124. Anderson GR, Wardell SE, Cakir M, Yip C, Ahn YR, Ali M, et al. Dysregulation of mitochondrial dynamics proteins are a targetable feature of human tumors. *Nat Commun*. (2018) 9:1677. doi: 10.1038/s41467-018-04033-x
125. Sun X, Cao H, Zhan L, Yin C, Wang G, Liang P, et al. Mitochondrial fission promotes cell migration by Ca2+ /CaMKII/ERK/FAK pathway in hepatocellular carcinoma. *Liver Int*. (2018) 38:1263–72. doi: 10.1111/liv.13660
126. Tondera D, Czauderna F, Paulick K, Schwarzer R, Kaufmann J, Santel A. The mitochondrial protein MTP18 contributes to mitochondrial fission in mammalian cells. *J Sci*. (2005) 118:3049–59. doi: 10.1242/jcs.02415
127. Morita M, Prudent J, Basu K, Goyon V, Katsumura S, Hulea L, et al. mTOR controls mitochondrial dynamics and cell survival via MTFP1. *Mol Cell*. (2017) 67:922–35.e5. doi: 10.1016/j.molcel.2017.08.013
128. Gomes LC, Di BG, Scorrano L. During autophagy mitochondria elongate, are spared from degradation and sustain cell viability. *Nat Cell Biol*. (2011) 13:589–98. doi: 10.1038/ncb2220
129. Rambold AS, Kostelecky B, Elia N, Lippincott-Schwartz J. Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. *Proc Natl Acad Sci USA*. (2011) 108:10190–5. doi: 10.1073/pnas.1107402108
130. Tran Q, Jung JH, Park J, Lee H, Hong Y, Cho H, et al. S6 Kinase 1 Plays a Key Role in Mitochondrial Morphology and Cellular Energy Flow. *Cell. Signal*. (2018) 48:13-24. doi: 10.1016/j.cellsig.2018.04.002
131. Ferro F, Servais S, Besson P, Roger S, Dumas J-F, Brisson L. Autophagy and mitophagy in cancer metabolic remodelling. *Semin Cell Dev Biol*. (2019). doi: 10.1016/j.semcdb.2019.05.029. [Epub ahead of print].
132. Puigserver P, Spiegelman BM. Peroxisome proliferator-activated receptor-γ coactivator 1α. (PGC-1α): transcriptional coactivator and metabolic regulator. *Endocr Rev*. (2003) 24:78–90. doi: 10.1210/er.2002-0012
133. Lebleu VS, O'Connell JT, Gonzalez Herrera KN, Wikman H, Pantel K, Haigis MC, et al. PGC-1α mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis. *Nat Cell Biol*. (2014) 16:992–1003. doi: 10.1038/ncb3039
134. Yun CW, Han YS, Lee SH. PGC-1α controls mitochondrial biogenesis in drug-resistant colorectal cancer cells by regulating endoplasmic reticulum stress. *Int J Mol Sci*. (2019) 20:1–14. doi: 10.3390/ijms20071707
135. Scarpulla RC. Nuclear activators and coactivators in mammalian mitochondrial biogenesis. *Biochim Biophys Acta*. (2002) 1576:1–14. doi: 10.1016/S0167-4781(02)00343-3
136. Wu S, Kasim V, Kano MR, Tanaka S, Ohba S, Miura Y, et al. Transcription factor YY1 contributes to tumor growth by stabilizing hypoxia factor HIF-1 in a p53-independent manner. *Cancer Res*. (2013) 73:1787–99. doi: 10.1158/0008-5472.CAN-12-0366
137. Shen X, Zhong J, Yu P, Zhao Q, Huang T. YY1-regulated LINC00152 promotes triple negative breast cancer progression by affecting on stability of PTEN protein. *Biochem Biophys Res Commun*. (2019) 509:448–54. doi: 10.1016/j.bbrc.2018.12.074
138. Ye Y, Gu B, Wang Y, Shen S, Huang W. YY1-induced upregulation of long noncoding RNA ARAP1-AS1 promotes cell migration and invasion in colorectal cancer through the Wnt/β-catenin signaling pathway. *Cancer Biother Radiopharm*. (2019) 34:519–28. doi: 10.1089/cbr.2018.2745
139. Khachigian L, Hulett MD, Li J-M, Santiago FS, Parish CR, Khachigian LM. Yin Yang-1 inhibits tumor cell growth and inhibits p21WAF1/Cip1 complex formation with cdk4 and cyclin D1. *Int J Oncol*. (2012) 40:1575–80. doi: 10.3892/ijo.2012.1362
140. Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK. mTOR controls mitochondrial oxidative function through a YY1

– PGC-1 a transcriptional complex. *Nature*. (2007) 450:736–40. doi: 10.1038/nature06322
141. Blattler SM, Verdeguer F, Liesa M, Cunningham JT, Vogel RO, Chim H, et al. Defective mitochondrial morphology and bioenergetic function in mice lacking the transcription factor yin yang 1 in skeletal muscle. *Mol Cell Biol*. (2012) 32:3333–46. doi: 10.1128/MCB.00337-12
142. Lin J, Puigserver P, Donovan J, Tarr P, Spiegelman BM. Peroxisome proliferator-activated receptor γ coactivator 1β. (PGC-1β), a novel PGC-1-related transcription coactivator associated with host cell factor. *J Biol Chem*. (2002) 277:1645–8. doi: 10.1074/jbc.C100631200
143. Wang L, Liu Q, Li F, Qiu J, Fan H, Ma H, et al. Apoptosis induced by PGC-1β in breast cancer cells is mediated by the mTOR pathway. *Oncol Rep*. (2013) 30:1631–8. doi: 10.3892/or.2013.2628
144. Whang YM, Kim MJ, Cho MJ, Yoon H, Choi YW, Kim TH, et al. Rapamycin enhances growth inhibition on urothelial carcinoma cells through LKB1 deficiency-mediated mitochondrial dysregulation. *J Cell Physiol*. (2019) 234:13083–96. doi: 10.1002/jcp.27979
145. Jager S, Handschin C, St.-Pierre J, Spiegelman BM. AMP-activated protein kinase. (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1. *Proc Natl Acad Sci USA*. (2007) 104:12017–22. doi: 10.1073/pnas.0705070104
146. Gilkerson RW, De vries RLA, Lebot P, Wikstrom JD, Torgyekes E, Shirihai OS, et al. Mitochondrial autophagy in cells with mtDNA mutations results from synergistic loss of transmembrane potential and mTORC1 inhibition. *Hum Mol Genet*. (2012) 21:978–90. doi: 10.1093/hmg/ddr529
147. Windmueller HG, Spaeth AE. Uptake and metabolism of plasma glutamine by the small intestine. *J Biol Chem*. (1974) 249:5070–9.
148. Yang L, Venneti S, Nagrath D. Glutaminolysis: a hallmark of cancer metabolism. *Annu Rev Biomed Eng*. (2017) 19:163–94. doi: 10.1146/annurev-bioeng-071516-044546
149. Matés JM, Segura JA, Campos-Sandoval JA, Lobo C, Alonso L, Alonso FJ, et al. Glutamine homeostasis and mitochondrial dynamics. *Int J Biochem Cell Biol*. (2009) 41:2051–61. doi: 10.1016/j.biocel.2009.03.003
150. Metallo CM, Gameiro PA, Bell EL, Mattaini KR, Yang J, Hiller K, et al. Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. *Nature*. (2012) 481:380–4. doi: 10.1038/nature10602
151. Altman BJ, Stine ZE, Dang CV. From krebs to clinic: glutamine metabolism to cancer therapy. *Nat Rev Cancer*. (2016) 16:619–34. doi: 10.1038/nrc.2016.71
152. Matés JM, Di Paola FJ, Campos-Sandoval JA, Mazurek S, Márquez J. Therapeutic targeting of glutaminolysis as an essential strategy to combat cancer. *Semin Cell Dev Biol*. (2019). doi: 10.1016/j.semcdb.2019.05.012. [Epub ahead of print].
153. Bhutia YD, Ganapathy V. Glutamine transporters in mammalian cells and their functions in physiology and cancer. *Biochim Biophys Acta*. (2016) 1863:2531–9. doi: 10.1016/j.bbamcr.2015.12.017
154. Villar VH, Merhi F, Djavaheri-Mergny M, Durán R V. Glutaminolysis and autophagy in cancer. *Autophagy*. (2015) 11:1198–208. doi: 10.1080/15548627.2015.1053680
155. DeBerardinis RJ, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, Thompson CB. Beyond aerobic glycolysis: Transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc Natl Acad Sci USA*. (2007) 104:19345–50. doi: 10.1073/pnas.0709747104
156. Durán RV, Hall MN. Glutaminolysis feeds mTORC1. *Cell Cycle*. (2012) 11:4107–8. doi: 10.4161/cc.22632
157. Gaglio D, Soldati C, Vanoni M, Alberghina L, Chiaradonna F. Glutamine deprivation induces abortive S-phase rescued by deoxyribonucleotides in K-ras transformed fibroblasts. *PLoS One*. (2009) 4:e4715. doi: 10.1371/journal.pone.0004715
158. Reitzer LJ, Wice BM, Kennell D. Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells. *J Biol Chem*. (1979) 254:2669–76.
159. Dornier E, Rabas N, Mitchell L, Novo D, Dhayade S, Marco S, et al. Glutaminolysis drives membrane trafficking to promote invasiveness of breast cancer cells. *Nat Commun*. (2017) 8:1–14. doi: 10.1038/s41467-017-02101-2
160. Zhang Y, Jia Q, Kadel D, Zhang X, Zhang Q. Targeting mTORC1/2 complexes inhibit tumorigenesis and enhance sensitivity to 5-flourouracil. (5-FU) in hepatocellular carcinoma: a preclinical study of mTORC1/2-targeted therapy in hepatocellular carcinoma. (HCC). *Med Sci Monit*. (2018) 24:2735–43. doi: 10.12659/MSM.907514
161. Nicklin P, Bergman P, Zhang B, Triantafellow E, Wang H, Nyfeler B, et al. Bidirectional transport of amino acids regulates mTOR and autophagy. *Cell*. (2009) 136:521–34. doi: 10.1016/j.cell.2008.11.044
162. Durán RV, Mackenzie ED, Boulahbel H, Frezza C, Heiserich L, Tardito S, et al. HIF-independent role of prolyl hydroxylases in the cellular response to amino acids. *Oncogene*. (2013) 32:4549–56. doi: 10.1038/onc.2012.465
163. Jung J, Genau HM, Behrends C. Amino acid-dependent mTORC1 regulation by the lysosomal membrane protein SLC38A9. *Mol Cell Biol*. (2015) 35:2479–94. doi: 10.1128/MCB.00125-15
164. Rebsamen M, Kandasamy RK, Snijder B, Fauster A, Rudashevskaya EL, Bruckner M, et al. SLC38A9 is a component of the lysosomal amino acid sensing machinery that controls mTORC1. *Nature*. (2015) 519:477–81. doi: 10.1038/nature14107
165. Wang S, Tsun Z-Y, Wolfson R, Shen K, Wyant GA, Plovanich ME, et al. The amino acid transporter SLC38A9 is a key component of a lysosomal membrane complex that signals arginine sufficiency to mTORC1. *Science*. (2015) 347:188–94. doi: 10.1126/science.1257132
166. Jewell JL, Kim YC, Russell RC, Yu F-X, Park HW, Plouffe SW, et al. Metabolism. Differential regulation of mTORC1 by leucine and glutamine. *Science*. (2015) 347:194–8. doi: 10.1126/science.1259472
167. Csibi A, Fendt SM, Li C, Poulogiannis G, Choo AY, Chapski DJ, et al. The mTORC1 pathway stimulates glutamine metabolism and cell proliferation by repressing SIRT4. *Cell*. (2013) 153:840–54. doi: 10.1016/j.cell.2013.04.023
168. Choo AY, Kim SG, Heiden MG Vander, Sarah J, Vu H, Yoon S, et al. Dependent balancing of metabolic demand with supply. *Mol Cell*. (2011) 38:487–99. doi: 10.1016/j.molcel.2010.05.007
169. Ye X, Zhou Q, Matsumoto Y, Moriyama M, Kageyama S, Komatsu M, et al. Inhibition of glutaminolysis inhibits cell growth via down-regulating mtorc1 signaling in lung squamous cell carcinoma. *Anticancer Res*. (2016) 36:6021–30. doi: 10.21873/anticanres.11191
170. Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen H-Y, et al. Autophagy suppresses tumorigenesis through elimination of p62. *Cell*. (2009) 137:1062–75. doi: 10.1016/j.cell.2009.03.048
171. Guo JY, Chen HY, Mathew R, Fan J, Strohecker AM, Karsli-Uzunbas G, et al. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. *Genes Dev*. (2011) 25:460–70. doi: 10.1101/gad.2016311
172. Weichhart T. mTOR as regulator of lifespan, aging, and cellular senescence: a mini-review. *Gerontology*. (2018) 64:127–34. doi: 10.1159/000484629
173. Papadopoli D, Boulay K, Kazak L, Pollak M, Mallette F, Topisirovic I, et al. mTOR as a central regulator of lifespan and aging. *F1000Research*. (2019) 8:998. doi: 10.12688/f1000research.17196.1
174. Shen C, Houghton PJ. The mTOR pathway negatively controls ATM by up-regulating miRNAs. *Proc Natl Acad Sci USA*. (2013) 110:11869–74. doi: 10.1073/pnas.1220898110
175. Durán RV, Oppliger W, Robitaille AM, Heiserich L, Skendaj R, Gottlieb E, et al. Glutaminolysis activates Rag-mTORC1 signaling. *Mol Cell*. (2012) 47:349–58. doi: 10.1016/j.molcel.2012.05.043
176. Villar VH, Nguyen TL, Delcroix V, Terés S, Bouchecareilh M, Salin B, et al. MTORC1 inhibition in cancer cells protects from glutaminolysis-mediated apoptosis during nutrient limitation. *Nat Commun*. (2017) 8:14124. doi: 10.1038/ncomms14124
177. Collins RRJ, Patel K, Putnam WC, Kapur P, Rakheja D. Oncometabolites: a new paradigm for oncology, metabolism, and the clinical laboratory. *Clin Chem*. (2017) 63:1812–20. doi: 10.1373/clinchem.2016.267666
178. Zhou Z, Ibekwe E, Chornenkyy Y. Metabolic alterations in cancer cells and the emerging role of oncometabolites as drivers of neoplastic change. *Antioxidants*. (2018) 7:16. doi: 10.3390/antiox7010016
179. Chin RM, Fu X, Pai MY, Vergnes L, Hwang H, Deng G, et al. The metabolite α-ketoglutarate extends lifespan by inhibiting ATP synthase and TOR. *Nature*. (2014) 510:397–401. doi: 10.1038/nature13264
180. Kaminska B, Czapski B, Guzik R, Król S, Gielniewski B. Consequences of IDH1/2 mutations in gliomas and an assessment of inhibitors

targeting mutated IDH proteins. *Molecules*. (2019) 24:968. doi: 10.3390/molecules24050968
181. Lin P, Luo Y, Zhu S, Maggio D, Yang H, Hu C, et al. Isocitrate dehydrogenase 2 mutations correlate with leukemic transformation and are predicted by 2-hydroxyglutarate in myelodysplastic syndromes. *J Cancer Res Clin Oncol*. (2018) 144:1037–47. doi: 10.1007/s00432-018-2627-3
182. Miyake K, Baba Y, Ishimoto T, Hiyoshi Y, Iwatsuki M, Miyamoto Y, et al. Isocitrate dehydrogenase gene mutations and 2-hydroxyglutarate accumulation in esophageal squamous cell carcinoma. *Med Oncol*. (2019) 36:11. doi: 10.1007/s12032-018-1229-x
183. Ward PS, Patel J, Wise DR, Abdel-Wahab O, Bennett BD, Coller HA, et al. The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting α-ketoglutarate to 2-hydroxyglutarate. *Cancer Cell*. (2010) 17:225–34. doi: 10.1016/j.ccr.2010.01.020
184. Sim H-W, Nejad R, Zhang W, Nassiri F, Mason W, Aldape KD, et al. Tissue 2-hydroxyglutarate as a biomarker for *Isocitrate Dehydrogenase* mutations in gliomas. *Clin Cancer Res*. (2019) 25:3366–73. doi: 10.1158/1078-0432.CCR-18-3205
185. Seok J, Yoon S, Lee S, Jung JH, Lee YM. The oncometabolite d-2-hydroxyglutarate induces angiogenic activity through the vascular endothelial growth factor receptor 2 signaling pathway. *Int J Oncol*. (2018) 54:753–63. doi: 10.3892/ijo.2018.4649
186. Fu X, Chin RM, Vergnes L, Hwang H, Deng G, Xing Y, et al. 2-hydroxyglutarate inhibits ATP synthase and mTOR Signaling. *Cell Metab*. (2015) 22:508–15. doi: 10.1016/j.cmet.2015.06.009
187. Carbonneau M, Gagne LM, Lalonde ME, Germain MA, Motorina A, Guiot MC, et al. The oncometabolite 2-hydroxyglutarate activates the mTOR signalling pathway. *Nat Commun*. (2016) 7:12700. doi: 10.1038/ncomms12700
188. Hujber Z, Petovári G, Szoboszlai N, Dankó T, Nagy N, Kriston C, et al. Rapamycin. (mTORC1 inhibitor) reduces the production of lactate and 2-hydroxyglutarate oncometabolites in IDH1 mutant fibrosarcoma cells. *J Exp Clin Cancer Res*. (2017) 36:1–12. doi: 10.1186/s13046-017-0544-y
189. Batsios G, Viswanath P, Gillespie AM, Subramani E, Phillips JJ, Pieper RO, et al. PI3K/mTOR inhibition of IDH1 mutant glioma leads to reduced 2HG production that is associated with increased survival. *Proc Jt Annu Meet ISMRM-ESMRMB*. (2018) 9:10521. doi: 10.1038/s41598-019-47021-x
190. Vissers MCM, Kuiper C, Dachs GU. Regulation of the 2-oxoglutarate-dependent dioxygenases and implications for cancer. *Biochem Soc Trans*. (2014) 42:945–51. doi: 10.1042/BST20140118
191. Isaacs JS, Jung YJ, Mole DR, Lee S, Torres-Cabala C, Chung Y-L, et al. HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: novel role of fumarate in regulation of HIF stability. *Cancer Cell*. (2005) 8:143–53. doi: 10.1016/j.ccr.2005.06.017
192. Pezzuto A, Carico E. Role of HIF-1 in cancer progression: novel insights. a review. *Curr Mol Med*. (2019) 18:343–51. doi: 10.2174/1566524018666181109121849
193. Singh D, Arora R, Kaur P, Singh B, Mannan R, Arora S. Overexpression of hypoxia-inducible factor and metabolic pathways: possible targets of cancer. *Cell Biosci*. (2017) 7:62. doi: 10.1186/s13578-017-0190-2
194. Shanmugasundaram K, Nayak B, Shim EH, Livi CB, Block K, Sudarshan S. The oncometabolite fumarate promotes pseudohypoxia through noncanonical activation of NF-κB signaling. *J Biol Chem*. (2014) 289:24691–9. doi: 10.1074/jbc.M114.568162
195. Wentzel JF, Lewies A, Bronkhorst AJ, van Dyk E, du Plessis LH, Pretorius PJ. Exposure to high levels of fumarate and succinate leads to apoptotic cytotoxicity and altered global DNA methylation profiles *in vitro*. *Biochimie*. (2017) 135:28–34. doi: 10.1016/j.biochi.2017.01.004
196. Drusian L, Nigro EA, Mannella V, Pagliarini R, Pema M, Costa ASH, et al. mTORC1 upregulation leads to accumulation of the oncometabolite fumarate in a mouse model of renal cell carcinoma. *Cell Rep*. (2018) 24:1093–04.e6. doi: 10.1016/j.celrep.2018.06.106
197. Husseinzadeh N, Husseinzadeh HD. mTOR inhibitors and their clinical application in cervical, endometrial and ovarian cancers: a critical review. *Gynecol Oncol*. (2014) 133:375–81. doi: 10.1016/j.ygyno.2014.02.017
198. Faivre S, Kroemer G, Raymond E. Current development of mTOR inhibitors as anticancer agents. *Nat Rev Drug Discov*. (2006) 5:671–88. doi: 10.1038/nrd2062
199. Benjamin D, Colombi M, Moroni C, Hall MN. Rapamycin passes the torch: a new generation of mTOR inhibitors. *Nat Rev Drug Discov*. (2011) 10:868–80. doi: 10.1038/nrd3531
200. Dantal J, Morelon E, Rostaing L, Goffin E, Brocard A, Tromme I, et al. Sirolimus for secondary prevention of skin cancer in kidney transplant recipients: 5-year results. *J Clin Oncol*. (2018) 36:2612–20. doi: 10.1200/JCO.2017.76.6691
201. Beuvink I, Boulay A, Fumagalli S, Zilbermann F, Ruetz S, Reilly TO, et al. The mTOR inhibitor RAD001 sensitizes tumor cells to DNA-damaged induced apoptosis through inhibition of p21 translation. (2005) 120:747–59. doi: 10.1016/j.cell.2004.12.040
202. Oh K, Kalinina A, Park N, Bagchi S. Deregulation of eIF4E : 4E-BP1 in differentiated human papillomavirus-containing cells leads to high levels of expression of the e7 oncoprotein. *J Virol*. (2006) 80:7079–88. doi: 10.1128/JVI.02380-05
203. Shaughnessy JO, Beck JT, Royce M. Everolimus-based combination therapies for HR +, HER2 – metastatic breast cancer. *Cancer Treat Rev*. (2018) 69:204–14. doi: 10.1016/j.ctrv.2018.07.013
204. Safra T, Kaufman B, Kadouri L, Ben-baruch NE, Ryvo L, Nisenbaum B, et al. Everolimus plus letrozole for treatment of patients with HR+, HER2– advanced breast cancer progressing on endocrine therapy: an open-label, phase 2 trial. *Clin Breast Cancer*. (2017) 18:e197–203. doi: 10.1016/j.clbc.2017.09.004
205. Matsubara N, Naito Y, Nakano K, Fujiwara Y, Ikezawa H, Yusa W, et al. Lenvatinib in combination with everolimus in patients with advanced or metastatic renal cell carcinoma: a phase 1 study. *Int J Urol*. (2018) 25:922–8. doi: 10.1111/iju.13776
206. Huijts CM, Werter IM, Lougheed SM, Goedegebuure RS, Herpen CM Van, Hamberg P, et al. Phase 1 study of everolimus and low-dose oral cyclophosphamide in patients with metastatic renal cell carcinoma. *Cancer Immunol Immunother*. (2018) 68:319–29. doi: 10.1007/s00262-018-2248-3
207. de Wit D, Schneider TC, Moes DJ, Roozen CF, den Hartigh J, Gelderblom H, et al. Everolimus pharmacokinetics and its exposure–toxicity relationship in patients with thyroid cancer. *Cancer Chemother Pharmacol*. (2016) 78:63–71. doi: 10.1007/s00280-016-3050-6
208. Zhou H, Luo Y, Huang S. Updates of mTOR inhibitors. *Anticancer Agents Med Chem*. (2012) 10:571–81. doi: 10.2174/187152010793498663
209. Assad DX, Borges GA, Avelino SR, Guerra ENS. Additive cytotoxic effects of radiation and mTOR inhibitors in a cervical cancer cell line. *Pathol - Res Pract*. (2018) 214:259–62. doi: 10.1016/j.prp.2017.10.019
210. Dong Y, Eskandari R, Ray C, Granlund KL, Santos-Cunha L Dos, Miloushev VZ, et al. Hyperpolarized MRI visualizes warburg effects and predicts treatment response to mTOR inhibitors in patient-derived CCRCC xenograft models. *Cancer Res*. (2019) 79:242–50. doi: 10.1158/0008-5472.CAN-18-2231
211. Chinnaiyan P, Won M, Wen PY, Rojiani AM, Werner-Wasik M, Shih HA, et al. A randomized phase II study of everolimus in combination with chemoradiation in newly diagnosed glioblastoma: results of NRG Oncology RTOG 0913. *Neuro Oncol*. (2018) 20:666–73. doi: 10.1093/neuonc/nox209
212. Dan VM, Muralikrishnan B, Sanawar R, Vinodh JS, Burkul B, Srinivas KP, et al. Streptomyces sp metabolite. (s) promotes Bax mediated intrinsic apoptosis and autophagy involving inhibition of mTOR pathway in cervical cancer cell lines. *Sci Rep*. (2018) 8:2810. doi: 10.1038/s41598-018-21249-5
213. Tinker A V, Ellard S, Welch S, Moens F, Allo G, Tsao MS, et al. Gynecologic Oncology Phase II study of temsirolimus. (CCI-779) in women with recurrent, unresectable, locally advanced or metastatic carcinoma of the cervix. A trial of the NCIC Clinical Trials Group. (NCIC CTG IND 199). *Gynecol Oncol*. (2013) 130:269–74. doi: 10.1016/j.ygyno.2013.05.008
214. Ariaans G, Jalving M, de Vries EGE, de Jong S. Anti-tumor effects of everolimus and metformin are complementary and glucose-dependent in breast cancer cells. *BMC Cancer*. (2017) 17:1–13. doi: 10.1186/s12885-017-3230-8
215. Zhang JW, Zhao F, Sun Q. Metformin synergizes with rapamycin to inhibit the growth of pancreatic cancer in vitro and *in vivo*. *Oncol Lett*. (2018) 15:1811–6. doi: 10.3892/ol.2017.7444

216. Alzahrani AS. PI3K/Akt/mTOR inhibitors in cancer: at the bench and bedside. *Semin Cancer Biol*. (2019). doi: 10.1016/j.semcancer.2019.07.009. [Epub ahead of print].
217. Caumanns JJ, van Wijngaarden A, Kol A, Meersma GJ, Jalving M, Bernards R, et al. Low-dose triple drug combination targeting the PI3K/AKT/mTOR pathway and the MAPK pathway is an effective approach in ovarian clear cell carcinoma. *Cancer Lett*. (2019) 461:102–11. doi: 10.1016/j.canlet.2019.07.004
218. Bendell JC, Kelley RK, Shih KC, Grabowsky JA, Bergsland E, Jones S, et al. A phase i dose-escalation study to assess safety, tolerability, pharmacokinetics, and preliminary efficacy of the dual mTORC1/mTORC2 kinase inhibitor CC-223 in patients with advanced solid tumors or multiple myeloma. *Cancer*. (2015) 121:3481–90. doi: 10.1002/cncr.29422
219. Moore KN, Bauer TM, Falchook GS, Chowdhury S, Patel C, Neuwirth R, et al. Phase i study of the investigational oral mTORC1/2 inhibitor sapanisertib. (TAK-228): Tolerability and food effects of a milled formulation in patients with advanced solid tumours. *ESMO Open*. (2018) 3:e000291. doi: 10.1136/esmoopen-2017-000291
220. Heinzen D, Divé I, Lorenz NI, Luger A-L, Steinbach JP, Ronellenfitsch MW. Second generation mTOR inhibitors as a double-edged sword in malignant glioma treatment. *Int J Mol Sci*. (2019) 20:4474. doi: 10.3390/ijms20184474
221. Wolin E, Mita A, Mahipal A, Meyer T, Bendell J, Nemunaitis J, et al. A phase 2 study of an oral mTORC1/mTORC2 kinase inhibitor. (CC-223) for non-pancreatic neuroendocrine tumors with or without carcinoid symptoms. *PLoS ONE*. (2019) 14:e0221994. doi: 10.1371/journal.pone.0221994
222. Eyre TA, Hildyard C, Hamblin A, Ali AS, Houlton A, Hopkins L, et al. A phase II study to assess the safety and efficacy of the dual mTORC1/2 inhibitor vistusertib in relapsed, refractory DLBCL. *Hematol Oncol*. (2019) 37:hon.2662. doi: 10.1002/hon.2662
223. Wicki A, Brown N, Xyrafas A, Bize V, Hawle H, Berardi S, et al. First-in human, phase 1, dose-escalation pharmacokinetic and pharmacodynamic study of the oral dual PI3K and mTORC1/2 inhibitor PQR309 in patients with advanced solid tumors. (SAKK 67/13). *Eur J Cancer*. (2018) 96:6–16. doi: 10.1016/j.ejca.2018.03.012

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Targeting Metabolic Deregulation Landscapes in Breast Cancer Subtypes

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Metabolic deregulation is an emergent hallmark of cancer. Altered patterns of metabolic pathways result in exacerbated synthesis of macromolecules, increased proliferation, and resistance to treatment via alteration of drug processing. In addition, molecular heterogeneity creates a barrier to therapeutic options. In breast cancer, this broad variation in molecular metabolism constitutes, simultaneously, a source of prognostic and therapeutic challenges and a doorway to novel interventions. In this work, we investigated the metabolic deregulation landscapes in breast cancer molecular subtypes. Such landscapes are the regulatory signatures behind subtype-specific metabolic features. $n = 735$ breast cancer samples of the Luminal A, Luminal B, Her2+, and Basal subtypes, as well as $n = 113$ healthy breast tissue samples were analyzed. By means of a single-sample-based algorithm, deregulation for all metabolic pathways in every sample was determined. Deregulation levels match almost perfectly with the molecular classification, indicating that metabolic anomalies are closely associated with gene-expression signatures. Luminal B tumors are the most deregulated but are also the ones with higher within-subtype variance. We argued that this variation may underlie the fact that Luminal B tumors usually present the worst prognosis, a high rate of recurrence, and the lowest response to treatment in the long term. Finally, we designed a therapeutic scheme to regulate purine metabolism in breast cancer, independently of the molecular subtype. This scheme is founded on a computational tool that provides a set of FDA-approved drugs to target pathway-specific differentially expressed genes. By providing metabolic deregulation patterns at the single-sample level in breast cancer subtypes, we have been able to further characterize tumor behavior. This approach, together with targeted therapy, may open novel avenues for the design of personalized diagnostic, prognostic, and therapeutic strategies.

Keywords: cancer metabolism, pathway deregulation, breast cancer subtypes, therapeutic targets, steroid and fatty acid metabolism, purine metabolism

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1. INTRODUCTION

Breast cancer is a complex, heterogeneous disease. Manifestations of this heterogeneity can be observed at the transcriptomic, molecular, or histological level (1). The origins of such manifestations can be traced back by looking at different levels of molecular control within the cells and tissues. The mechanisms behind gene expression, cell signaling, and metabolism are highly

intertwined, and cross-regulation patterns appear (2, 3), which strongly determine the phenotypic variance observed in clinical practice (4–6). In fact, this broad variance in molecular metabolism in breast cancer constitutes, simultaneously, a source of prognostic and therapeutic challenges and a doorway to novel interventions (7–9).

In order to face the challenges posed by tumor heterogeneity, it is customary to classify or *subtype* tumors according to their feature similarity. One currently used classification method in breast cancer, which has been particularly useful for capturing biological functional features, is the so-called molecular subtyping (10). The default classification scheme in this regard is given by the PAM50 (10, 11) algorithm, which groups breast tumors into molecular classes or subtypes according to a gene-expression signature of 50 genes relevant to the patho-physiology of the tumor. These subtypes are *Luminal A*, *Luminal B*, *Her2+*, and *Basal*. Some authors include a fifth subtype, the so-called *Normal-like*, but its use is controversial, and its use has been in decline lately (12).

These subtypes have been able to capture relevant differences in the origin, prognosis, response to treatment, and relapse probability of breast tumors. In general, it is considered that luminal subtypes are less aggressive and have better prognosis and better response to treatment than non-luminal ones (11). However, under certain circumstances, Luminal B tumors may have a higher recurrence, less response to treatment, and worse long-term prognosis (13). This variation in response is not clear and is of the utmost importance for the understanding of the disease at the personalized level.

Genomic alterations (mutations, copy number variations, chromosomal aberrations) often derive into anomalous cell functioning, including deregulation of metabolism—an important emergent hallmark of cancer (14) via abnormal gene regulatory programs. Aberrant gene-expression patterns are currently studied using next-generation sequencing (NGS) techniques such as RNA-Seq.

The analysis of these gene deregulation signatures provides a comprehensive (genome-wide) approach to dig into the molecular basis of disease. In the case of tumor metabolism, one may argue that metabolomics and phospho-proteomics would be closer proxies to the actual underlying molecular mechanisms. However, despite important advances in experimental-omic techniques, comprehensive metabolomic mapping and fluxomics are still under-developed for the task of describing cellular metabolic processes comprehensively, although this should change in the upcoming years. Approaches to analyzing metabolic deregulation in cancer based on gene expression have been developed (15, 16). Those extensive studies used differentially expressed genes for more than 20 types of cancer to distinguish deregulated metabolic pathways. In both cases, specific pathways were identified as deregulated in particular types of cancer. However, those studies performed phenotype-specific analyses and did not focus on single-sample deregulation.

To overcome this issue, an appealing way to study deregulation of metabolism is by analyzing metabolism-related gene-expression signatures at a single-sample level. In this work, we used TCGA gene-expression data from 735 tumor samples (17, 18), classified according to their molecular signature, to investigate the pathway deregulation patterns for the four PAM50 molecular subtypes, to determine subtype-specific metabolic landscapes. We used a single-sample-based algorithm (19) to quantify metabolic anomalies. This algorithm provides a pathway deregulation score for each pathway at a sample level. For validation purposes, we used a 2,000-sample cohort (20) with the same pipeline. Analyzing metabolic deregulation patterns at the subtype and individual sample levels provides a means of characterizing tumor behavior with a view to designing personalized diagnostic, prognostic, and therapeutic strategies.

2. MATERIALS AND METHODS

2.1. RNASeq Data Acquisition and Processing

Data were acquired from the Genome Data Commons Data Portal (https://bit.ly/2lJJrgi).

Briefly, 1,102 primary breast tumors and 113 normal solid tissues (normal solid tissue refers to healthy tumor-adjacent tissue taken from some of the tumors) samples were acquired and pre-processed to obtain log_2 normalized gene-expression values (21). Data were pre-processed to eliminate intrinsic experimental biases (22).

2.1.1. Integration

The following pipeline was already used and reported in Espinal-Enríquez et al. (21). Basically, an integrity check had to be carried out on raw expression files to ensure that all of them both had the same dimensions and provided TCGA identifiers before complementary annotation could be incorporated.

2.1.2. Quality Control

The `NOISeq` R library was used for global quality control (23, 24). All samples reached saturation for the number of detected features at the corresponding sequencing depth. Global expression quantification for each experimental condition yielded a feature sensitivity >60% for 10 counts per million (CPM). **Bias** detection assessment showed the presence of gene length, %GC, and RNA patterns.

The `EDASeq` R library was used for batch-effect removal (25). Before normalization, genes with mean counts <10 were filtered, resulting in 17,215 genes, as suggested in Risso et al. (25). Different within/between **normalization** strategies were tested to remove bias.

Exploration of sample log_2(normalized count) expression densities showed a consistent bi-modal pattern, corresponding to **noisy** lower-expressed genes and global sample behavior. Filtering out features with low counts ($CPM < 10$ cut-off) retained 15,281 genes, removing the undesired lower density peak. Finally, `ARSyN` R library was used for multidimensional noise reduction using default parameters (22).

2.1.3. Subtyping

We classified the 1,112 breast cancer samples into the four molecular subtypes using the pbcmc R package (26), a variation of the PAM50 algorithm, which characterizes the assessment

of the uncertainty in gene-expression-based classifiers (e.g., PAM50) based on permutation tests (12). Tumor samples with a non-reliable breast cancer subtype call were removed from the analysis. The number of removed samples was 377, giving a final number of 735 reliable samples.

2.2. Differential Expression Analysis and Pathway Discrimination

To determine overexpressed or underexpressed genes, we used the limma" R package (27), considering an absolute difference of Log_2FoldChange > 1 and a B-statistic > 5. The False Discovery Rate-adjusted p-value threshold was 10^{-3}. Since the main goal of this work is to establish the extent of deregulation in the metabolism for each breast cancer sample/subtype, we kept 80 metabolic pathways present in the KEGG database (28) (the Pathifier algorithm needs a minimum number of molecules to be performed).

2.3. Pathway Deregulation Analysis

Metabolic pathway deregulation in each sample was quantified by using the Pathifier algorithm (19). This algorithm integrates the expression data of genes involved in a given metabolic pathway into a single deregulation value at the individual-sample level. the algorithm assigns a score between 0 and 1, called the Pathway Deregulation Score (PDS)." Values close to 0 correspond to samples whose expression levels are similar to controls (29). Samples with higher values present higher differences in expression levels compared to the control group. Pathifier quantifies the level of deregulation of a metabolic pathway in a single tumor sample by measuring the deviation of said sample from control behavior.

In some cases, a single sample with extreme gene-expression changes (majorly different from those of other samples) for genes in a given pathway may give rise to a really high (assigning PDS = 1 to that sample) score, making all other deregulated samples (with large but comparatively low gene-expression changes) close to zero, thus appearing to be minorly deregulated. In other words, several deregulated pathways/samples would be missing. In such cases, the outlier sample was removed from the analysis. Finally, an unsupervised clustering method was used to group samples with similar PDS. A graphical representation of the pipeline is presented here in **Figure 1**.

2.4. Identification of Potential Pharmacological Targets

Genes being commonly over/underexpressed in all breast cancer subtypes would suggest that there should be subtype-independent drugs. In order to assess this idea, we performed data mining on transcriptomic/drug data by using a previously developed (by our group) computational pipeline to find differentially expressed pharmacological targets of FDA-approved drugs (31) for those shared DEGs. This tool performs all possible combinations of differentially expressed targets and FDA-approved drugs in public pharmacological databases, as well as their two-drug interactions. So, for the more than 2611 drugs annotated in the DrugBank database and the 660 drugs annotated in PharmGKB, all subtype-specific differentially expressed genes were interrogated.

2.5. Validation

For validation purposes, we used 2,000 microarray samples from the METABRIC cohort (20), performed the same analysis with the already classified samples, obtained the single-sample PDS, and compared them with the TCGA cohort.

3. RESULTS AND DISCUSSION

3.1. Subtype-Specific Deregulated Genes Are Associated With Characteristic Metabolic Pathways

As has been observed previously (1, 6), gene-expression signatures differ between all subtypes (**Figure 2**). The signatures presented here include only genes associated with metabolic pathways. **Figure 2** shows the overexpressed and underexpressed metabolism-associated genes for each subtype in the form of a Venn diagram. It can be observed that all subtypes have a non-shared set of differentially expressed genes (DEGs) but also a small subset of shared deregulated genes.

By using $|\log_2 FoldChange| > 1$ and $B - statistic > 5$ as significance thresholds, the number of DEGs in all the tumors is 204 overexpressed and 287 underexpressed. The numbers of overexpressed and underexpressed genes for each subtype are very similar. Interestingly, the subset of shared overexpressed genes ($n = 10$) is substantially smaller than that of the underexpressed genes ($n = 79$). This difference between the number of shared underexpressed and overexpressed genes may be associated with the fact that some metabolic pathways are silenced or decreased in all subtypes; on the other hand, metabolic pathways with incremental activity are subtype-specific.

To evaluate whether shared overexpressed genes influence the regulation of metabolism, we associated them with the metabolic processes in which they participate. **Figure 3** shows the relationships between the overexpressed genes (in red), and their associated metabolic processes (in pink) in the form of a bipartite network–a network composed by nodes of different nature, in this case, genes and pathways. Analogously, we constructed a network composed of the common underexpressed genes and their associated metabolic pathways.

As can be seen from the structure of the bipartite network, there are central molecules involved in several interrelated metabolic processes, giving rise to the so-called pathway-crosstalk events. This is a result of the utmost importance, since crosstalk phenomena have been associated with anomalous therapeutic responses and pharmacological resistance in breast cancer subtypes (32).

We can see, for instance, how the Interleukin 4-induced 1 gene (IL4I1) is the one with the most associated metabolic processes ($n = 7$), all related to amino acid biosynthesis (**Figure 3A**). This gene is often overexpressed in B-cell lymphomas (33) and has also been associated with cancer by promoting tumor growth

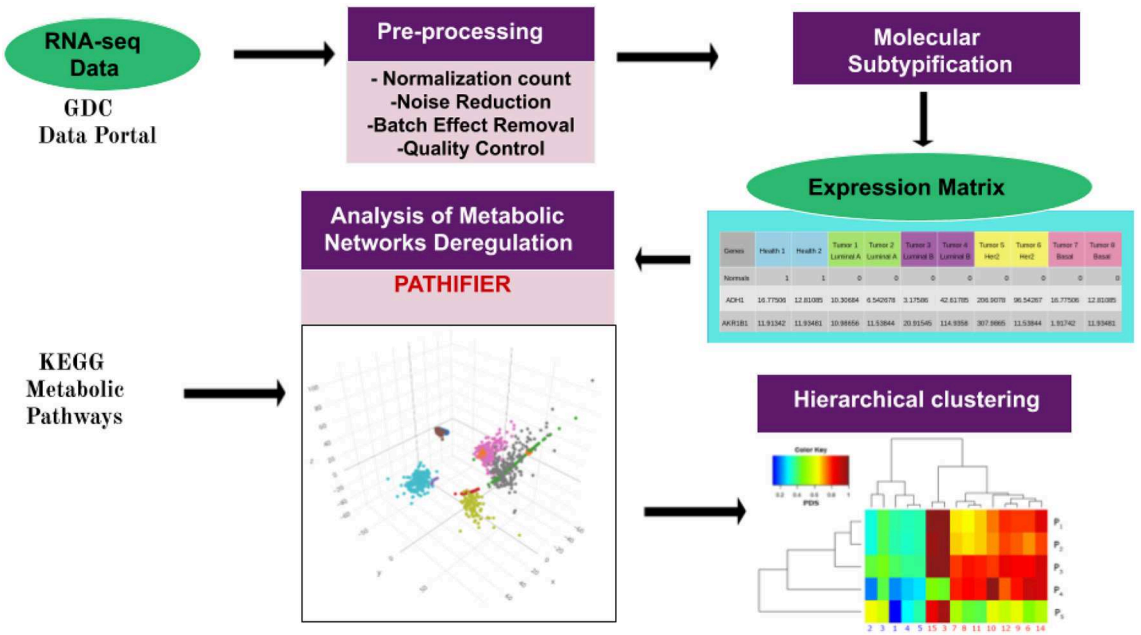

FIGURE 1 | Pipeline of the work presented here. The workflow starts with data acquisition from the TCGA Genome Data Commons Data Portal. Pre-processing of gene-expression files was performed as in Espinal-Enríquez et al. (21). Breast cancer molecular classification was made by using the pcbcmc R package (12). Molecular subtype classification of normalized samples provides us with a gene-expression matrix, which is used to run the Pathifier algorithm (19). This algorithm assigns a Pathway Deregulation Score (PDS) to every metabolic pathway in each sample. The PDS is a score between 0 and 1. Here, 0 corresponds to the centroid of the control samples of a given pathway. The score increases according to the distance of the sample from this centroid along a principal curve, spanning the cloud of data points. The pathways used to perform Pathifier were obtained by filtering the metabolism-associated KEGG pathways. Finally, PDSs were grouped by using unsupervised hierarchical clustering. Hierarchical clustering modified from García-Campos et al. (30).

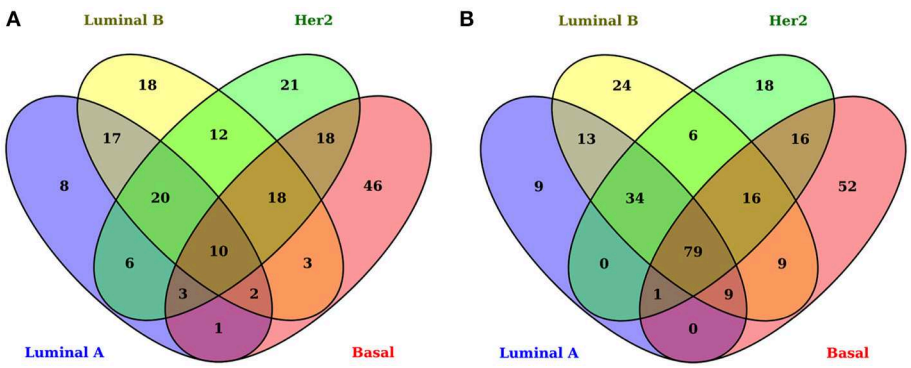

FIGURE 2 | Differentially expressed genes associated with metabolism in breast cancer molecular subtypes. In these Venn diagrams, each ellipse corresponds to the DEGs appearing in each subtype. The left set **(A)** corresponds to overexpressed genes and the right set **(B)** to underexpressed genes. The number inside each subset represents the number of genes appearing in each subset. Notice that the center of both figures corresponds to the common DEGs for all subtypes. There are only 10 overexpressed shared genes, while, for the underexpressed subset, 79 genes appear.

and shaping the immune microenvironment in melanoma (34). Autoimmune suppression and the inhibition of *CD*8+ cells are also pro-tumor-associated mechanisms regulated by IL4I1 (35, 36). Such processes are ultimately linked to the metabolic activity of IL4I1 as a phenylalanine oxidase. Crosstalk events involving cross-regulation via IL4I1 and non-coding RNAs have also been reported to play a role in triple-negative breast cancer (37).

As can be observed from **Figure 3B**, common underexpressed genes participate collectively in specific metabolic processes, such as purine metabolism. This pathway provides the metabolites needed for survival and cell proliferation and DNA and RNA production (38). ATP and GTP are also products of this metabolic pathway.

Among the underexpressed genes, we may find ADCY genes (ADCY4 and ADCY5), which regulate the nucleotide proportion

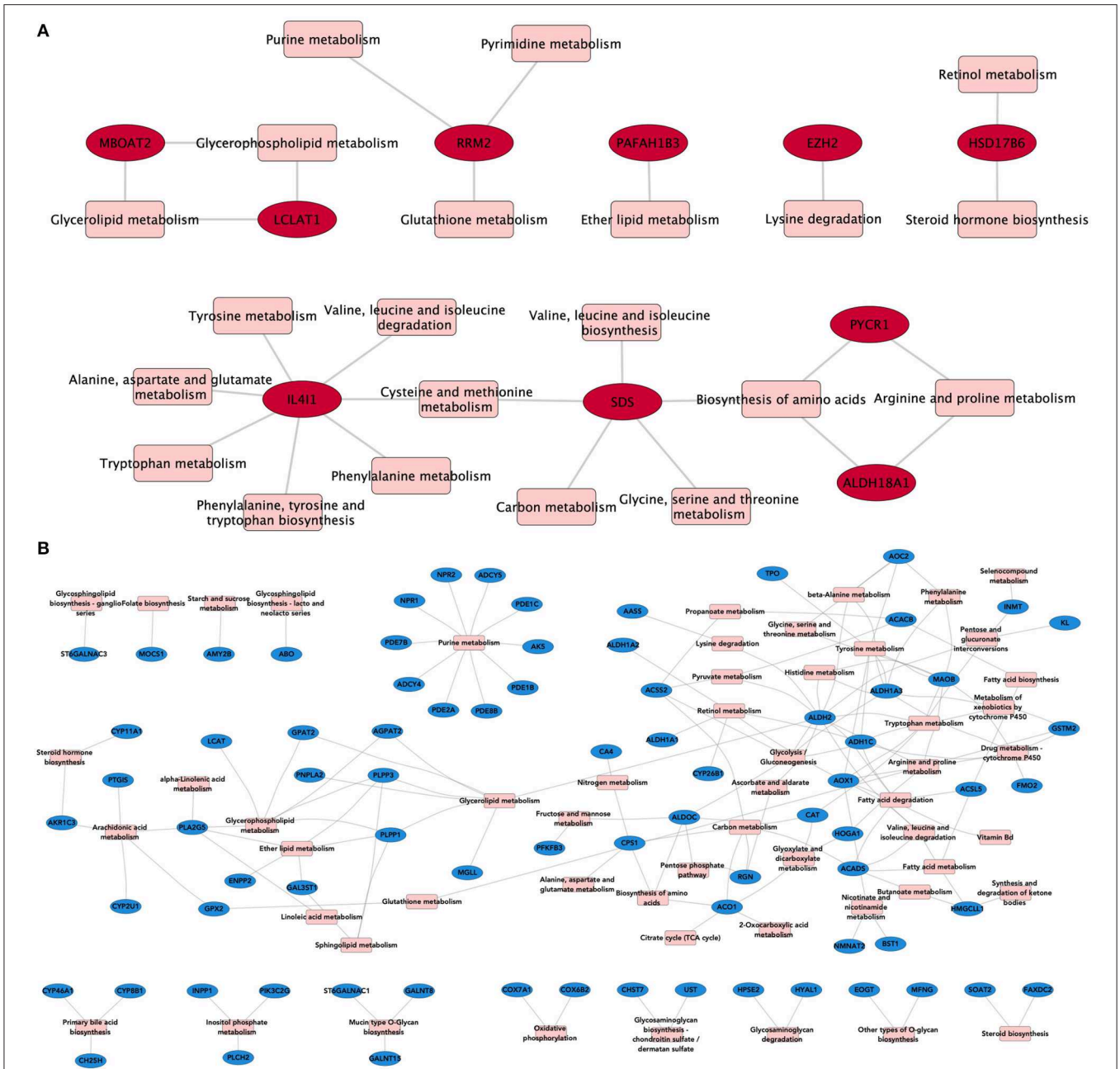

FIGURE 3 | Metabolic processes associated with DEGs. In these bipartite network representations, overexpressed **(A)** and underexpressed **(B)** genes are depicted in red and blue, respectively. Metabolic processes are shown in pink squares. Links between both types of nodes appear if the gene is present in the corresponding pathway. For this network, we kept only those DEGs that appear in the four subtypes.

(39), AK5, which catalyzes degradation reactions of ATP (40), or PDE and NPR, which control the proportion of second messengers, strongly implicated in signal transduction (41).

The majority of these genes are involved in the formation/degradation of ATP. Since cell proliferation is a hallmark of cancer, we argue that underexpression of these genes may enable the tumors to avoid ATP/GTP degradation, thus providing energetic fuel to cell proliferation.

3.2. Metabolic Deregulation Patterns Are Characteristic of Each Breast Cancer Subtype

Once it has been shown that common deregulated genes induce regulation patterns in some metabolic processes, the remaining question is whether variations in the whole gene-expression signature correspond to changes in specific metabolic deregulation.

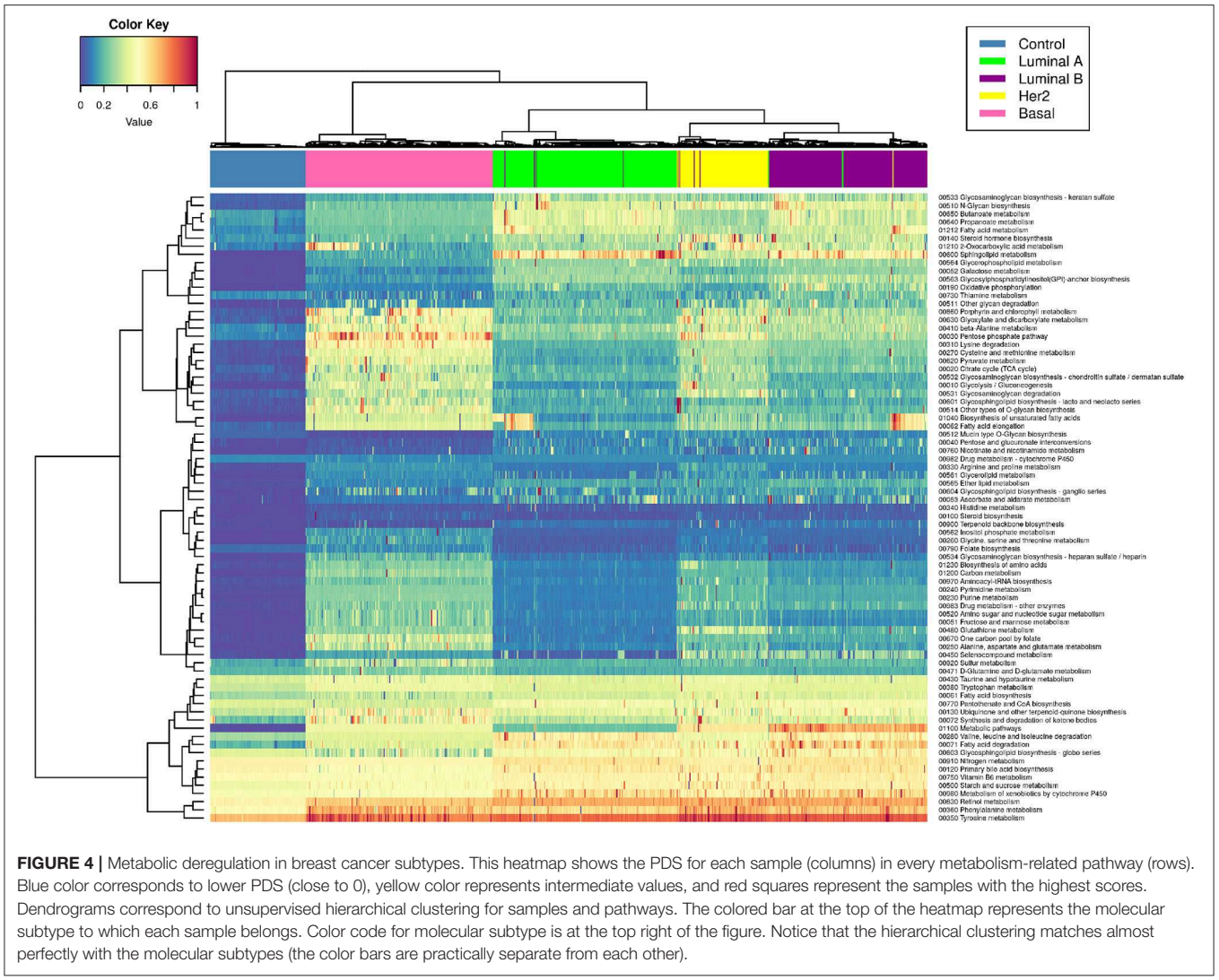

FIGURE 4 | Metabolic deregulation in breast cancer subtypes. This heatmap shows the PDS for each sample (columns) in every metabolism-related pathway (rows). Blue color corresponds to lower PDS (close to 0), yellow color represents intermediate values, and red squares represent the samples with the highest scores. Dendrograms correspond to unsupervised hierarchical clustering for samples and pathways. The colored bar at the top of the heatmap represents the molecular subtype to which each sample belongs. Color code for molecular subtype is at the top right of the figure. Notice that the hierarchical clustering matches almost perfectly with the molecular subtypes (the color bars are practically separate from each other).

Figure 4 shows a heatmap of the PDS values (see methods) grouped by PDS similarity. Rows correspond to all pathways associated with metabolism, while columns correspond to samples. There are subsets of samples that present a similar metabolic deregulation among subgroups and differ from the other samples.

Interestingly, unsupervised hierarchical clustering of PDS coincides almost absolutely with the PAM50 classification. The colored bars in the upper part of the figure correspond to each subtype, and, as can be appreciated, each color of the bar is grouped together. This phenomenon reflects the high specificity of metabolic deregulation for each molecular subtype.

Figure 5 shows that only one KEGG pathway: 01100 Metabolic Pathways" contains the full set of 1,142 genes present in every metabolism-associated KEGG pathway. Hence, the PDS in this particular process summarizes (to a certain degree) information about the rest of the metabolic-related pathways. The PDS for each subtype again presents a subtype-specific behavior, but more widespread than in **Figure 4**.

The PDS values are different between all subtypes, but more importantly, it is clear to observe that Luminal B is the subtype with the highest PDS. This result was unexpected, since it is usually considered that the most aggressive and with worst prognosis is the Basal subtype (42). In this case, the order of deregulation is as follows: Luminal A, Basal, HER2+, and, finally, Luminal B (**Figure 5**). From the PDS distributions, it can be noticed that the Luminal B subtype has the highest values but also the largest variance between samples. The rest of the subtypes are highly concentrated in a narrow range of PDS.

Previous reports have also analyzed the relationship between transcriptional deregulation and metabolic changes in cancer (15, 16). From these studies, some commonalities and differences arise. The work of Rosario uses differentially expressed genes for several phenotypes, breast cancer subtypes included. There, a score is based on LogFoldChange and adjusted *p*-values, measures that have not been derived with pathway-level assessment in mind, in contrast with the PDS, which is a specific pathway-level measure.

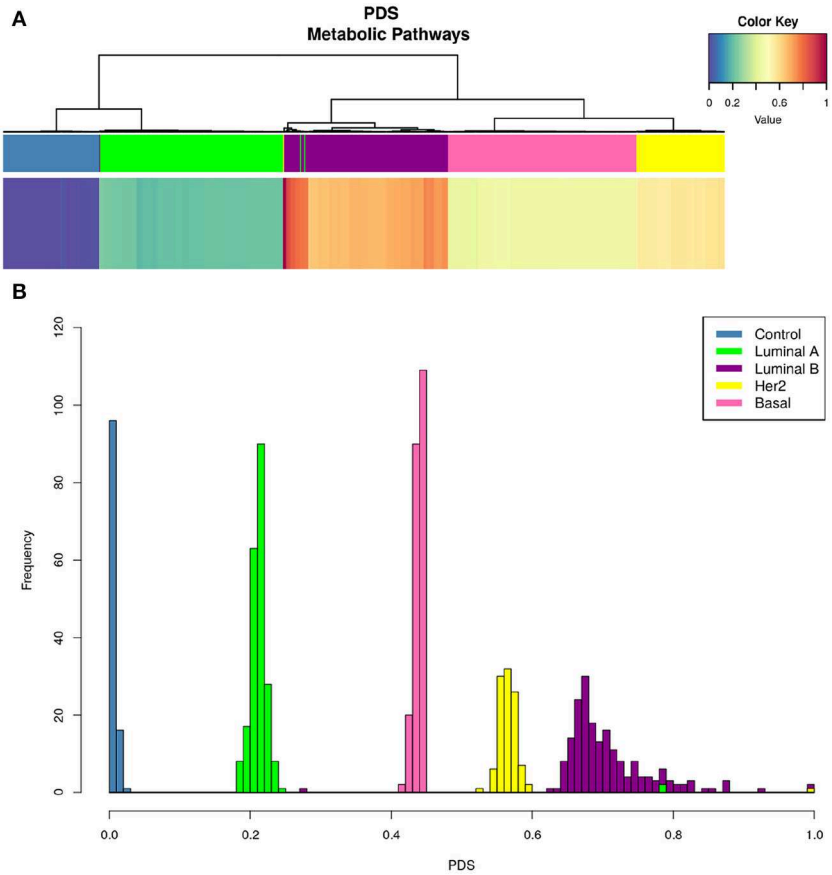

FIGURE 5 | KEGG 00100 Metabolic pathways PDS in breast cancer subtypes. Upper figure shows the PDS for only one Kegg entry: 00100 Metabolic pathways. As in **Figure 4**, upper bar **(A)** shows that hierarchical clustering matches PAM50 subtypes even better than the whole set of pathways. From the color of the heatmap, it is possible to observe that deregulation per subtype follows this pattern: Luminal A, Basal, Her2+, and Luminal B. At the bottom **(B)**, we present the distributions indicating the frequency of PDS according to each subtype. Notice that the Luminal B histogram presents the largest variance, while the rest of phenotypes are, in general, confined to a narrow PDS range.

Regarding commonalities, metabolic pathways are found to be differentially regulated in all subtypes in both manuscripts, in spite of the different approaches to pathway scoring. Purine and retinol metabolism are also found to be highly deregulated in both studies, particularly in the Luminal B and Basal subtypes. Interestingly enough, the Luminal B and Basal subtypes are the most deregulated phenotypes in both studies. This is reflected in Figure 6d from Rosario's paper and in **Figure 4** in our manuscript.

Another point in common between both studies is the coincidence of the Citric acid cycle as a unique pathway observed in the Basal subtype, with the TCA cycle found in our Basal samples (**Figure 4**). Interestingly, the categories reported in Figures 6d–f of Rosario's paper correspond to those of the Reactome database and not the ones described in the KEGG database. This is relevant since the categories are similar but not identical. This may be an additional source of some apparent discrepancies between Rosario's results and ours.

Regarding differences, Rosario et al. found different pathway scores for the Basal and Luminal A subtypes. However, as can be seen from Figure 6C of Rosario's paper, the low specificity of the average gene-expression Z-scores results in a non-conclusive depiction, as it is hard to distinguish signal from background noise. This is also reflected in the density plot of the Figure 6C heatmap. Additionally, the hierarchical clustering on top of the heatmap reflects a large degree of heterogeneity, resulting from the broad variance of the average gene-expression profiles. However, a clear phenotypic fingerprint of basal tumors is actually captured in terms of average gene-expression profiles, likely due to a reduced heterogeneity in these tumors.

3.3. Luminal B Tumors Present Higher Pathway Deregulation Scores

Figure 6 represents the PDSs for the Luminal B subtype only. It can be observed that several metabolic processes are highly deregulated (reddish rows), such as is the case of pyruvate metabolism, tyrosine metabolism, fatty acid degradation, and the pentose phosphate pathway.

In some cases, only a small subgroup of samples presents high PDSs (scattered red pixels), which in turn reflects the intrinsic

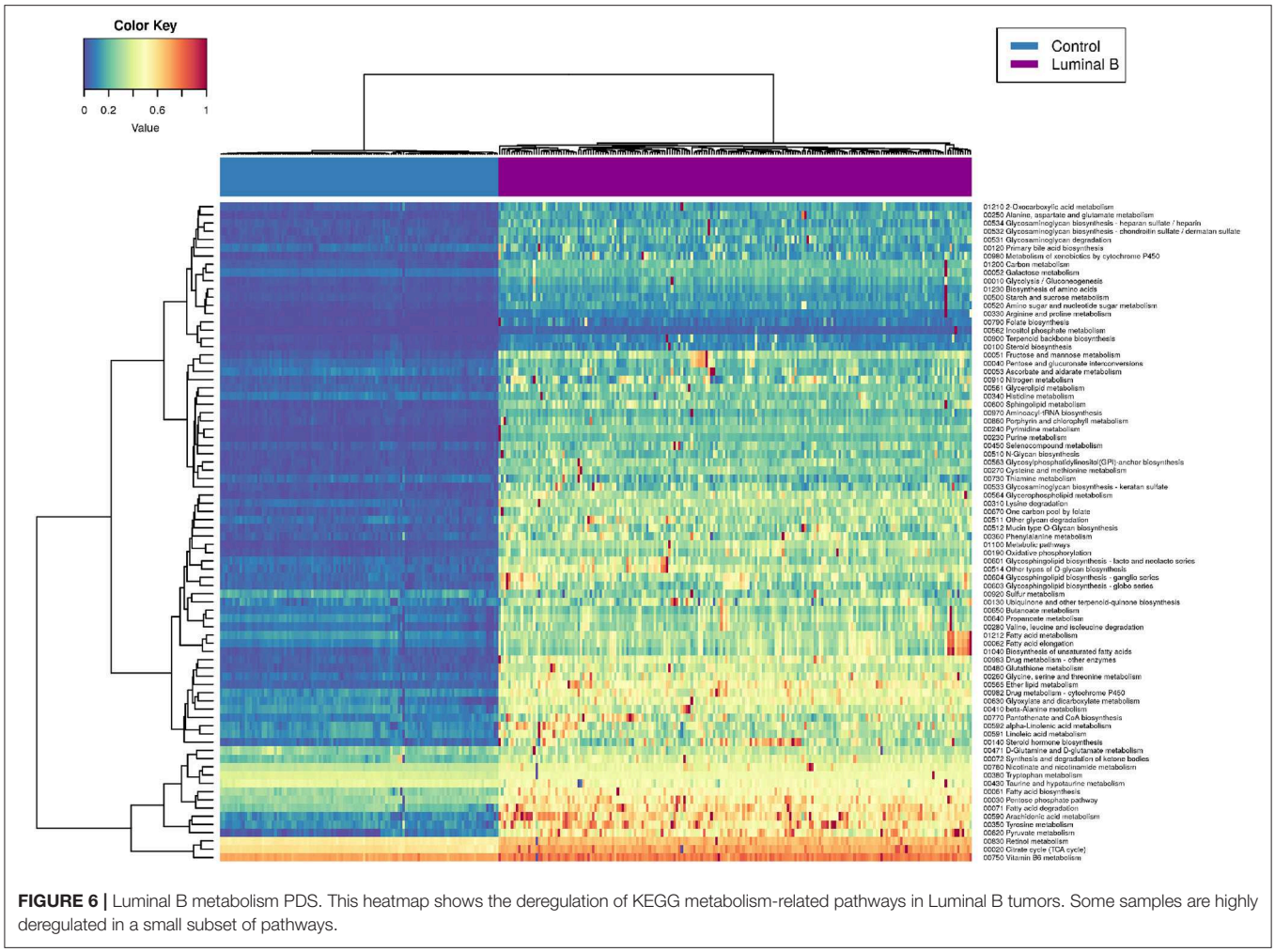

FIGURE 6 | Luminal B metabolism PDS. This heatmap shows the deregulation of KEGG metabolism-related pathways in Luminal B tumors. Some samples are highly deregulated in a small subset of pathways.

heterogeneity of samples, even if they belong to the same subtype. In the following, we will make some remarks regarding the most deregulated metabolic pathways observed in Luminal B tumors.

Pyruvate-related metabolic reprogramming has been associated with metastatic potential and treatment resistance in cancer (43). Pyruvate is a central metabolite for glucose, lactate, lipids, and amino acids. In breast cancer, liver-metastatic breast cancer cells exhibit a unique metabolic program compared to bone- or lung-metastatic cells, converting glucose-derived pyruvate into lactate, with a concomitant reduction in glutamine. This metabolic reprogramming results in a higher metastatic potential (44). Deregulation of fatty acid metabolism is crucial for malignant transformation in breast cancer. Proteins involved in the synthesis and oxidation of fatty acids play a pivotal role in the proliferation, migration, and invasion of breast cancer cells. Additionally, it has been shown that molecular subtypes display specific fatty acid metabolism (45). Deregulation of fatty acid metabolism has been associated with non-luminal tumors. Luminal subtypes rely on a balance between de novo fatty acid synthesis and oxidation as sources for biomass and energy. On the other hand, triple-negative basal breast cancer often uses exogenous fatty acids. In terms of targeted, personalized therapy, it is desirable to take such differences into account. In the case of the pentose phosphate pathway (PPP), it has been shown that PPP-associated proteins, such as 6PGL, 6PGDH, or NRF2, are not differentially expressed among breast cancer subtypes but are overexpressed relative to control samples (46). Glucose 6-phosphate dehydrogenase G6PD has been closely associated with prognosis in Basal tumors (47). It has been demonstrated that G6PD silencing increases the glycolytic flux, reduces lipid synthesis, and increases glutamine uptake in breast cancer cells. This effect has also been strongly related to poor prognosis (48). Her2-positive Luminal B tumors present overexpression of G6PDH (49). However, even if the presence of PPP-related proteins in Luminal B breast cancer has been established, a global analysis of this pathway is still lacking.

As we have said, the Luminal B subtype is the one with the highest metabolic deregulation. It is known that, in the long-term, the Luminal B subtype presents higher drug resistance, metastasis, and relapses (50, 51). This could be, in part, due to the individual heterogeneity at the gene-expression level. The metabolic deregulation in this subtype could also underlie drug resistance.

TABLE 1 | Overexpressed genes with FDA-approved inhibitors to regulate purine metabolism.

Search term	Drug	Interaction type
RRM2	FLUDARABINE PHOSPHATE	Inhibitor
RRM2	GALLIUM NITRATE	Inhibitor
RRM2	CLADRIBINE	Inhibitor
RRM2	CLOFARABINE	Inhibitor
RRM2	FLUDARABINE	Inhibitor
RRM2	GEMCITABINE	Inhibitor
RRM2	HYDROXYUREA	Inhibitor
RRM2	MOTEXAFIN GADOLINIUM	Inhibitor
RRM2	TEZACITABINE	Inhibitor
RRM2	GEMCITABINE HYDROCHLORIDE	Inhibitor
EZH2	CHEMBL3287735	Inhibitor

TABLE 2 | Underexpressed genes with FDA-approved activators to regulate purine metabolism.

Search term	Drug	Interaction type
ACACB	METFORMIN	Activator
NPR1	ATACIGUAT	Activator
PDE1C	BEPRIDIL	Activator
PDE2A	CHEMBL395336	Activator

To our knowledge, a profound study regarding metabolism in the Luminal B subtype is still necessary. However, we suggest that the long-term malignancy and poor prognosis of the Luminal B subtype are due, in part, to global metabolic deregulation more than to any single-molecule alteration. Further analyses in this regard are required to assess the metabolic deregulation patterns observed here with higher accuracy.

3.4. Purine Metabolism as a Potential Target in All Breast Cancer Subtypes

For the more than 2,611 drugs annotated in the DrugBank database and the 660 drugs annotated in PharmGKB, all subtype-specific differentially expressed genes were matched. The top 20 identified potential pharmacological targets obtained by the pipeline performed in Mejía-Pedroza et al. (31) are reported in **Table 1**. It contains those drugs that inhibit overexpressed genes. **Table 2** lists those drugs that activate underexpressed ones.

As can be observed in **Table 1**, RRM2, which participates in purine, pyrimidine, and glutathione metabolism, is the most targeted gene. EZH2, involved in lysine degradation, is another target that may be inhibited.

It is worth noticing that this computational tool provides all FDA-approved drugs that target a list of molecules, together with the effect that is produced in the target. **Supplementary Tables 1**, **2** contain comprehensive lists of drugs and their targets for commonly overexpressed and underexpressed breast cancer genes.

In the case of underexpressed genes, three of the four targets of activator drugs participate in purine metabolism: NPR1, PDE1C, and PDE2A. This result appears to be relevant in terms of the potential therapeutic options that breast cancer patients may have. There is a common deregulated metabolic pathway (purine metabolism) that can be targeted by specific drugs that have activator and inhibitory actions over underexpressed/overexpressed genes, respectively.

3.5. Deregulation of Metabolism Is Consistent in a Different Cohort

We performed a comparison with data from METABRIC (20), another large breast cancer cohort study. Our validation analysis shows a separation between groups as in the discovery group. A heatmap of the validation cohort is presented in **Supplementary Figure 1**, and the distribution of PDS in the METABRIC dataset is presented in **Supplementary Figure 2**. Some of our findings replicate those of METABRIC, although there were also differences, some of which may be attributable to METABRIC being a microarray-based experimental approach, whereas TCGA included data from RNA-sequencing experiments.

4. CONCLUSIONS

Heterogeneity is a crucial factor that impedes the understanding, diagnosis, and treatment of breast cancer tumors. Manifestations of this heterogeneity can be observed at the genomic, histological, or clinical level. In this work, we have provided another instance of this heterogeneity: metabolic deregulation.

Each breast cancer subtype has its own pattern of deregulation in metabolism, with Luminal B having the highest deregulation scores. This subtype presents alterations to metabolic processes such as pyruvate metabolism, tyrosine metabolism, fatty acid degradation, and the pentose phosphate pathway.

To our knowledge, this is the first time that a single-sample-based pathway analysis in breast cancer subtypes has been performed to identify differences in metabolic regulation. At the same time, this work has allowed us to design a common therapeutic FDA-approved scheme to regulate purine metabolism, independently of the subtype. With this kind of approach, it is possible to determine global deregulation patterns while, at the same time, finding individual signatures that may represent a further step toward personalized medicine.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the (Genomic Data Commons Data Portal).

AUTHOR CONTRIBUTIONS

EH-L and JE-E contributed to the conception and design of the study. ES-C collected, organized the database, preprocessed the data, performed the computational analysis, and performed results visualization. ES-C, JE-E, and EH-L discussed and contextualized the results. JE-E and EH-L wrote the first draft of the manuscript. All authors

contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.00097/full#supplementary-material

Supplementary Figure 1 | PDS heatmap of validation cohort. This heatmap showing the deregulation of KEGG metabolism-related pathways in the METABRIC cohort. Color code is the same as in the figures in the main manuscript.

Supplementary Figure 2 | Distribution of PDS in the validation cohort. Distributions indicating the frequency of PDS according to each subtype in the METABRIC cohort.

Supplementary Table 1 | Comprehensive lists of drugs and their targets for commonly overexpressed breast cancer genes.

Supplementary Table 2 | Comprehensive lists of drugs and their targets for commonly underexpressed breast cancer genes.

REFERENCES

1. Melchor L, Honrado E, Garcia M, Alvarez S, Palacios J, Osorio A, et al. Distinct genomic aberration patterns are found in familial breast cancer associated with different immunohistochemical subtypes. *Oncogene*. (2008) 27:3165. doi: 10.1038/sj.onc.1210975
2. Alcalá-Corona SA, de Anda-Jáuregui G, Espinal-Enríquez J, Hernández-Lemus E. Network modularity in breast cancer molecular subtypes. *Front Physiol*. (2017) 8:915. doi: 10.3389/fphys.2017.00915
3. de Anda-Jáuregui G, Velázquez-Caldelas TE, Espinal-Enríquez J, Hernández-Lemus E. Transcriptional network architecture of breast cancer molecular subtypes. *Front Physiol*. (2016) 7:568. doi: 10.3389/fphys.2016.00568
4. Habermann JK, Doering J, Hautaniemi S, Roblick UJ, Bündgen NK, Nicorici D, et al. The gene expression signature of genomic instability in breast cancer is an independent predictor of clinical outcome. *Int J Cancer*. (2009) 124:1552–64. doi: 10.1002/ijc.24017
5. Hicks J, Krasnitz A, Lakshmi B, Navin NE, Riggs M, Leibu E, et al. Novel patterns of genome rearrangement and their association with survival in breast cancer. *Genome Res*. (2006) 16:1465–79. doi: 10.1101/gr.5460106
6. Chin K, DeVries S, Fridlyand J, Spellman PT, Roydasgupta R, Kuo WL, et al. Genomic and transcriptional aberrations linked to breast cancer pathophysiologies. *Cancer Cell*. (2006) 10:529–41. doi: 10.1016/j.ccr.2006.10.009
7. Sims AH, Howell A, Howell SJ, Clarke RB. Origins of breast cancer subtypes and therapeutic implications. *Nat Rev Clin Oncol*. (2007) 4:516. doi: 10.1038/ncponc0908
8. Yersal O, Barutca S. Biological subtypes of breast cancer: prognostic and therapeutic implications. *World J Clin Oncol*. (2014) 5:412. doi: 10.5306/wjco.v5.i3.412
9. Telang N. Stem cell targeted therapeutic approaches for molecular subtypes of clinical breast cancer. *World Acad Sci J*. (2018) 1:20–4. doi: 10.3892/wasj.2018.3
10. Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA*. (2001) 98:10869–74. doi: 10.1073/pnas.191367098
11. Prat A, Perou CM. Deconstructing the molecular portraits of breast cancer. *Mol Oncol*. (2011) 5:5–23. doi: 10.1016/j.molonc.2010.11.003
12. Fresno C, González GA, Merino GA, Flesia AG, Podhajcer OL, Llera AS, et al. A novel non-parametric method for uncertainty evaluation of correlation-based molecular signatures: its application on PAM50 algorithm. *Bioinformatics*. (2017) 33:693–700. doi: 10.1093/bioinformatics/btw704
13. Olsen SN, Wronski A, Castaño Z, Dake B, Malone C, De Raedt T, et al. Loss of RasGAP tumor suppressors underlies the aggressive nature of luminal B breast cancers. *Cancer Discovery*. (2017) 7:202–17. doi: 10.1158/2159-8290.CD-16-0520
14. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
15. Hu J, Locasale JW, Bielas JH, O'sullivan J, Sheahan K, Cantley LC, et al. Heterogeneity of tumor-induced gene expression changes in the human metabolic network. *Nat Biotechnol*. (2013) 31:522. doi: 10.1038/nbt.2530
16. Rosario SR, Long MD, Affronti HC, Rowsam AM, Eng KH, Smiraglia DJ. Pan-cancer analysis of transcriptional metabolic dysregulation using The Cancer Genome Atlas. *Nat Commun*. (2018) 9:5330. doi: 10.1038/s41467-018-07232-8
17. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature*. (2011) 5:61–70. doi: 10.1038/nature11412
18. Tomczak K, Czerwińska P, Wiznerowicz M. The Cancer Genome Atlas (TCGA): an immeasurable source of knowledge. *Contempor Oncol*. (2015) 19:A68–77. doi: 10.5114/wo.2014.47136
19. Drier Y, Sheffer M, Domany E. Pathway-based personalized analysis of cancer. *Proc Natl Acad Sci USA*. (2013) 110:6388–93. doi: 10.1073/pnas.1219651110
20. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature*. (2012) 486:346–52. doi: 10.1038/nature10983
21. Espinal-Enríquez J, Fresno C, Anda-Jáuregui G, Hernández-Lemus E. RNA-Seq based genome-wide analysis reveals loss of inter-chromosomal regulation in breast cancer. *Sci Rep*. (2017) 7:1760. doi: 10.1038/s41598-017-01314-1
22. Nueda MJ, Ferrer A, Conesa A. ARSyN: a method for the identification and removal of systematic noise in multifactorial time course microarray experiments. *Biostatistics*. (2012) 13:553–66. doi: 10.1093/biostatistics/kxr042
23. Tarazona S, García-Alcalde F, Dopazo J, Ferrer A, Conesa A. Differential expression in RNA-seq: a matter of depth. *Genome Res*. (2011) 21:2213–23. doi: 10.1101/gr.124321.111
24. Tarazona S, García F, Ferrer A, Dopazo J, Conesa A. NOIseq: a RNA-seq differential expression method robust for sequencing depth biases. *EMBnet J*. (2012) 17:18–9. doi: 10.14806/ej.17.B.265
25. Risso D, Schwartz K, Sherlock G, Dudoit S. GC-content normalization for RNA-Seq data. *BMC Bioinform*. (2011) 12:480. doi: 10.1186/1471-2105-12-480

26. Fresno C, González GA, Llera AS, Fernández EA. *pbcmc: Permutation-Based Confidence for Molecular Classification*. Saitama: R package version 1. (2016).
27. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. (2015) 43:e47. doi: 10.1093/nar/gkv007
28. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res*. (2000) 28:27–30. doi: 10.1093/nar/28.1.27
29. Drier Y. Quantify deregulation of pathways in cancer. *Bioconductor*. (2013). doi: 10.18129/B9.bioc.pathifier
30. García-Campos MA, Espinal-Enríquez J, Hernández-Lemus E. Pathway analysis: state of the art. *Front Physiol*. (2015) 6:383. doi: 10.3389/fphys.2015.00383
31. Mejía-Pedroza RA, Espinal-Enríquez J, Hernández-Lemus E. Pathway-based drug repositioning for breast cancer molecular subtypes. *Front Pharmacol*. (2018) 9:905. doi: 10.3389/fphar.2018.00905
32. de Anda-Jáuregui G, Mejía-Pedroza RA, Espinal-Enríquez J, Hernández-Lemus E. Crosstalk events in the estrogen signaling pathway may affect tamoxifen efficacy in breast cancer molecular subtypes. *Comput Biol Chem*. (2015) 59:42–54. doi: 10.1016/j.compbiolchem.2015.07.004
33. Carbonnelle-Puscian A, Copie-Bergman C, Baia M, Martin-Garcia N, Allory Y, Haioun C, et al. The novel immunosuppressive enzyme IL4I1 is expressed by neoplastic cells of several B-cell lymphomas and by tumor-associated macrophages. *Leukemia*. (2009) 23:952. doi: 10.1038/leu.2008.380
34. Bod L, Lengagne R, Wrobel L, Ramspott JP, Kato M, Avril MF, et al. IL4-induced gene 1 promotes tumor growth by shaping the immune microenvironment in melanoma. *Oncoimmunology*. (2017) 6:e1278331. doi: 10.1080/2162402X.2016.1278331
35. Lasoudris F, Cousin C, Prevost-Blondel A, Martin-Garcia N, Abd-Alsamad I, Ortonne N, et al. IL4I1: an inhibitor of the CD8+ antitumor T-cell response *in vivo*. *Eur J Immunol*. (2011) 41:1629–38. doi: 10.1002/eji.201041119
36. Castellano F, Molinier-Frenkel V. IL4I1: an emerging target to reinvigorate antitumor immune responses. *Immunother Open Access*. (2017) 3:132–6. doi: 10.4172/2471-9552.1000132
37. Yuan N, Zhang G, Bie F, Ma M, Ma Y, Jiang X, et al. Integrative analysis of lncRNAs and miRNAs with coding RNAs associated with ceRNA crosstalk network in triple negative breast cancer. *OncoTargets Therapy*. (2017) 10:5883. doi: 10.2147/OTT.S149308
38. Pedley AM, Benkovic SJ. A new view into the regulation of purine metabolism: the purinosome. *Trends Biochem Sci*. (2017) 42:141–54. doi: 10.1016/j.tibs.2016.09.009
39. Klepinin A, Ounpuu L, Guzun R, Chekulayev V, Timohhina N, Tepp K, et al. Simple oxygraphic analysis for the presence of adenylate kinase 1 and 2 in normal and tumor cells. *J Bioenerget Biomembran*. (2016) 48:531–48. doi: 10.1007/s10863-016-9687-3
40. Sadana R, Dessauer CW. Physiological roles for G protein-regulated adenylyl cyclase isoforms: insights from knockout and overexpression studies. *Neurosignals*. (2009) 17:5–22. doi: 10.1159/000166277
41. Jeon Y, Heo YS, Kim C, Hyun YL, Lee T, Ro S, et al. Phosphodiesterase: overview of protein structures, potential therapeutic applications and recent progress in drug development. *Cell Mol Life Sci CMLS*. (2005) 62:1198–220. doi: 10.1007/s00018-005-4533-5
42. Sasmita A, Wong Y. Organoids as reliable breast cancer study models: an update. *Int J Oncol Res*. (2018) 1:008. doi: 10.23937/ijor-2017/1710008
43. Corbet C. Stem cell metabolism in cancer and healthy tissues: pyruvate in the limelight. *Front Pharmacol*. (2018) 8:958. doi: 10.3389/fphar.2017.00958
44. Dupuy F, Tabariès S, Andrzejewski S, Dong Z, Blagih J, Annis MG, et al. PDK1-dependent metabolic reprogramming dictates metastatic potential in breast cancer. *Cell Metabol*. (2015) 22:577–89. doi: 10.1016/j.cmet.2015.08.007
45. Monaco ME. Fatty acid metabolism in breast cancer subtypes. *Oncotarget*. (2017) 8:29487. doi: 10.18632/oncotarget.15494
46. Choi J, Kim ES, Koo JS. Expression of pentose phosphate pathway-related proteins in breast cancer. *Disease Mark*. (2018) 2018:9369358. doi: 10.1155/2018/9369358
47. Dong T, Kang X, Liu Z, Zhao S, Ma W, Xuan Q, et al. Altered glycometabolism affects both clinical features and prognosis of triple-negative and neoadjuvant chemotherapy-treated breast cancer. *Tumor Biol*. (2016) 37:8159–68. doi: 10.1007/s13277-015-4729-8
48. Benito A, Polat IH, Noé V, Ciudad CJ, Marin S, Cascante M. Glucose-6-phosphate dehydrogenase and transketolase modulate breast cancer cell metabolic reprogramming and correlate with poor patient outcome. *Oncotarget*. (2017) 8:106693. doi: 10.18632/oncotarget.21601
49. Cha YJ, Jung WH, Koo JS. Differential site-based expression of pentose phosphate pathway-related proteins among breast cancer metastases. *Disease Mark*. (2017) 2017:7062517. doi: 10.1155/2017/7062517
50. Tobin N, Harrell J, Lövrot J, Egyhazi Brage S, Frostvik Stolt M, Carlsson L, et al. Molecular subtype and tumor characteristics of breast cancer metastases as assessed by gene expression significantly influence patient post-relapse survival. *Ann Oncol*. (2014) 26:81–8. doi: 10.1093/annonc/mdu065.1
51. Prat A, Pineda E, Adamo B, Galván P, Fernández A, Gaba L, et al. Clinical implications of the intrinsic molecular subtypes of breast cancer. *Breast*. (2015) 24:S26–35. doi: 10.1016/j.breast.2015.07.008

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REVIEW
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Autophagy Regulation by the Translation Machinery and Its Implications in Cancer

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Various metabolic pathways and molecular processes in the cell act intertwined, and dysregulating the interplay between some of them may lead to cancer. It is only recently that defects in the translation process, i.e., the synthesis of proteins by the ribosome using a messenger (m)RNA as a template and translation factors, have begun to gain strong attention as a cause of autophagy dysregulation with effects in different maladies, including cancer. Autophagy is an evolutionarily conserved catabolic process that degrades cytoplasmic elements in lysosomes. It maintains cellular homeostasis and preserves cell viability under various stress conditions, which is crucial for all eukaryotic cells. In this review, we discuss recent advances shedding light on the crosstalk between the translation and the autophagy machineries and its impact on tumorigenesis. We also summarize how this interaction is being the target for novel therapies to treat cancer.

Keywords: autophagy, translation initiation, cancer, mTOR, PERK, eIF2alpha, endoplasmic reticulum, ATG

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INTRODUCTION

Cancer often results from glitching the interconnection between different metabolic networks and molecular processes (1), such as translation and autophagy. Translation is a fundamental process for all forms of life because it plays a central role in gene expression, and translational control critically contributes to the composition and quantity of a cell's proteome (2–5). Recently, dysregulation of translational control has been recognized as a cause of malfunctioning of other key cellular processes, which may lead to the onset and development of different types of cancer (6–10). Here, we discuss current research shedding light on the interplay between translation and autophagy and its involvement in cancer. We finally discuss new drugs targeting these processes to treat this malady.

TRANSLATION INITIATION AND ITS REGULATION

An Overview

Translation consists of initiation, elongation, termination, and a final stage of ribosome recycling that drives to a new round of translation. It is one of the most energy-consuming process in the cell. The whole process is largely controlled at the initiation step and, in consequence, defects in the translation initiation machinery or the signaling pathways regulating this step have different consequences on the cell that lead to numerous diseases, including cancer (11, 12).

The initiation step of translation consists in the recruitment of the small (40S) ribosome subunit to the 5′-UTR (see **Table 1** for abbreviations) of an mRNA and the selection of the translation start site, usually an AUG codon (depicted in **Figure 1**) (5, 13, 14).

Translation initiation starts when the cap structure (m^7GpppN, where N is any nucleotide) located at the 5′-end of an mRNA is recognized by the cap-binding protein, the eukaryotic initiation factor (eIF) 4E (**Figure 1**). In a parallel set of reactions, a free 40S ribosomal subunit interacts with eIF1, eIF1A, eIF3, eIF5, and a ternary complex (consisting of eIF2 bound to GTP and an initiator Met-$tRNA_i^{Met}$) to form a 43S pre-initiation complex (PIC). This step loosely positions the initiator Met-$tRNA_i^{Met}$ in the peptidyl (P) decoding site of the ribosome.

The scaffold protein eIF4G performs simultaneous interactions with the cap-bound eIF4E, the ATP-dependent RNA-helicase eIF4A, the poly(A)-binding protein (PABP) and the ribosome-bound eIF3, to coordinate recruitment of the 43S PIC to the mRNA 5′-UTR. Afterward, 43S PIC scans base-by-base the mRNA 5′-UTR to reach the AUG start codon, a process in which eIF4A, assisted by eIF4B, unwinds secondary structures of the 5′-UTR. Fidelity in the recognition of the correct mRNA AUG start codon is driven by eIF1 and eIF1A, which stabilize Watson-Crick base-pairing between the AUG codon and the Met-$tRNA_i^{Met}$ CAU anticodon. Selection of the start codon establishes the open reading frame for mRNA decoding, and results in a 48S PIC with the Met-$tRNA_i^{Met}$ and eIF1A tightly positioned within the P-site. Then, a GTP-eIF5B complex promotes release of eIF1 and eIF5B, facilitating joining of a 60S ribosomal subunit to the 48S PIC to assemble an 80S initiation complex, which is ready to start the elongation step of translation (13–15).

Ribosomal proteins, RNA binding proteins and miRNAs regulate protein synthesis either targeting global mRNAs by inhibiting or activating general translational machinery, or targeting specific mRNAs. Although this type of regulation can take place at initiation, elongation, and termination of translation, the rate-limiting step is initiation, and hence the most common and effective target (13–15).

Regulation of Translation Initiation

Different signaling cascades control protein synthesis in response to various stimuli, such as the MAPK pathway and the PI3K/Akt/TSC/RHEB/mTORC1 pathway (16, 17). A third pathway also regulates translation at the initiation step via phosphorylation of the eIF2 alpha subunit at Ser51 by four different protein kinases detailed below (18, 19). The MAPK pathway was not considered in this review.

mTOR is a serine/threonine kinase that dimerizes and forms the catalytic subunit of two functionally distinct multiprotein complexes, namely mTORC1 and mTORC2 (16, 17, 20–22) (**Figure 2**). mTORC1 is composed by three subunits that cooperate to phosphorylate substrates: mTOR itself, RAPTOR and mLST8; and by two inhibitory subunits: DEPTOR and PRAS40. The mTORC1 signaling pathway senses nutrient availability, growth factors, and cellular energy levels to promote cellular growth, survival, and proliferation, as well as translation, ribosome biogenesis, and lipid synthesis. It also blocks key catabolic processes such as autophagy and lysosome biogenesis. It is sensitive to rapamycin, a compound that forms a gain of

TABLE 1 | Abbreviations.

Abbreviation	Definition
3′-UTR	3′ untranslated region
5′-UTR	5′ untranslated region
4E-BPs	eIF4E-binding proteins
Akt	Protein kinase B
AMPK	Adenosine monophosphate–activated protein kinase
ATF4	Activating Transcription Factor 4
Atg	Autophagy related genes
BECN1	Beclin-1
CHOP	C/EBP Homologous Protein
DDX6	DEAD-Box Helicase 6
DEPTOR	DEP domain-containing mTOR-interacting protein
Dhh1	DExD/H-box helicase
eEF2K	elongation factor 2 kinase
eIF	eukaryotic initiation factor
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GCN2	General control non-repressed 2 kinase
hnRPA1	Heterogeneous Nuclear Ribonucleoprotein A1
HRI	Heme-regulated inhibitor
Hu	Human antigen
LC3	Microtubule-associated protein 1A/1B-light chain 3
MAPK	Mitogen-activated protein kinases
mLST8	mammalian lethal with SEC13 protein 8
mSIN1	mammalian stress-activated protein kinase interacting protein 1
mTOR	mammalian/mechanistic target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
Orb	Oo18 RNA-binding protein
p62/SQSTM1	p62/Sequetosome1
PABP	Poly(A)-binding protein
PDCD4	Programmed cell death 4 protein
PERK	PKR-like endoplasmic reticulum kinase
PI3K	Phosphatidylinositol 3-kinase
PI3KC3/VPS34	Class III Phosphatidylinositol 3-kinase
PIC	Pre-initiation complex
PIK3R4	Phosphoinositide 3-kinase regulatory subunit 4
PKR	Double-stranded RNA activated protein kinase
PRAS40	Proline-rich Akt substrate 40 kDa
PROTOR	Protein observed with RICTOR
Psp2	Polymerase suppressor protein 2
PtdIns3P	Phosphatidylinositol 3-phosphate
RACK1	Receptor for activated C kinase 1
RAPTOR	Regulatory associated protein of mTOR
RHEB	Ras homolog enriched in brain
RICTOR	Rapamycin-insensitive companion of TOR
RPS27L	Ribosomal protein S27-like
S6Ks	Protein kinases S6 kinases
TSC1/2	Tuberous sclerosis complex 1/2
ULK1/2	Unc51-like kinase 1/2
WIPI	WD-repeat protein interacting with phosphoinositides
ZFP36/TTP	Zinc finger protein 36 homolog/Tristetraprolin

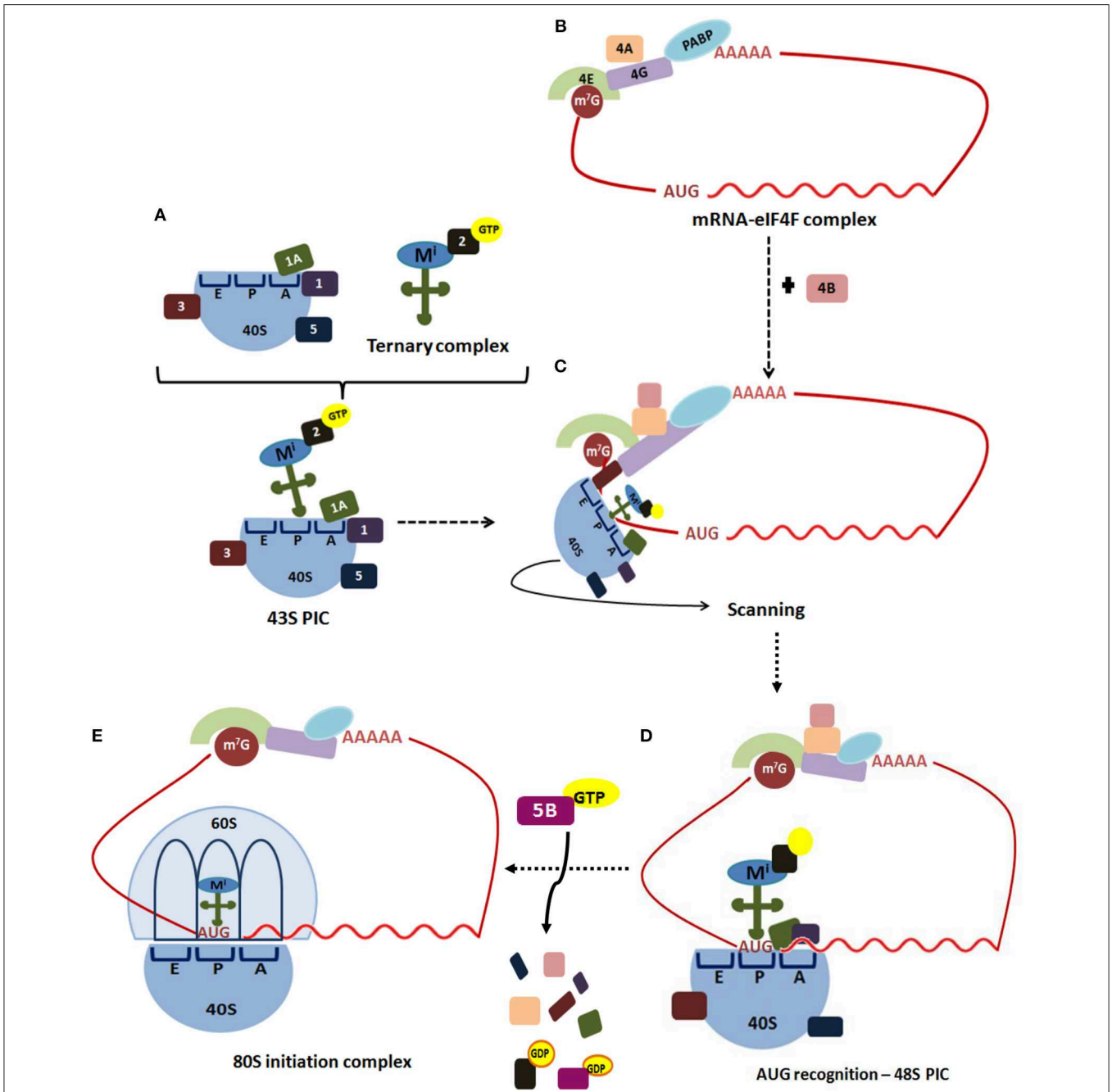

FIGURE 1 | Translation initiation in eukaryotes. Translation of most eukaryotic mRNAs is mediated by the eukaryotic initiation factors (eIFs). **(A)** This process begins when the free 40S ribosomal subunit, which is stabilized by eIF3 *(3)*, eIF1 *(1)*, eIF1A *(1A)*, and eIF5 *(5)*, binds to a ternary complex consisting of eIF2-GTP bound to an initiator Met-tRNAi, forming 43S pre-initiation complex *(PIC)*. **(B)** Simultaneously, the cap structure *(m^7G)* located at the 5′-end of an mRNA is recognized by the cap-binding protein, eIF4E *(4E)*. The scaffold protein eIF4G *(4G)* performs simultaneous interactions with the cap-bound eIF4E, the ATP-dependent RNA-helicase eIF4A *(4A)* and PABP bound to poliA, circularizing the mRNA to form the mRNA-eIF4F complex. **(C)** The ribosome-bound eIF3 coordinates the recruitment of the 43S PIC to the mRNA 5′-UTR. The 43S PIC scans base-by-base the mRNA 5′-UTR to reach the AUG start codon, a process in which eIF4A, assisted by eIF4B *(4B)*, unwinds secondary structures of the 5′-UTR. **(D)** Selection of the start codon establishes the open reading frame for mRNA decoding, and results in a 48S PIC with the Met-$tRNA_i^{Met}$ and eIF1A tightly positioned within the P-site. **(E)** Then, a GTP-eIF5B complex promotes release of eIF1 and eIF5B, facilitating joining of a 60S ribosomal subunit to the 48S PIC to assemble an 80S initiation complex, which is ready to start the elongation step of translation.

function complex with the peptidyl-prolyl isomerase FKBP12 that binds to mTOR and inhibits mTORC1 signaling. Therefore, rapamycin is an inducer of autophagy.

When amino acids are abundant, mTORC1 stimulates protein translation and inhibits autophagy by phosphorylating ULK1/2 at S757 and S637 residues, resulting in its catalytic activity

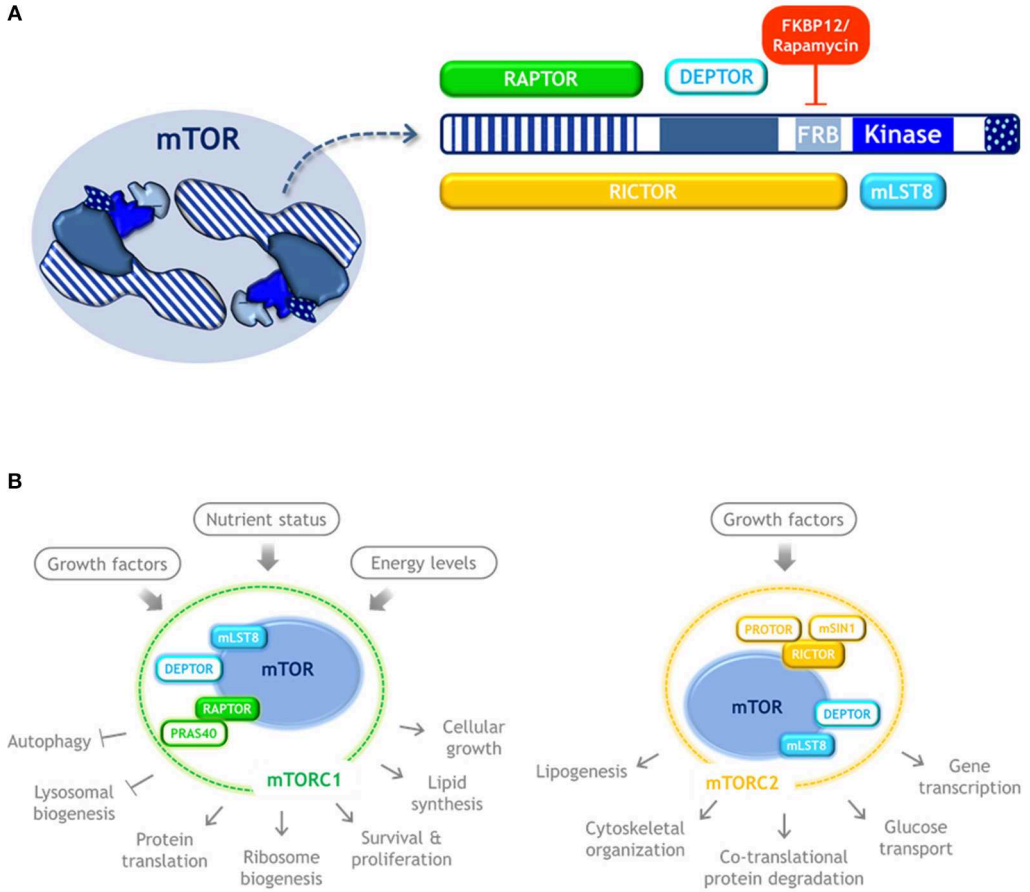

FIGURE 2 | mTOR kinase structure and complexes. **(A)** Schematic representation of mTOR kinase domains and its interacting proteins. mTOR possess 5 main domains (highlighted in blue). As an active form, mTOR dimerizes and may form two distinct complexes. mTORC1 is composed by three subunits that cooperate to phosphorylate substrates: mTOR, RAPTOR, and mLST8, and by the inhibitory subunits DEPTOR and PRAS40. Rapamycin forms a complex with FKBP12 that binds to mTOR and inhibits mTORC1 signaling. mTORC2 also contains mTOR, DEPTOR, and mLST8, but instead of RAPTOR it contains RICTOR, as well as the regulatory subunits mSIN1, and PROTOR. **(B)** mTORC1 and mTORC2 respond to distinct stimulus and control different cellular process. Color code: blue, mTOR kinase; cyan, components of both mTOR complexes; green, MTORC1 exclusive components; yellow, MTORC2 exclusive components.

suppression. Phosphorylation in these sites also disrupts the interaction of ULK1 with AMPK (23), a kinase activated by low glucose and ATP levels, and is the main activator of autophagy. AMPK activates ULK1 by phosphorylation at different serine residues (24), and inactivates mTORC1 phosphorylating RAPTOR and indirectly, by activating TSC2 (which in turn inhibits RHEB, a mTOR activator) (25) (schematized in **Figure 3**). When the amino acids pool is reduced, mTOR is inactivated allowing ULK1 dephosphorylation by the PP2A-B55α complex (26), while upon autophagy induction by genotoxic agents, ULK1 is dephosphorylated by PPM1D phosphatase (27).

mTORC2 also contains mLST8 and DEPTOR, but instead of RAPTOR it contains RICTOR, as well as mSIN1, and PROTOR. mTORC2 regulates co-translational protein degradation, lipogenesis, glucose transport, gene transcription, and cytoskeletal organization (21, 22). Since mTORC1 is the one involved in the control of translation and autophagy, only mTORC1 is further reviewed here.

To regulate protein synthesis, mTORC1 phosphorylates eIF4E-binding proteins (4E-BPs) that directly regulate eIF4E: hypophosphorylated 4E-BPs bind eIF4E with high affinity, which precludes eIF4E association with eIF4G, thus repressing cap-dependent translation. On the contrary, the hyperphosphorylated species of 4E-BPs dissociate from eIF4E to relieve translational repression. mTORC1 also phosphorylates S6Ks and eEF2K, that phosphorylate translation factors eIF4B, eIF4G, elongation factor eEF2, the ribosomal protein S6 and PDCD4, a negative regulator of eIF4A (16, 17, 20).

eIF2 phosphorylation at the alpha subunit is a key mechanism to regulate translation initiation. Upon mRNA AUG start codon recognition by the ribosome, ternary complex GTP/eIF2/Met-$tRNA_i^{Met}$ delivers methionyl-$tRNA_i^{Met}$ to the ribosomal P-site to arrest scanning, form the 80S Initiation Complex, and further initiates mRNA decoding. eIF2alpha activity relays on its phosphorylation status: whereas non-phosphorylated eIF2alpha promotes translation, phosphorylated eIF2alpha at Ser51 binds with high affinity to the guanine nucleotide exchange factor

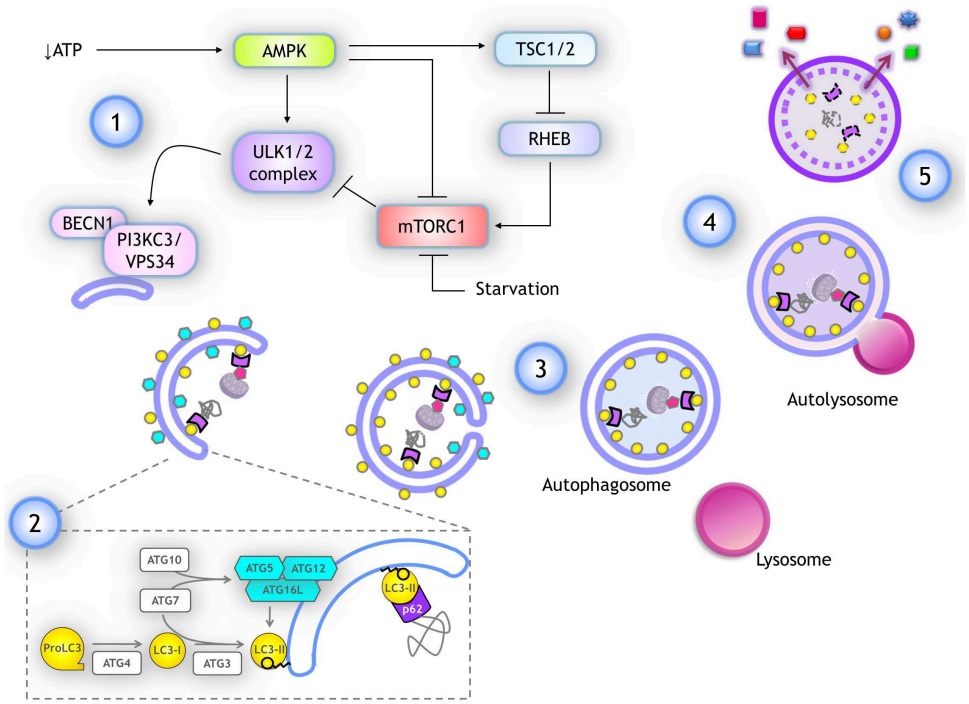

FIGURE 3 | Autophagy: overview and key molecular components. (1) Several stimuli promoting autophagy, like a drop in ATP, lead to AMPK activation, which stimulates autophagy by activating ULK1/2 complex and inhibiting mTORC1 through TSC1/2 activation, which in turn inactivates RHEB, a negative regulator of mTORC1. ULK1/2 complex activates the class III PI3K complex I by phosphorylating PIK3C3/VPS34. (2) For phagophore elongation, conjugation of ATG5-ATG12 complex is catalyzed by ATG7 and ATG10. ATG5-ATG12 covalently linked then interact with ATG16L forming a complex that is recruited at the phagophore. LC3 is proteolytically cleaved upon its translation by ATG4, producing LC3-I form. When autophagy is induced, LC3-I is covalently bound to phosphatidylethanolamine (PE) at the membrane of the phagophore. This reaction is catalyzed by ATG3 (E1-like) and ATG7 (E2-like) again, while ATG12-ATG5/ATG16L complex already recruited at the phagophore surface functions as an E3-like enzyme. Lipidated LC3-I is named LC3-II and it remains anchored to the elongating phagophore. LC3-II associates to both inner and outer membranes of the phagophore in expansion. Cargo is recognized by adaptor proteins like p62/SQSTM1, which also binds to LC3-II. (3) After elongation is completed the tips of the vesicle fuse giving rise to a double membrane vesicle named autophagosome. Autophagosomes maintain LC3-II at the inner membrane. (4) Autophagosomes fuse with lysosomes and the autophagosome inner membrane is degraded with the cargo, LC3-II, and adaptor proteins. (5) Finally, some of the products of degradation could be recycled, being released back into the cytoplasm.

eIF2B, leading to the formation of inactive eIF2B–eIF2–GDP complex that represses global translation. Upon diverse stimuli, mammalian eIF2alpha can be phosphorylated by four stress-responsive protein-serine/threonine kinases, namely PKR, that responds to virus infection; GCN2, that becomes activated in response to amino acids depletion, UV radiation, high salinity, and viral infection; HRI, that responds to oxidative agents, heat shock, and heme groups deficiency; and PERK, a transmembrane protein that becomes activated in response to perturbations in endoplasmic reticulum and unfolded proteins (18, 19).

AUTOPHAGY

An Overview

Autophagy is mainly a catabolic process that delivers cytoplasmic components for lysosomal degradation. In mammals, there are three pathways to deliver the cargo into the lysosomes: (1) Macroautophagy, where cargoes are first recognized and engulfed into a specialized double-membrane vesicle termed the "autophagosome." Afterward, it fuses with lysosomes to create the "autolysosome." This review focuses on this mechanism, which for simplicity will be referred to as "autophagy." Other mechanisms delivering cytoplasmic material into lysosomes are (2) Chaperone-mediated autophagy, where specific proteins are translocated into the lysosome; and (3) Endosomal microautophagy, where cytoplasmic cargoes get engulfed directly by late endosomes or multivesicular bodies. The latter processes have been revised elsewhere (28).

Autophagy can degrade all kind of macromolecules, whole organelles, and even intracellular pathogens. The physiological function of autophagy depends on the inducer and the fate of the degraded cargo. Autophagy is not merely a catabolic process but rather functions as a metabolic integrator, sometimes inducing anabolism. For instance, under a lack of nutrients, autophagy is triggered to degrade long-lived proteins for amino acids recycling for the synthesis of essential proteins; lipid droplets can also be degraded to release free fatty acids or even glycogen is degraded to release glucose, hence fostering anabolic biochemical pathways (29). Cancerous cells in solid tumors benefit from these functions, as autophagy allows them to resist under low oxygen and nutrients availability, maintaining the metabolic pathways necessary for aggressive tumor growth (30).

Autophagy is also induced in response to several stressors, such as genotoxic compounds. In this case, autophagy maintains genome integrity and consequently, autophagy malfunctioning leads to tumorigenesis (31). Nevertheless, autophagy plays a dual role in cancer, as some cancerous cells acquire chemotherapy resistance through activating autophagy (32). Since autophagy prevents early tumor formation but also is able to promote tumor cells survival, more comprehensive understanding of the autophagy involvement in carcinogenesis is needed before a therapy can be established.

Molecular Mechanisms of Autophagy

The regulation and execution of autophagy are mediated by several proteins known as ATG (autophagy related) (33). Here, we review only key proteins whose mRNAs are a target for translational regulation. The process of autophagy is divided into five steps (an overview is depicted in **Figure 3**):

1) *Initiation*. Upon autophagy induction, the ULK1/2 complex is activated. It is composed of ATG13, RB1CC1 and ATG101. ULK1/2 is a serine/threonine kinase that phosphorylates and activates the Class III PI3K complex I (composed of PIK3C3/VPS34, BECN1, PIK3R4, ATG14). This complex generates PtdIns3P at the surface of the membrane where the phagophore will form, most commonly at the endoplasmic reticulum membrane. PtdIns3P recruits WIPI family proteins, setting up the site of nucleation to further recruit molecules that give rise to the autophagosome.
2) *Elongation*. Two ubiquitin-like complexes are conjugated to promote phagophore elongation around the engulfed cargo. The first conjugation forms the ATG12-ATG5 complex. ATG12 is a small protein with structural similarity to ubiquitin, which is covalently bound to ATG5 by ubiquitin-like biochemical reactions catalyzed by ATG7 (E1-like) and ATG10 (E2-like) enzymes. This complex seems to be constitutively formed after ATG5 and ATG12 translation. When autophagy is induced, ATG12-ATG5 complex interacts with several molecules of ATG16L, forming a multiprotein complex that is recruited to the phagophore. Separately, upon its translation, protein LC3 (encoded by *MAP1LC3B* gene) is cleaved by the protease ATG4, producing the LC3-I isoform. When autophagy is induced, LC3-I is covalently bound to phosphatidylethanolamine at the phagophore's membrane. This reaction is catalyzed again by ATG7 (E1-like) and by ATG3 (E2-like), while ATG12-ATG5/ATG16L complex already recruited to the phagophore surface functions as an E3-like enzyme. Lipidated LC3-I is termed LC3-II and it remains anchored to the elongating phagophore.

   Detecting LC3-II abundance is a common way to monitor autophagy induction. It is also common to follow intracellular localization of GFP-LC3, since the unlipidated form (corresponding to LC3-I) is diffused in the cytoplasm. As it gets lipidated and anchored to the phagophore (corresponding to LC3-II), upon autophagy induction, LC3-II displays a punctuated pattern when observed by fluorescence microscopy.

   Cargo recognition occurs during phagophore elongation. Cytoplasmic material to be degraded is labeled by specific proteins, such as ubiquitin. Adaptor proteins serve as autophagic receptors to bridge the labeled cargo with the surrounding phagophore, leading the direction of membrane elongation around the cargo. Autophagic receptors have a domain to interact with the protein label and another domain to interact with LC3-II (or members of the family LC3/GABARAP) at the phagophore's membrane. The most common autophagic receptor is p62/SQSTM1.
3) *Closure*. Phagophore continues elongating around the cargo until its tips fuse, giving rise to the double-membrane vesicle termed autophagosome. Other proteins and lipids contribute to the autophagosome closure and have been recently reviewed (34). Once autophagosome forms, LC3-II is detached from the outer membrane giving rise to a mature autophagosome, ready to fuse with a lysosome (35).
4) *Fusion*. Autophagosomes travel through microtubules to reach and fuse with lysosomes, giving rise to autolysosomes. The molecular machinery for autolysosomes fusion has been recently reviewed (34).
5) *Degradation and recycling*. Within the autolysosomes, lysosome hydrolases digest cytoplasmic cargoes, the inner membrane of the autophagosome, and associated proteins like LC3-II and p62/SQSTM1 as well. If autophagy was induced by a lack of nutrients, macromolecules building blocks are released into the cytoplasm through specific transporters and permeases that are recruited during autophagosome formation. Then, the lysosome membrane segregates in the autolysosome and elongates until a new lysosome is detached and reconstituted (34). The fate of the remaining autolysosome is poorly understood.

When analyzing autophagy, it is essential to study not only the accumulation of LC3-II or autophagosomes, but also to verify the cargo degradation. An increase in the abundance of LC3-II, for example, could be a consequence of an interruption of the autophagic flux instead of a true autophagic induction. The most common way to evaluate the autophagic flux is by comparing the abundance of an autophagic adapter such as p62/SQSTM1 or verifying cargo degradation. For a full description of methods to monitor autophagy see (36).

In the next section, we review how translation machinery modulates autophagy in normal and cancerous cells.

REGULATION OF AUTOPHAGY BY TRANSLATION

Since its discovery, autophagy regulation has been broadly studied with focus on understanding *ATG* genes transcriptional regulation and ATG proteins post-translational modifications. In recent years, however, a new level of integration of information has emerged: the post transcriptional regulation of *ATG* mRNAs expression by the translation machinery. Here we summarize investigations that use gain- or loss- of-function approaches to learn about the regulation of *ATG* mRNAs translation by eIFs, ribosomal proteins and RNA

binding proteins, and how these interventions affect autophagy (**Figure 4**). It is important to consider that in several of these studies only LC3-II or autophagosomes abundance were studied, without distinguishing whether there was an autophagic flux blockage or a true autophagy induction. In those cases, it is not possible to conclude that a functional autophagy takes place. We review in **Table 2** specific experiments performed to analyze autophagy.

Translation Initiation Factors Control Autophagy

In vertebrates, the family of 4E-BPs contains three members: 4E-BP1, 4EB-P2, and 4E-BP3, and all of them function as repressors of cap dependent translation by sequestering eIF4E thus preventing its interaction with eIF4G (14). Among them, 4E-BP1 is the best characterized. The first study that suggested an inhibition of autophagy by 4E-BP1 was done in genetically engineered immortalized and tumorigenic human prostate epithelial cells (PrEC) that overexpressed *MYC* oncogene. MYC binds to the regulatory region of *4EBP1* gene increasing its expression, which leads to a decreased autophagy. The inhibitory role of 4E-BP1 over autophagy was confirmed by the observation that cells with reduced expression of 4E-BP1 accumulate autophagosomes (37). A negative regulation of this translation repressor over autophagy is also true in human hepatoblastoma cells with stable expression of hepatitis B virus (HepG2.2.15), since again, silencing 4E-BP1 expression increases LC3-II, and blocking autophagic flux with chloroquine results in an even greater accumulation, indicating that LC3-II accumulates due to an activation of autophagy (40).

Tumor cells have to adapt to hypoxia by altering their gene expression and protein synthesis; while general translation is inhibited, selected mRNAs remain efficiently translated. A study searching for such hypoxia-regulated genes found translational

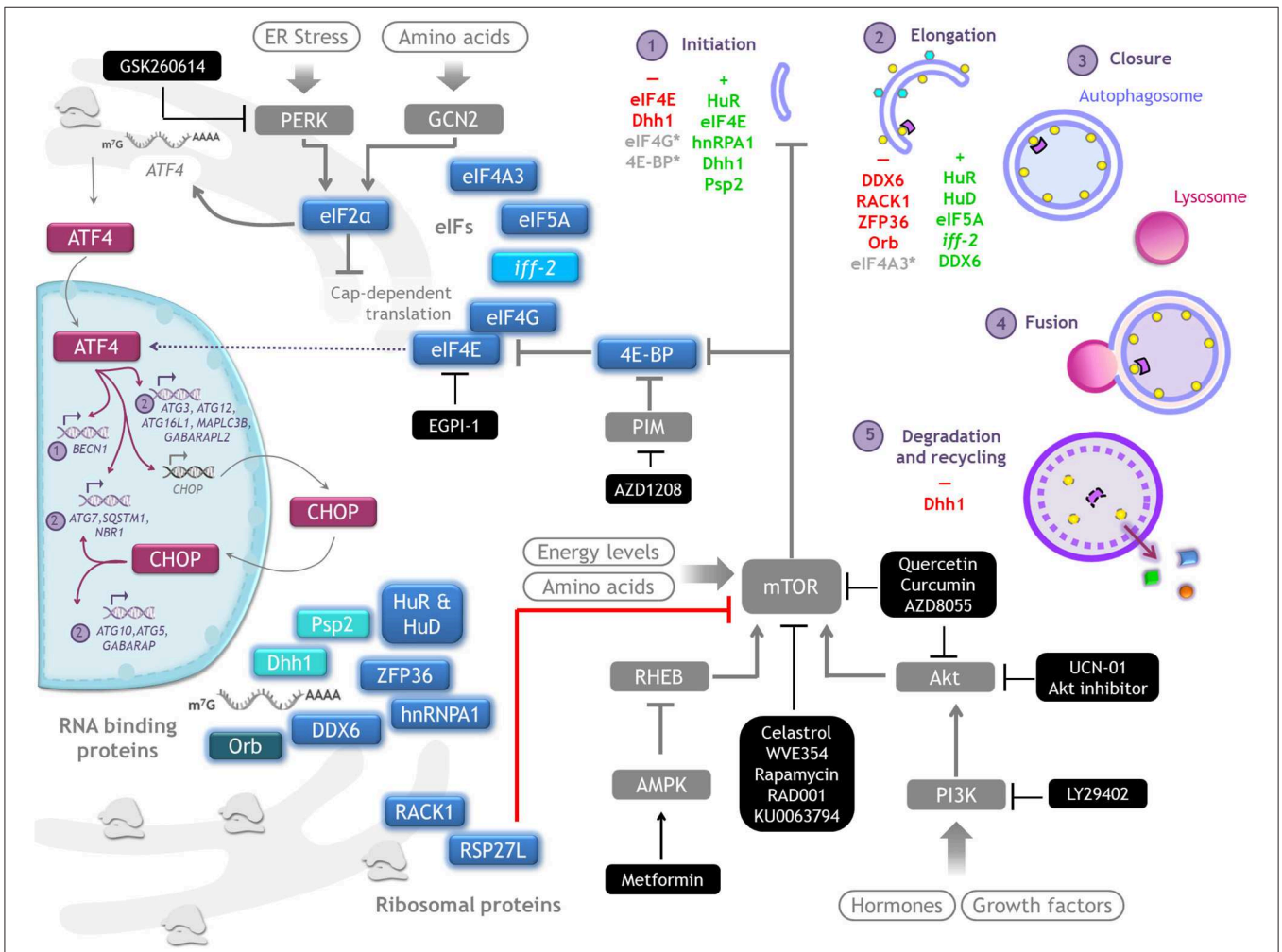

FIGURE 4 | Autophagy regulation by translation machinery, and therapeutics targets. Integrative scheme of the examples of autophagy regulation described on conditions found in tumor environment such as hypoxia, starvation, or cell death resistance. Although the main control of autophagy occurs at translational level, eIF4E and eIF2alpha are able to regulate the transcription of some *ATG* genes through ATF4/CHOP. Color code: *magenta*, transcriptional regulators of *ATG* genes; blue, proteins that control translation of *ATG* mRNAs (a different intensity of blue denotes observations made on different species); gray, signaling pathways upstream of autophagy. Therapeutics agents against cancer targeting key molecules for protein translation and autophagy regulation are shown in black boxes.

TABLE 2 | *ATG* mRNAs expression regulated by translation machinery.

Protein Studied	Model	Autophagy evaluation	Autophagy flux assessment	Additional observations	References
4E-BP1	LHMB-AR PrEC sh4E-BP1	↑LC3-II, ↑acridine orange			(37) #122
4E-BP1	HL60 or HeLa parthenolide HEK293 Parthenolide + sh4E-BP1 HeLa Parthenolide + plasmid 4E-BP1	↑LC3-II, ↑GFP-LC3 ↑LC3-II ↓LC3-II		↓ 4EB-P1 ↓ Ⓟ4E-BP1	(38) #127
4EB-P1	HCT-1116 hypoxia	↑ translation 35 lysosomal mRNAs ↑ acridine orange ↑ LysoTracker^{+} ↑ LC3-II ↓p62		↑Ⓟ eIF2α; ↓Ⓟ4EB-P1 ↑ translation *EIF4EBP3* and *EIF2AK3*; *EIF4E*, *RPS6K* subunits	(39) #40
4E-BP1 Akt S6K1	HepG2.2.15 si4E-BP1 siAkt or Akt inhibitors siS6K1	↑LC3-II ↓LC3-II ↓GFP-LC3 puncta ↑p62 ↓LC3-II	CQ ↑ LC3-II CQ ≠ LC3-II		(40) #161
eIF4E	T-ALL Jurkat selenite selenite +sieIF4E	↑LC3-II ↑GFP-LC3 puncta ↑ATF4 on *MAP1LC3B* and *CHOP* promoters ↓GFP-LC3 puncta ↓ATF4 on *MAP1LC3B* and *CHOP* promoters	Baf A1 ↑LC3-II ↑p62	↑CHOP, ↑Ⓟ eIF4E ↓ATF4 + si_p38 or p38 inhibitors prevent selenite effects	(41) #124
eIF4E eIF2α	NB-4 selenite selenite+ sieIF2α selenite+ plasmid eIF4E	↓LC3-II, ↑p62 ↓GFP-LC3 puncta ↓ATF4 on *MAP1LC3B* promoter ↑LC3-II, ≠ GFP-LC3 ↑LC3-II ↑GFP-LC3 puncta ↑ATF4 on *MAP1LC3B* promoter		↑CHOP, ↑ATF4 ↑Ⓟ eIF2α, ↓Ⓟ eIF4E ↓CHOP ↑CHOP	(42) #43
eIF5A eIF4A3	MCF-7 sieIF5A sieIF5A +Torin-1	↓GFP-LC3 puncta ↓autophagosome (TEM) ↓ATG3 ↓GFP-LC3 puncta	Baf A1 ↑LC3-II		(43)
iff-2 (eIF5A homlog)	*C. elegans iff-2* RNAi	↓GFP::LGG-1 puncta			(43)
eIF4G1 eIF4G2	MCF10A or HEK293T sheIF4G1 or sh eIF4G2	↑LC3-II ↑GFP-LC3 puncta ↑MDC^{+} Vesicles			(44) #216
eIF4G1	MCF10A sheIF4G1 γ irradiation	↑LC3-II ↑GFP-LC3 puncta			(45) #2
RACK1	HT1080 siRACK1 HepG2, Hep3B, U2OS, HeLa, MCF-7 and MDAMB231	↑LC3-II ↑LAMP1 and LAMP2 ↑GFP-LC3 puncta and colocalization with LysoTracker ↑BCL-XL and BECN1 interaction ↓p62 ↑polysomal fraction on *MAP1LC3* and *BCL-XL* mRNA	Baf A1 ↑LC3-II ↑p62		(46) #126
RPS27L	MB231 or SK-BR3+ siRPS27L	↑LC3-II ↑EGFP-LC3 puncta ↓p62	+CQ or Baf A1: ↑LC3-II and ↑p62	↑DEPTOR, ↓Ⓟ S6K1 and Ⓟ 4EBP1 ↓Ⓟ S6K1 and Ⓟ 4EBP1	(47) #131
	MEFs *RSP27L*$^{-/-}$	↑LC3-II ↓p62			
HuD	βTC6 or U2OS siHuD plasmid HuD	≠ *ATG5 mRNA*; ↓ATG5 ↓LC3-II ↓GFP-LC3 puncta ↓autophagosomes (TEM) ≠ *ATG5 mRNA* ↑ATG5 ↑LC3-II ↑GFP-LC3 puncta		+miR-181 ↓EGFP-*3'UTR Atg5 mRNA* ↑EGFP-*3'UTR Atg5 mRNA*	(48) #125

(Continued)

TABLE 2 | Continued

Protein Studied	Model	Autophagy evaluation	Autophagy flux assessment	Additional observations	References
HuR	HSC-LX2 or HSC-T6 erastin+ siHuR HSC-LX2 or HSC-T6 erastin+ plasmid HuR	↓LC3-II ↓BECN1 ↑p62 ↑LC3-II ↑BECN1 ↑autophagosome (TEM), ↓p62	CQ: ↑LC3-II	RIP: *3'UTR BECN1 mRNA* enrichment	(49)
HuR	L-02 or Hep3B siHuR	↓ATG5, ATG12 and ATG16 ↓polysome association to *ATG5, ATG12 and ATG16 mRNAs* ↓LC3-II ↓Autophagosome and autolysosome (TEM) ↓GFP-LC3 puncta	colchicine modest ↑LC3-II	RIP: *3'UTR ATG5, ATG12, ATG16 mRNAs* enrichment	(50) #36
HuR	HK-2 hypoxia HK-2 hypoxia+ shHuR	↑LC3-II, ATG7 and ATG16 ↓LC3-II, ATG7 and ATG16		↑TUNEL^{+} cells RIP: *ATG7, ATG16 mRNAs* enrichment	(51)
HuR	MCF-7 starvation MCF-7, MDA-MB 231, PC3, HaCat siHur	↑LC3-II, ↑BECN1, ↑polysomes association of *BECN1 mRNA* & HuR, ↓LC3-II ↓BECN1 ↓*BECN1 mRNA*		RIP: *3'UTR BEC1 mRNA* enrichment	(52)
HuR	Intestinal epithelium IE_HuR$^{-/-}$ mice	↓LC3-II ↓BECN1, ↓ATG16L1 ↓ATG7		RIP: *ATG16 mRNA* enrichment	(53)
	IECs siHuR	↓LC3-II ↓ATG16L1 ↓ newly synthesized ATG16L1			
ZFP36	HSC-LX2 or HSC-T6 erastin + plasmid ZFP36	↓ LC3-II ↓GFP-LC3 puncta ↓ ATG16L1 ↓ ATG5-ATG12 ↓ Autophagosome (TEM) ↓ *ATG16 mRNA,* ↑*SQSTM11 mRNA*		RIP: *ATG16 mRNA* enrichment Luc-*3'ÚTR Atg16 mRNA*: ↓Luc activity	(54)
	HSC-LX2 or HSC-T6 erastin + plasmid FXBW7	↑ LC3-II, ↑ ATG16L1, ↑ ATG5-ATG12 ↑ Autophagosome (TEM)			
hnRNPA1	HCT-116 sihnRNPA1 plasmid hnRNPA1	↓BECN1, ≠ *Becn1 mRNA* ↑BECN1, ≠ *Becn1 mRNA*		Luc-*3'ÚTR Becn1 mRNA*: ↓Luc activity Luc-*3'ÚTR Becn1 mRNA*: ↑ Luc activity Biotin-*3'UTR Benc1 mRNA* RIP: *Becn1 mRNA* enrichment	(55)
Orb	*Drosophila* germarium Orb mutant	↑*Atg12 (mRNA);* ↑Atg12 and Atg8 (protein); ↑LysoTracker^{+} structures		RIP: *Atg12 mRNA* enrichment	(56) #8
Dhh1 (DDX6)	Yeast Δ*dhh1* nutrient replete	↑ *Atg3, Atg7, Atg8, Atg19, Atg20, Atg22 and Atg24* mRNA	GFP- ATG8 processing assay Δ*dhh1*+ *starvation*: ↑ GFP free		(57) #123
	Mouse ESC DDX6 $^{+/-}$	↑*Map1lc3* mRNA ↑LC3-II ↓LC3 puncta ↓p62			
	HeLa+ siDDX6	↑ *MAP1LC3* mRNA ↑ LC3 puncta			
	HeLa+ plasmid DDX6	↓ *MAP1LC3* mRNA ↓ LC3-II ↓LC3 puncta ↑p62			
Dhh1 (DDX6) Eap1	Yeast Δ*dhh1* Nitrogen starvation HEK293A DDX6 $^{-/-}$ Amino acid starvation	↓Atg1, Atg13 (protein) ≠ *ATG1 and ATG13* mRNAs ↓ATG16L1 ↑*ATG16L1* mRNA	Pgi-GFP processing assay: ↓ free GFP		(58) #24 ó 1

(Continued)

TABLE 2 | Continued

Protein Studied	Model	Autophagy evaluation	Autophagy flux assessment	Additional observations	References
Psp2	Yeast Δ*psp2*+ Nitrogen starvation	↓Atg1 ≠ *ATG1* mRNA ↓polysomes association of *ATG1* mRNA, ↓Atg13	Pgi-GFP processing assay: ↓ free GFP Pho8Δ60 assay: ↓ vacuolar Pho8Δ60	RIP: *ATG1 & ATG13* mRNA enrichment	(59) #274
ATF4 CHOP	MEFs *ATF4* $^{-/-}$ leucine starvation	≠ *Atg16l1, Map1lc3b, Atg12, Atg3, Becn1, Gabarapl2, p62, Nbr1, Atg7* mRNAs		↑Ⓟ eIF2α	(60) #3
	MEFs *CHOP* $^{-/-}$ leucine starvation	≠ *Atg10, Gabarap, Atg5, p62, Nbr1, Atg7 mRNAs*		↑Ⓟ eIF2α	
PERK ATF4	LNCaP Tunicamycin	↑ LC3-II mTagRFP-mWasabi-LC3: ↑ red punctate ↑ *MAP1LC3B, GABARAPL1, WIPI1, MAPLC3B2, MAPLC3A, ATG13, ATG16L1, GAGARAP, ATG12, ATG5, ATG3, BECN1* mRNA	BafA1: ↑ LC3-II mTagRFP-mWasabi-LC3: ↑ yellow puncta LDH sequestration assay: ↑ sequestration rate LLPD assay: ↑ Valine release		(61) #128
	LNCaP Tunicamycin siATF4	↓ *MAP1LC3B, GABARAPL1, WIPI1, MAPLC3B2, MAPLC3A, ATG13* mRNA	↓↓ Valine release		
eIF2α ATF4	MEFs eIF2Aα non-phosphorylable mutant or *ATF4*$^{-}$/$^{-}$ Rapamycin	↓*Map1lc3 and Atg5* mRNA ↓LC3-II and LC3 puncta	GFP-LC3 processing assay: ↓ free GFP		(62) #130

up-regulation of lysosomal proteins in human colon cancer cells, associated with 4EB-P1 dephosphorylation. The study of autophagy induction was more complete in this work, since in addition of detecting an increased number of autophagosomes, they also found more autolysosomes and lysosomes, as well as a decrease of the adaptor protein p62/SQSTM1, demonstrating the autophagic flux is not interrupted (39). However, further experiments are necessary to elucidate the mechanisms by which 4E-BP1 inhibits autophagy, since Lan et al. found that neither its phosphorylation nor its binding to eIF4E are necessary for the regulation of autophagy (38). Nevertheless, the relevance of 4E-BPs phosphorylation should not yet be ruled out since in the cited work only two out of seven phosphorylation sites were mutated, and other kinases additional to mTOR could also phosphorylate 4E-BPs (63). An alternative mechanism for the negative effect of 4E-BP1 over autophagy could be by stabilization of the mTORC1-ATG13-RB1CC1 complex, leading to autophagy repression at the initiation step. Interestingly, it has recently been described in yeast a repression of translation role for the eIF4E-interacting protein p20 in an eIF4E-independent manner, where p20 remains bound to its mRNAs targets (64).

eIF4E is a key component of the eIF4F complex, and its level and availability limit the translation process. eIF4E phosphorylation is important to promote selective translation of a subset of mRNAs related to proliferation, inflammation, and survival (7). Since autophagy contributes to mitigate various types of stress to avoid cell death, *ATG* mRNAs might belong to the subset of selected mRNAs translated when global translation is inhibited.

Transcriptional Control of Autophagy Mediated by Translation Initiation Factors

During Unfolding Protein Response PERK phosphorylates eIF2alpha, leading to global protein translation shut down but allowing *ATF4* translation. ATF4 is a transcription factor that upregulates expression of stress-responsive genes, including *ATG* genes and *CHOP*. CHOP, likewise, plays a critical role in adaptation to stress and also induces transcription of some *ATG* genes, while a subset of genes needs to be upregulated by both ATF4 and CHOP (60), possibly to ensure a rapid stress relief.

eIF4E regulates ATF4 binding to some promoters, being *MAP1LC3B* (gene coding for LC3) among them. A couple of observations studying different leukemia cell lines suggest that eIF4E can be both a negative and a positive regulator of autophagy by modulating the transcription of *MAP1LC3B,* apparently depending on the function of the tumor suppressor p53 (41, 42). *In vivo*, in a leukemia cell line (NB4) xenograft model treated with the anti-cancer agent selenite, tumors show a reduction of LC3-II and an increase of p62/SQSTM1, which is indicative of autophagy inhibition. Concomitantly, there is activation of caspase 3, indicative of apoptosis induction. In this model p53 signals to induce eIF4E dephosphorylation, preventing the binding of ATF4 to *MAP1LC3B* promoter and hence hampering autophagy (42). In contrast, in a study

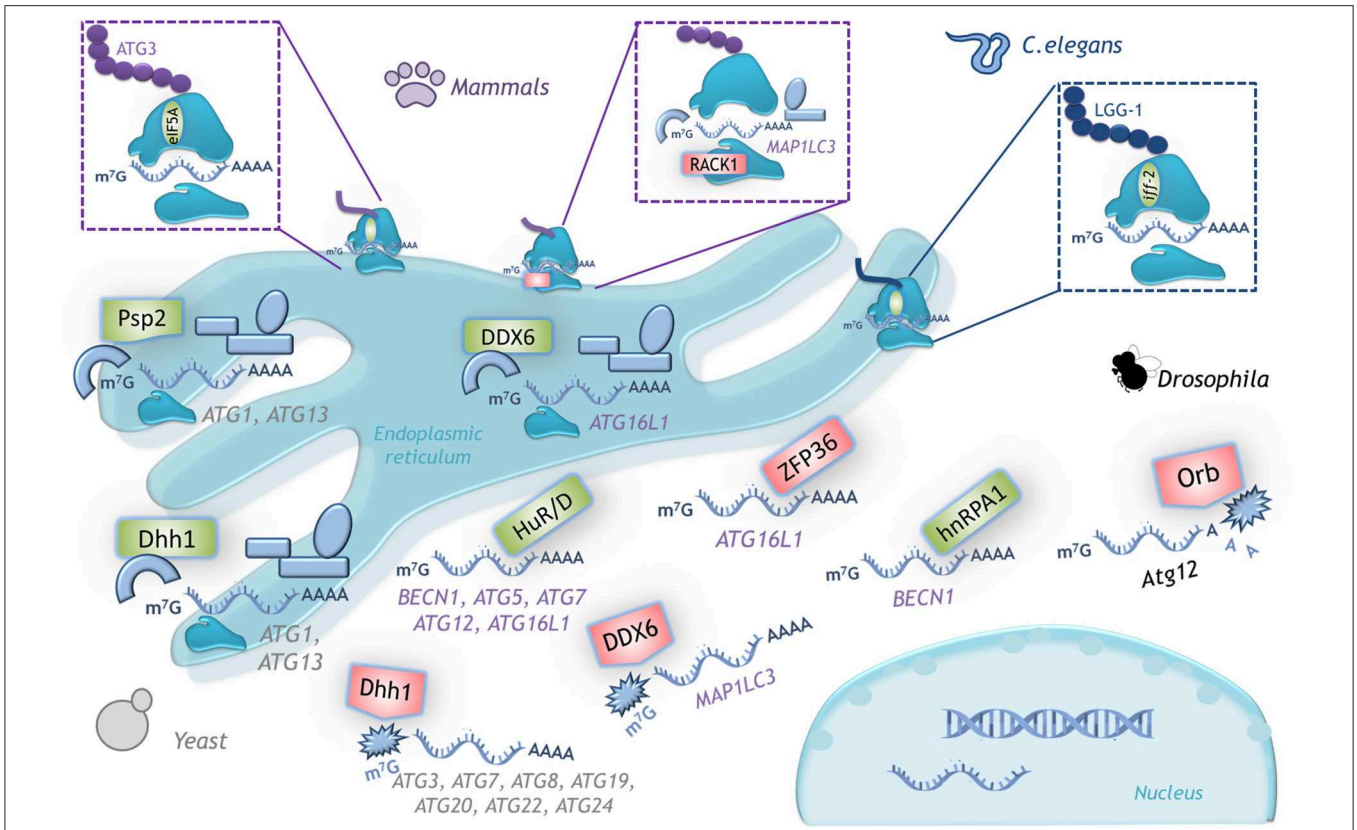

FIGURE 5 | Examples of translational control of *ATG* mRNAs with conserved function in several organisms. A schematic representation of the translation factors that regulate positively (green) or negatively (red) translation of the indicated mRNAs. In yeast, Dhh1 either promotes or represses *ATG* mRNA translation according to the cell nutritional status. In mammals the dual function of DDX6 (Dhh1 homolog) is conserved. RNA binding proteins HuD, HuR and hnRPA1 are positive regulators and ZFP36 is a negative regulator of translation of the indicated mRNAs. The ribosomal protein RACK1 limits LC3 translation, while eIF5A-hypusine targets *ATG3* mRNA to favor autophagosome formation. In *C. elegans iff-2* (eIF5A homolog) is also a positive autophagy regulator. In *Drosophila* Orb promotes deadenylation and decay of its target mRNA. (See text for details).

of selenite treatment of T-cell acute lymphoblastic leukemia, which are p53-deficient, eIF4E is phosphorylated and ATF4 mediates *MAP1LC3B* transcription, leading to an increase of autophagosomes. In this case apoptosis follows autophagy activation (41).

Initially, it was thought that transcriptional regulation of *ATG* genes depends entirely on the PERK/eIF2alpha/ATF4 axis, since upon ER stress, starvation or viral infection of cells bearing an eIF2alpha mutation non-responsive to PERK are incompetent to induce autophagy (62, 65, 66). However, a recent work with a functional assay to evaluate autophagosomes formation as well as cargo degradation, showed that ATF4 indeed induces the transcription of *ATG* genes involved in the formation of autophagosomes, but independent of PERK. PERK activates autophagy at steps subsequent to cargo sequestration in a transcriptional-independent way (61). Although these distinct roles could be cell- or context- dependent, it is important to consider them.

Although it keeps the name, eIF5A acts at the translation elongation phase. It alleviates translational stalling of the ribosome at hard-to-translate motifs. eIF5A enhances *ATG3* mRNA translation, which enhances autophagosome formation, as ATG3 is an E2-like protein necessary for LC3 (and other family members like GABARAP) lipidation. eIF5A has a unique aminoacid, hypusine, formed by post-translational modification of a conserved Lysine residue that is important for ribosome binding and translation. Hypusination of eIF5A is also necessary for autophagy induction (43).

Depletion of members of the scaffold eIF4G protein family, such as eIF4G1 and eIF4G2 (44, 45), or the RNA helicase eIF4A3 (43) cause an accumulation of autophagosomes, but it is still necessary to determine whether this is due to stimulation of autophagy or an impairment of the autophagic flux.

Ribosomal Proteins Control Autophagy

RACK1 is a ribosomal protein component of the 40S subunit that promotes the formation of the 80S ribosome to allow translation. Depletion of RACK1 triggers autophagy induction in tumor-derived cell lines from breast, liver, connective tissue, and bone. Thus, RACK1 is a negative regulator of autophagy; this function depends on its localization at the ribosome, since a mutant unable to bind to the ribosome promotes *MAP1LC3B* mRNA-specific translation (46) (**Figure 5**).

Ribosomal protein RPS27L is also a negative regulator of autophagy. However, the mechanism to prevent autophagy is rather related with an upstream signaling that regulates the activation of autophagy. mTORC1, the main inhibitor of autophagy, is negatively regulated by DEPTOR. In the absence of RPS27L, DEPTOR is stabilized leading to its accumulation, inhibiting mTORC1 activity. Interestingly, RPS27L is reduced in human breast cancer cells compared with adjacent healthy tissue, perhaps having its reduction a promoting role during breast tumorigenesis (47).

RNA Binding Proteins Control Autophagy

The Hu family of RNA binding proteins is effector of several post-transcriptional process of RNA metabolism, ranging from splicing to translation (67, 68). Hu family is composed of four members: HuR, HuB, HuC, and HuD. Interestingly, at least HuR regulates many processes such as inflammation, differentiation, migration, cell death, and as recently found, autophagy (50, 51).

Several *ATG* mRNAs coding for key proteins involved in initiation or elongation phases of autophagy are targets of Hu (**Figure 5**). In non-cancerous and cancerous human liver cells HuR depletion impairs the autophagic flux, with cells having smaller autophagosomes and lysosomes. By ribonucleotide immunoprecipitation it was demonstrated the interaction of HuR with *ATG5*, *ATG12,* and *ATG16* mRNAs; HuR binds to AU-rich elements (AREs) located at the 3'UTR of these mRNAs (50). That HuR enhances *ATG16* mRNA translation was also demonstrated in intestinal epithelium cells *in vitro* and *in vivo* in a mice line with intestinal epithelium-specific ablation of HuR (*IE-HuR*$^{-/-}$); human intestinal mucosa from patients with Inflammatory Bowel Disease exhibit decreased levels of both HuR and ATG16L1, this is an interesting finding since autophagy is frequently defective in those patients (53). HuR induction of *ATG7* and *ATG16* mRNA translation was demonstrated in renal proximal tubular cells during hypoxia-induced autophagy; HuR binds to motifs located within *ATG7* mRNA coding region (51). *BECN1* mRNA also poses AREs at its 3′UTR, and upon starvation HuR stimulates BECN1 translation in non-cancerous keratinocyte, in breast and prostate cancer cells (52), and in human and rat liver stellate cells (49). *BECN1* mRNA translation is also enhanced by RNA binding protein hnRPA1 in human colon cancer cells (55). HuD also induces translation of *ATG5* mRNA. In pancreatic β cells silencing of HuD decreases *ATG5* mRNA translation, and conversely, HuD overexpression enhances *ATG5* mRNA translation (48).

Translational regulation of *ATG* mRNAs by RNA binding proteins is not always positive. The RNA binding protein ZFP36/TTP acts as a negative regulator of *Atg16* mRNA translation during ferroptosis, a type of cell death mediated by autophagy. ZFP36/TTP binds to AREs located at 3′UTR of *ATG16* mRNA and recruits deadenylation and degradations factors (54).

The examples of autophagy regulation by modulating *ATG* mRNAs translation reviewed above refer to conditions found in tumor environment, such as hypoxia and starvation. In some situations autophagy induction favors cancerous cells survival, for example in response to starvation (52) or hypoxia (51), while in other situations autophagy is rather inhibited to evade cell death (49). It is currently unknown what regulates the binding of Hu proteins to target mRNAs. Recently, it was reported that the circular RNA *circPABPN1* blocks the interaction between HuR and *Atg16 mRNA* (53). Whether other Hu/mRNA interactions are also regulated by circRNAs or other mechanisms, such as post-translational modifications (69), or whether it is constitutive under certain circumstances, need to be further studied.

Translational Control of Autophagy in Other Organisms

Autophagy is an evolutionarily conserved process, therefore, it is reasonable to think that its regulation is also conserved across species. Here we review some examples (**Figure 5**).

During *Drosophila* oogenesis, protein Orb negatively modulates translation of *Atg12* mRNA, and thus autophagy (56). Orb belongs to a highly conserved RNA-binding protein family that recognizes cytoplasmic polyadenylation elements located in the 3′-UTR, and can both upregulate or downregulate its target depending on its association with polyadenylases or deadenylases, respectively. Several other autophagy mRNAs also contain cytoplasmic polyadenylation elements (*Atg1, Atg2, Atg5, Atg7, Atg8a,* and *Atg18*), suggesting that Orb might control autophagy at different steps. It has not yet been investigated whether members of the CPEB-family, orthologs of Orb in vertebrates, maintain this regulation. It would be interesting to study if under stress conditions CPEBs associate with polyadenylases to induce autophagy instead of repressing it.

In yeast there is an autophagy regulator with a dual role that can either repress or promote the translation of *ATG* mRNAs, depending on the nutritional status. The RNA helicase Dhh1 under nutrient replete conditions acts as a negative regulator of *ATG* mRNAs that code for proteins participating in almost all stages of the autophagic pathway: initiation (Atg20, Atg24), elongation (Atg3, Atg7, Atg8, Atg19), and recycling (Atg22) (57). Unexpectedly, under nitrogen starvation-conditions Dhh1 switches its function to become a positive regulator of autophagy, and promotes the translation of *ATG1* and *ATG3* mRNAs (58). In mammalian cells there is an ortholog of Dhh1 known as DDX6 that conserved this dual role, however the mRNAs targets are different (57, 58). Also in yeast, the RNA-binding protein Psp2 is a positive translational regulator of autophagy. Under nitrogen-starvation, Psp2 binds the eIF4E/eIF4G complex and the 5′-UTR of *ATG1* and *ATG13* mRNAs to promote their translation (70).

The positive relationship between eIF5A and autophagy stimulation is also conserved in *C. elegans*. Worms deficient on *iff-2* (eIF5A homolog) show a decreased punctate pattern of the GFP::LGG-1 (an LC3 ortholog) fusion protein (43). Considering that protein translation integrates signaling from a wide variety of stimuli, to couple autophagy regulation with protein synthesis is essential.

TABLE 3 | Autophagy induction by the PERK/eIF2alpha/ATF4 axis in different cancer models.

Neoplasia	Cells/model	Inducer	Reference
Glioblastoma	Multiple human glioblastoma cells	Melanoma differentiation associated gene-7/interleukin 24 (GST-MDA-7/IL-24)	(72)
	U87MG	Glucosamine-induced ER stress	(73)
	Primary glioblastoma human multiforme cells	Recombinant Melanoma differentiation associated gene-7 (*mda-7*) adenovirus (Ada. *mda-7*)	(74)
Ovarian cancer	Epitelial human Pa-1 cells	Metformin-induced ER stress	(75)
Breast cancer	Human MCF-7 cells	Ursolic acid-induced ER stress	(76)
Neural radiation myelitis (spinal metastasis)	Banna mini-pigs spinal cord cells	Iodine-125-induced ER stress	(77)
Leukemia	Human acute promyelitic leukemia NB4 cells	Selenite-induced ER stress	(42)
Bone cancer	Human osteosarcoma MG63 and KHOS cells	2-methoxyestradiol	(78)
	Human osteosarcoma MG63 cells	Thapsigargin-induced ER stresses	(79)
MYC-induced tumorigenesis	Human B-cell lymphoma P493-6B cells and mouse embryonic fibroblast	c-Myc-induced ER stresses	(80)

TARGETING TRANSLATION AND AUTOPHAGY IN CANCER

Traditionally, most studies on cancer have focused on the processes occurring at the DNA level, such as mutations and chromosomal rearrangements, variation in chromatin methylation, and transcriptional dysregulation. In the last years, new evidence has emerged supporting the notion that cancer may also result from failures in the interconnection among different metabolic networks and molecular processes that underlie even disparate diseases (1). Studies on the interplay between translation and autophagy have led to the identification of new molecules that can be chemically targeted with clinical implications in the treatment of several types of cancer. Here we mention few examples.

Targeting the PERK/eIF2alpha/ATF4 Axis

Recently, the PERK/eIF2alpha/ATF4 axis has been involved in the onset and development of different types of cancer. For example, ER stress-mediated PKR activation regulates the induction of autophagy during tumorigenesis in carcinoma, gastric adenocarcinoma, and melanoma cells. When PERK is inhibited either pharmacologically with the drug GSK2606414 or genetically by using siRNA to silence PERK expression, decreased both LC3 expression and LC3-II lipidation (71). Additional examples of autophagy induction by the PERK/eIF2alpha/ATF4 axis in different cancer models are summarized in **Table 3**.

The Akt/mTOR Pathway

Research in different kinds of cancer has focused mainly on mTOR or the Akt/mTOR pathway (81–84), which are signaling cascades shared between translation and autophagy. Here we review examples of molecules currently tested targeting this pathway (squematized in **Figure 4**).

Studies in glioma cells have shown that celastrol possess antitumor effects. It inactivates mTOR, drives cell cycle G2/M phase arrest, autophagosomes accumulation apparently due to lysosomes impaired function, and apoptosis (85). Studies with rapamycin in various cancer cell lines showed that it increases the number of LC3 puncta suggesting autophagy induction (86, 87), but not apoptosis, and this effect is synergized in combination with PI3K or AKT inhibitors (86). However, neuroblastoma or squamous cell carcinoma seem to be resistant to autophagy induction mediated by rapamycin, apparently because RAPTOR maintains bound to mTOR, and these cells are sensitized only when they are treated with mTOR catalytic inhibitors (87). This finding suggests that using combined therapies could be more effective or even necessary to treat certain types of cancer.

The use of quercetin, a flavonoid present in fruits and vegetables, inactivates the AKT/mTOR pathway and induces HIF-1alpha signaling in gastric cancer, promoting simultaneously apoptosis and protective autophagy. In this case inhibition of autophagy reduces cell viability (88). Also in a study of breast cancer, quercetin reduced cell invasion, and migration by inactivating also Akt/mTOR pathway and leading to an apparently autophagy induction. It is interesting to note that the mechanism to reduce breast cancer cells migration could be due to a quercetin-reduced expression of matrix metalloproteinase 9, and this reduction is abrogated when autophagy is inhibited, suggesting a role of autophagy regulating metalloproteinases availability (89). Since autophagy machinery can also contribute to alternative secretion (90), autophagy could regulate metalloproteinasses maturation and/or secretion. This particular non-catabolic function of autophagy needs to be further investigated in cancer research. Since autophagy in these cases is induced in response to quercetin and favors tumor progression, a pharmacological combination with autophagy inhibitors could increase quercetine effectivity.

On the other hand, the Akt inhibitor 1L-6-hydroxymethyl-chiro-inositol 2(R)-2-O-methyl-3-O-octadecylcarbonate shows radiosensitizing effects in malignant glioma cells by apparently inducing autophagy, with an overall outcome of anti-tumorigenesis (91). Curcumin also inhibits the Akt/mTOR/p70S6K pathway and activates the ERK1/2 pathway,

TABLE 4 | Therapeutic compounds used for autophagy induction or inhibition in cancer.

Compound	Target	Model system	Autophagy evaluation	References
GSK2606414	PERK	Basal cell carciona (BCC/KMC1) Gastric Adenocarcinoma (AGS) Melanoma (A375) Imiquimod	↓LC3-II ↓EGFP-LC3-II puncta	(71)
Celastrol	mTOR	Glioma (U251, U87 and C6) Pre-treatment CQ	↑LC3, BECN1,p62 ↑LC3 puncta ≠ LC3-II	(85)
LY294002 UCN-01 (7-hydroxystaurosporine)	PI3K Akt	Glioma (U87-MG, U373-MG and T98G) Rapamycin	↑MDC stain	(86)
Rapamycin RAD001 (rapalogue) KU-0063794 (catalytic mTOR inhibitor) WYE-354 (catalytic mTOR inhibitor)	mTOR	Bladder carcinona (RT112) osteosarcoma (U2OS) neuroblastoma (SK-N-SH) squamous cell carcinoma (HN10)	↑LC3 punctate (all treatments) ↑LC3 punctate (only with catalytic mTOR inhibitors)	(87)
AZD8055	mTOR	lung cancer H838 and A549 +E64d/pepstatinA	↑LC3-II ↑LC3 puncta ↑Acridine orange stain ↑↑LC3-II	(93)
Metformin	AMPK	Melanoma (A375, and SKMel28) Xenograft model	↑LC3-II, BECN1 ↑LC3 puncta ↑Autophagosomes (TEM) ↑ CatB activity ↑LC3-II ↑LC3 puncta	(94)
Metformin	mTOR	Esophageal squamous cancer cells (ESCC) Pre-treatment 3-MA or CQ Xenograft	↑LC3-II, BECN1 ↑GFP-LC3 puncta ↑ Autophagosomes (TEM) ↑ Acridine orange and MDC stain ↓LC3-II, BECN1 ↓LC3-II, ↓p62 (IHC)	(95)
Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol 2(R)-2-O-methyl-3-O-octadecylcarbonate)	AKT	Glioma U87-MG U87-MG Δ*EGFR*	↑GFP-LC3 punctate ↑autophagosomes (TEM), ↑Acridine orange stain	(91)
Curcumin	Akt/mTOR/p70S6K/4E-BP	Glioma U87-MG and U373-MG Xenograft	↑GFP-LC3 puncta ↑LC3-II ↑autophagosomes (TEM) ↑ Acridine orange stain ↑LC3-II, ↑LC3 (IHC)	(92)
Quercetin	Akt-mTOR	Breast cancer MCF-7 and MB-231	↑LC3-II, LC3 puncta	(89)
Quercetin	Akt-mTOR	Gastric adenocarcinoma AGS and MKN28	↑ LC3-II, BECN1, ATG7, ATG5/12 ↑ GFP-LC3 puncta ↑ Autophagosomes (TEM) ↑ Acridine orange stain	(88)
AZD1208	pan-PIM kinase inhibitor	Chronic lymphocytic leukemia (CLL) BafA1	↑Acridine orange stain, ↑LC3-II ↑↑LC3-II	(96)
Parthenolide	Oxidative stress downregulation of 4E-BP1	See **Table 2**	See **Table 2**	(38)
Selenite	eIF2alpha phosphorylation by ROS or ER stress	See **Table 2**	See **Table 2**	(41, 42)
Erastin	Ferroptosis inducer	See **Table 2**	See **Table 2**	(49, 54)

resulting in autophagy induction both *in vitro* and *in vivo*. In a subcutaneous xenograft model of U87-MG cells, curcumin induces autophagy and inhibits tumor growth (92).

A summary of compounds targeting translation and autophagy in cancer is presented in **Table 4**.

CONCLUDING REMARKS

A common feature of cancerous cells is having aberrant translation, as many oncogenes and tumor suppressors affect the translation machinery. Many translation initiation factors

are dysregulated in various cancers, and increased levels of eIF4F complex render cancer cells resistant to chemotherapeutics (7). Considering also that protein synthesis is coupled to autophagy regulation, targeting translation factors is a promising therapy that could at same time reduce autophagy induction. Nevertheless, as reviewed above, even though in early tumor environments under hypoxia and low nutrients availability autophagy induction favors cancerous cell survival, in other cancerous cells autophagy is rather inhibited to evade cell death. Therefore, it is not possible to generalize the use of autophagy inhibitors to treat cancer. A characterization of the function of autophagy in particular types of cancer is necessary.

Once the specific function of autophagy is known, targeting autophagy machinery to modulate its function could complement chemotherapy to increase its effectiveness. Most recently, novel strategies to treat cancer have been developed that utilize nanoparticles to target mTOR and AMP-activated protein kinase (AMPK) pathways. These nanoparticles, made up of different metal or silica materials, are designed to overcome obstacles usually encountered with traditional drugs, such as low specificity, irregular distribution in tissues and organs, rapid drug clearance, and biodegradation. The clinical relevance of these innovative therapies is currently being evaluated (97).

AUTHOR CONTRIBUTIONS

GH conceived the manuscript, gathered information, and wrote part of the paper. PA-R gathered information, wrote part of the paper, assembled **Tables 1**, **2**, **4**, and did **Figures 2**–**5**. GM gathered information, and contributed to assemble **Tables 2**–**4**, and did **Figure 1**. SC-O gathered information, wrote part of the paper, and integrated the information.

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REFERENCES

1. Le A, Udupa S, Zhang C. *The metabolic interplay between cancer and other diseases. Trends Cancer*. (2019) 5:809–21. doi: 10.1016/j.trecan.2019.10.012
2. Maier T, Güell M, Serrano L. Correlation of mRNA and protein in complex biological samples. *FEBS Lett.* (2009) 583:3966–73. doi: 10.1016/j.febslet.2009.10.036
3. Hernández G, Tettweiler G. Protein abundance variation. In: Meyer RA, editor. *Systems Biology*. Weinheim: Wiley-Blackwell (2012). p. 117–37. doi: 10.1002/3527600906.mcb.201100039
4. de Sousa Abreu R, Penalva LO, Marcotte EM, Vogel C. Global signatures of protein and mRNA expression levels. *Mol BioSyst.* (2014) 5:1512–26. doi: 10.1039/b908315d
5. Hershey JWB, Sonenberg N, Mathews MB. Principles of translational control. *Cold Spring Harb Perspect Biol.* (2018) 2018:a032607. doi: 10.1101/cshperspect.a032607
6. Villa N, Fraser CS. Diverse mechanisms of translation regulation and their role in cancer. In: Parsyan A, editor. *Translation and Its Regulation in Cancer Biology and Medicine*. (Dodrecht; New York, NY; London: Springer (2014). p. 39–72. doi: 10.1007/978-94-017-9078-9_3
7. Bhat M, Robichaud N, Hulea L, Sonenberg N, Pelletier J, Topisirovic I. Targeting the translation machinery in cancer. *Nat Rev Drug Discovery*. (2015) 14:261–78. doi: 10.1038/nrd4505
8. Truitt ML, Ruggero D. New frontiers in translational control of the cancer genome. *Nat Rev Cancer*. (2016) 16:288–304. doi: 10.1038/nrc.2016.27
9. Ali MU, Rahman MS, Jia Z, Jiang C. Eukaryotic translation initiation factors and cancer. *Tumor Biol.* (2017) 39:1010428317709805. doi: 10.1177/1010428317709805
10. Robichaud N, Sonenberg N, Ruggero D, Schneider RJ. Translational control in cancer. *Cold Spring Harb Perspect Biol.* (2018) 11:a032896. doi: 10.1101/cshperspect.a032896
11. Sonenberg N, Hinnebusch AG. New modes of translational control in development, behavior, and disease. *Mol Cell.* (2007) 28:721–9. doi: 10.1016/j.molcel.2007.11.018
12. Tahmasebi S, Khoutorsky A, Mathews MB, Sonenberg N. Translation deregulation in human disease. *Nat Rev Mol Cell Biol.* (2018) 19:791–807. doi: 10.1038/s41580-018-0034-x
13. Hinnebusch AG. The scanning mechanism of eukaryotic translation initiation. *Annu Rev Biochem.* (2014) 83:779–812. doi: 10.1146/annurev-biochem-060713-035802
14. Pelletier J, Sonenberg N. The organizing principles of eukaryotic ribosome recruitment. *Annu Rev Biochem.* (2019) 88:307–35. doi: 10.1146/annurev-biochem-013118-111042
15. Jackson RJ, Hellen CU, Pestova TV. The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat Rev Mol Cell Biol.* (2010) 11:113–27. doi: 10.1038/nrm2838
16. Proud CG. Phosphorylation and signal transduction pathways in translational control *Cold Spring Harb Perspect Biol.* (2018) 2018:a033050. doi: 10.1101/cshperspect.a033050
17. Roux PP, Topisirovic I. Signaling pathways involved in the regulation of mRNA translation. *Mol Cell Biol.* (2018) 38:e00070–18. doi: 10.1128/MCB.00070-18
18. Berlanga JJ, de Haro C, Rodríguez-Gabriel MA, Ventoso I. eIF2alpha kinases and the evolution of stress response in eukaryotes. In: Hernández G, Jagus R, editors. *Evolution of the Protein Synthesis Machinery and Its Regulation*. Switzerland: Springer (2016). p. 261–76. doi: 10.1007/978-3-319-39468-8_12
19. Wek R. Role of eIF2α kinases in translational control and adaptation to cellular stress. *Cold Spring Harb Perspect Biol.* (2018) 10:a032870. doi: 10.1101/cshperspect.a032870
20. Fonseca B, Graber TE, Hoang H-D, González A, Soukas AA, Hernández G, et al. TOR and translation control in evolution. In: Hernández G, Jagus R, editors. *Evolution of the Protein Synthesis Machinery and Its Regulation*. Springer (2016) 327–412. doi: 10.1007/978-3-319-39468-8_15
21. Gaubitz C, Prouteau M, Kusmider B, Loewith R. TORC2 structure and function. *Trends Biochem Sci.* (2016) 41:532–45. doi: 10.1016/j.tibs.2016.04.001
22. Xie J, Wang X, Proud CG. Who does TORC2 talk to? *Biochem J.* (2018) 475:1721–38. doi: 10.1042/BCJ20180130

23. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol.* (2011) 13:132–41. doi: 10.1038/ncb2152
24. Zachari M, Ganley IG. The mammalian ULK1 complex and autophagy initiation. *Essays Biochem.* (2017) 61:585–96. doi: 10.1042/EBC20170021
25. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, et al. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol Cell.* (2008) 30:214–26. doi: 10.1016/j.molcel.2008.03.003
26. Wong PM, Feng Y, Wang J, Shi R, Jiang X. Regulation of autophagy by coordinated action of mTORC1 and protein phosphatase 2A. *Nat Commun.* (2015) 6:8048. doi: 10.1038/ncomms9048
27. Torii S, Yoshida T, Arakawa S, Honda S, Nakanishi A, Shimizu S. Identification of PPM1D as an essential Ulk1 phosphatase for genotoxic stress-induced autophagy. *EMBO Rep.* (2016) 17:1552–64. doi: 10.15252/embr.201642565
28. Tekirdag K, Cuervo AM. Chaperone-mediated autophagy and endosomal microautophagy: joint by a chaperone. *J Biol Chem.* (2018) 293:5414–24. doi: 10.1074/jbc.R117.818237
29. Kaur J, Debnath J. Autophagy at the crossroads of catabolism and anabolism. *Nat Rev Mol Cell Biol.* (2015) 16:461–72. doi: 10.1038/nrm4024
30. Kenific CM, Debnath J. Cellular and metabolic functions for autophagy in cancer cells. *Trends Cell Biol.* (2015) 25:37–45. doi: 10.1016/j.tcb.2014.09.001
31. Bishop E, Bradshaw TD. Autophagy modulation: a prudent approach in cancer treatment? *Cancer Chemother Pharmacol.* (2018) 82:913–22. doi: 10.1007/s00280-018-3669-6
32. Santana-Codina N, Mancias JD, Kimmelman AC. The role of autophagy in cancer. *Annu Rev Cancer Biol.* (2017) 1:19–39. doi: 10.1146/annurev-cancerbio-041816-122338
33. Dikic I, Elazar Z. Mechanism and medical implications of mammalian autophagy. *Nat Rev Mol Cell Biol.* (2018) 19:349–64. doi: 10.1038/s41580-018-0003-4
34. Yu L, Chen Y, Tooze SA. Autophagy pathway: cellular and molecular mechanisms. *Autophagy.* (2018) 14:207–15. doi: 10.1080/15548627.2017.1378838
35. Takahashi Y, He H, Tang Z, Hattori T, Liu Y, Young MM, et al. An autophagy assay reveals the ESCRT-III component CHMP2A as a regulator of phagophore closure. *Nat Commun.* (2018) 9:2855. doi: 10.1038/s41467-018-05254-w
36. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy.* (2016) 12:1–222. doi: 10.1080/15548627.2015.1100356
37. Balakumaran BS, Porrello A, Hsu DS, Glover W, Foye A, Leung JY, et al. MYC Activity mitigates response to rapamycin in prostate cancer through eukaryotic initiation factor 4E-binding protein 1-mediated inhibition of autophagy. *Cancer Res.* (2009) 69:7803–10. doi: 10.1158/0008-5472.CAN-09-0910
38. Lan BL, Wan YJ, Pan S, Wang Y, Yang Y, Leng QL, et al. Parthenolide induces autophagy via the depletion of 4E-BP1. *Biochem Biophys Res Comm.* (2015) 456:434–9. doi: 10.1016/j.bbrc.2014.11.102
39. Lai MC, Chang CM, Sun HS. Hypoxia induces autophagy through translational up-regulation of lysosomal proteins in human colon cancer cells. *PLoS ONE.* (2016) 11:e0153627. doi: 10.1371/journal.pone.0153627
40. Gao Q, Hou B, Yang H, Jiang X. Distinct role of 4E-BP1 and S6K1 in regulating autophagy and hepatitis B virus (HBV) replication. *Life Sci.* (2019) 220:1–7. doi: 10.1016/j.lfs.2019.01.039.s
41. Jiang Q, Li F, Shi K, Wu P, An J, Yang Y, et al. ATF4 activation by the p38MAPK-eIF4E axis mediates apoptosis and autophagy induced by selenite in Jurkat cells. *FEBS Lett.* (2013) 587:2420–9. doi: 10.1016/j.febslet.2013.06.011
42. Jiang Q, Li F, Shi K, Wu PJ, An J, Yang Y, et al. (2014). Involvement of p38 in signal switching from autophagy to apoptosis via the PERK/eIF2α/ATF4 axis in selenite-treated NB4 cells. *Cell Death Dis.* 5:e1270. doi: 10.1038/cddis.2014.200
43. Lubas M, Harder LM, Kumsta C, Tiessen I, Hansen M, Andersen JS, et al. eIF5A is required for autophagy by mediating ATG3 translation. *EMBO Rep.* (2018) 19:e46072. doi: 10.15252/embr.201846072
44. Ramírez-Valle F, Braunstein S, Zavadil J, Formenti SC, Schneider RJ. eIF4GI links nutrient sensing by mTOR to cell proliferation and inhibition of autophagy. *J Cell Biol.* (2008) 181:293–307. doi: 10.1083/jcb.200710215
45. Badura M, Braunstein S, Zavadil J, Schneider RJ. DNA damage and eIF4G1 in breast cancer cells reprogram translation for survival and DNA repair mRNAs. *Proc Natl Acad Sci USA.* (2012) 109:18767–72. doi: 10.1073/pnas.1203853109
46. Kim HD, Kong E, Kim Y, Chang JS, Kim J. RACK1 depletion in the ribosome induces selective translation for non-canonical autophagy. *Cell Death Dis.* (2017) 8:e2800. doi: 10.1038/cddis.2017.204
47. Xiong X, Liu X, Li H, He H, Sun Y, Zhao Y. Ribosomal protein S27-like regulates autophagy via the beta-TrCP-DEPTOR-mTORC1 axis. *Cell Death Dis.* (2018) 9:1131. doi: 10.1038/s41419-018-1168-7
48. Kim C, Kim W, Lee H, Ji E, Choe YJ, Martindale J, et al. The RNA-binding Protein HuD regulates autophagosome formation in pancreatic beta cells by promoting autophagy-related gene 5 expression. *J Biol Chem.* (2014) 289:112–21. doi: 10.1074/jbc.M113.474700
49. Zhang Z, Yao Z, Wang L, Ding H, Shao J, Chen A, et al. Activation of ferritinophagy is required for the RNA-binding protein ELAVL1/HuR to regulate ferroptosis in hepatic stellate cells. *Autophagy.* (2018) 14:2083–103. doi: 10.1080/15548627.2018.1503146
50. Ji E, Kim C, Kang H, Ahn S, Jung M, Hong Y, et al. RNA binding protein HuR promotes autophagosome formation by regulating expression of autophagy-related proteins 5, 12, and 16 in human hepatocellular carcinoma cells. *Mol Cell Biol.* (2019) 39:18. doi: 10.1128/MCB.00508-18
51. Palanisamy K, Tsai TH, Yu TM, Sun KT, Yu SH, Lin FY, et al. RNA-binding protein, human antigen R regulates hypoxia-induced autophagy by targeting ATG7/ATG16L1 expressions and autophagosome formation. *J Cell Physiol.* (2019) 234:7448–58. doi: 10.1002/jcp.27502
52. De S, Das S, Sengupta S. Involvement of HuR in the serum starvation induced autophagy through regulation of Beclin1 in breast cancer cell-line, MCF-7. *Cell Signal.* (2019) 61:78–85. doi: 10.1016/j.cellsig.2019.05.008
53. Li XX, Xiao L, Chung HK, Ma XX, Liu X, Song JL, et al. Interaction between HuR and circPABPN1 modulates autophagy in the intestinal epithelium by altering ATG16L1 translation. *Mol Cell Biol.* (2019). doi: 10.1128/MCB.00492-19
54. Zhang Z, Guo M, Li Y, Shen M, Kong D, Shao J, et al. RNA-binding protein ZFP36/TTP protects against ferroptosis by regulating autophagy signaling pathway in hepatic stellate cells. *Autophagy.* (2019). doi: 10.1080/15548627.2019.1687985. [Epub ahead of print].
55. Ji E, Lee H, Ahn S, Jung M, Lee SH, Lee JH, et al. Heterogeneous nuclear ribonucleoprotein A1 promotes the expression of autophagy-related protein 6 in human colorectal cancer. *Biochem Biophys Res Commun.* (2019) 513:255–60. doi: 10.1016/j.bbrc.2019.03.179
56. Rojas-Ríos P, Chartier A, Pierson S, Severac D, Dantec C, Busseau I, et al. Translational control of autophagy by Orb in the *Drosophila* germline. *Dev Cell.* (2015) 35:622–31. doi: 10.1016/j.devcel.2015.11.003
57. Hu G, McQuiston T, Bernard A, Park YD, Qiu J, Vural A, et al. A conserved mechanism of TOR-dependent RCK-mediated mRNA degradation regulates autophagy. *Nat Cell Biol.* (2015) 17:930–42. doi: 10.1038/ncb3189
58. Liu X, Jin M, Yao Z, Bernard A, Klionsky DJ. Bidirectional roles of Dhh1 in regulating autophagy. *Autophagy.* (2019) 15:1838–9. doi: 10.1080/15548627.2019.1621632
59. Yin Z, Liu X, Ariosa A, Huang H, Jin M, Karbstein K, et al. Psp2, a novel regulator of autophagy that promotes autophagy-related protein translation. *Cell Res.* (2019) 29:994–1008. doi: 10.1038/s41422-019-0246-4
60. B'chirt W, Maurin AC, Carraro V, Averous J, Jousse C, Muranishi Y, et al. The eIF2a/ATF4 pathway is essential for stress-induced autophagy gene expression. *Nucleic Acid Res.* (2013) 41:7683–99. doi: 10.1093/nar/gkt563
61. Luhr M, Torgersen ML, Szalai P, Hashim A, Brech A, Staerk J, et al. The kinase PERK and the transcription factor ATF4 play distinct and essential roles in autophagy resulting from tunicamycin-induced ER stress. *J Biol Chem.* (2019) 294:8197–217. doi: 10.1074/jbc.RA118.002829
62. Wengrod J, Wang D, Weiss S, Zhong H, Osman I, Gardner LB. Phosphorylation of eIF2alpha triggered by mTORC1 inhibition and PP6C activation is required for autophagy and is aberrant in PP6C-mutated melanoma. *Sci Signal.* (2015) 8:27. doi: 10.1126/scisignal.aaa0899
63. Qin X, Jiang B, Zhang Y. 4E-BP1, a multifactor regulated multifunctional protein. *Cell Cycle.* (2016) 15:781–6. doi: 10.1080/15384101.2016.1151581

64. Castelli LM, Talavera D, Kershaw CJ, Mohammad-Qureshi SS, Costello JL, Rowe W, et al. The 4E-BP Caf20p mediates both eIF4E-dependent and independent repression of translation. *PLoS Genet.* (2015) 11:e1005233. doi: 10.1371/journal.pgen.1005233
65. Tallóczy Z, Jiang W, Virgin HW, Leib DA, Scheuner D, Kaufman RJ, et al. Regulation of starvation- and virus-induced autophagy by the eIF2alpha kinase signaling pathway. *Proc Natl Acad Sci USA*. (2002) 99:190–5. doi: 10.1073/pnas.012485299
66. Pakos-Zebrucka K, Koryga I, Mnich K, Ljujic M, Samali A, Gorman AM. The integrated stress response. *EMBO Rep.* (2016) 17:1374–95. doi: 10.15252/embr.201642195
67. Srikantan S, Gorospe M. HuR function in disease. *Front Biosci.* (2015) 17:189–205. doi: 10.2741/3921
68. Velasco MX, Kosti A, Penalva LOF, Hernández G. The diverse roles of RNA-binding proteins in glioma development. *Adv Exp Med Biol.* (2018) 1157:29–39. doi: 10.1007/978-3-030-19966-1_2
69. Grammatikakis I, Abdelmohsen K, Gorospe M. Posttranslational control of HuR function. *Wiley Interdiscip Rev RNA*. (2017) 8:1372. doi: 10.1002/wrna.1372
70. Yin X, Hu L, Feng X, Wang H, Zhang C, Wang H, et al. Simultaneous activation of impaired autophagy and the mammalian target of rapamycin pathway in oral squamous cell carcinoma. *J Oral Pathol Med*. (2019) 48:12884. doi: 10.1111/jop.12884
71. Chang SH, Huang SW, Wang ST, Chung KC, Hsieh CW, Kao JK, et al. Imiquimod-induced autophagy is regulated by ER stress-mediated PKR activation in cancer cells. *J Dermatol Sci.* (2017) 87:138–48. doi: 10.1016/j.jdermsci.2017.04.011
72. Park MA, Yacoub A, Sarkar D, Emdad L, Rahmani M, Spiegel S, et al. PERK-dependent regulation of MDA-7/IL-24-induced autophagy in primary human glioma cells. *Autophagy.* (2008) 4:513–5. doi: 10.4161/auto.5725
73. Hwang MS, Baek WK. Glucosamine induces autophagic cell death through the stimulation of ER stress in human glioma cancer cells. *Biochem Biophys Res Commun.* (2010) 399:111–6. doi: 10.1016/j.bbrc.2010.07.050
74. Yacoub A, Hamed HA, Allegood J, Mitchell C, Spiegel S, Lesniak MS, et al. PERK-dependent regulation of ceramide synthase 6 and thioredoxin play a key role in mda-7/IL-24-induced killing of primary human glioblastoma multiforme cells. *Cancer Res.* (2010) 70:1120–9. doi: 10.1158/0008-5472.CAN-09-4043
75. Moon HS, Kim B, Gwak H, Suh DH, Song YS. Autophagy and protein kinase RNA-like endoplasmic reticulum kinase (PERK)/eukaryotic initiation factor 2 alpha kinase (eIF2α) pathway protect ovarian cancer cells from metformin-induced apoptosis. *Mol Carcinog.* (2015) 55:346–56. doi: 10.1002/mc.22284
76. Zhao C, Yin S, Dong Y, Guo X, Fan L, Ye M, et al. Autophagy-dependent EIF2AK3 activation compromises ursolic acid-induced apoptosis through upregulation of MCL1 in MCF-7 human breast cancer cells. *Autophgy.* (2013) 9:196–207. doi: 10.4161/auto.22805
77. Yang Z, Xu Y, Xu L, Maccauro G, Rossi B, Chen Y, et al. Regulation of autophagy via PERK-eIF2α effectively relieve the radiation myelitis induced by iodine-125. *PLoS ONE.* (2013) 8:e76819. doi: 10.1371/journal.pone.0076819
78. Yang C, Shogren KL, Goyal R, Bravo D, Yaszemski MJ, Maran A. RNA-dependent protein kinase is essential for 2-methoxyestradiol-induced autophagy in osteosarcoma cells. *PLoS ONE.* (2013) 8:e59406. doi: 10.1371/journal.pone.0059406
79. Ji GR, Yu NC, Xue X, Li ZG. PERK-mediated autophagy in osteosarcoma cells resists ER stress-induced cell apoptosis. *Int J Biol Sci.* (2015) 11:803–12. doi: 10.7150/ijbs.11100
80. Hart LS, Cunningham JT, Datta T, Dey S, Tameire F, Lehman SL, et al. ER stress-mediated autophagy promotes Myc-dependent transformation and tumor growth. *J Clin Inv.* (2012) 122:4621–34. doi: 10.1172/JCI62973
81. Dowling RJ, Pollak M, Sonenberg N. Current status and challenges associated with targeting mTOR for cancer therapy. *BioDrugs.* (2009) 23:77–91. doi: 10.2165/00063030-200923020-00002
82. Dowling RJO, Topisirovic I, Fonseca B, Sonenberg N. Dissecting the role of mTOR: Lessons from mTOR inhibitors. *Biochim Biophys Acta.* (2009) 1804:433–9. doi: 10.1016/j.bbapap.2009.12.001
83. Chiarini F, Evangelisti C, McCubrey JA, Martelli AM. Current treatment strategies for inhibiting mTOR in cancer. *Trends Pharmacol. Sci.* (2015) 36:124–35. doi: 10.1016/j.tips.2014.11.004
84. Cuomo F, Altucci L, Cobellis G. Autophagy function and dysfunction: potential drugs as anti-cancer therapy. *Cancers.* (2019) 11:1465. doi: 10.3390/cancers11101465
85. Liu X, Zhao P, Wang X, Wang L, Zhu Y, Song Y, et al. Celastrol mediates autophagy and apoptosis via the ROS/JNK and Akt/mTOR signaling pathways in glioma cells. *J Exp Clin Cancer Res.* (2019) 38:184. doi: 10.1186/s13046-019-1285-x
86. Takeuchi H, Kondo Y, Fujiwara K, Kanzawa T, Aoki H, Mills G, et al. Synergistic augmentation of rapamycin-induced autophagy in malignant glioma cells by phosphatidylinositol 3-kinase/protein kinase B inhibitors. *Cancer Res.* (2005) 65:3336–46. doi: 10.1158/0008-5472.CAN-04-3640
87. Nyfeler B, Bergman P, Triantafellow E, Wilson CJ, Zhu Y, Radetich B, et al. Relieving autophagy and 4EBP1 from rapamycin resistance. *Mol Cell Biol.* (2011) 31:2867–76. doi: 10.1128/MCB.05430-11
88. Wang K, Liu R, Li J, Mao J, Lei Y, Wu J, et al. Quercetin induces protective autophagy in gastric cancer cells: involvement of Akt-mTOR- and hypoxia-induced factor 1alpha-mediated signaling. *Autophagy.* (2011) 7:966–78. doi: 10.4161/auto.7.9.15863
89. Jia L, Huang S, Yin X, Zan Y, Guo Y, Han L. Quercetin suppresses the mobility of breast cancer by suppressing glycolysis through Akt-mTOR pathway mediated autophagy induction. *Life Sci.* (2018) 208:123–30. doi: 10.1016/j.lfs.2018.07.027
90. Ponpuak M, Mandell MA, Kimura T, Chauhan S, Cleyrat C, Deretic V. Secretory autophagy. *Curr Opin Cell Biol.* (2015) 35:106–16. doi: 10.1016/j.ceb.2015.04.016
91. Fujiwara K, Iwado E, Mills GB, Sawaya R, Kondo S, Kondo Y. Akt inhibitor shows anticancer and radiosensitizing effects in malignant glioma cells by inducing autophagy. *Int J Oncol.* (2007) 31:753–60. doi: 10.3892/ijo.31.4.753
92. Aoki H, Takada Y, Kondo S, Sawaya R, Aggarwal BB, Kondo Y. Evidence that curcumin suppresses the growth of malignant gliomas in vitro and in vivo through induction of autophagy: role of Akt and extracellular signal-regulated kinase signaling pathways. *Mol Pharmacol.* (2007) 72:29–39. doi: 10.1124/mol.106.033167
93. Chresta CM, Davies BR, Hickson I, Harding T, Cosulich S, Critchlow SE, et al. AZD8055 is a potent, selective, and orally bioavailable ATP-competitive mammalian target of rapamycin kinase inhibitor with *in vitro* and *in vivo* antitumor activity. *Cancer Res.* (2010) 70:288–98. doi: 10.1158/0008-5472.CAN-09-1751
94. Tomic T, Botton T, Cerezo M, Robert G, Luciano F, Puissant A, et al. Metformin inhibits melanoma development through autophagy and apoptosis mechanisms. *Cell Death Dis.* (2011) 2:e199. doi: 10.1038/cddis.2011.86
95. Feng Y, Ke C, Tang Q, Dong H, Zheng X, Lin W, et al. Metformin promotes autophagy and apoptosis in esophageal squamous cell carcinoma by downregulating Stat3 signaling. *Cell Death Dis.* (2014) 5:e1088. doi: 10.1038/cddis.2014.59
96. Cervantes-Gomez F, Stellrecht CM, Ayres ML, Keating MJ, Wierda WG, Gandhi V. PIM kinase inhibitor, AZD1208, inhibits protein translation and induces autophagy in primary chronic lymphocytic leukemia cells. *Oncotarget.* (2019) 10:2793–809. doi: 10.18632/oncotarget.26876
97. Cordani M, Somoza A. Targeting autophagy using metallic nanoparticles: a promising strategy for cancer treatment. *Cell Mol Life Sci.* (2019) 76:1215–42. doi: 10.1007/s00018-018-2973-y

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LC-MS-Based Plasma Metabolomics and Lipidomics Analyses for Differential Diagnosis of Bladder Cancer and Renal Cell Carcinoma

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Bladder cancer (BC) and Renal cell carcinoma(RCC) are the two most frequent genitourinary cancers in China. In this study, a comprehensive liquid chromatography—mass spectrometry (LC-MS) based method, which utilizes both plasma metabolomics and lipidomics platform, has been carried out to discriminate the global plasma profiles of 64 patients with BC, 74 patients with RCC, and 141 healthy controls. Apparent separation was observed between cancer (BC and RCC) plasma samples and controls. The area under the receiving operator characteristic curve (AUC) was 0.985 and 0.993 by plasma metabolomics and lipidomics, respectively (external validation group: AUC was 0.944 and 0.976, respectively). Combined plasma metabolomics and lipidomics showed good predictive ability with an AUC of 1 (external validation group: AUC = 0.99). Then, separation was observed between the BC and RCC samples. The AUC was 0.862, 0.853 and 0.939, respectively, by plasma metabolomics, lipidomics and combined metabolomics and lipidomics (external validation group: AUC was 0.802, 0.898, and 0.942, respectively). Furthermore, we also found eight metabolites that showed good predictive ability for BC, RCC and control discrimination. This study indicated that plasma metabolomics and lipidomics may be effective for BC, RCC and control discrimination, and combined plasma metabolomics and lipidomics showed better predictive performance. This study would provide a reference for BC and RCC biomarker discovery, not only for early detection and screening, but also for differential diagnosis.

Keywords: bladder cancer, renal cell carcinoma, metabolomics, lipidomics, biomarker

INTRODUCTION

Bladder cancer (BC) and Renal cell carcinoma(RCC) are, respectively, the second and third most common genitourinary cancers in Europe and North America, and the first two most commonly occurring genitourinary cancers in China (1). Currently, cystoscopy and cytology are the standards for initial BC diagnosis and recurrence, but they have some limitations. Cystoscopy may fail to visualize certain areas within the bladder, and may also fail to detect some cancers, particularly cases of carcinoma *in situ* (2). Cytology has high specificity and

selectivity for high-grade tumors, but fails to provide a strong predictive value for low-grade tumors (3). With regard to RCC, computed tomography, magnetic resonance imaging, and positron emission tomography are commonly used diagnostic techniques (4). However, even with the combination of these three techniques, it remains difficult to detect early tumors because of their small size (5). Therefore, developing convenient and novel techniques for early detection of BC and RCC with high sensitivity and specificity is urgently required. There are increasing numbers of studies evaluating the use of metabolomic analyses in the diagnosis of a number of pathologies (6–8) and in the elucidation of the clinical pathogenesis of various diseases (9, 10). Lipidomics is an emerging independent branch of metabolomics (11), and lipid metabolism dysfunction has been found to be associated with the pathogenesis of many diseases, such as ovarian cancer (12), prostate cancer (13), and breast cancer (14), among others.

Metabolomics has also been used to study BC and RCC, especially to identify biomarkers in urine and serum (15–23). In 2014, Jin et al. (23) applied LC-MS to profile urinary metabolites of 138 patients with BC and 121 control subjects. The study identified 12 putative markers that were involved in glycolysis and beta-oxidation; Wittmann et al. (19) applied LC-MS to profile urinary metabolites of 66 BC and 266 non-BC subjects. They suggested that metabolites (palmitoyl sphingomyelin, phosphocholine, and arachidonate) related to lipid metabolism may be potential BC markers. In 2016, Zhou et al. (20) developed a plasma pseudotargeted method based on GC-MS SIM and found metabolites involved in the PPP, nucleic acid, and fatty acid biosynthesis were disordered in BC patients. For RCC research, in 2011, Kim et al. (16) analyzed urine metabolomics of 29 kidney cancer patients and 33 control patients and identified 13 significant differentially expressed metabolites (hexanoylglycine, 4-hydroxybenzoate, gentisate, etc) that involved in amino acid metabolism and fatty acid beta-oxidation metabolism. In 2017, Falegan et al. (18) applied an NMR and GC-MS platform to perform urine and serum metabolomics for 40 RCC patients and 13 benign patients. The results showed alterations in levels of glycolytic and tricarboxylic acid (TCA) cycle intermediates in RCC relative to benign masses. In addition, Lin et al. (5) have utilized both RPLC-MS and HILIC-MS to discriminate the global serum profiles of BC, RCC, and non-cancer controls. The study identified some cancer-specific potential biomarkers for BC and RCC, and they also found acetylphenylalanine, methyl hippuric acid, PC(40:7) and PC(40:6) were common differential biomarkers for both BC and RCC. As described, these studies showed the same changes of pathways, including glycolysis, amino acid metabolism and fatty acid metabolism in BC and RCC patients, but there is less consistency in identified metabolites in these studies (**Table 5**).

As mentioned above, previous studies have identified some potential disease biomarkers in urine and serum for BC or RCC diagnosis, but some issues remain to be addressed. First, most of these studies focus on one kind of cancer. However, in clinic there is great interest in the possibility of distinguishing different types of cancer based on metabolomics and to acquire deeper insight into the tumor biology and cancer type-specific biomarker discovery (5, 24, 25). Up to now, only one study worked on above issue. Lin et al. (5) utilized serum metabolomics to discriminate the global serum profiles of BC, RCC, and non-cancer controls. The results indicated that serum metabolic profiling could be used for BC or RCC diagnosis. They also identified some metabolites that were common differential biomarkers for both BC and RCC. Lin et al. study provided very useful metabolomic clues for BC and RCC common biomarker discovery, but their conclusions and results needed more work to be proved. In addition, it remains to explore whether serum metabolomics could be used for differential diagnosis of genitourinary cancer (16, 18, 19, 23). Second, to our knowledge, urinary metabolomics has been extensively investigated for BC and RCC biomarker discovery (15, 18, 19, 26–28), but there are few studies on blood metabolomics and lipidomics for BC biomarker discovery. Blood has fewer intra- and inter-individual variations, and it is less susceptible to dietary changes than urine (29). Moreover, blood is rich in lipids, which plays an essential role in many biological processes (30). Lipidomics is proposed as a viable method to monitor the prognosis, diagnosis, and treatment of cancer and acts as a new method of cancer biomarker discovery (31). Therefore, the combination of metabolomics and lipidomics may be a significant platform for BC and RCC biomarker discovery.

In this study, we tried to explore potential biomarkers for BC and RCC, which can not only screen BC and/or RCC before subjective symptom in non-metastatic stage of cancer, but also provide differential diagnostic clues for BC or RCC in the clinical stage, so that the proper following tests (cystoscopy or computed tomography) can be used. Plasma metabolomics and lipidomics were utilized, first to explore potential biomarkers between cancer (BC and RCC) and non-cancer. Then, differential metabolites were explored between BC and RCC to find cancer-specific biomarker for differential diagnosis. Furthermore, we also explored common differential metabolites among BC, RCC, and control groups to find whether it is a panel of metabolites biomarker could be as potential biomarker for discrimination of BC, RCC, and control. Our study will provide a reference for BC and RCC biomarker discovery, not only for early detection and screening, but also for differential diagnosis.

MATERIALS AND METHODS

Sample Collection and Preparation

The consent procedure and the research protocol for this study were approved by the Institutional Review Board of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Project NO: 047-2019). And all participants provided informed consent and took a series of physical examinations and laboratory tests before participating in this study, including blood pressure, body mass index (BMI), fasting blood glucose (FBG), total cholesterol (TC), triglyceride (TG) etc. Finally, a total of 141 participants aged 27–74 years with health standard were recruited in this study. Meanwhile, the BC and RCC patients also took above tests, and only the patients with normal results were recruited.

The plasma samples from 64 bladder cancer (BC) patients, 74 Renal cell carcinoma(RCC) patients and 141 healthy controls

TABLE 1 | Demographics of cancer (BC and RCC) patients and healthy controls.

Sample group	Discovery group			Validation group		
	Healthy controls	BC patients	RCC patients	Healthy controls	BC patients	RCC patients
No. plasma samples	95	42	53	46	22	21
Mean age ± SD	59.25 ± 11.19	64.21 ± 14.18	56.96 ± 15.09	61.32 ± 9.43	62.59 ± 12.77	53.66 ± 12.35
No. Males	65	31	36	30	14	16
No. Females	30	11	17	16	8	5

were collected from Peking Union Hospital (**Table 1**, the detailed clinical information was shown in **Table S1**). All the plasma samples in our study were collected before any treatments. The plasma samples were collected in the morning from 07:00 a.m.−09:00 a.m. after an overnight fast to eliminate dietary disturbances. After collected, all plasma samples were separated following centrifugation at 1,024 g for 10 min at 4°C and were stored at −80°C.

Sample Preparation

For plasma metabolomics, 50 μL of sample were mixed with 150 μL of H_2O by vortexed for 30 s to dilute the sample, then acetonitrile (400 μl) was added into each sample (200 μl), the mixture was vortexed for 1 min. The mixture was allowed to stand for 30 min at −20°C and was centrifuged at 14,000 × g for 10 min. The supernatant was dried under vacuum and then reconstituted with 100 μL of 2% acetonitrile. For plasma lipidomics, 200 μL plasma samples were precipitated by the addition of 600 μL of isopropanol (IPA) precooled to −20°C. Samples were stored for 2 h at −20°C to improve protein precipitation and then centrifuged at 14,000 × g for 20 min. The supernatant was dried under vacuum and then reconstituted with 100 μL of 50% IPA. The quality control (QC) (32) sample was a pooled sample prepared by mixing aliquots of two hundred samples across different groups. And the two hundred samples were randomly selected from BC, RCC and control groups.

LC-MS Analysis

Ultra-performance LC-MS analyses of samples were conducted using a Waters ACQUITY H-class LC system coupled with an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, MA, USA). An HSS C18 column (3.0 × 100 mm, 1.7 μm) (Waters, Milford, MA, USA) was used for reversed phase separation. Plasma metabolites were separated with an 18 min gradient at a flow rate of 0.5 mL/min. Mobile phase A was 0.1% formic acid in H_2O and mobile phase B was acetonitrile. The gradient was set as follows: 0–1 min, 2% solvent B; 1–3 min, 2–55% solvent B; 3–8 min, 55–100% solvent B; 8–13 min, 100% solvent B; 13–13.1 min, 100–2% solvent B; 13.1–18 min, 2% solvent B. The column temperature was set as 50°C. Plasma lipids were separated with a 23 min gradient at a flow rate of 0.4 mL/min. Mobile phase A was 10 mM ammonium acetate in acetonitrile (4:6) and mobile phase B was 10 mM ammonium acetate in isopropanol/acetonitrile (9:1). The gradient was set as follows: 0 min, 40% solvent B; 0–2 min, 40–43% solvent B; 2–8 min, 43–85% solvent B; 8–15 min, 85–99% solvent B; 15–18 min, 99% solvent B; 18–18.1 min, 99–40% solvent B; 18.1–23 min, 40% solvent B. The column temperature was set as 55°C.

The mass spectrometer was operated in positive ion mode using the m/z range 100–1,000 m/z at a resolution of 60 K. Automatic gain control (AGC) target was 1×10^6 and maximum injection time (IT) was 100 ms. Subsequently differential metabolites identification was performed by UPLC targeted-MS/MS analyses of QC sample. It acquired at a resolution of 15 K with AGC target of 5×10^5, maximum IT of 50 ms, and isolation window of 3 m/z. Collision energy was optimized as 20, 40, 60 for each target with higher-energy collisional dissociation (HCD) fragmentation.

Data Processing

Raw data files (**Figure S6**) were processed by the Progenesis QI 2.2 (Waters, Milford, MA, USA) software (33). The detailed workflow for QI data processing and metabolites identification was given in **Supplementary Methods**. Further data pre-processing including missing value estimation, Log transformation and Pareto scaling were performed to make features more comparable using MetaboAnalyst 4.0 (34) (http://www.metaboanalyst.ca). Pattern recognition analysis (principal component analysis, PCA; orthogonal partial least squares discriminant analysis, OPLS-DA) was carried out using SIMCA 14.0 software (Umetrics, Sweden). The differential variables were selected according to three conditions: (1) adjusted $P < 0.05$; 2) Fold change between two groups >1.5; 3) VIP value obtained from OPLS-DA > 1.

Metabolite Annotation and Pathway Analysis

Significantly differential metabolites were further determined from the exact mass composition, from the goodness of the isotopic fit for the predicted molecular formula and from MS/MS fragmentation matching with databases (HMDB (35), LIPID MAPS, METLIN, and mzCloud), using Progenesis QI 2.2 (Waters, Milford, MA, USA). In addition, homocysteine thiolactone, hypoxanthine, 4-Ethylphenol, L-Octanoylcarnitine and acetylcysteine were confirmed by standard compounds (**Figure S7**). Metabolic pathways were analyzed using Mummichog (36) and MetaboAnalyst 4.0 (34). Identified differential metabolites were subjected to MetaboAnalyst 4.0 to perform exploratory ROC analysis. Random Forest algorithms were used for ROC curve construction. Detailed methods were listed in the **Supplemental Methods**.

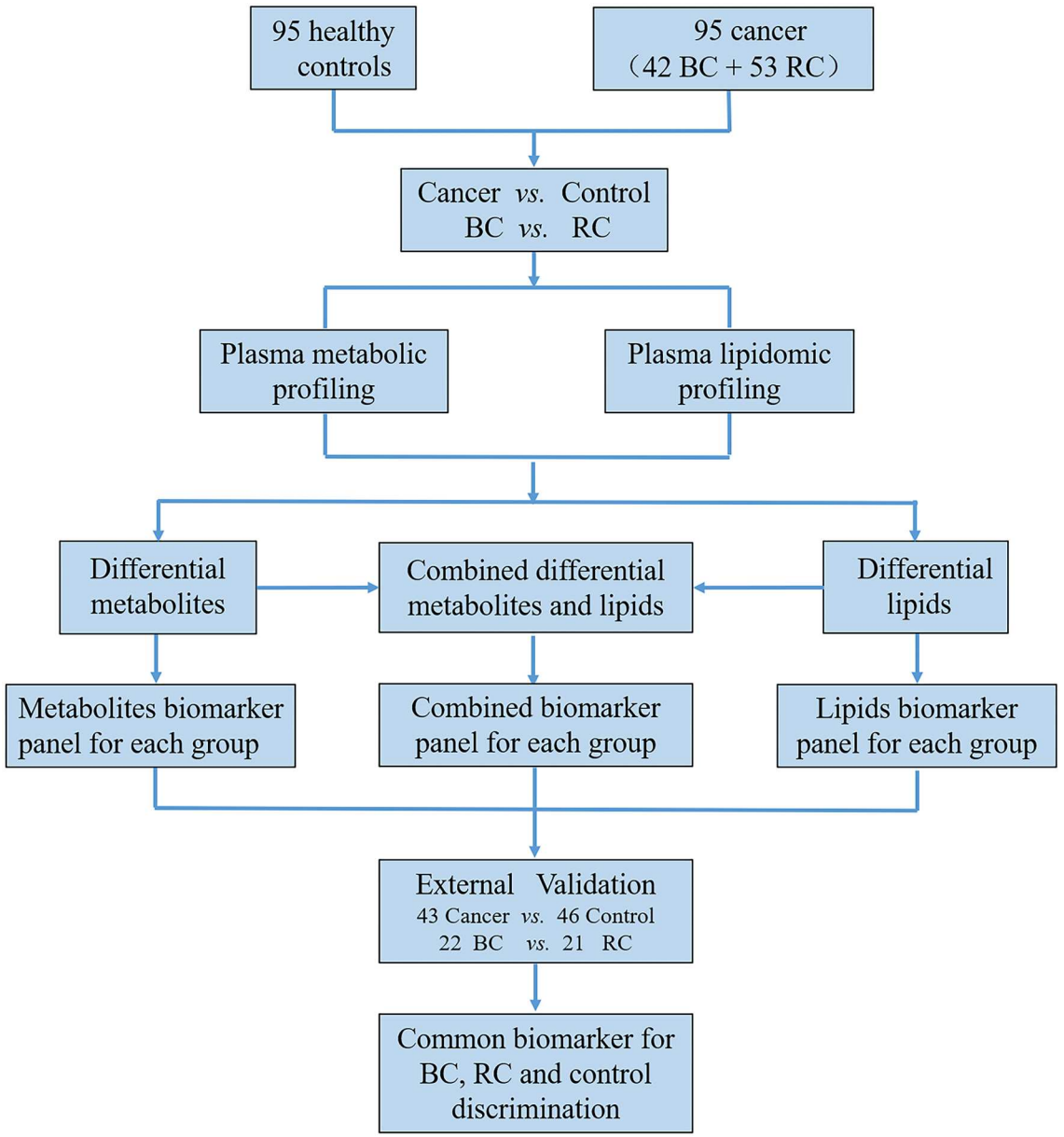

FIGURE 1 | The workflow of this study.

RESULTS

The workflow of this study is shown in **Figure 1** total of 279 subjects were enrolled in our study, with 141 volunteers with a normal clinically healthy index, 64 patients who were clinically diagnosed with bladder cancer and 74 patients who were clinically diagnosed with Renal cell carcinoma. First, LC-MS based plasma metabolomics and lipidomics were performed based on 95 healthy controls, 42 patients with BC and 53 patients with RCC. Differential metabolites were found through a critical selection criterion. Potential biomarkers for cancer vs. control and BC vs. RCC were explored and discovered tentatively. Moreover, the identified differential metabolites were also combined for better predictive ability. Then, the potential biomarkers were further externally validated using an independent batch of 22 BC, 21 RCC and 46 control samples. Furthermore, common differential metabolites were explored for BC, RCC, and control discrimination.

Quality Control

This large cohort of samples was analyzed randomly in a single batch. QC is important in large-scale metabolomics studies to ensure stable system performance and to limit experimental

bias. A QC standard was prepared as a pooled mixture of aliquots from representative plasma samples in each group. For plasma metabolomics and lipidomics analysis, the QC sample was injected 5 times before the analytical run and was frequently injected once every ten samples throughout the analytical run to monitor instrument stability. Metabolomics technical reproducibility was assessed by analyzing the QC sample variations with time. The injections showed a stable condition with small variation ($< \pm$ 2SD) in plasma metabolomics and lipidomics (**Figures S1A,B**). Tight clustering of QC samples (**Figures S1C,D**) further demonstrated the quality of the QC data and the essential repeatability and stability throughout the analytical run.

Distinction Cancer (BC and RCC) From Control by Plasma Metabolomics and Lipidomics

Distinction Cancer (BC and RCC) From Control by Plasma Metabolomics

LC-MS-based plasma metabolomics from cancer and control patients yielded 2,432 spectral features after removal of missing values and quality control. To select potential biomarkers for distinguishing cancer (BC and RCC) from control patients, multivariate statistical analysis models were applied. Apparent differences between the metabolic profiles of cancer and control subjects was observed from the PCA score plot (R2X = 0.624, Q2 = 0.416; **Figure S2A**). The OPLS-DA model achieved better separation (R2X = 0.263, R2Y = 0.953, Q2 = 0.931; **Figure 2A**; **Table S2**). Permutation tests were carried out to confirm the stability and robustness of the supervised models presented in this study (**Figure S2B**). Differential metabolites were assigned based on VIP value >1, $p < 0.05$ and FC >1.5. Pathway enrichment analysis using Mummichog showed significant enrichment ($p < 0.05$) of several pathways related to tyrosine metabolism, linoleate metabolism, porphyrin metabolism, fructose, and mannose metabolism, and phosphatidylinositol phosphate metabolism, among others (**Figure S2C**), in cancer compared with that in the healthy controls.

Further, significantly differential features obtained from "mummichog" and OPLS-DA predictions were submitted to MS/MS fragmentation and Progenesis QI identification. Overall, 25 significantly differential metabolites were identified as shown in **Table S3**. The diagnostic accuracy of identified differential metabolites for cancer (BC and RCC) from control samples was evaluated. A total of 22 metabolites had a good diagnostic value with the AUC above 0.8 (37) (**Table S4**). Combined biomarkers are more valuable for diagnosing disease progression than just one biomarker (23). Multivariate ROC curve-based exploratory analysis was tried to achieve a better predictive model (https://www.metaboanalyst.ca/faces/upload/RocUploadView.xhtml) using these differential metabolites. A panel consisting of 9,10,13-TriHOME, 12,13-DHOME and linolenelaidic acid showed the best predictive ability with a ROC area of 0.985 for the testing dataset (**Figure S2D**) and 0.944 for the external validation dataset (**Figure S2E**).

Distinction Cancer (BC and RCC) From Control by Plasma Lipidomics

LC-MS-based plasma lipidomics from cancer and control samples was analyzed using similar multiple statistic methods as above. In total, 1421 spectral features were retained after quality control. PCA analysis showed apparent discrimination of cancer and control samples (R2X = 0.682, Q2 = 0.406; **Figure S3A**). Further, the OPLS-DA model achieved significant separation (R2X = 0.296, R2Y = 0.949, Q2 = 0.924; **Figure 2B**). Permutation tests showed stability and robustness of the supervised models (**Figure S3B**). Pathway enrichment analysis using Mummichog showed significant enrichment pathways related to the carnitine shuttle, the urea cycle/amino group metabolism, and fatty acid metabolism, among others (**Figure S3C**), in cancer compared with control samples. Overall, 26 significantly differential lipids were identified as shown in **Table S5**, and a total of 20 lipids had potential useful diagnostic values with the AUC above 0.7 (**Table S6**). A panel consisting of 11Z-Eicosenal, 6Z-Heneicosen-9-one, behenic acid and 7Z-Tricosen-11-one showed the best predictive ability with ROC area of 0.993 for the testing dataset (**Figure S3D**) and 0.976 for the external validation dataset (**Figure S3E**).

Distinction Cancer (BC and RCC) From Control by Combination of Plasma Metabolomics and Lipidomics

Combining the results of identified differential metabolites, the relative intensity was plotted as a heatmap in **Figure 2C**. It showed that the metabolites involved in amino acid metabolism and fatty acid metabolism were up-regulated in cancer patients, including dipeptides, bile acid metabolites, and some fatty acyls (FAs). While the down-regulated metabolites included some carnitines (3-hydroxyoctanoyl carnitine, L-Octanoylcarnitine, 2-Hydroxylauroylcarnitine, O-decanoyl-L-carnitine, Undecanoylcarnitine), glycerophospholipids (GPs), sphingolipids (SPs), and sterol lipids (STs). Multivariate ROC curve-based exploratory analysis was tried to achieve a better predictive model using these combined differential metabolites. A panel consisting of 9,10,13-TriHOME, 11Z-Eicosenal, 12,13-DHOME, 6Z-Heneicosen-9-one, linolenelaidic acid, behenic acid, and 16-Hydroxy-10-oxohexadecanoic acid (**Table 2**) showed the best predictive ability with ROC area of 1 for the testing dataset (**Figure 2D**) and 0.99 for the external validation dataset (**Figure 2E**).

Distinction BC and RCC by Plasma Metabolomics and Lipidomics

BC and RCC are the first two most frequent genitourinary cancers in China. The above analysis explored potential differential metabolites to discriminate cancer (BC and RCC) from control samples, and the feasibility of using plasma metabolomics and lipidomics to discover potential biomarkers for differential diagnosis of the two types of cancer was evaluated.

Distinction BC and RCC by Plasma Metabolomics

Herein, using similar multiple statistic methods as above, metabolic profiling differentiation was explored between BC and

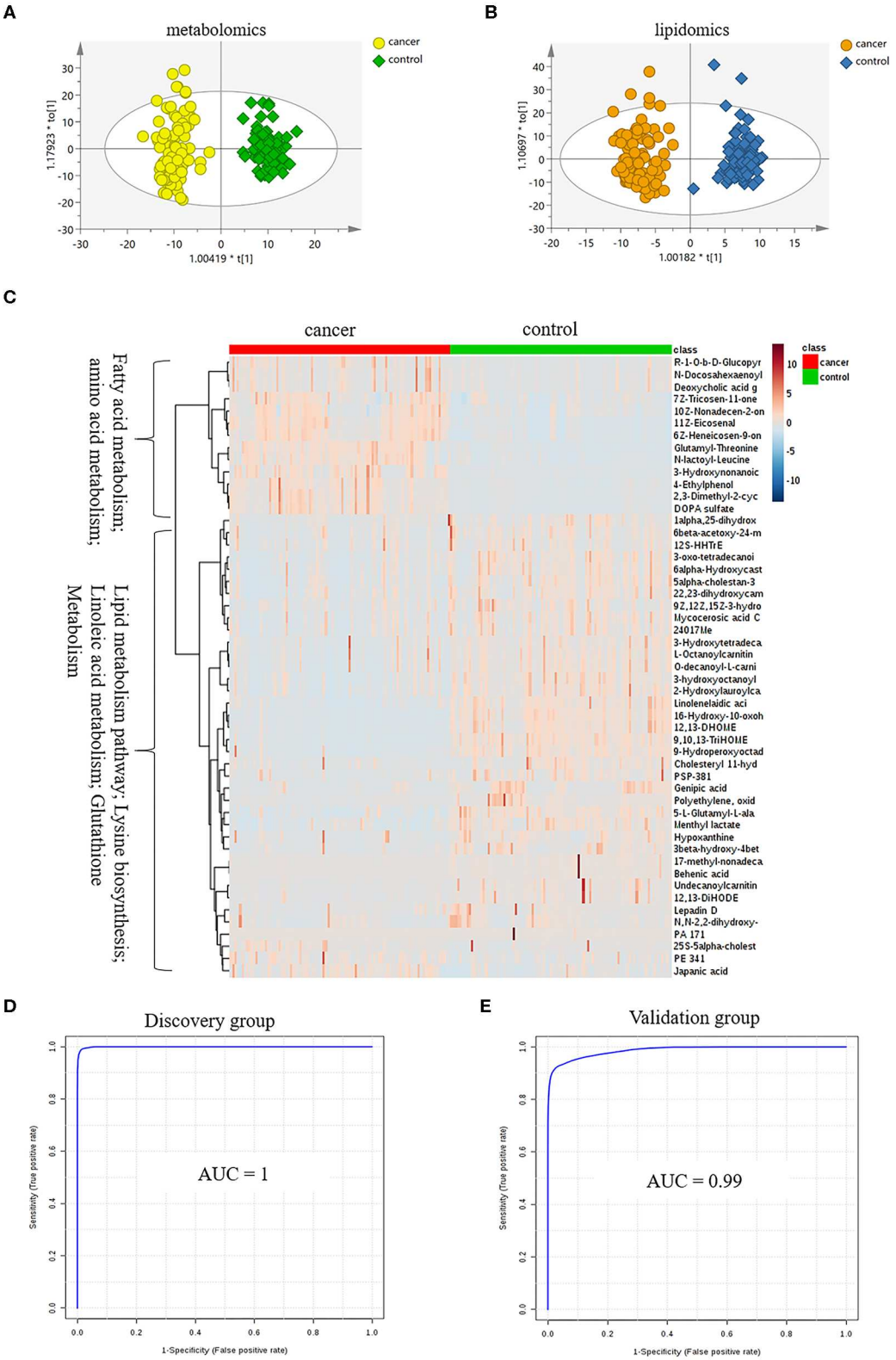

FIGURE 2 | Analysis of plasma metabolomics and lipidomics of 95 cancer samples (42 BC and 53 RCC) and 95 healthy control samples. **(A)** Score plot of OPLS-DA based on plasma metabolic profiling of cancer and control. **(B)** Score plot of OPLS-DA based on plasma lipidomic profiling of cancer and control.

(Continued)

FIGURE 2 | (C) Relative intensity of differential metabolites in cancer and control. **(D)** ROC plot with discovery group for distinction of cancer and control based on combined metabolites panel of 9,10,13-TriHOME, 11Z-Eicosenal, 12,13-DHOME, 6Z-Heneicosen-9-one, linolenelaidic acid, behenic acid and 16-Hydroxy-10-oxohexadecanoic acid. **(E)** ROC plot with external validation group for distinction of cancer and control based on combined metabolites panel.

TABLE 2 | Differential metabolites for distinction of cancer (BC and RCC) and control.

Features	Metabolites ID	Description	Score	*p*-value	Fold change (cancer/HC)	AUC
5.81_269.2104m/z	HMDB41287	16-Hydroxy-10-oxohexadecanoic acid[a]	45.3	2.97E-29	0.0988	0.9948
5.60_331.2470m/z	HMDB04710	9,10,13-TriHOME[a]	42.3	1.09E-28	0.1001	0.9853
6.60_314.2448n	HMDB04705	12,13-DHOME[a]	42.7	6.90E-27	0.1859	0.9675
6.59_279.2309m/z	HMDB30964	Linolenelaidic acid[a]	38.1	5.15E-23	0.4242	0.9313
9.09_311.3170n	LMFA06000248	11Z-Eicosenal[b]	50.9	5.89E-27	2.2601	0.9788
9.24_325.3325n	LMFA12000215	6Z-Heneicosen-9-one[b]	41.5	1.27E-24	2.2569	0.9535
8.19_358.3658m/z	LMFA01020019	Behenic acid[b]	48.9	2.53E-17	0.161	0.8726
9.71_354.3710m/z	LMFA12000222	7Z-Tricosen-11-one[b]	48.7	7.95E-12	1.6531	0.8049

[a]*Metabolites identified by the chemical structure analysis matching with The Human Metabolome Database.*
[b]*Metabolites identified by the chemical structure analysis matching with LIPID MAPS.*

RCC plasma samples. First, the metabolic profiles of BC and RCC subjects showed separation trend to some extent from the PCA score plot (R2X = 0.557, Q2 = 0.324; **Figure S4A**). The OPLS-DA model achieved better separation (R2X = 0.322, R2Y = 0.941, Q2 = 0.652; **Figure 3A**). Permutation tests showed stability and robustness of the supervised models (**Figure S4B**). Differential metabolites were assigned based on VIP value > 1 and $p < 0.05$. Pathway enrichment analysis using Mummichog showed significant enrichment ($p < 0.05$) of several pathways related to caffeine metabolism, porphyrin metabolism, chondroitin sulfate degradation, heparan sulfate degradation, and vitamin H (biotin) metabolism, among others (**Figure S4C**), in BC samples compared with those in RCC samples. Overall, 24 differential metabolites were identified as shown in **Table S7**. ROC analysis showed that 9 metabolites have a potentially useful diagnostic value for BC and RCC discrimination (**Table S8**). Further metabolite panels were explored to achieve better predictive ability. Using Random Forest algorithms, a metabolite panel consisting of 7,8-Dihydropteroic acid, Avenoleic acid, and 3,4-Dimethyl-5-pentyl-2-furanundecanoic acid showed the best predictive ability with ROC area of 0.862 for the testing dataset (**Figure S4D**) and of 0.802 for the external validation dataset (**Figure S4E**) for BC and RCC discrimination.

Distinction BC and RCC by Plasma Lipidomics

Lipidomic profiling differentiation was explored between BC and RCC plasma samples using similar multiple statistic methods. PCA analysis also showed separation trend to some extent of BC and RCC (R2X = 0.602, Q2 = 0.272; **Figure S5A**). Further, the OPLS-DA model achieved better separation (R2X = 0.339, R2Y = 0.959, Q2 = 0.715; **Figure 3B**). Hundred permutation tests showed no over-fitting of the models (**Figure S5B**). Pathway enrichment analysis using Mummichog showed significant enrichment in pathways related to aspartate and asparagine metabolism, pentose phosphate pathway, hexose phosphorylation and vitamin H (biotin) metabolism, among others (**Figure S5C**), in BC samples compared with those in RCC samples. Further, a total of 17 differential metabolites were identified as shown in **Table S9**. Using Random Forest algorithms, a panel consisting of PS(P-38:0), 4E,14Z-Sphingadiene, Tetrapedic acid A (**Table S10**) showed the best predictive ability with ROC area of 0.853 for the testing dataset (**Figure S5D**) and 0.898 for the external validation dataset (**Figure S5E**) for BC and RCC discrimination.

Distinction BC and RCC by Combination of Plasma Metabolomics and Lipidomics

Combining the results of the identified differential metabolites, the relative intensity was plotted as a heatmap in **Figure 3C**. The up-regulated metabolites in BC compared to RCC included some acyl carnitines, fatty acids, amino acids, and derivatives and glycerophospholipids (GPs). The down-regulated metabolites included some dipeptides, sterol lipids (STs), sphingolipids (SPs), and fatty acyls (FAs) in BC compared with those in RCC. Multivariate ROC curve-based exploratory analysis was tried to achieve a better predictive model using these combined differential metabolites. A panel consisting of 7,8-Dihydropteroic acid, PS(P-38:0), 9,10,13-TriHOME, Avenoleic acid, 3,4-Dimethyl-5-pentyl-2-furanundecanoic acid and 4E,14Z-Sphingadiene (**Table 3**) showed the best predictive ability with ROC area of 0.939 for the testing dataset (**Figure 3D**) and 0.942 for the external validation dataset (**Figure 3E**).

Common Differential Metabolites for Differential Diagnosis Among BC, RCC, and Control

According to the above analysis, plasma metabolites could diagnose cancer (BC and RCC) from controls with high accuracy, and another panel of plasma metabolites could also discriminate BC and RCC with high accuracy. We further tried to find common differential metabolites among BC, RCC and control groups. Then, differential metabolites were selected in BC vs. control groups and RCC vs. control groups using

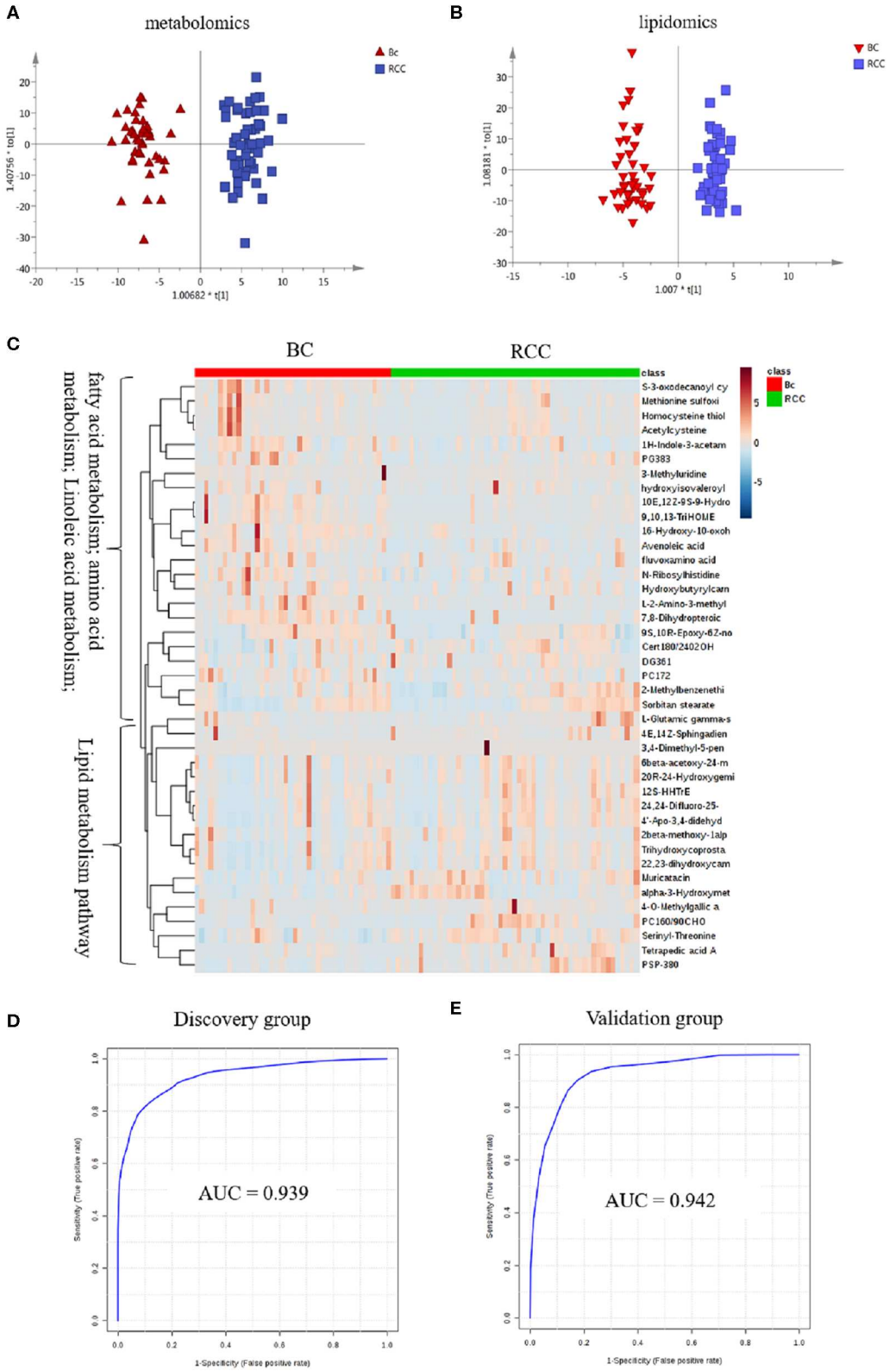

FIGURE 3 | Analysis of plasma metabolomics and lipidomics between 42 BC and 53 RCC. **(A)** Score plot of OPLS-DA based on plasma metabolic profiling of BC and RCC. **(B)** Score plot of OPLS-DA based on plasma lipidomic profiling of BC and RCC. **(C)** Relative intensity of differential metabolites in BC and RCC.

(Continued)

FIGURE 3 | (D) ROC plot with discovery group for distinction of BC and RCC based on combined metabolites panel of 7,8-Dihydropteroic acid, PS(P-38:0), 9,10,13-TriHOME, Avenoleic acid, 3,4-Dimethyl-5-pentyl-2-furanundecanoic acid and 4E,14Z-Sphingadiene. **(E)** ROC plot with external validation group for discrimination of BC and RCC based on combined metabolites panel.

TABLE 3 | Differential metabolites for distinction of BC and RCC.

Features	Metabolites ID	Description	Score	*p*-value	Fold change (BC/RCC)	AUC
1.15_297.1068m/z	HMDB01412	7,8-Dihydropteroic acid[a]	47.3	3.29E-04	3.41	0.8055
5.60_331.2470m/z	HMDB04710	9,10,13-TriHOME[a]	42.3	3.74E-05	4.93	0.7857
6.60_314.2448n	HMDB29978	Avenoleic acid[a]	39.5	1.47E-03	1.73	0.7556
4.85_372.2654n	HMDB31126	3,4-Dimethyl-5-pentyl-2-furanundecanoic acid[a]	53.1	2.61E-05	0.64	0.7300
8.39_826.5905m/z	LMGP03030046	PS(P-38:0)[b]	43.9	7.74E-07	0.45	0.7925
3.23_320.2539m/z	LMSP01080002	4E,14Z-Sphingadiene[b]	40.3	1.92E-04	0.6	0.7089
2.18_367.2823m/z	LMFA01050426	Tetrapedic acid A[b]	47.8	1.89E-04	0.34	0.7048

[a]*Metabolites identified by the chemical structure analysis matching with The Human Metabolome Database.*
[b]*Metabolites identified by the chemical structure analysis matching with LIPID MAPS.*

similar multiple statistic methods as above. In all, 8 metabolites presented different levels in BC, RCC, and control groups. The relative content of the 8 metabolites in the BC, RCC, and control groups was plotted in **Figure 4A**. Non-parameter test was performed and the *p*-values from different groups were all <0.05, which showed in **Figure 4A**. Herein, homocysteine thiolactone, acetylcysteine, methionine sulfoximine, 9,10,13-TriHOME, avenoleic acid, (10E,12Z)-(9S)-9-Hydroperoxyoctadeca-10,12-dienoic acid, 16-Hydroxy-10-oxohexadecanoic acid were down-regulated in cancer groups compared with the control group, and the relative content in the RCC group was lower than that in the BC group. In addition, 9S,10R-Epoxy-6Z-nonadecene was up-regulated in the cancer groups compared with the control group, and the relative content in the RCC group was lower than that in the BC group. Further PCA score plot indicated that a panel of 8 common differential metabolites showed good predictive ability for BC, RCC and control discrimination, with an AUC of 0.8456 for the BC group, 0.88 for the RCC group, and 0.986 for the control group (**Figure 4B**).

DISCUSSION

Disease-related metabolomics is currently a hot area of research, and numerous metabolites have been proposed as potential biomarkers (5). Lipidomics, a specific component of metabolomics, has attracted increased attention due to its unique biological significance (38), and it is widely studied for the identification and validation of disease-specific biomarkers (12–14).

Within metabolomics, three analytical techniques are most used: nuclear magnetic resonance spectroscopy (NMR), and gas or liquid chromatography coupled to mass spectrometry (GC/MS and LC/MS, respectively), but they have different operational performance characteristics(**Table S12**). NMR is known for its reproducibility, minimal sample preparation requirements and its non-destructive nature, but MS methods possess much higher levels of sensitivity and are certainly more accessible to most laboratories (18, 39). While several metabolites cannot be analyzed by GC-MS because they are prone to thermal decomposition or are unable to be volatilized. In contrast, a LC-MS based platform can detect a wider range of chemical species, and reversed phase liquid chromatography (RPLC)-MS is the most widely used platform in metabonomic studies (27). Therefore, we applied RPLC-MS analysis in this study.

In this study, plasma metabolomics and lipidomics were utilized, first to explore potential biomarkers between cancer (BC and RCC) and non-cancer for early detection of genitourinary cancer (BC and RCC). Then, differential metabolites were explored between BC and RCC to find cancer-specific biomarker for differential diagnosis (**Table 4**). Furthermore, 8 common differential metabolites were also found that showed good predictive ability for BC, RCC, and control plasma sample discrimination.

BC and RC are two different types of genitourinary cancers differing in their cellular origins, which BC occurs on the mucous membrane of the bladder and RCC originates in the urinary tubular epithelial system of the renal parenchyma, thus, they represent distinct clinical entities (25, 40, 41). However, proteomics and metabolomics studies showed that similar pathway dysregulation could be found in both cancers, such as glycolysis, TCA cycle, fatty acid oxidation, etc (42, 43). We compared the main findings found in this study with previous reports (**Table 5**) and found some common dysregulation pathways, including glycolysis, lipid metabolism, and fatty acid beta-oxidation in BC and RCC patients. Among them, a massive shift in fatty acid metabolism and the carnitine shuttle was found in both cancers compared with that in the healthy controls. Fatty acids are involved in energy metabolism and cell membrane molecule synthesis (20). In tumors tissues, free fatty acids (FFA) are esterified to fatty acyl-CoAs and then transported into the mitochondria by carnitine palmitoyltransferase-1 (CPT1) and the carnitine system, while in normal tissue, they are subjected to b-oxidation as fatty acyl-CoAs to feed into the TCA cycle (42). Carnitine is essential in mediating the transport of acyl groups across the mitochondrial inner membrane (45). Disturbances in fatty acid metabolism and in the carnitine shuttle may contribute to energy metabolism disorders in cancer patients (42). Our

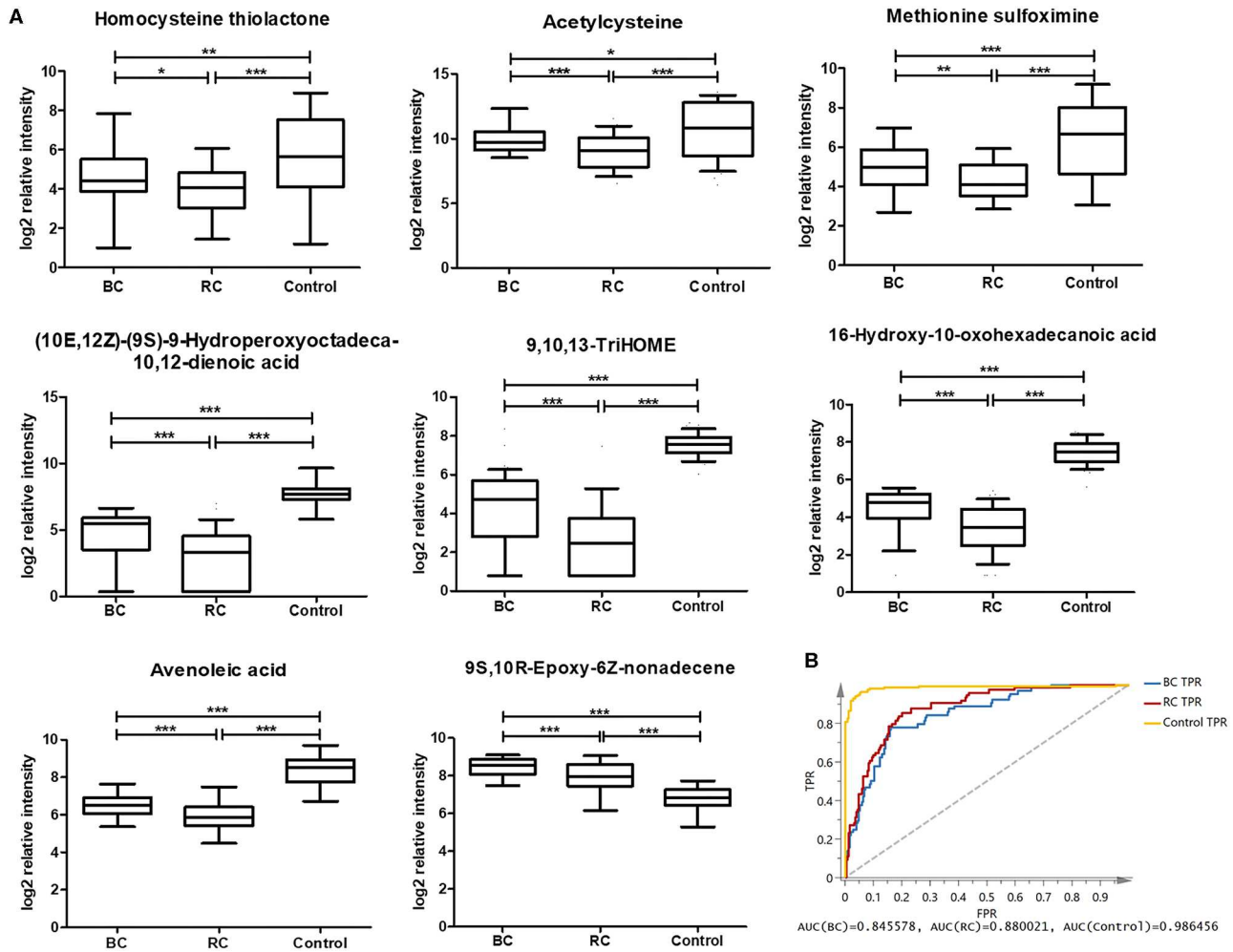

FIGURE 4 | Analysis of 8 common differential metabolites in BC (64 samples), RCC (74 samples), and control (141 samples) group. Homocysteine thiolactone and acetylcysteine were confirmed by standard compounds. **(A)** Relative intensity of 8 common differential metabolites in BC, RCC, and control group. *, **, and *** represent *p*-value less than 0.05, 0.01, and 0.001 between two groups, respectively. **(B)** Score plot based on 8 common differential metabolites for BC, RCC, and control discrimination.

metabolomics studies have led to the identification of carnitine derivatives as being significantly altered in the plasma of affected patients. This finding was validated *in vitro* using several RCC cell lines and show that these acylcarnitines, as a function of carbon chain length, affect cell survival, and markers of inflammation (46).

In addition, linoleate metabolism was found to be disturbed in cancer samples compared with that in controls. Linoleate metabolism is involved in the generation of inflammatory mediators (47) and in the regulation of lipid metabolism by activation of the peroxisome proliferators-activated receptor alpha (PPARa) (48). 9,10,13-TriHOME is an important inflammatory mediator which has the ability to aggregate neutrophils (49). 12,13-DHOME is known to directly affect cell differentiation through its PPAR binding activity (50). Taken together, there is a common regulatory mechanism among these metabolic pathways that contributes to disturbances of energy supply, to inflammation, to activation of the immune response and to oxidative stress in cancer (BC and RCC) patients.

Though similar pathways dysregulations could be found in BC and RCC, significant different pathways also could be found between them, such as pentose phosphate pathway (22, 51), amino acid metabolism (43, 52). In this study, pathway analysis between BC and RCC showed disturbed aspartate and asparagine metabolism, pentose phosphate pathway, linoleic acid metabolism, and vitamin H (biotin) metabolism in BC compared with that in RCC. Pentose phosphate pathway (PPP) is a major pathway for glucose catabolism. Emerging evidence suggests that the PPP directly or indirectly provides reducing power to fuel the biosynthesis of lipids and nucleotides and sustains antioxidant responses to support cell survival and proliferation (53). Zhou et al. (20) also found that pentose phosphate pathway(PPP) were significantly upregulated in bladder cancer. Previous multi-omics analysis showed that pentose phosphate pathway, fatty

TABLE 4 | Performance of metabolomics/lipidomics panels for groups discrimination.

ROC analysis	Plasma metabolomics		Plasma lipidomics		Combined plasma metabolomics and lipidomics	
Groups	**Discovery group**	**Validation group**	**Discovery group**	**Validation group**	**Discovery group**	**Validation group**
Cancer vs. Control	0.985[a]	0.944[a]	0.993[b]	0.976[b]	1[c]	0.99[c]
BC vs. RCC	0.862[d]	0.802[d]	0.853[e]	0.898[e]	0.939[f]	0.942[f]

[a]*A panel consists of 9,10,13-TriHOME, 12,13-DHOME and linolenelaidic acid.*
[b]*A panel consists of 11Z-Eicosenal, 6Z-Heneicosen-9-one, behenic acid and 7Z-Tricosen-11-one.*
[c]*A panel consists of 9,10,13-TriHOME, 11Z-Eicosenal, 12,13-DHOME, 6Z-Heneicosen-9-one, linolenelaidic acid, behenic acid and 16-Hydroxy-10-oxohexadecanoic acid.*
[d]*A panel consists of 7,8-Dihydropteroic acid, Avenoleic acid and 3,4-Dimethyl-5-pentyl-2-furanundecanoic acid.*
[e]*A panel consists of PS(P-38:0), 4E,14Z-Sphingadiene and Tetrapedic acid A.*
[f]*A panel consists of 7,8-Dihydropteroic acid, PS(P-38:0), 9,10,13-TriHOME, Avenoleic acid, 3,4-Dimethyl-5-pentyl-2-furanundecanoic acid and 4E,14Z-Sphingadiene.*

TABLE 5 | The comparison of the main findings found in this study with previous related reports.

Author (year)	Analytical platform	Sample type	BC[a]	RCC [a]	Control[a]	Pathways dysregulated in cancer compared to control[b]					
						Glycolysis	**TCA cycle**	**Fatty acid beta-oxidation**	**Pentose phosphate pathway**	**Amino acid metabolism**	**Lipid metabolism**
Cao et al. (44)	NMR	Serum	37		45	↑				↓	↑
Jin et al. (23)	RPLC-MS	Urine	138		121	↑	↑	↑			
Wittmann et al. (19)	LC-MS and GC-MS	Urine	66		266	↑	↑			*	↑
Zhou et al. (20)	GC-MS	plasma	92		48		↑		↑	↑	↑
Kim et al. (16)	LC-MS and GC-MS	Urine		29	33	↑		↑		↓	
Lin et al. (17)	LC-MS	Serum		33	25			*		↓	*
Falegan et al. (18)	NMR and GC-MS	Urine and serum		40	13	↑	↑			*	*
Lin et al. (5)	LC-MS	Serum	24	24	24					*	*
Liu et al. (this study)	LC-MS	Serum	64	73	141			*	*	*	*

[a]*The number of patients recruited in the study.*
[b]*Change trend of the Pathways dysregulated in cancer compared to control. (↑): up-regulated; (↓): down-regulated; (*): dysregulated.*

acid b-oxidation, glutamine pathway and tryptophan metabolism are reprogrammed in RCC, and the changes are related to energy metabolism, oxidative stress and immunosuppression (42, 51, 54). These alterations in glucose metabolism and pentose phosphate pathway were in accordance with previous findings that oncogenic signaling pathways may promote cancer through rerouting the sugar metabolism (51, 53). (10E,12Z)-(9S)-9-Hydroperoxyoctadeca-10,12-dienoic acid and 9,10,13-TriHOME are involved in linoleic acid metabolism, and they are both up-regulated in BC compared with RCC. Linoleic acid has previously been reported to induce carcinogenesis through oxidative damage and pro-inflammatory mechanisms (55). Trihydroxyoctadecenoic acids (TriHOMEs) are linoleic acid-derived oxylipins with potential physiological relevance in inflammatory processes as well as in maintaining an intact skin barrier (56). 9,10,13-TriHOME is an important inflammatory mediator that has the ability to aggregate neutrophils (49), which suggested that inflammation may be higher in BC than in RCC. Previous mRNA expression analysis showed that BC samples showed strong immune expression signature, including T cell markers and inflammation genes (57). Inflammation occurs during all stages of the tumor and inflammation establishes cancer invasion metastasis by reducing apoptosis and increasing angiogenesis (58, 59).

In this study, 8 metabolites were found to show different levels in BC, RCC, and control groups. The relative intensity results (**Figure 4A**) showed that the 8 metabolites were significantly statistical different between the two kinds of cancers and control group, though the difference between the BC and RCC was less obvious. Herein, homocysteine thiolactone, acetylcysteine, and methionine sulfoximine are amino acids. 16-Hydroxy-10-oxohexadecanoic acid and 9S,10R-Epoxy-6Z-nonadecene are fatty acids that are involved in lipid transport and fatty acid metabolism. (10E,12Z)-(9S)-9-Hydroperoxyoctadeca-10,

12-dienoic acid, avenoleic acid and 9,10,13-TriHOME are linoleic acids and their derivatives. Homocysteine (Hcy) was converted to Hcy-thiolactone by methionyl-tRNA synthetase (60). The relevance of cysteine metabolism in cancer has been reported, but these reports have been largely focused on its role in generating the antioxidant glutathione (61). Linoleic acid metabolites have previously been reported to have relevance in inflammatory processes (49, 55, 56), and 9S,10R-Epoxy-6Z-nonadecene is one of the unsaturated fatty acid metabolites. The 9S,10R-Epoxy-6Z-nonadecene level in the cancer group was obviously higher than that in the control group, and the relative content in the BC group was higher than that in the RCC group, as shown in **Figure 4A**. That finding was consistent with previous results that saturated fatty acyls decrease and that highly unsaturated fatty acyls increase in tumor tissues (30). However, the specific biological function of 9S,10R-Epoxy-6Z-nonadecene remains to be uncovered.

Among the above 8 metabolites, though the fold changes of two metabolites (Avenoleic acid and 9S,10R-Epoxy-6Z-nonadecene) in BC and RCC distinguish were <1.5, their performances in the difference between BC or RCC and control were better (**Table S11**). Moreover, the PCA score plot of the panel consisting of these 8 metabolites showed good predictive ability for BC, RCC, and control discrimination, with an AUC of 0.8456 for the BC group, 0.88 for the RCC group and 0.986 for the control group. Therefore, the panel of 8 common differential metabolites might be used as potential biomarker for early detection of BC and RCC from control. On the other hand, present study was a relative small sample size and single-center pilot study, further larger sample cohorts and multiple-center study will be performed in the future for more comprehensive validation. The prediction of prognosis after surgery was an important issue for clinical research. The performances of the panel on this issue need to be evaluated by follow-up data in the future.

LIMITATION OF THIS STUDY

The results of BC and RCC plasma metabolome in this study indicated that it was feasible to utilize plasma metabolomics and lipidomics for discriminating cancer from non-cancer and for differential diagnose of BC and RCC. However, this study also has the following limitations to be considered. (1) The sample size of the present study was relatively small, further larger sample cohorts and multiple-center study should be performed for more comprehensive validation. (2) In this study, the differential metabolites were discovered by non-targeted LC/MS/MS analysis. This approach provided a preliminary result in potential candidate biomarkers. To validate the above results, a targeted approach with authentic standards should be used in future validation study. (3) The samples recruited in this study were only from non-metastatic stage, thus the grades and stages of cancer were not taken into consideration. Whether different grades and stages of cancer will present different serum metabolomic pattern or not is of great importance, which should be thoroughly evaluated by a large-scale cohort in the future. (4) Due to the short follow-up time of the cohort in this study, we could not evaluate the relationship of the differential metabolites and clinical parameters, which should be comprehensively analyzed in future work. (5) In this study, the potential metabolite biomarkers of BC and RCC were discovered, but their function and mechanism in cancers had not been investigated, which should be presented by cell lines or animal model analysis in the future.

CONCLUSION

In conclusion, we have for the first time utilized data from a combination of plasma metabolomics and lipidomics analysis for BC and RCC early detection and screening, and provided a new insight into the differential diagnosis of BC and RCC. The results suggested that the plasma metabolome and lipidome could differentiate BC and RCC patients from controls, and panels of plasma metabolites were discovered to have potential value for BC and RCC discrimination. Moreover, the results suggested that combining plasma metabolomics and lipidomics has better predictive performance than either method alone. We also identified 8 metabolites might be used as potential biomarker panel to distinguish BC, RCC, and control.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

XianL, MZ, and XC prepared the first draft. XiaoL and WS conceived and designed the experiments. JL, XT, and ZW performed the experiments. XianL, XiaoL, HS, and ZG analyzed the data. MZ, XC, YZ, and ZJ contributed to collect clinical samples. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Issaq HJ, Nativ O, Waybright T, Luke B, Veenstra TD, Issaq EJ, et al. Detection of bladder cancer in human urine by metabolomic profiling using high performance liquid chromatography/mass spectrometry. *J Urol*. (2008) 179:2422–6. doi: 10.1016/j.juro.2008.01.084
2. van der Poel HG, Debruyne FM. Can biological markers replace cystoscopy? An update. *Curr Opin Urol*. (2001) 11:503–9. doi: 10.1097/00042307-200109000-00009
3. Konety BR. Molecular markers in bladder cancer: a critical appraisal. *Urol Oncol*. (2006) 24:326–37. doi: 10.1016/j.urolonc.2005.11.023
4. Siu KW, DeSouza LV, Scorilas A, Romaschin AD, Honey RJ, Stewart R, et al. Differential protein expressions in renal cell carcinoma: new biomarker discovery by mass spectrometry. *J Proteome Res*. (2009) 8:3797–807. doi: 10.1021/pr800389e
5. Lin L, Huang Z, Gao Y, Chen Y, Hang W, Xing J, et al. LC-MS-based serum metabolic profiling for genitourinary cancer classification and cancer type-specific biomarker discovery. *Proteomics*. (2012) 12:2238–46. doi: 10.1002/pmic.201200016
6. Lewis GD, Wei R, Liu E, Yang E, Shi X, Martinovic M, et al. Metabolite profiling of blood from individuals undergoing planned myocardial infarction reveals early markers of myocardial injury. *J Clin Invest*. (2008) 118:3503–12. doi: 10.1172/JCI35111
7. Spratlin JL, Serkova NJ, Eckhardt SG. Clinical applications of metabolomics in oncology: a review. *Clin Cancer Res*. (2009) 15:431–40. doi: 10.1158/1078-0432.CCR-08-1059
8. Sreekumar A, Poisson LM, Rajendiran TM, Khan AP, Cao Q, Yu J, et al. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature*. (2009) 457:910–4. doi: 10.1038/nature07762
9. Hirayama A, Kami K, Sugimoto M, Sugawara M, Toki N, Onozuka H, et al. Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry. *Cancer Res*. (2009) 69: 4918–25. doi: 10.1158/0008-5472.CAN-08-4806
10. Zhang A, Sun H, Yan G, Wang P, Wang X. Mass spectrometry-based metabolomics: applications to biomarker and metabolic pathway research. *Biomed Chromatogr*. (2016) 30:7–12. doi: 10.1002/bmc.3453
11. Herzog K, Pras-Raves ML, Ferdinandusse S, Vervaart MAT, Luyf ACM, van Kampen AHC, et al. Plasma lipidomics as a diagnostic tool for peroxisomal disorders. *J Inherit Metab Dis*. (2017). doi: 10.1007/s10545-017-0114-7
12. Sutphen R, Xu Y, Wilbanks GD, Fiorica J, Grendys EC, LaPolla JP, et al. Lysophospholipids are potential biomarkers of ovarian cancer. *Cancer Epidemiol Biomarkers Prev*. (2004) 13:1185–91.
13. Giskeødegård GF, Hansen AF, Bertilsson H, Gonzalez SV, Kristiansen KA, Bruheim P, et al. Metabolic markers in blood can separate prostate cancer from benign prostatic hyperplasia. *Br J Cancer*. (2015) 113:1712–9. doi: 10.1038/bjc.2015.411
14. Nagahashi M, Tsuchida J, Moro K, Hasegawa M, Tatsuda K, Woelfel IA, et al. High levels of sphingolipids in human breast cancer. *J Surg Res*. (2016) 204:435–44. doi: 10.1016/j.jss.2016.05.022
15. Kim K, Aronov P, Zakharkin SO, Anderson D, Perroud B, Thompson IM, et al. Urine metabolomics analysis for kidney cancer detection and biomarker discovery. *Mol Cell Proteomics*. (2009) 8:558–70. doi: 10.1074/mcp.M800165-MCP200
16. Kim K, Taylor SL, Ganti S, Guo L, Osier MV, Weiss RH. Urine metabolomic analysis identifies potential biomarkers and pathogenic pathways in kidney cancer. *OMICS*. (2011) 15:293–303. doi: 10.1089/omi.2010.0094
17. Lin L, Huang Z, Gao Y, Yan X, Xing J, Hang W. LC-MS based serum metabonomic analysis for renal cell carcinoma diagnosis, staging, and biomarker discovery. *J Proteome Res*. (2011) 10:1396–405. doi: 10.1021/pr101161u
18. Falegan OS, Ball MW, Shaykhutdinov RA, Pieroraio PM, Farshidfar F, Vogel HJ, et al. Urine and serum metabolomics analyses may distinguish between stages of renal cell carcinoma. *Metabolites*. (2017) 7. doi: 10.3390/metabo7010006
19. Wittmann BM, Stirdivant SM, Mitchell MW, Wulff JE, McDunn JE, Li Z, et al. Bladder cancer biomarker discovery using global metabolomic profiling of urine. *PLoS ONE*. (2014) 9:e115870. doi: 10.1371/journal.pone.0115870
20. Zhou Y, Song R, Zhang Z, Lu X, Zeng Z, Hu C, et al. The development of plasma pseudotargeted GC-MS metabolic profiling and its application in bladder cancer. *Anal Bioanal Chem*. (2016) 408:6741–9. doi: 10.1007/s00216-016-9797-0
21. Shao CH, Chen CL, Lin JY, Chen CJ, Fu SH, Chen YT, et al. Metabolite marker discovery for the detection of bladder cancer by comparative metabolomics. *Oncotarget*. (2017) 8:38802–10. doi: 10.18632/oncotarget.16393
22. Zhou Y, Song R, Ma C, Zhou L, Liu X, Yin P, et al. Discovery and validation of potential urinary biomarkers for bladder cancer diagnosis using a pseudotargeted GC-MS metabolomics method. *Oncotarget*. (2017) 8:20719–28. doi: 10.18632/oncotarget.14988
23. Jin X, Yun SJ, Jeong P, Kim IY, Kim WJ, Park S. Diagnosis of bladder cancer and prediction of survival by urinary metabolomics. *Oncotarget*. (2014) 5:1635–45. doi: 10.18632/oncotarget.1744
24. Cimadamore A, Gasparrini S, Santoni M, Cheng L, Lopez-Beltran A, Battelli N, et al. Biomarkers of aggressiveness in genitourinary tumors with emphasis on kidney, bladder, and prostate cancer. *Expert Rev Mol Diagn*. (2018) 18:645–55. doi: 10.1080/14737159.2018.1490179
25. Arora HC, Fascelli M, Zhang JH, Isharwal S, Campbell SC. Kidney, ureteral, and bladder cancer: a primer for the internist. *Med Clin North Am*. (2018) 102:231–49. doi: 10.1016/j.mcna.2017.10.002
26. Pasikanti KK, Esuvaranathan K, Ho PC, Mahendran R, Kamaraj R, Wu QH, et al. Noninvasive urinary metabonomic diagnosis of human bladder cancer. *J Proteome Res*. (2010) 9:2988–95. doi: 10.1021/pr901173v
27. Huang Z, Lin L, Gao Y, Chen Y, Yan X, Xing J, et al. Bladder cancer determination via two urinary metabolites: a biomarker pattern approach. *Mol Cell Proteomics*. (2011) 10:M111.007922. doi: 10.1074/mcp.M111.007922
28. Peng J, Chen YT, Chen CL, Li L. Development of a universal metabolome-standard method for long-term LC-MS metabolome profiling and its application for bladder cancer urine-metabolite-biomarker discovery. *Anal Chem*. (2014) 86:6540–7. doi: 10.1021/ac5011684
29. Walsh MC, Brennan L, Malthouse JP, Roche HM, Gibney MJ. Effect of acute dietary standardization on the urinary, plasma, and salivary metabolomic profiles of healthy humans. *Am J Clin Nutr*. (2006) 84:531–9. doi: 10.1093/ajcn/84.3.531
30. Cífková E, Holčapek M, Lísa M, Vrána D, Melichar B, Študent V. Lipidomic differentiation between human kidney tumors and surrounding normal tissues using HILIC-HPLC/ESI-MS and multivariate data analysis. *J Chromatogr B Analyt Technol Biomed Life Sci*. (2015) 1000:14–21. doi: 10.1016/j.jchromb.2015.07.011
31. Yan F, Zhao H, Zeng Y. Lipidomics: a promising cancer biomarker. *Clin Transl Med*. (2018) 7:21. doi: 10.1186/s40169-018-0199-0
32. Sangster T, Major H, Plumb R, Wilson AJ, Wilson ID. A pragmatic and readily implemented quality control strategy for HPLC-MS and GC-MS-based metabonomic analysis. *Analyst*. (2006) 131:1075–8. doi: 10.1039/b604498k
33. Zhang J, Yang W, Li S, Yao S, Qi P, Yang Z, et al. An intelligentized strategy for endogenous small molecules characterization and quality evaluation

of earthworm from two geographic origins by ultra-high performance HILIC/QTOF MS(E) and Progenesis QI. *Anal Bioanal Chem*. (2016) 408:3881–90. doi: 10.1007/s00216-016-9482-3
34. Chong J, Soufan O, Li C, Caraus I, Li S, Bourque G, et al. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic Acids Res*. (2018) 46:W486–94. doi: 10.1093/nar/gky310
35. Wishart DS, Feunang YD, Marcu A, Guo AC, Liang K, Vázquez-Fresno R, et al. HMDB 4.0: the human metabolome database for 2018. *Nucleic Acids Res*. (2018) 46:D608–17. doi: 10.1093/nar/gkx1089
36. Li S, Park Y, Duraisingham S, Strobel FH, Khan N, Soltow QA, et al. Predicting network activity from high throughput metabolomics. *PLoS Comput Biol*. (2013) 9:e1003123. doi: 10.1371/journal.pcbi.1003123
37. van Erkel AR, Pattynama PM. Receiver operating characteristic (ROC) analysis: basic principles and applications in radiology. *Eur J Radiol*. (1998) 27:88–94. doi: 10.1016/S0720-048X(97)00157-5
38. Xuan Q, Hu C, Yu D, Wang L, Zhou Y, Zhao X, et al. Development of a high coverage pseudotargeted lipidomics method based on ultra-high performance liquid chromatography-mass spectrometry. *Anal Chem*. (2018) 90:7608–16 doi: 10.1021/acs.analchem.8b01331
39. Emwas AH. The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research. *Methods Mol Biol*. (2015) 1277:161–93. doi: 10.1007/978-1-4939-2377-9_13
40. Park JC, Hahn NM. Bladder cancer: a disease ripe for major advances. *Clin Adv Hematol Oncol*. (2014) 12:838–45.
41. Rini BI, Campbell SC, Escudier B. Renal cell carcinoma. *Lancet*. (2009) 373:1119–32. doi: 10.1016/S0140-6736(09)60229-4
42. Wettersten HI, Hakimi AA, Morin D, Bianchi C, Johnstone ME, Donohoe DR, et al. Grade-dependent metabolic reprogramming in kidney cancer revealed by combined proteomics and metabolomics analysis. *Cancer Res*. (2015) 75:2541–52. doi: 10.1158/0008-5472.CAN-14-1703
43. Cheng Y, Yang X, Deng X, Zhang X, Li P, Tao J, et al. Metabolomics in bladder cancer: a systematic review. *Int J Clin Exp Med*. (2015) 8:11052–63.
44. Cao M, Zhao L, Chen H, Xue W, Lin D. NMR-based metabolomic analysis of human bladder cancer. *Anal Sci*. (2012) 28:451–6. doi: 10.2116/analsci.28.451
45. Massari F, Ciccarese C, Santoni M, Iacovelli R, Mazzucchelli R, Piva F, et al. Metabolic phenotype of bladder cancer. *Cancer Treat Rev*. (2016) 45:46–57. doi: 10.1016/j.ctrv.2016.03.005
46. Ganti S, Taylor SL, Kim K, Hoppel CL, Guo L, Yang J, et al. Urinary acylcarnitines are altered in human kidney cancer. *Int J Cancer*. (2012) 130:2791–800. doi: 10.1002/ijc.26274
47. Salmon JA, Higgs GA. Prostaglandins and leukotrienes as inflammatory mediators. *Br Med Bull*. (1987) 43:285–96. doi: 10.1093/oxfordjournals.bmb.a072183
48. Caligiuri SP, Love K, Winter T, Gauthier J, Taylor CG, Blydt-Hansen T, et al. Dietary linoleic acid and alpha-linolenic acid differentially affect renal oxylipins and phospholipid fatty acids in diet-induced obese rats. *J Nutr*. (2013) 143:1421–31. doi: 10.3945/jn.113.177360
49. Nording ML, Yang J, Hegedus CM, Bhushan A, Kenyon NJ, Davis CE, et al. Endogenous levels of five fatty acid metabolites in exhaled breath condensate to monitor asthma by high-performance liquid chromatography: electrospray tandem mass spectrometry. *IEEE Sens J*. (2010) 10:123–30. doi: 10.1109/JSEN.2009.2035736
50. Edwards LM, Lawler NG, Nikolic SB, Peters JM, Horne J, Wilson R, et al. Metabolomics reveals increased isoleukotoxin diol (12,13-DHOME) in human plasma after acute Intralipid infusion. *J Lipid Res*. (2012) 53:1979–86. doi: 10.1194/jlr.P027706
51. Lucarelli G, Rutigliano M, Sallustio F, Ribatti D, Giglio A, Lepore Signorile M, et al. Integrated multi-omics characterization reveals a distinctive metabolic signature and the role of NDUFA4L2 in promoting angiogenesis, chemoresistance, and mitochondrial dysfunction in clear cell renal cell carcinoma. *Aging*. (2018) 10:3957–85. doi: 10.18632/aging.101685
52. Hakimi AA, Reznik E, Lee CH, Creighton CJ, Brannon AR, Luna A, et al. An integrated metabolic atlas of clear cell renal cell carcinoma. *Cancer Cell*. (2016) 29:104–16. doi: 10.1016/j.ccell.2015.12.004
53. Jiang P, Du W, Wu M. Regulation of the pentose phosphate pathway in cancer. *Protein Cell*. (2014) 5:592–602. doi: 10.1007/s13238-014-0082-8
54. Cancer Genome Atlas Research N. Comprehensive molecular characterization of clear cell renal cell carcinoma. *Nature*. (2013) 499:43–9. doi: 10.1038/nature12222
55. Brinkman MT, Karagas MR, Zens MS, Schned AR, Reulen RC, Zeegers MP. Intake of alpha-linolenic acid and other fatty acids in relation to the risk of bladder cancer: results from the New Hampshire case-control study. *Br J Nutr*. (2011) 106:1070–7. doi: 10.1017/S0007114511001346
56. Fuchs D, Hamberg M, Sköld CM, Wheelock ÅM, Wheelock CE. An LC-MS/MS workflow to characterize 16 regio- and stereoisomeric trihydroxyoctadecenoic acids (TriHOMEs). *J Lipid Res*. (2018). doi: 10.1194/jlr.D087429
57. Robertson AG, Kim J, Al-Ahmadie H, Bellmunt J, Guo G, Cherniack AD, et al. Comprehensive molecular characterization of muscle-invasive bladder cancer. *Cell*. (2017) 171:540–56.e25.
58. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature*. (2008) 454:436–44. doi: 10.1038/nature07205
59. Gorgel SN, Akin Y, Koc EM, Kose O, Ozcan S, Yilmaz Y. Retrospective study of systemic immune-inflammation index in muscle invasive bladder cancer: initial results of single centre. *Int Urol Nephrol*. (2020) 52:469–73. doi: 10.1007/s11255-019-02325-9
60. Jakubowski H. Pathophysiological consequences of homocysteine excess. *J Nutr*. (2006) 136:1741s—9s. doi: 10.1093/jn/136.6.1741S
61. Prabhu A, Sarcar B, Kahali S, Yuan Z, Johnson JJ, Adam KP, et al. Cysteine catabolism: a novel metabolic pathway contributing to glioblastoma growth. *Cancer Res*. (2014) 74:787–96. doi: 10.1158/0008-5472.CAN-13-1423

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