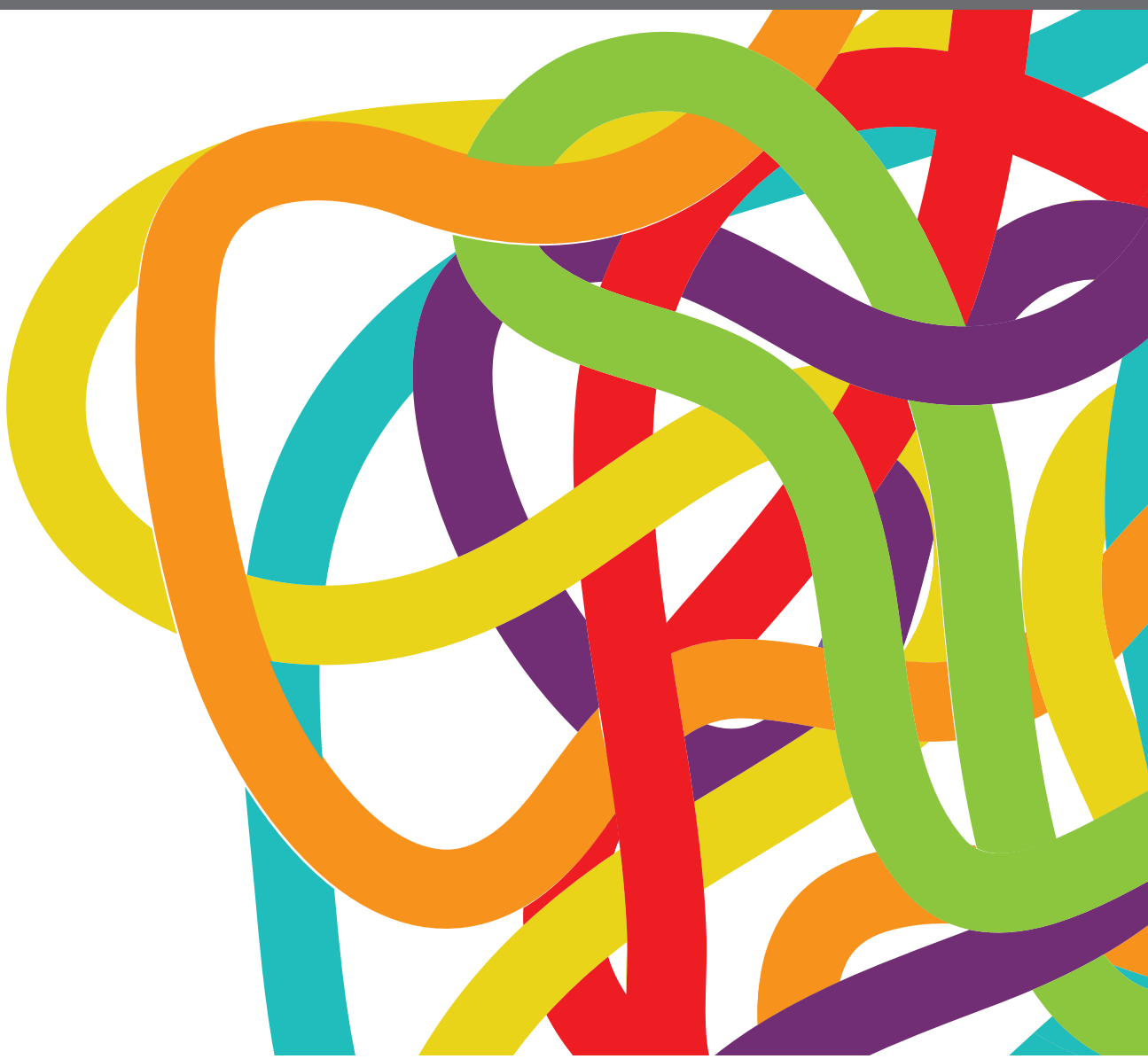
CANCER METABOLISM: CURRENT KNOWLEDGE AND PERSPECTIVES

EDITED BY: Leonardo Freire-de-Lima, Lucia Mendonça-Previato and Luciana Boffoni Gentile

PUBLISHED IN: Frontiers in Oncology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88945-989-6
DOI 10.3389/978-2-88945-989-6

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

CANCER METABOLISM: CURRENT KNOWLEDGE AND PERSPECTIVES

Topic Editors:
Leonardo Freire-de-Lima, Universidade Federal do Rio de Janeiro, Brazil
Lucia Mendonça-Previato, Universidade Federal do Rio de Janeiro, Brazil
Luciana Boffoni Gentile, Universidade Federal Fluminense, Brazil

Citation: Freire-de-Lima, L., Mendonça-Previato, L., Gentile, L. B., eds. (2019). Cancer Metabolism: Current Knowledge and Perspectives. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-989-6

Table of Contents

frontiers in Oncology

EDITORIAL
published: 16 April 2019
doi: 10.3389/fonc.2019.00287

Editorial: Cancer Metabolism: Current Knowledge and Perspectives

Leonardo Freire-de-Lima[1], Lucia Mendonça-Previato[1]* and Luciana Boffoni Gentile[2]**

[1] Laboratorio de Glicobiologia, Instituto de Biofisica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [2] Laboratório de Patologia Clínica Veterinária, Hospital Universitário de Medicina Veterinária Professor Firmino Márcico Filho (Huvet), Universidade Federal Fluminense, Rio de Janeiro, Brazil

Keywords: cancer, metabolism, glycolysis (glycolytic pathway), multidrug resistance phenotype, invasiviness

Editorial on the Research Topic

Cancer Metabolism: Current Knowledge and Perspectives

Over the first half of the twentieth century, it has been demonstrated that an important hallmark of cancer cells is the reprogramming of cellular metabolism. The capacity to obtain nutrients from an extremely poor microenvironment and utilize it to both generate biomass and maintain the cellular viability is a major feature of transformed cells. Several papers have proposed new mechanisms by which cancer cells undergo substantial metabolic reprogramming to endure within the hostile microenvironment. However, the metabolic profile adopted by cancer cells with invasive and multidrug-resistance phenotypes is still poorly understood. The research topic "Cancer Metabolism: Current Knowledge and Perspectives" provides exciting articles on the valuable field of cancer metabolism, where one hypothesis and theory, two original researches and five outstanding reviews are presented.

In their original research, Herrero and Gutierrez demonstrated that multiple myeloma cells with ongoing endogenous DNA damage depend on a homologous recombination (HR) pathway, which may be used as therapeutic proposals. They suggested that obstructing HR after the initial step of end resection might be more suitable to promote cell death, since it avoids a compensatory non-homologous end joining repair mechanism. The authors claim that these preclinical observations afford the basis for its clinical assessment.

Sant'Anna-Silva et al. analyzed the metabolic changes to the metastatic phenotype of human tongue squamous cell carcinoma lineages. By using both metabolomic and fluorescence lifetime imaging microscopy analysis, the authors demonstrated that many pathways linked to lipid metabolism seem to be associated to metastatic phenotype. On the other hand, amino acid metabolism and cell cycle regulation are most correlated to cells with low invasive phenotype.

In their review article, Turgeon et al. addressed evidence that link DNA damage/repair mechanisms with cancer cell metabolism. The authors claim that such connection is progressively apparent, granting opportunities to better understand the metabolic susceptibilities of a considerable fraction of tumors.

Prakasam et al., summarized recent progress in the understanding of many posttranslation modifications (PTMs) in cancer, in particular the PTMs in the M2 isoform of pyruvate kinase (PKM2). The authors believe that such knowledge will be crucial to evaluate their therapeutic potential for the treatment of different types of cancer.

Morrot et al. provided a snapshot of metabolic reprogramming in cancer cells, describing how, even in aerobic conditions, transformed cells opt for glycolysis instead of oxidative phosphorylation (OXPHOS). They discussed how this metabolic reprogramming is able to induce a high-lactate output, then promoting mmunosuppressive events. The authors believe that further studies are needed to better understand the effect of lactate and other "waste" metabolites on cancer progression.

OPEN ACCESS

Edited and reviewed by:
Paolo Pinton,
University of Ferrara, Italy

****Correspondence:***
Leonardo Freire-de-Lima
leolima@biof.ufrj.br
Lucia Mendonça-Previato
luciamp@biof.ufrj.br
Luciana Boffoni Gentile
luboffoni@id.uff.br

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *12 March 2019*
Accepted: *29 March 2019*
Published: *16 April 2019*

Citation:
Freire-de-Lima L, Mendonça-Previato L and Gentile LB (2019) Editorial: Cancer Metabolism: Current Knowledge and Perspectives. Front. Oncol. 9:287. doi: 10.3389/fonc.2019.00287

Coelho et al. presented an overview of the metabolic reprogramming in thyroid cancer, emphasizing factors that promote enhanced glycolysis in transformed cells. The authors also discussed about promising metabolic targets that might be useful to treat patients with thyroid cancer.

Snyder et al. discussed about the metabolic phenotype of cancer stem cells (CSCs), especially taking into account both glycolytic and OXPHOS pathways. Since CSCs present metabolic peculiarities when compared to most of cancer cells in a tumor, the authors believe that such singularities might offer a great potential for developing improved treatments for cancer patients.

Finally, in their hypothesis and theory article, Vidal et al. addressed the connection between multidrug-resistance phenotype and metabolic reprogramming in cancer cells, taking into consideration the functions mediated by ATP-binding cassette transporters, as well as the numerous non-metabolic roles mediated by enzymes that are part of the glycolytic pathway, with special attention to glyceraldehyde-3-phosphate dehydrogenase.

Taken together, the published papers in this research topic strengthen the concept that cancer cell metabolism is an important field in cancer biology, and further studies in this lively research area might provide important information, which may be useful for treatment, diagnostic and therapeutic purposes.

AUTHOR CONTRIBUTIONS

LF, LM-P and LG wrote the paper. All the authors read and approved the final version of the manuscript.

FUNDING

The authors are supported by grants from the Brazilian agencies: CAPES, FAPERJ, CNPq, and Cancer Foundation.

ACKNOWLEDGMENTS

The authors would like to express their deep gratitude to all authors who kindly contributed in this Research Topic.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

MINI REVIEW
published: 05 June 2018
doi: 10.3389/fonc.2018.00203

Cancer Stem Cell Metabolism and Potential Therapeutic Targets

Vusala Snyder[1], Tamika C. Reed-Newman[2], Levi Arnold[1], Sufi Mary Thomas[1,3,4] and Shrikant Anant[2,4]**

[1] Department of Otolaryngology, University of Kansas Medical Center, Kansas City, KS, United States, [2] Department of General Surgery, University of Kansas Medical Center, Kansas City, KS, United States, [3] Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, KS, United States, [4] Cancer Biology, University of Kansas Medical Center, Kansas City, KS, United States

Malignant tumors contain heterogeneous populations of cells in various states of proliferation and differentiation. The presence of cancer stem or initiating cells is a well-established concept wherein quiescent and poorly differentiated cells within a tumor mass contribute to drug resistance, and under permissive conditions, are responsible for tumor recurrence and metastasis. A number of studies have identified molecular markers that are characteristic of tissue-specific cancer stem cells (CSCs). Isolation of CSCs has enabled studies on the metabolic status of CSCs. As metabolic plasticity is a hallmark of cancer cell adaptation, the intricacies of CSC metabolism and their phenotypic behavior are critical areas of research. Unlike normal stem cells, which rely heavily on oxidative phosphorylation (OXPHOS) as their primary source of energy, or cancer cells, which are primarily glycolytic, CSCs demonstrate a unique metabolic flexibility. CSCs can switch between OXPHOS and glycolysis in the presence of oxygen to maintain homeostasis and, thereby, promote tumor growth. Here, we review key factors that impact CSC metabolic phenotype including heterogeneity of CSCs across different histologic tumor types, tissue-specific variations, tumor microenvironment, and CSC niche. Furthermore, we discuss how targeting key players of glycolytic and mitochondrial pathways has shown promising results in cancer eradication and attenuation of disease recurrence in preclinical models. In addition, we highlight studies on other potential therapeutic targets including complex interactions within the microenvironment and cellular communications in the CSC niche to interfere with CSC growth, resistance, and metastasis.

Keywords: stem cells, metabolism, microenvironment, targets, cancer stem cell markers

OPEN ACCESS

Edited by:
Leonardo Freire-de-Lima, Universidade Federal do Rio de Janeiro, Brazil

Reviewed by:
Jane Frimodig, University of Louisville, United States
Dhiraj Kumar, University of Texas MD Anderson Cancer Center, United States
Federica Barbieri, Università di Genova, Italy

****Correspondence:***
Sufi Mary Thomas
sthomas7@kumc.edu;
Shrikant Anant
sanant@kumc.edu

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *04 March 2018*
Accepted: *21 May 2018*
Published: *05 June 2018*

Citation:
Snyder V, Reed-Newman TC, Arnold L, Thomas SM and Anant S (2018) Cancer Stem Cell Metabolism and Potential Therapeutic Targets. Front. Oncol. 8:203. doi: 10.3389/fonc.2018.00203

INTRODUCTION

Despite the advances in modern medicine, some of the major challenges currently confronted in treating cancer patients include the development of therapeutic drug resistance and disease recurrence. Traditional treatments target cancer cells as a means to eradicate tumors and treat the patients. These methods are largely based on the stochastic model—a theory that suggests that cancer cells can arise from a cell that undergoes gene mutations resulting in the acquisition of a highly proliferative state (1, 2). Each progenitor cell bears the mutation and phenotypic profile of the parent cell and is capable of reconstituting a tumor. Several studies have since challenged this theory by demonstrating the existence of a subpopulation of cells called cancer stem cells (CSCs) or tumor-initiating cells, which are typically quiescent but under certain conditions, capable of proliferating to self-renew

the CSC population and generate progenitor tumor cells (3, 4). CSCs are resistant to therapies that target rapidly proliferating tumor cells and are primarily responsible for tumor relapse. In the 1990s, the theory of a hierarchical organization within tumors was introduced in acute myeloid leukemia (AML), identifying leukemia-initiating cells *via* their expression of a $CD34^{++}CD38^{-}$ phenotype. This hierarchical model postulates that individual tumor cells have distinct mutational profiles and epigenetic modifications contributing to cellular heterogeneity. In the years to follow, researchers have used molecular markers to identify and isolate CSCs of various solid tumors (5–7).

Currently, there are more than 40 established CSC markers (**Table 1**); however, much controversy surrounds the scientific techniques employed to identify surface markers. Moreover, majority of the markers established for the identification of CSCs were previously described in human embryonic stem cells and/or adult stem cells of normal tissue cells (5, 8). This shared feature may suggest two possibilities: CSCs could originate from genetic alterations in normal stem cells or could be the result of dedifferentiation of mutated cancer cells into stem-like cells. Despite the shared properties, CSCs differ from normal stem cells in that unlike CSCs, cell proliferation is rigidly controlled in normal stem cells (9). Glycosylation of glycoprotein markers has also been suggested to impact the biological behavior of CSCs (8). It is important to focus future investigation on the mutations, metabolic phenotype, and other aspects of the microenvironment that distinguish CSCs from normal stem cells.

Normal stem cells are unique in their ability to self-renew, proliferate, and differentiate into various tissue types, as well as reproduce progeny essential to maintain and repair the organ system in which they are found (35, 36). Embryonic stem cells, hematopoietic stem cells, and mesenchymal stem cells have a low mitochondrial DNA copy number, as well as poorly developed mitochondrial morphology and reduced oxidative capacity. On the other hand, glycolytic pathways are highly active in these stem cells. Hypoxia-induced glycolysis in pluripotent stem cells and inhibition of mitochondrial respiration promote stemness, whereas inhibition of glycolysis disrupts proliferation and promotes cell death (37).

Although CSCs share many of the characteristics of normal stem cells, the differ in that, they contribute to tumor progression, drug resistance, and recurrence (38). In addition, several reports suggest that CSCs preferentially use glycolysis. However, other reports suggest a propensity for mitochondrial oxidative phosphorylation (OXPHOS) suggesting a possible metabolic plasticity. The aim of this review is to summarize and emphasize some of

TABLE 1 | Biomarkers reported to characterize CSCs.

Marker	Cancers identified	Metabolic phenotype	Reference
ABCG2	HNSCC, retinoblastoma, lung cancer, liver cancer, pancreatic cancer, melanoma	Hypoxia induced	(10)
Aldehyde dehydrogenase 1-A1/ALDH1A1	Liver, kidney, red blood cells, skeletal muscle, lung, breast, lens, stomach, brain, pancreas, testis, prostate, ovary	Converts acetaldehyde to acetate, maintains low ROS	(11)
Alpha-methylacyl-CoA racemase/AMACR	Prostate cancer, gastric cancer, nasopharyngeal cancer, CRC	Facilitates metabolic switch to fatty acid β-oxidation	(12)
CD24	Gastric cancer	CD24 is a hypoxia-inducible factor	(13)
CD27	Lymphoma, multiple myeloma, B-cell chronic lymphocytic leukemia, renal cell carcinoma, glioblastoma, mesothelioma, HCC, cancers of the pancreas, breast and ovary, CRC, melanoma, neuro-endocrine carcinoma	Not specified	(14)
CD44	Most epithelial cancers, leukemia	Promotes glycolysis *via* PKM2 suppression	(15)
CD47	AML, ALL, breast cancer, esophageal cancer	Regulates glycolytic metabolic pathways	(16)
CD133	Brain, breast, CRC, HNSCC, kidney, liver, lung, ovary, pancreas, prostate, stomach, bone/soft tissue, eye, skin	Decreased hexokinase II expression, promoted by hypoxia	(17, 18)
Connexin 43/GJA1	Prostate cancer, nasopharyngeal cancer, glioblastoma, HCC	Increased glucose uptake	(19)
c-Met	HNSCC, breast cancer, thyroid cancer, HCC	Prevents excessive ROS	(20, 21)
ErbB2/Her2	Breast cancer, endometrial cancer, gastric cancer	Promotes aerobic glycolysis	(22, 23)
GLI-1	Leukemia, breast cancer, glioma	Hypoxia induced	(24)
GLI-2	Leukemia, breast cancer, glioma, osteosarcoma, HCC, pancreatic cancer	Hypoxia induced	(25)
HIF-2 alpha/EPAS1	HCC, lung cancer, renal cancer, CRC, melanoma, glioblastoma, gastric cancer	Hypoxia induced	(26)
IL-3 R alpha/CD123	AML, pancreatic cancer, non-small cell lung cancer, breast cancer, ovarian cancer	Promotes glycolytic enzyme activity	(27, 28)
IL-6 R alpha	Most epithelial cancers	Promotes glycogenolysis	(29, 30)
Integrin alpha 6/CD49f	Prostate cancer, breast cancer, glioblastoma	Not specified	(31, 32)
Lgr5/GPR49	HNSCC, HCC, CRC, ovarian cancer, basal cell carcinoma	Promotes mitochondrial OXPHOS	(33, 34)

AML, acute myeloid leukemia; ALL, acute lymphocytic leukemia; CRC, colorectal carcinoma; HNSCC, head and neck squamous cell carcinoma; HCC, hepatocellular carcinoma; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; CSCs, cancer stem cells: PKM2, pyruvate kinase M2.

the key aspects currently known about CSC metabolism and the potential therapeutic targets contributing to cancer progression.

CELLULAR AND CANCER METABOLISM

All cells require energy for growth, division, and survival, which they acquire through the absorption of nutrients including glucose that are broken down in a series of metabolic reactions involving glycolysis and cellular respiration through OXPHOS. Under normal physiological conditions, cells rely on both glycolysis and OXPHOS for efficient energy production (39). The process of glycolysis involves the breakdown of glucose through a series of reactions to produce pyruvate, two molecules of adenosine 5′-triphosphate (ATP), and nicotinamide adenine dinucleotide (NADH). Under normoxic conditions (oxygen is readily available), pyruvate is transported to the mitochondria where it is converted into acetyl coenzyme A. Acetyl CoA enters the tricarboxylic acid cycle to produce high amounts of energy in the form of NADH and flavin adenine dinucleotide (FADH2) molecules. The hydrogen ions from NADH trigger the electron transport chain and generation of up to 32 molecules of ATP through OXPHOS (40, 41).

Since OXPHOS generates more ATP molecules than glycolysis, normal cells rely primarily on OXPHOS as an efficient source of energy. This process, however, is impaired in hypoxic conditions due a dearth in oxygen.

Rapidly proliferating cancer cells outpace angiogenesis resulting in areas of low oxygen. However, increasing evidence suggests that cancer cells engage in glycolysis even in the presence of oxygen (42). As a result, tumor cells demonstrate enhanced glycolytic production of ATP (43). Otto Warburg first described this phenomenon now known as the Warburg effect of aerobic glycolysis (41, 44, 45). The increased glycolysis was attributed to mitochondrial damage in cancer cells. Subsequent studies found that most cancer cells do not demonstrate mitochondrial damage, but rather suggest that aerobic glycolysis can occur simultaneously to enhance energy production for the maintenance of cancer cell homeostasis (43). In fact, several studies demonstrate that acceleration of glycolysis provides a source of metabolites and other essential factors required for rapidly dividing cells (41, 43, 46, 47).

METABOLIC PHENOTYPE OF CSCs

Due to the highly proliferative, tumorigenic, and drug-resistant properties of CSCs, in-depth investigation of CSC metabolic phenotype has comprised the cornerstone of numerous recent studies. Although metabolic adaptation or plasticity is one of the hallmarks of cancer, the majority of reports suggest that CSCs are primarily glycolytic (48–55). However, examination of CSCs isolated from patient tumors suggests that OXPHOS is the main source of energy (56, 57). We describe other multifactorial causes contributing to the apparent differences in CSC metabolism across tumor types in the following sections. Emerging evidence suggests the existence of specific metabolic phenotypes of CSCs based on their location, such as those in actively growing regions of the tumor that have adequate levels of oxygen, hypoxic areas of the tumor, or those in a distant metastatic site summarized in **Figure 1**.

GLYCOLYTIC PATHWAY

A number of studies performed in various tumor types, such as glioblastoma, lung cancer, osteosarcoma, breast cancer, ovarian cancer, and colon cancer, suggest that CSCs more strongly favor the glycolytic pathway than other differentiated cancer cells *in vitro* and *in vivo* (58–62). Rationale for investigating the role of glycolytic metabolism in CSCs is due to its proposed phenotypic similarity to normal stem cells with self-renewal characteristics. Earlier studies paved the way by illustrating the low activity of mitochondrial respiration in brain tumor CSCs, as well as higher rates of glycolysis in CSCs than other tumor cells (63, 64). Further investigations revealed that upregulation of glycolytic enzymes (GLUT1, HK-1, and PDK-1) and stimulation of glycolysis are necessary for cell immortalization and is sufficient to increase cellular lifespan (65). Comparing glucose utilization by CSCs and non-CSCs has revealed differentially elevated glucose consumption, lactate synthesis, and ATP content in CSCs, thus suggesting distinct metabolic profiles of CSCs in comparison to non-CSCs (66–68). Glycolysis has also been identified as the preferred metabolic pathway of CSCs in nasopharyngeal carcinoma and of tumor-initiating stem-like cells in hepatocellular carcinoma (69, 70). In addition, cellular metabolism is thought to control stemness characteristics; in particular, the glycolytic switch has a causal relation in induced pluripotent stem cell reprogramming and acquisition of pluripotent markers (71). Reprogramming the metabolic switch from OXPHOS to glycolysis was shown to enhance stemness and CSC properties in $CD44^{+}CD24^{low}EPCAM^{+}$ cells of basal-like breast cancer by reducing reactive oxygen species (ROS) levels (48). Glycolysis-driven induction of pluripotency is consistent with the finding that hypoxia maintains the stem cell state and a hypoxic environment promotes the reprogramming process (72).

OXPHOS PATHWAY

Growing evidence suggests mitochondrial oxidative metabolism as the preferred form of energy production in CSCs. Several studies in numerous tumor types, such as $CD133^{+}$ cells of glioblastoma and pancreatic ductal adenocarcinoma, ROS^{low} quiescent leukemia stem cells, lung cancer side population cells, and breast cancer, strongly support an OXPHOS phenotype and less glycolytic profile (49, 50, 54, 73). In contrast to the non-CSC cancer cells, which mainly utilize glycolysis for energy production, CSCs have an enhanced mitochondrial ROS, higher rates of oxygen consumption, and overall increased mitochondrial function, as evidenced by increased mitochondrial mass and membrane potential (50, 52, 53, 73–76). Moreover, this increased mitochondrial bulk in a subpopulation of breast cancer cells induces stem-like characteristics and confers metastatic potential and resistance to DNA damage (77). In addition, CSCs may depend on mitochondrial fatty acid oxidation (FAO) for the generation of ATP and NADH. A population of isolated ovarian CSCs revealed upregulated expression of genes associated with

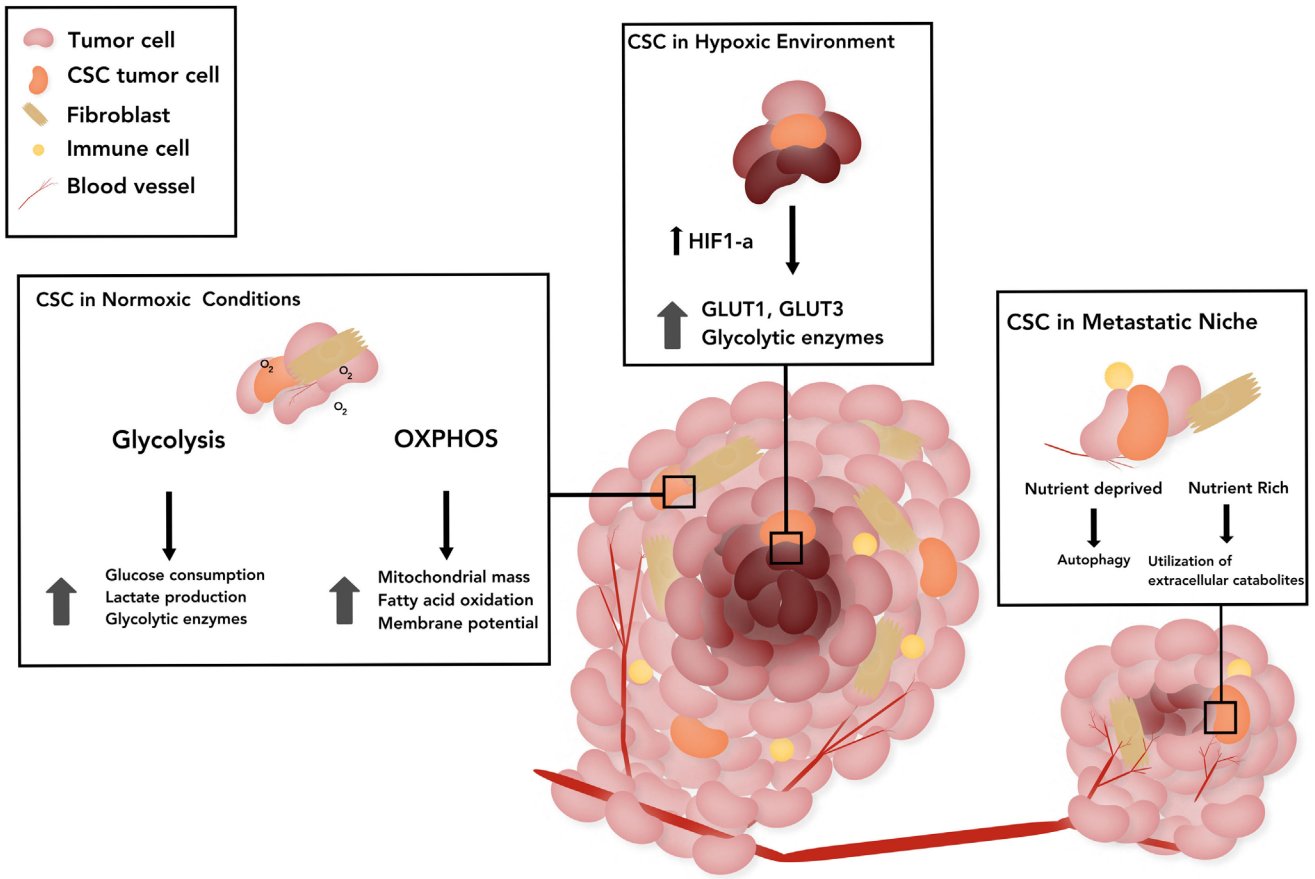


FIGURE 1 | The schematic represents the metabolic status of cancer stem cells (CSCs) in three broad categories based on the location. CSCs in a normoxic tumor: stemness has been associated with upregulation of glycolytic enzymes in those CSCs that rely on glucose pathway, as well as with dependence on mitochondrial pathway as evidenced by increased mitochondrial mass, membrane potential in CSCs and mitochondrial fatty acid oxidation (FAO) for generation of adenosine 5′-triphosphate and nicotinamide adenine dinucleotide. CSCs in tumor under hypoxia: hypoxia-inducible factor-1α (HIF-1α) promotes upregulation and potentiated activity of several glycolytic proteins, such as transporters (GLUT1, GLUT3) and various isoforms of glycolytic enzymes. CSCs in the metastatic niche: CSCs induced by epithelial-to-mesenchymal transition, have augmented utilization of extracellular catabolites, such as pyruvate, lactate, glutamine, glutamate, alanine, or ketone bodies. In nutrient poor states, quiescent disseminated tumor cells rely on alternative energy sources such as autophagy. The CSC stem cell model of treatment proposes the prevention of recurrence if all CSCs are eliminated.

FAO and OXPHOS (52). FAO is instrumental in self-renewal processes of hematopoietic stem cells and leukemia-initiating cells, as in the survival of ablation-resistant pancreatic CSCs and survival of epithelial cancer cells subsequent to matrix detachment (78–80). An oxidative phenotype confers resistance to treatment modalities and evasion of apoptosis as evidenced by the vastly tumorigenic and chemoresistant metabolism found in hepatocellular CSCs, upon NANOG-induced expression of FAO genes (70). The powerful antioxidant defense mechanism of CSCs contributes to therapy resistance, by maintaining a significantly lower ROS levels and preserving stemness and tumorigenic properties of CSCs (52, 81, 82).

FACTORS AFFECTING THE METABOLIC STATUS OF CSCs

The reported differences in the metabolic profile of CSCs from various tumor types are due to multifactorial causes. One such explanation is the suggested plasticity of these cells and the potential harvest of them at various stages of differentiation/dedifferentiation during experiments (2). Another cause may be the lack of uniformity and precision in definition of CSCs and varying techniques utilized to isolate CSCs, such as specific markers, Hoechst staining-based sorting, chemoresistance-based isolation, and reoxygenation sorting post-hypoxic exposure (83–87). This is due to a vast heterogeneity of CSCs across various histologic tumor types. Another potential contributing factor that may explain the contradictory results is the contribution of the microenvironment. We broadly distinguish the metabolic status of CSCs in three locations, namely, regions with normoxic tumor, hypoxic tumor, and at metastatic sites (**Figure 1**).

As mentioned in the preceding sections, under normoxic conditions, CSCs can engage in glycolysis and/or OXPHOS. Furthermore, the metabolic status of CSCs can be affected by cross-talk between CSCs and cancer-associated stroma in the microenvironment. For example, cancer-associated fibroblast-secreted

metabolites including lactate and ketone bodies drive OXPHOS in cancer cells (88). The role of cancer-associated stroma in regulating CSC metabolism is unknown.

Similar to embryonic stem cell maintenance, tumor hypoxia promotes the persistence of an undifferentiated, stem cell phenotype (89). Ductal breast carcinoma cells revert to a stem cell-like phenotype through dedifferentiation under hypoxic conditions (90). Other studies showed that hypoxic exposure altered gene expression in human neuroblastoma cells toward a neural crest-like, immature profile and caused upregulation of the stem cell surface marker CD-133 in medulloblastoma (90, 91). Under hypoxic conditions, overexpression of hypoxia-inducible factor-1α (HIF-1α) promotes upregulation and potentiated activity of several glycolytic proteins, such as transporters (GLUT1 and GLUT3) and various isoforms of glycolytic enzymes (92). In addition, HIF-1α regulates pyruvate dehydrogenase kinase 1 levels which facilitate glycolysis in breast CSCs under hypoxic conditions (93).

Metastatic cancer cells undergo epithelial-to-mesenchymal transition (EMT) upregulating a number of factors associated with a stem-like phenotype. EMT-associated factors including HIF-1α, Wnt, and Snail regulate cellular metabolism (94). Furthermore, EMT-associated metabolites—glutamine, glutamate, and alanine—as well as high lactate concentrations are associated with poor survival and higher metastatic potential in breast cancers (95, 96). CSCs have augmented utilization of extracellular catabolites, such as pyruvate, lactate, glutamine, glutamate, alanine, or ketone bodies to support OXPHOS (97–99). In nutrient poor states, quiescent disseminated tumor cells rely on alternative energy sources such as autophagy, yet metabolic plasticity demonstrated by their ability to produce energy through various pathways is instrumental for metastatic growth and proliferation (100–102). Finally, a recent study of metabolic dependencies of non-small cell lung cancers highlighted the significant contribution of the microenvironment as a determinant of the metabolic phenotype of cancer cells, as evidenced by varying profiles *in vitro* and *in vivo* settings. *KRAS*-driven lung cancer cells in mice models showed preferential glutamine utilization *in vitro*, but did not depend on glutamine metabolism *in vivo* (103).

TARGETING CELLULAR METABOLISM

A strong association between tumors with high CSC fractions and recurrence, poorer overall survival, and higher incidence of metastasis, underscores the significant prognostic and therapeutic implications of CSCs (104, 105). Defining characteristics of CSCs such as surface markers, metabolic phenotypes, resistance to chemoradiotherapy, and regulatory factors in microenvironment compile the bulk of therapeutic targets. For instance, CD44, a receptor for hyaluronic acid-mediated motility, is shown to induce CSC attachment to extracellular matrix and cell migration, promoting metastasis and invasion (106). Treatment of breast, colon, esophageal, gastric, lung, and ovarian cancers overexpressing CD44, with ONCOFID™-S which is a conjugate of hyaluron and chemotherapeutic agent SN38 (7-ethyl-10-hydroxycamptothecin, active metabolite of CPT-11) revealed a strong *in vitro* anti-proliferative activity (107, 108). In addition, use of anti-CD44 antibodies H90 and A3D8 inhibited proliferation and induced apoptosis, by promoting the differentiation of AML blasts (108–111). Finally, CD44 interacts with pyruvate kinase M2 (PKM2), enhancing the glycolytic profile of cancer cells deficient in p53 or exposed to hypoxia. Subsequent ablation of CD44 led to inhibition of glycolysis, increase in ROS and enhancement of chemotherapeutic drug effect in these cancer cells (110). Therefore, preferentially targeting of identified CSC markers, such as CD44, can be utilized for an effective cytotoxic drug delivery. In addition, inhibition of glycolysis can be achieved by targeting various glycolytic enzymes, transporters, and other complex regulators, such as GLUT 1–4, hexokinase, PKM2, and lactate dehydrogenase A (111–113).

Previously discussed evidence for OXPHOS dependence of CSCs in numerous cancer lines proposes mitochondrial metabolism to be a potential target for an effective elimination of CSCs. Inhibition of the OXPHOS pathway reduces sphere formation and tumor formation potential demonstrating vulnerability of CSC to mitochondria-targeted therapies (54, 114, 115). Pharmacological agents targeting CSCs through inhibition of mitochondrial biogenesis and OXPHOS are currently under investigation for cancer treatment. Several FDA-approved compounds known to inhibit mitochondrial function have been reported to achieve a more effective eradication of CSCs. Salinomycin, erythromycins, tetracyclines, and glycylcyclines are some of the approved agents to have already demonstrated efficacy in eradicating CSCs *via* reduction of stemness properties (115–118). Metformin, an inhibitor of OXPHOS complex I, has demonstrated anti-tumoral activity by reducing mammosphere formation, delaying *in vivo* tumor growth, and inducing apoptosis in pancreatic CSCs unable to switch to glycolysis (54, 119, 120). However, emergence of a small subset of resistant CSCs with an intermediate glycolytic/OXPHOS phenotype could be prevented/reversed by utilizing a mitochondrial ROS inducer such as menadione (54). Dual mechanism of menadione inhibition of Complex I and induction of mitochondrial ROS points out the superior efficacy of multi-modal targeted therapy. Studies have shown that inhibition of mitochondrial respiration not only induces apoptosis in pancreatic CSCs with OXPHOS phenotype but also effectively eliminates primarily glycolytic breast and nasopharyngeal CSCs (53, 54, 121). These data highlight the extended role of mitochondria beyond energy production in CSCs, such as acquiring metabolites from glutamine *via* reductive carboxylation to support growth in tumor cells with defective mitochondria (122). A novel compound 3,5-bis(2,4-difluorobenzylidene)-4-piperidone (DiFiD) has been shown to inhibit pancreatic cancer growth by targeting a CSC marker, doublecortin and CaM kinase-like-1 (DCLK-1) (59, 66, 123). However, the role of DCLK-1 and the impact of DiFiD on CSC metabolism have not been studied.

Evident from the data reviewed, the CSC phenotype varies between cancer subtypes and among populations of the same subtypes. Preferred energy-producing metabolic pathways depend on various factors, including metastatic site highlighting vast metabolic variability and patterns (124). In addition to studies supporting metabolic plasticity, simultaneous enhancement of glycolysis and OXPHOS pathways was observed in highly metastatic breast cancer cell lines relative to non-metastatic

cell lines (49, 124). Consequently, dual inhibition of glycolytic and mitochondrial energy pathways has proven to be effective against tumor growth in a number of preclinical cancer models (125). One such study elegantly demonstrated sarcoma cells to be twofold to fivefold more sensitive than normal cells to dual inhibition of glycolysis with 2-deoxyglucose and OXPHOS with oligomycin or metformin (126). Therefore, dual inhibition of metabolic pathways may be a superior approach to eradicating heterogeneous CSCs rather than singularly targeting glycolysis or OXPHOS pathways. Finally, other factors directly affecting metabolic status of CSCs may represent potential targets for pharmacological treatments. These developments may include promoting CSC differentiation, targeting complex interactions within the microenvironment, and disrupting cellular communications in the CSC niche to interfere with CSC growth, resistance, and metastasis (97, 127–130).

CONCLUSION

Substantial evidence suggests that the CSCs are pluripotent, self-renewing, "original cells" of a tumor capable of differentiation into more specialized cancer cell types. CSCs are responsible for tumor formation, differentiation, maintenance, spread, and recurrence, making them an attractive therapeutic target for a potential permanent cure or long-term disease-free survival (127, 131). Regardless of the controversy about the metabolic phenotype of CSCs, metabolism is not only a key player but also a regulatory instigator of stemness.

Metabolic singularities that distinguish CSCs need to be further investigated, as they offer a great potential for developing improved treatments to eradicate them. In particular, streamlining and standardization of CSC identification methods is important. Development of CSC marker combinations would contribute to better delineation of CSCs from non-CSC cancer cells and normal stem cells. Interactions between CSCs and their microenvironment also provide a fertile ground for advance investigations. Chronicity and causality of these complex interactions needs to be established. Moving forward, CSC metabolic pathways and principal players of metabolism comprise potential therapeutic targets with a great promise for improved cancer treatments.

AUTHOR CONTRIBUTIONS

VS carried out the literature review and wrote and edited the manuscript. TR-N carried out the literature review and wrote a section of the manuscript. LA carried out literature review and prepared the figure and table included in the manuscript. SA and ST conceptualized the manuscript, supervised the writing, and edited the manuscript.

FUNDING

This work was supported by the NIH grants CA135559 and CA109269, the Kansas Bioscience Authority, Tom O'Sullivan Foundation Supported Research and Braden's Hope Foundation Grant to SA. This study was supported in part by University of Kansas Cancer Center under CCSG 1-P30-CA168524-0 (to SA and ST), an NIH Clinical and Translational Science Award grant (UL1 TR000001, formerly UL1RR033179) awarded to the University of Kansas Medical Center, and an internal Lied Basic Science Grant Program of the KUMC Research Institute (to ST).

REFERENCES

1. Odoux C, Fohrer H, Hoppo T, Guzik L, Stolz DB, Lewis DW, et al. A stochastic model for cancer stem cell origin in metastatic colon cancer. *Cancer Res* (2008) 68(17):6932–41. doi:10.1158/0008-5472.CAN-07-5779
2. Peiris-Pagès M, Martinez-Outschoorn UE, Pestell RG, Sotgia F, Lisanti MP. Cancer stem cell metabolism. *Breast Cancer Res* (2016) 18(1):55. doi:10.1186/s13058-016-0712-6
3. Badri H, Leder K. Optimal treatment and stochastic modeling of heterogeneous tumors. *Biol Direct* (2016) 11:40. doi:10.1186/s13062-016-0142-5
4. Kreso A, Dick JE. Evolution of the cancer stem cell model. *Cell Stem Cell* (2014) 14(3):275–91. doi:10.1016/j.stem.2014.02.006
5. Kim WT, Ryu CJ. Cancer stem cell surface markers on normal stem cells. *BMB Rep* (2017) 50(6):285–98. doi:10.5483/BMBRep.2017.50.6.039
6. Zheng S, Xin L, Liang A, Fu Y. Cancer stem cell hypothesis: a brief summary and two proposals. *Cytotechnology* (2013) 65(4):505–12. doi:10.1007/s10616-012-9517-3
7. Langenkamp U, Siegler U, Jörger S, Diermayr S, Gratwohl A, Kalberer CP, et al. Human acute myeloid leukemia CD34(+)CD38(−) stem cells are susceptible to allorecognition and lysis by single KIR-expressing natural killer cells. *Haematologica* (2009) 94(11):1590–4. doi:10.3324/haematol.2009.005967
8. Karsten U, Goletz S. What makes cancer stem cell markers different? *Springerplus* (2013) 2(1):301. doi:10.1186/2193-1801-2-301
9. Li L, Neaves WB. Normal stem cells and cancer stem cells: the niche matters. *Cancer Res* (2006) 66(9):4553–7. doi:10.1158/0008-5472.CAN-05-3986
10. Ding XW, Wu JH, Jiang CP. ABCG2: a potential marker of stem cells and novel target in stem cell and cancer therapy. *Life Sci* (2010) 86(17):631–7. doi:10.1016/j.lfs.2010.02.012
11. Tomita H, Tanaka K, Tanaka T, Hara A. Aldehyde dehydrogenase 1A1 in stem cells and cancer. *Oncotarget* (2016) 7(10):11018–32. doi:10.18632/oncotarget.6920
12. Lloyd MD, Yevglevskis M, Lee GL, Wood PJ, Threadgill MD, Woodman TJ. α-Methylacyl-CoA racemase (AMACR): metabolic enzyme, drug metabolizer and cancer marker P504S. *Progr Lipid Res* (2013) 52(2):220–30. doi:10.1016/j.plipres.2013.01.001
13. Fujikuni N, Yamamoto H, Tanabe K, Naito Y, Sakamoto N, Tanaka Y, et al. Hypoxia-mediated CD24 expression is correlated with gastric cancer aggressiveness by promoting cell migration and invasion. *Cancer Sci* (2014) 105(11):1411–20. doi:10.1111/cas.12522
14. Jacobs J, Deschoolmeester V, Zwaenepoel K, Rolfo C, Silence K, Rottey S, et al. CD70: an emerging target in cancer immunotherapy. *Pharmacol Ther* (2015) 155:1–10. doi:10.1016/j.pharmthera.2015.07.007
15. Yan Y, Zuo X, Wei D. Concise review: emerging role of CD44 in cancer stem cells: a promising biomarker and therapeutic target. *Stem Cells Transl Med* (2015) 4(9):1033–43. doi:10.5966/sctm.2015-0048
16. Suzuki S, Yokobori T, Tanaka N, Sakai M, Sano A, Inose T, et al. CD47 expression regulated by the miR-133a tumor suppressor is a novel prognostic marker in esophageal squamous cell carcinoma. *Oncol Rep* (2012) 28(2):465–72. doi:10.3892/or.2012.1831
17. Grosse-Gehling P, Fargeas CA, Dittfeld C, Garbe Y, Alison MR, Corbeil D, et al. CD133 as a biomarker for putative cancer stem cells in solid tumours: limitations, problems and challenges. *J Pathol* (2013) 229(3):355–78. doi:10.1002/path.4086
18. Li Z. CD133: a stem cell biomarker and beyond. *Exp Hematol Oncol* (2013) 2:17. doi:10.1186/2162-3619-2-17
19. Wang W-K, Chen M-C, Leong H-F, Kuo Y-L, Kuo C-Y, Lee C-H, et al. Connexin 43 suppresses tumor angiogenesis by down-regulation of vascular

endothelial growth factor via hypoxic-induced factor-1α. *Int J Mol Sci* (2015) 16(1):439–51. doi:10.3390/ijms16010439
20. Lim YC, Kang HJ, Moon JH. C-Met pathway promotes self-renewal and tumorigenecity of head and neck squamous cell carcinoma stem-like cell. *Oral Oncol* (2014) 50(7):633–9. doi:10.1016/j.oraloncology.2014.04.004
21. Gómez-Quiroz LE, Factor VM, Kaposi-Novak P, Coulouarn C, Conner EA, Thorgeirsson SS. Hepatocyte-specific c-Met deletion disrupts redox homeostasis and sensitizes to Fas-mediated apoptosis. *J Biol Chem* (2008) 283(21):14581–9. doi:10.1074/jbc.M707733200
22. Contino F, Mazzarella C, Ferro A, Lo Presti M, Roz E, Lupo C, et al. Negative transcriptional control of ERBB2 gene by MBP-1 and HDAC1: diagnostic implications in breast cancer. *BMC Cancer* (2013) 13:81. doi:10.1186/1471-2407-13-81
23. Walsh A, Cook RS, Rexer B, Arteaga CL, Skala MC. Optical imaging of metabolism in HER2 overexpressing breast cancer cells. *Biomed Opt Express* (2012) 3(1):75–85. doi:10.1364/BOE.3.000075
24. Lei J, Fan L, Wei G, Chen X, Duan W, Xu Q, et al. Gli-1 is crucial for hypoxia-induced epithelial-mesenchymal transition and invasion of breast cancer. *Tumor Biol* (2015) 36(4):3119–26. doi:10.1007/s13277-014-2948-z
25. Im S, Choi HJ, Yoo C, Jung JH, Jeon YW, Suh YJ, et al. Hedgehog related protein expression in breast cancer: Gli-2 is associated with poor overall survival. *Korean J Pathol* (2013) 47(2):116–23. doi:10.4132/KoreanJPathol.2013.47.2.116
26. Zhao J, Du F, Luo Y, Shen G, Zheng F, Xu B. The emerging role of hypoxia-inducible factor-2 involved in chemo/radioresistance in solid tumors. *Cancer Treat Rev* (2015) 41(7):623–33. doi:10.1016/j.ctrv.2015.05.004
27. Dentelli P, Rosso A, Olgasi C, Camussi G, Brizzi MF. IL-3 is a novel target to interfere with tumor vasculature. *Oncogene* (2011) 30(50):4930–40. doi:10.1038/onc.2011.204
28. Bauer DE, Harris MH, Plas DR, Lum JJ, Hammerman PS, Rathmell JC, et al. Cytokine stimulation of aerobic glycolysis in hematopoietic cells exceeds proliferative demand. *FASEB J* (2004) 18(11):1303–5. doi:10.1096/fj.03-1001fje
29. Krishnamurthy S, Warner KA, Dong Z, Imai A, Nör C, Ward BB, et al. Endothelial interleukin-6 defines the tumorigenic potential of primary human cancer stem cells. *Stem Cells* (2014) 32(11):2845–57. doi:10.1002/stem.1793
30. Mauer J, Denson JL, Brüning JC. Versatile functions for IL-6 in metabolism and cancer. *Trends Immunol* (2015) 36(2):92–101. doi:10.1016/j.it.2014.12.008
31. Kacsinta AD, Rubenstein CS, Sroka IC, Pawar S, Gard JM, Nagle RB, et al. Intracellular modifiers of integrin alpha 6p production in aggressive prostate and breast cancer cell lines. *Biochem Biophys Res Commun* (2014) 454(2):335–40. doi:10.1016/j.bbrc.2014.10.073
32. Lathia JD, Gallagher J, Heddleston JM, Wang J, Eyler CE, Macswords J, et al. Integrin alpha 6 regulates glioblastoma stem cells. *Cell Stem Cell* (2010) 6(5):421–32. doi:10.1016/j.stem.2010.02.018
33. Chen Q, Zhang X, Li WM, Ji YQ, Cao HZ, Zheng P. Prognostic value of LGR5 in colorectal cancer: a meta-analysis. *PLoS One* (2014) 9(9):e107013. doi:10.1371/journal.pone.0107013
34. Rodríguez-Colman MJ, Schewe M, Meerlo M, Stigter E, Gerrits J, Pras-Raves M, et al. Interplay between metabolic identities in the intestinal crypt supports stem cell function. *Nature* (2017) 543(7645):424–7. doi:10.1038/nature21673
35. Botelho M, Alves H. Significance of cancer stem cells in anti-cancer therapies. *Int J Immunother Cancer Res* (2016) 2(1):14–6. doi:10.17352/2455-8591.000010
36. NIH Stem Cell Information Home Page. *Stem Cell Information*. Bethesda, MD: National Institutes of Health, U.S. Department of Health and Human Services (2016). Available from: https://stemcells.nih.gov/info/basics/1.htm (Accessed: May 28, 2018).
37. Folmes CD, Dzeja PP, Nelson TJ, Terzic A. Metabolic plasticity in stem cell homeostasis and differentiation. *Cell Stem Cell* (2012) 11(5):596–606. doi:10.1016/j.stem.2012.10.002
38. Rahman M, Deleyrolle L, Vedam-Mai V, Azari H, Abd-El-Barr M, Reynolds BA. The cancer stem cell hypothesis: failures and pitfalls. *Neurosurgery* (2011) 68(2):531–45; discussion 545. doi:10.1227/NEU.0b013e3181ff9eb5
39. Baudot A, de la Torre V, Valencia A. Mutated genes, pathways and processes in tumours. *EMBO Rep* (2010) 11(10):805–10. doi:10.1038/embor.2010.133
40. Sancho P, Barneda D, Heeschen C. Hallmarks of cancer stem cell metabolism. *Br J Cancer* (2016) 114(12):1305–12. doi:10.1038/bjc.2016.152
41. San-Millan I, Brooks GA. Reexamining cancer metabolism: lactate production for carcinogenesis could be the purpose and explanation of the Warburg effect. *Carcinogenesis* (2017) 38(2):119–33. doi:10.1093/carcin/bgw127
42. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* (2001) 414(6859):105–11. doi:10.1038/35102167
43. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* (2009) 324(5930):1029–33. doi:10.1126/science.1160809
44. Warburg O. On the origin of cancer cells. *Science* (1956) 123(3191):309–14. doi:10.1126/science.123.3191.309
45. Jang M, Kim SS, Lee J. Cancer cell metabolism: implications for therapeutic targets. *Exp Mol Med* (2013) 45(10):e45. doi:10.1038/emm.2013.85
46. Lee M, Yoon JH. Metabolic interplay between glycolysis and mitochondrial oxidation: the reverse Warburg effect and its therapeutic implication. *World J Biol Chem* (2015) 6(3):148–61. doi:10.4331/wjbc.v6.i3.148
47. De Preter G, Neveu MA, Danhier P, Brisson L, Payen VL, Porporato PE, et al. Inhibition of the pentose phosphate pathway by dichloroacetate unravels a missing link between aerobic glycolysis and cancer cell proliferation. *Oncotarget* (2016) 7(3):2910–20. doi:10.18632/oncotarget.6272
48. Dong C, Yuan T, Wu Y, Wang Y, Fan TW, Miriyala S, et al. Loss of FBP1 by Snail-mediated repression provides metabolic advantages in basal-like breast cancer. *Cancer Cell* (2013) 23(3):316–31. doi:10.1016/j.ccr.2013.01.022
49. Ye XQ, Li Q, Wang GH, Sun FF, Huang GJ, Bian XW, et al. Mitochondrial and energy metabolism-related properties as novel indicators of lung cancer stem cells. *Int J Cancer* (2011) 129(4):820–31. doi:10.1002/ijc.25944
50. Janiszewska M, Suvà ML, Riggi N, Houtkooper RH, Auwerx J, Clément-Schatlo V, et al. Imp2 controls oxidative phosphorylation and is crucial for preserving glioblastoma cancer stem cells. *Genes Dev* (2012) 26(17):1926–44. doi:10.1101/gad.188292.112
51. Anderson AS, Roberts PC, Frisard MI, Hulver MW, Schmelz EM. Ovarian tumor-initiating cells display a flexible metabolism. *Exp Cell Res* (2014) 328(1):44–57. doi:10.1016/j.yexcr.2014.08.028
52. Pasto A, Bellio C, Pilotto G, Ciminale V, Silic-Benussi M, Guzzo G, et al. Cancer stem cells from epithelial ovarian cancer patients privilege oxidative phosphorylation, and resist glucose deprivation. *Oncotarget* (2014) 5(12):4305–19. doi:10.18632/oncotarget.2010
53. Shen YA, Lin CH, Chi WH, Wang CY, Hsieh YT, Wei YH, et al. Resveratrol impedes the stemness, epithelial-mesenchymal transition, and metabolic reprogramming of cancer stem cells in nasopharyngeal carcinoma through p53 activation. *Evid Based Complement Alternat Med* (2013) 2013:590393. doi:10.1155/2013/590393
54. Sancho P, Burgos-Ramos E, Tavera A, Bou Kheir T, Jagust P, Schoenhals M, et al. MYC/PGC-1alpha balance determines the metabolic phenotype and plasticity of pancreatic cancer stem cells. *Cell Metab* (2015) 22(4):590–605. doi:10.1016/j.cmet.2015.08.015
55. Dando I, Dalla Pozza E, Biondani G, Cordani M, Palmieri M, Donadelli M. The metabolic landscape of cancer stem cells. *IUBMB Life* (2015) 67(9):687–93. doi:10.1002/iub.1426
56. Feng W, Gentles A, Nair RV, Huang M, Lin Y, Lee CY, et al. Targeting unique metabolic properties of breast tumor initiating cells. *Stem Cells* (2014) 32(7):1734–45. doi:10.1002/stem.1662
57. Vlashi E, Lagadec C, Vergnes L, Matsutani T, Masui K, Poulou M, et al. Metabolic state of glioma stem cells and nontumorigenic cells. *Proc Natl Acad Sci U S A* (2011) 108(38):16062–7. doi:10.1073/pnas.1106704108
58. Ciavardelli D, Rossi C, Barcaroli D, Volpe S, Consalvo A, Zucchelli M, et al. Breast cancer stem cells rely on fermentative glycolysis and are sensitive to 2-deoxyglucose treatment. *Cell Death Dis* (2014) 5:e1336. doi:10.1038/cddis.2014.285
59. Liao J, Qian F, Tchabo N, Mhawech-Fauceglia P, Beck A, Qian Z, et al. Ovarian cancer spheroid cells with stem cell-like properties contribute to tumor generation, metastasis and chemotherapy resistance through hypoxia-resistant metabolism. *PLoS One* (2014) 9(1):e84941. doi:10.1371/journal.pone.0084941
60. Palorini R, Votta G, Balestrieri C, Monestiroli A, Olivieri S, Vento R, et al. Energy metabolism characterization of a novel cancer stem cell-like line 3AB-OS. *J Cell Biochem* (2014) 115(2):368–79. doi:10.1002/jcb.24671

61. Zhou Y, Zhou Y, Shingu T, Feng L, Chen Z, Ogasawara M, et al. Metabolic alterations in highly tumorigenic glioblastoma cells: preference for hypoxia and high dependency on glycolysis. *J Biol Chem* (2011) 286(37):32843–53. doi:10.1074/jbc.M111.260935
62. Chen KY, Liu X, Bu P, Lin CS, Rakhilin N, Locasale JW, et al. A metabolic signature of colon cancer initiating cells. *Conf Proc IEEE Eng Med Biol Soc* (2014) 2014:4759–62. doi:10.1109/EMBC.2014.6944688
63. Wu M, Neilson A, Swift AL, Moran R, Tamagnine J, Parslow D, et al. Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. *Am J Physiol Cell Physiol* (2007) 292(1):C125–36. doi:10.1152/ajpcell.00247.2006
64. Liu PP, Liao J, Tang ZJ, Wu WJ, Yang J, Zeng ZL, et al. Metabolic regulation of cancer cell side population by glucose through activation of the Akt pathway. *Cell Death Differ* (2014) 21(1):124–35. doi:10.1038/cdd.2013.131
65. Kondoh H, Lleonart ME, Gil J, Wang J, Degan P, Peters G, et al. Glycolytic enzymes can modulate cellular life span. *Cancer Res* (2005) 65(1):177–85.
66. Emmink BL, Verheem A, Van Houdt WJ, Steller EJ, Govaert KM, Pham TV, et al. The secretome of colon cancer stem cells contains drug-metabolizing enzymes. *J Proteomics* (2013) 91:84–96. doi:10.1016/j.jprot.2013.06.027
67. Hammoudi N, Ahmed KB, Garcia-Prieto C, Huang P. Metabolic alterations in cancer cells and therapeutic implications. *Chin J Cancer* (2011) 30(8):508–25. doi:10.5732/cjc.011.10267
68. Liu Y, Meyer C, Xu C, Weng H, Hellerbrand C, ten Dijke P, et al. Animal models of chronic liver diseases. *Am J Physiol Gastrointest Liver Physiol* (2013) 304(5):G449–68. doi:10.1152/ajpgi.00199.2012
69. Shen YA, Wang CY, Hsieh YT, Chen YJ, Wei YH. Metabolic reprogramming orchestrates cancer stem cell properties in nasopharyngeal carcinoma. *Cell Cycle* (2015) 14(1):86–98. doi:10.4161/15384101.2014.974419
70. Chen CL, Uthaya Kumar DB, Punj V, Xu J, Sher L, Tahara SM, et al. NANOG metabolically reprograms tumor-initiating stem-like cells through tumorigenic changes in oxidative phosphorylation and fatty acid metabolism. *Cell Metab* (2016) 23(1):206–19. doi:10.1016/j.cmet.2015.12.004
71. Folmes CD, Nelson TJ, Martinez-Fernandez A, Arrell DK, Lindor JZ, Dzeja PP, et al. Somatic oxidative bioenergetics transitions into pluripotency-dependent glycolysis to facilitate nuclear reprogramming. *Cell Metab* (2011) 14(2):264–71. doi:10.1016/j.cmet.2011.06.011
72. Mohyeldin A, Garzon-Muvdi T, Quinones-Hinojosa A. Oxygen in stem cell biology: a critical component of the stem cell niche. *Cell Stem Cell* (2010) 7(2):150–61. doi:10.1016/j.stem.2010.07.007
73. Lagadinou ED, Sach A, Callahan K, Rossi RM, Neering SJ, Minhajuddin M, et al. BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. *Cell Stem Cell* (2013) 12(3):329–41. doi:10.1016/j.stem.2012.12.013
74. De Luca A, Fiorillo M, Peiris-Pagès 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(17): 14777–95. doi:10.18632/oncotarget.4401
75. Lamb R, Bonuccelli G, Ozsvári B, Peiris-Pagès M, Fiorillo M, Smith DL, et al. Mitochondrial mass, a new metabolic biomarker for stem-like cancer cells: understanding WNT/FGF-driven anabolic signaling. *Oncotarget* (2015) 6(31):30453–71. doi:10.18632/oncotarget.5852
76. Vlashi E, Lagadec C, Vergnes L, Reue K, Frohnen P, Chan M, et al. Metabolic differences in breast cancer stem cells and differentiated progeny. *Breast Cancer Res Treat* (2014) 146(3):525–34. doi:10.1007/s10549-014-3051-2
77. Farnie G, Sotgia F, Lisanti MP. High mitochondrial mass identifies a sub-population of stem-like cancer cells that are chemo-resistant. *Oncotarget* (2015) 6(31):30472–86. doi:10.18632/oncotarget.5401
78. Samudio I, Harmancey R, Fiegl M, Kantarjian H, Konopleva M, Korchin B, et al. Pharmacologic inhibition of fatty acid oxidation sensitizes human leukemia cells to apoptosis induction. *J Clin Invest* (2010) 120(1):142–56. doi:10.1172/JCI38942
79. Ito K, Carracedo A, Weiss D, Arai F, Ala U, Avigan DE, et al. A PML-PPAR-delta pathway for fatty acid oxidation regulates hematopoietic stem cell maintenance. *Nat Med* (2012) 18(9):1350–8. doi:10.1038/nm.2882
80. Prigione A, Fauler B, Lurz R, Lehrach H, Adjaye J. The senescence-related mitochondrial/oxidative stress pathway is repressed in human induced pluripotent stem cells. *Stem Cells* (2010) 28(4):721–33. doi:10.1002/stem.404
81. Schafer ZT, Grassian AR, Song L, Jiang Z, Gerhart-Hines Z, Irie HY, et al. Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. *Nature* (2009) 461(7260):109–13. doi:10.1038/nature08268
82. Diehn M, Cho RW, Lobo NA, Kalisky T, Dorie MJ, Kulp AN, et al. Association of reactive oxygen species levels and radioresistance in cancer stem cells. *Nature* (2009) 458(7239):780–3. doi:10.1038/nature07733
83. Tang C, Ang BT, Pervaiz S. Cancer stem cell: target for anti-cancer therapy. *FASEB J* (2007) 21(14):3777–85. doi:10.1096/fj.07-8560rev
84. Bomken S, Fiser K, Heidenreich O, Vormoor J. Understanding the cancer stem cell. *Br J Cancer* (2010) 103(4):439–45. doi:10.1038/sj.bjc.6605821
85. Schatton T, Frank NY, Frank MH. Identification and targeting of cancer stem cells. *Bioessays* (2009) 31(10):1038–49. doi:10.1002/bies.200900058
86. Li HZ, Yi TB, Wu ZY. Suspension culture combined with chemotherapeutic agents for sorting of breast cancer stem cells. *BMC Cancer* (2008) 8:135. doi:10.1186/1471-2407-8-135
87. Louie E, Nik S, Chen JS, Schmidt M, Song B, Pacson C, et al. Identification of a stem-like cell population by exposing metastatic breast cancer cell lines to repetitive cycles of hypoxia and reoxygenation. *Breast Cancer Res* (2010) 12(6):R94. doi:10.1186/bcr2773
88. Zhang D, Wang Y, Shi Z, Liu J, Sun P, Hou X, et al. Metabolic reprogramming of cancer-associated fibroblasts by IDH3alpha downregulation. *Cell Rep* (2015) 10(8):1335–48. doi:10.1016/j.celrep.2015.02.006
89. Quail DF, Taylor MJ, Postovit LM. Microenvironmental regulation of cancer stem cell phenotypes. *Curr Stem Cell Res Ther* (2012) 7(3):197–216. doi:10.2174/157488812799859838
90. Axelson H, Fredlund E, Ovenberger M, Landberg G, Påhlman S. Hypoxia-induced dedifferentiation of tumor cells – a mechanism behind heterogeneity and aggressiveness of solid tumors. *Semin Cell Dev Biol* (2005) 16(4–5): 554–63. doi:10.1016/j.semcdb.2005.03.007
91. Platet N, Liu SY, Atifi ME, Oliver L, Vallette FM, Berger F, et al. Influence of oxygen tension on CD133 phenotype in human glioma cell cultures. *Cancer Lett* (2007) 258(2):286–90. doi:10.1016/j.canlet.2007.09.012
92. Keith B, Simon MC. Hypoxia-inducible factors, stem cells, and cancer. *Cell* (2007) 129(3):465–72. doi:10.1016/j.cell.2007.04.019
93. Peng F, Wang JH, Fan WJ, Meng YT, Li MM, Li TT, et al. Glycolysis gatekeeper PDK1 reprograms breast cancer stem cells under hypoxia. *Oncogene* (2018) 37(8):1119. doi:10.1038/onc.2017.407
94. Lee SY, Jeong EK, Ju MK, Jeon HM, Kim MY, Kim CH, et al. Induction of metastasis, cancer stem cell phenotype, and oncogenic metabolism in cancer cells by ionizing radiation. *Mol Cancer* (2017) 16(1):10. doi:10.1186/s12943-016-0577-4
95. Bhowmik SK, Ramirez-Peña E, Arnold JM, Putluri V, Sphyris N, Michailidis G, et al. EMT-induced metabolite signature identifies poor clinical outcome. *Oncotarget* (2015) 6(40):42651–60. doi:10.18632/oncotarget.4765
96. 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(17):3506–14. doi:10.4161/cc.9.17.12731
97. LaBarge MA. The difficulty of targeting cancer stem cell niches. *Clin Cancer Res* (2010) 16(12):3121–9. doi:10.1158/1078-0432.CCR-09-2933
98. Moore KA, Lemischka IR. Stem cells and their niches. *Science* (2006) 311(5769):1880–5. doi:10.1126/science.1110542
99. Cuyas E, Corominas-Faja B, Menendez JA. The nutritional phenome of EMT-induced cancer stem-like cells. *Oncotarget* (2014) 5(12):3970–82. doi:10.18632/oncotarget.2147
100. Aguilar E, Marin de Mas I, Zodda E, Marin S, Morrish F, Selivanov V, et al. Metabolic reprogramming and dependencies associated with epithelial cancer stem cells independent of the epithelial-mesenchymal transition program. *Stem Cells* (2016) 34(5):1163–76. doi:10.1002/stem.2286
101. Chen EI, Hewel J, Krueger JS, Tiraby C, Weber MR, Kralli A, et al. Adaptation of energy metabolism in breast cancer brain metastases. *Cancer Res* (2007) 67(4):1472–86. doi:10.1158/0008-5472.CAN-06-3137
102. Sosa MS, Bragado P, Aguirre-Ghiso JA. Mechanisms of disseminated cancer cell dormancy: an awakening field. *Nat Rev Cancer* (2014) 14(9):611–22. doi:10.1038/nrc3793
103. 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(3):517–28. doi:10.1016/j.cmet.2016.01.007
104. Abraham BK, Fritz P, McClellan M, Hauptvogel P, Athelogou M, Brauch H. Prevalence of CD44+/CD24-/low cells in breast cancer may not be associated

with clinical outcome but may favor distant metastasis. *Clin Cancer Res* (2005) 11(3):1154–9.
105. Glinsky GV, Berezovska O, Glinskii AB. Microarray analysis identifies a death-from-cancer signature predicting therapy failure in patients with multiple types of cancer. *J Clin Invest* (2005) 115(6):1503–21. doi:10.1172/JCI23412
106. Moitra K, Lou H, Dean M. Multidrug efflux pumps and cancer stem cells: insights into multidrug resistance and therapeutic development. *Clin Pharmacol Ther* (2011) 89(4):491–502. doi:10.1038/clpt.2011.14
107. Serafino A, Zonfrillo M, Andreola F, Psaila R, Mercuri L, Moroni N, et al. CD44-targeting for antitumor drug delivery: a new SN-38-hyaluronan bioconjugate for locoregional treatment of peritoneal carcinomatosis. *Curr Cancer Drug Targets* (2011) 11(5):572–85. doi:10.2174/156800911795655976
108. Mattheolabakis G, Milane L, Singh A, Amiji MM. Hyaluronic acid targeting of CD44 for cancer therapy: from receptor biology to nanomedicine. *J Drug Target* (2015) 23(7–8):605–18. doi:10.3109/1061186X.2015.1052072
109. Deonarain MP, Kousparou CA, Epenetos AA. Antibodies targeting cancer stem cells: a new paradigm in immunotherapy? *MAbs* (2009) 1(1):12–25. doi:10.4161/mabs.1.1.7347
110. Tamada M, Nagano O, Tateyama S, Ohmura M, Yae T, Ishimoto T, et al. Modulation of glucose metabolism by CD44 contributes to antioxidant status and drug resistance in cancer cells. *Cancer Res* (2012) 72(6):1438–48. doi:10.1158/0008-5472.CAN-11-3024
111. Annibaldi A, Widmann C. Glucose metabolism in cancer cells. *Curr Opin Clin Nutr Metab Care* (2010) 13(4):466–70. doi:10.1097/MCO.0b013e32833a5577
112. Krasnov GS, Dmitriev AA, Snezhkina AV, Kudryavtseva AV. Deregulation of glycolysis in cancer: glyceraldehyde-3-phosphate dehydrogenase as a therapeutic target. *Expert Opin Ther Targets* (2013) 17(6):681–93. doi:10.1517/14728222.2013.775253
113. Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, et al. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat Med* (2004) 10(8):858–64. doi:10.1038/nm1075
114. Roesch A, Vultur A, Bogeski I, Wang H, Zimmermann KM, Speicher D, et al. Overcoming intrinsic multidrug resistance in melanoma by blocking the mitochondrial respiratory chain of slow-cycling JARID1B(high) cells. *Cancer Cell* (2013) 23(6):811–25. doi:10.1016/j.ccr.2013.05.003
115. Lamb R, Ozsvari B, Lisanti CL, Tanowitz HB, Howell A, Martinez-Outschoorn UE, et al. Antibiotics that target mitochondria effectively eradicate cancer stem cells, across multiple tumor types: treating cancer like an infectious disease. *Oncotarget* (2015) 6(7):4569–84. doi:10.18632/oncotarget.3174
116. Lamb R, Harrison H, Hulit J, Smith DL, Lisanti MP, Sotgia F. Mitochondria as new therapeutic targets for eradicating cancer stem cells: quantitative proteomics and functional validation via MCT1/2 inhibition. *Oncotarget* (2014) 5(22):11029–37. doi:10.18632/oncotarget.2789
117. An H, Kim JY, Oh E, Lee N, Cho Y, Seo JH. Salinomycin promotes anoikis and decreases the CD44+/CD24- stem-like population via inhibition of STAT3 activation in MDA-MB-231 cells. *PLoS One* (2015) 10(11):e0141919. doi:10.1371/journal.pone.0141919
118. Chu DJ, Yao DE, Zhuang YF, Hong Y, Zhu XC, Fang ZR, et al. Azithromycin enhances the favorable results of paclitaxel and cisplatin in patients with advanced non-small cell lung cancer. *Genet Mol Res* (2014) 13(2):2796–805. doi:10.4238/2014.April.14.8
119. Mayer MJ, Klotz LH, Venkateswaran V. Metformin and prostate cancer stem cells: a novel therapeutic target. *Prostate Cancer Prostatic Dis* (2015) 18(4):303–9. doi:10.1038/pcan.2015.35
120. Jung JW, Park SB, Lee SJ, Seo MS, Trosko JE, Kang KS. Metformin represses self-renewal of the human breast carcinoma stem cells via inhibition of estrogen receptor-mediated OCT4 expression. *PLoS One* (2011) 6(11):e28068. doi:10.1371/journal.pone.0028068
121. Vazquez-Martin A, Oliveras-Ferraros C, Cufí S, Del Barco S, Martin-Castillo B, Menendez JA. Metformin regulates breast cancer stem cell ontogeny by transcriptional regulation of the epithelial-mesenchymal transition (EMT) status. *Cell Cycle* (2010) 9(18):3807–14. doi:10.4161/cc.9.18.13131
122. Mullen AR, Wheaton WW, Jin ES, Chen PH, Sullivan LB, Cheng T, et al. Reductive carboxylation supports growth in tumour cells with defective mitochondria. *Nature* (2011) 481(7381):385–8. doi:10.1038/nature10642
123. Subramaniam D, Nicholes ND, Dhar A, Umar S, Awasthi V, Welch DR, et al. 3,5-bis(2,4-difluorobenzylidene)-4-piperidone, a novel compound that affects pancreatic cancer growth and angiogenesis. *Mol Cancer Ther* (2011) 10(11):2146–56. doi:10.1158/1535-7163.MCT-11-0399
124. 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 Metab* (2015) 22(4):577–89. doi:10.1016/j.cmet.2015.08.007
125. Cheong JH, Park ES, Liang J, Dennison JB, Tsavachidou D, Nguyen-Charles C, et al. Dual inhibition of tumor energy pathway by 2-deoxyglucose and metformin is effective against a broad spectrum of preclinical cancer models. *Mol Cancer Ther* (2011) 10(12):2350–62. doi:10.1158/1535-7163.MCT-11-0497
126. Issaq SH, Teicher BA, Monks A. Bioenergetic properties of human sarcoma cells help define sensitivity to metabolic inhibitors. *Cell Cycle* (2014) 13(7):1152–61. doi:10.4161/cc.28010
127. Korkaya H, Liu S, Wicha MS. Breast cancer stem cells, cytokine networks, and the tumor microenvironment. *J Clin Invest* (2011) 121(10):3804–9. doi:10.1172/JCI57099
128. Garvalov BK, Acker T. Cancer stem cells: a new framework for the design of tumor therapies. *J Mol Med (Berl)* (2011) 89(2):95–107. doi:10.1007/s00109-010-0685-3
129. Gu W, Yeo E, McMillan N, Yu C. Silencing oncogene expression in cervical cancer stem-like cells inhibits their cell growth and self-renewal ability. *Cancer Gene Ther* (2011) 18(12):897–905. doi:10.1038/cgt.2011.58
130. Moserle L, Ghisi M, Amadori A, Indraccolo S. Side population and cancer stem cells: therapeutic implications. *Cancer Lett* (2010) 288(1):1–9. doi:10.1016/j.canlet.2009.05.020
131. Brandt WD, Matsui W, Rosenberg JE, He X, Ling S, Schaeffer EM, et al. Urothelial carcinoma: stem cells on the edge. *Cancer Metastasis Rev* (2009) 28(3–4):291–304. doi:10.1007/s10555-009-9187-6

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

HYPOTHESIS AND THEORY
published: 04 April 2018
doi: 10.3389/fonc.2018.00090

Metabolic Reprogramming During Multidrug Resistance in Leukemias

Raphael Silveira Vidal[1], *Julia Quarti*[1,2], *Mariana Figueiredo Rodrigues*[1], *Franklin D. Rumjanek*[1] *and Vivian M. Rumjanek*[1]*

[1]*Instituto de Bioquímica Médica Leopoldo de Meis, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil,*
[2]*Instituto de Nutrição Josué de Castro, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*

Cancer outcome has improved since introduction of target therapy. However, treatment success is still impaired by the same drug resistance mechanism of classical chemotherapy, known as multidrug resistance (MDR) phenotype. This phenotype promotes resistance to drugs with different structures and mechanism of action. Recent reports have shown that resistance acquisition is coupled to metabolic reprogramming. High-gene expression, increase of active transport, and conservation of redox status are one of the few examples that increase energy and substrate demands. It is not clear if the role of this metabolic shift in the MDR phenotype is related to its maintenance or to its induction. Apart from the nature of this relation, the metabolism may represent a new target to avoid or to block the mechanism that has been impairing treatment success. In this mini-review, we discuss the relation between metabolism and MDR resistance focusing on the multiple non-metabolic functions that enzymes of the glycolytic pathway are known to display, with emphasis with the diverse activities of glyceraldehyde-3-phosphate dehydrogenase.

Keywords: multidrug resistance, glycolysis, glyceraldehyde-3-phosphate dehydrogenase, leukemia, reactive oxygen species

OPEN ACCESS

Edited by:
Michael Breitenbach,
University of Salzburg, Austria

Reviewed by:
Johannes A. Mayr,
Paracelsus Medical University Salzburg, Austria
Hamid Morjani,
Université de Reims Champagne Ardenne, France
Carlo Vito Bruschi,
CVBiotechnologies, E.U., Austria

***Correspondence:**
Vivian M. Rumjanek
vivian@bioqmed.ufrj.br

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *10 January 2018*
Accepted: *15 March 2018*
Published: *04 April 2018*

Citation:
Vidal RS, Quarti J, Rodrigues MF, Rumjanek FD and Rumjanek VM (2018) Metabolic Reprogramming During Multidrug Resistance in Leukemias. Front. Oncol. 8:90. doi: 10.3389/fonc.2018.00090

BACKGROUND

Multidrug resistance (MDR) in cancer is the major factor impairing the success of conventional chemotherapy (1). Originally, many hopes were placed on the possibility that by inhibiting the activity of ABC transporters a reversal of the resistance would be attained. This was based on the knowledge that the transporters were shown to be capable of mediating the efflux of many chemotherapeutic drugs. Among the efflux transporters of the ABC superfamily, ABCB1 (P-gp), ABCC1 (MRP1), and ABCG2 (BCRP or MXR) have been described as major players in the development of MDR (2–4), with particular emphasis being given to ABCB1. However, it soon became apparent that inhibition of these transporters was not an effective approach since normal cells may also express these transporters and, therefore, inhibitors could sometimes generate an unacceptable toxicity. Furthermore, in experimental situations, where the down regulation of ABCB1 was achieved, a number of other effects were still observed. Now, it is quite clear that MDR is a multifactorial phenomenon that is involved in the regulation of survival and apoptosis, as well as a number of other cellular pathways. ABCB1 transporter expression, in cells with the MDR phenotype, is but one factor linked to pharmacological evasion of chemotherapeutic drugs (1, 5).

A case in point is the observation that hypoxia participates in the regulation of drug resistance. For instance, *ABCB1* gene expression and synthesis of functional proteins are induced by hypoxic environments (6). Furthermore, ABC transporters are expressed not only in MDR cancer cells, but

also in a number of stem and progenitor cells. Additionally, it has been reported that hypoxia promotes an undifferentiated cell state in various stem and precursor cell populations, as well as in cancer stem cells (7–9). In this respect, it has also been suggested that NOTCH signaling is involved. However, it must be recalled that when cells are under hypoxic conditions, there is a metabolic shift from oxidative phosphorylation to glycolysis (10). This situation contrasts with cells under normoxia, in which glucose is first anaerobically catabolized to pyruvate which is then further catabolized along the Krebs cycle where NADH and $FADH_2$ are reoxidized by the respiratory chain associated to the electron transport system. Incidentally, glycolysis is a hallmark in many types of tumor cells (11). This phenotype is in fact the basis of the so-called Warburg effect, also known as aerobic glycolysis. The Warburg effect describes a situation in which the glycolytic pathway is fully activated even in the presence of adequate oxygen supply (12). Although Warburg originally proposed that cancer was due to an impairment of mitochondrial function, it is accepted today that these organelles retain full oxidative capacities.

It must be mentioned, however, that apart from red blood cells, aerobic glycolysis is prevalent in highly proliferative cells, whether tumoral or not. Stem cells are a case in point (13). The common belief that cells undergoing glycolysis selected an inefficient form of energy production is misguided. Barring the comparative stoichiometry of ATP formation between glycolysis and OXPHOS, aerobic glycolysis is in fact an efficient form of ATP production due to the kinetic properties of the enzymes participating in the pathway which afford very fast fluxes compatible with the ATP demand of the rapidly growing cells. Beyond its role in bioenergetics, glycolysis constitutes a branch of the pentose phosphate pathway (PPP), since glucose-6-phosphate is also the substrate for glucose-6-phosphate dehydrogenase, the first enzyme of that pathway. Thus, glycolysis also contributes to the production of precursors for the biosynthesis of nucleotides (*via* generation of ribulose-5-phosphate). In addition, the PPP pathway promotes the formation of NADPH, an essential coenzyme for reductive biosynthetic processes such as that of fatty acids. NADPH also has an important role in maintaining the redox equilibrium. Similarly, glycolysis can be considered as an anaplerotic pathway by way of its participation in amino acids synthesis (*via* 3-phosphoglycerate or *via* pyruvate). Thus, from an energetic stand point glycolysis more than compensates the relatively small amounts of ATP produced when compared with oxidative phosphorylation.

However, it must be emphasized that tumors are in fact constituted by a mosaic of different cellular subpopulations. As such, from the biochemical point of view tumors can also be envisaged as being functionally heterogeneous. Accordingly, within the context of types of metabolism, tumors can be perceived as composed of subsets of resistant quiescent/slow-cycling cells that occasionally rely more on mitochondrial respiration and less on glycolysis. Likewise the same tumor could also harbor cells that are exclusively glycolytic (14, 15). Interestingly, the possibility of a switch that regulates mitochondrial function in the case of metastasis has been proposed. The results of Porporato et al. showed that overburdening the electron transport system may be an essential step in enhancing migration of cells *in vitro* and *in vivo* (16). The authors concluded that in order to achieve metastasis, mitochondria must be active, although not necessarily functional. By extension such findings suggest that in tumor cells there may be switches that constantly activate/inactivate mitochondrial function depending on changes dictated by the microenvironment that affect, for example, the availability of metabolites. The intermittent switching between anaerobic and oxidative metabolism seems to be a feature of metastasis. According to this scheme, accumulating data show that there is a tradeoff involving growth versus migration, i.e., cells which are proliferating prevalently exhibit a glycolytic type of metabolism, whereas migrating cells which proliferate less, rely more on mitochondria (16). Within this framework, it is known that the switch between the two main types of energy metabolism may be regulated by ATP demand. For example, cells expressing ABC transporters on their surfaces require a considerable amount of ATP in order to sustain the drug efflux activity. Additionally, it has been suggested that the transporter activity might suffer the impact of alterations in pH gradient due to the glycolytic phenotype (17). Normal cells maintain a gradient between acidic vesicular compartments and an alkaline cytoplasm a situation not observed in tumor cells that have an acidic cytoplasm. Nevertheless, it has been observed that MCF-7adr, that has an MDR phenotype, presents a similar pH gradient to that of normal cells (18, 19). This gradient contributes to the sequestration of drugs in acidic organelles and subsequent extrusion from the cell (19).

The mitochondrial electron transport chain generates reactive oxygen species (ROS) (20). In some instances, such as partial disintegration of complex I (21–23), oxidative stress may result from excessive production of ROS which is buffered by redox homeostasis. In turn, homeostasis is achieved by the participation of a number of enzymes such as catalase, superoxide dismutase, gluthatione peroxidase, and cofactors such as reduced gluthatione and NADPH. Control of redox equilibrium is important since an imbalance between the amount of ROS produced and of antioxidant systems may lead to DNA damage, particularly mitochondrial DNA, and other cell lesions.

Notwithstanding, the effect of ROS as agents of oxidative stress affecting the expression of ABCB1 is controversial. Both, downregulation and upregulation of the transporter have been reported (24, 25). Antioxidants (26), as well as products resulting from glycolysis can act as scavengers of free radicals. In this way, the energy metabolism could also play an indirect role as modulators of ABCB1 expression. For instance, it is known that under conditions leading to glycolysis inhibition, ABCB1 expression was observed to be downregulated. Conversely, when exogenous pyruvate was added to the tumor cells there was increased drug resistance and transporter expression (25).

Therefore, there is consensus that under normal conditions, glycolysis, and mitochondrial oxidative phosphorilation operate in concert in so far as energy production in the form of ATP is concerned.

Originally, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was thought to function exclusively as part of the glycolytic process in the cytoplasm, where its role is well established. However, under conditions of oxidative stress GAPDH may also redirect glyceraldehyde-3-P to glucose 6 phosphate (G6P) as a result from the reversal of part of the glycolytic reactions.

The formed G6P then becomes the initial substrate of the PPP leading to the increase of NADPH production (27). GAPDH is particularly sensitive to H_2O_2-induced oxidation and it has been suggested that cytosolic GAPDH might function as a sensor for redox signals and an information hub to transduce these signals (28). Oxidative stress may also promote GAPDH aggregation leading to mitochondrial dysfunction and necrotic cell death *via* the permeability transition pore (29). Interestingly, under some circumstances GAPDH was shown to bind to band 3 protein, an anion transporter located on the inner side of the red blood cell membrane. In this membrane bound state, GAPDH along with other glycolytic enzymes contributed toward ATP channeling thus allowing its direct consumption by ion pumps without release into the cytoplasm (30). Such a role for GAPDH would be in keeping with an accessory function within the context of drug efflux. GAPDH, however, plays a number of other roles in different cell compartments, where several pools of GAPDH sense cellular stresses and activate cognate pathways to maintain homeostasis or activate cell death (31). It has also been reported that following exposure to stressors GAPDH translocate into the nucleus (32) where it may suffer ADP-ribosylation by NO (33). Apart from GAPDH, other enzymes of the glycolytic pathway are known to display multiple non-metabolic functions. In fact, of the 10 enzymes that constitute glycolysis, at least 7 have been shown to display extra-glycolytic activities that may bear on the MDR phenotype (34). For example, hexokinase II (HKII) binds to the mitochondrial voltage-gated anion channels and thus relieves the negative feedback effect of G6P (35). HKII overexpression is regulated by HIF-1α and also by c-Myc oncogene. HKII is also under the control of many miRNAs. When phosphohexoseisomerase, or phosphoglucoisomerase (PGI) is secreted by cells it acquires the status of a cytokine and is renamed as the autocrine motility factor (AMF). As such, AMF stimulates cell motility and so PGI is thought to be one of the factors driving metastasis (36). Besides, PGI is involved in many other activities such as apoptosis and EMT. Other glycolytic enzymes such as phosphofructokinase 1, aldolase and triose phosphate isomerase, phosphoglycerate mutase, and pyruvate kinase have all been reported to take part in several cellular functions that have a direct relation to tumorigenesis. Hence, it is entirely plausible that individually or collectively, the glycolytic enzymes and in particular GAPDH may constitute integral parts of the MDR phenotype by acting in a non-canonical fashion (37).

Alternatively, the metabolic rewiring of tumor cells may also result from gene rearrangement. Using yeast as a model system, genomic instability and the reprogramming of central metabolism have been approached (38). Regarding gene rearrangement, factors that normally contribute toward the integrity of the replicative process may be compromised. Drug resistance has been observed in yeast in which genome translocants were investigated (39). A parallel between yeast and tumor cells could thus be established even though the detailed mechanisms are still not understood. Indeed in MDR cells the over expression of mini chromosome maintenance 7 (MCM7) was detected (40).

Oxidative stress induces severe damage to proteins, lipids, and DNA. Normally, the generation of oxyradicals is prevented by the mitochondrial antioxidant system; however, the degree of damage will also depend on the repair capacity of the cell. DNA damage leads to the activation of Poly(ADP-ribose) polymerases or PARPs that are nuclear enzymes responsible for catalyzing the attachment of ADP-ribose units from nicotinamide adenine dinucleotide (NAD^+) to acceptor proteins involved in the recognition and repair of DNA strands breaks. After repair is completed the Poly(ADP-ribose) (PAR) chains are degraded (41). During caspase mediated apoptosis PARP is cleaved into fragments that in turn inactivate the enzyme inhibiting repair to proceed (42, 43). It has been suggested that PARP inhibitors (PARPi) could be used in combination with chemotherapeutic drugs, but PARPi tends to be extruded from the cell by the ABCB1 transporter in MDR tumors (44).

Cell survival and energy metabolism in MDR tumor cells will be discussed in the next section.

MDR, CHRONIC MYELOID LEUKEMIA, AND ENERGY METABOLISM

Multidrug resistant cells may be very heterogeneous in their characteristics and survival pathways. In the present review, we will highlight, including our own data, some aspects related to the energy metabolism of two MDR chronic leukemia cell lines derived from the CML cell line, K562. The two cell lines were selected after exposure to different chemotherapeutic drugs. One cell line was exposed to vincristine, originating Lucena-1 (45), and the other exposed to daunorubicin, originating FEPS (46). Despite arising from the same parental cell, they were quite distinct. Comparative microarray analysis identified 130 differentially expressed genes between K562 versus Lucena-1, 1,932 between K562 versus FEPS, and 1,211 between Lucena-1 versus FEPS. *ABCB1* was overexpressed in both MDR cell lines, but highly overexpressed in FEPS, which is the most resistant line (47). Similarly, comparative proteomics of the parental cell line and the MDR counterparts indicated that K562 presented 560 unique proteins, Lucena-1 had 38 and FEPS 63 unique proteins. Lucena-1 and FEPS shared 929 proteins. From the latter, 112 were common only to Lucena-1 and FEPS (48). Results from another survey investigating the proteomic profiles of K562 and Lucena-1, identified 36 differentially expressed proteins between these two cell lines (40). From those, the leucine-rich PPR motif-containing protein and MCM7, as well as the expression of ABCB1 could be used as markers to identify patients that would respond or fail to therapy with the tirosine kinase inhibitor, Imatinib (40). The importance of ABCB1 in the resistance to Imatinib in the clinical setting has been demonstrated (49, 50), but it is not the only transporter involved. Tumor cells expressing ABCG2 are capable of Imatinib extrusion with great affinity (51, 52). In a different CML model, comparing the proteomic analysis of another MDR cell line derived from K562 selected with doxorubicin, Qinghong et al. highlighted 44 differentially expressed proteins. Some of the differentially expressed proteins were common to those observed in FEPS (53).

The possibility that overexpression of ABCB1 in these cell lines might reflect stem cell characteristics was analyzed by looking at the NOTCH pathway. Notch was overexpressed in FEPS,

the most resistant cell line displaying the highest expression of *ABCB1*, and slow replication time (47). Another pathway present in both, normal stem cells and CML stem cells is the canonical Wnt pathway. This signaling pathway was shown to be more strongly activated to positively regulate ABCB1 in Lucena-1 cells when compared with the non-MDR K562. However, FEPS was not studied on this occasion (40). Conversely, both MDR cell lines had an increased expression of carbonic anhydrase and hemoglobin (47). This was confirmed by proteomics (48) suggesting that these cells are more differentiated compared with the parental erythroleukemic cell line K562 (54). Furthermore, they have the potential to maintain the intracellular pH (17). It has been reported that Imatinib induces erythrocytic differentiation in K562 cells and this is independent of blockade of apoptosis being also observed in resistant clones (55).

One characteristic of the two MDR cell lines described above (Lucena-1 and FEPS) is their antioxidant capacity. The elevated catalase activity observed in Lucena-1 provides these cells with protection against cytotoxic chemicals as well as UV radiation (56). Catalase activity is also elevated in FEPS and these cells also present increased Glucose 6 phosphate dehydrogenase (G6PD) activity (Vidal RS, Faria G, Maia RC, and Rumjanek VM, unpublished data). Despite the well-known role in redox homeostasis, G6PD is also involved in cell growth and signaling and this might be an equally important role in resistant cells (57). Many redox changes are now perceived to allow, in a localized compartment, a rapid and physiological signaling event that may regulate the activity of certain proteins (26, 58). Important players in regulating these intracellular effects are members of the thioredoxin family, including thioredoxin that has increased levels in Lucena-1 and FEPS (48), glutaredoxins, and peroxiredoxins. The third most upregulated gene in Lucena-1 when compared with K562 is *SESN3* that catalyzes peroxiredoxins leading to ROS detoxification (47). Furthermore, several members of peroxiredoxins, a family of antioxidant enzymes, are increased in FEPS (PRDX1, PRDX2, PRDX3, and PRDX6) or Lucena-1 (PDRX1) compared with K562 (47, 48). Therefore, ROS generated by the MDR tumor cell lines Lucena-1 and FEPS, are rapidly reduced. In this way, many anticancer drugs that act *via* generation of oxidative stress become ineffective.

When the MDR cell lines and their parental counterpart, K562, were tested for oxygen consumption measured by high-resolution respirometry, the most resistant cell line, FEPS, reproducibly displayed comparatively lower values in all parameters measured (**Figure 1**). This result suggested that in FEPS, energy was probably being obtained *via* glycolysis. Gene expression (47) and protein expression (48) indicate an increase in pyruvate kinase levels in FEPS, whereas in Lucena-1 only increased protein expression was observed (48). Differences in the glycolytic pathway, with higher expression of hexoquinase 2, GAPDH and LDH, were observed using SKOV3 $_{TR}$, an ovarian cell line transfected with the *ABCB1* gene (59).

Proteomic analysis of another MDR line obtained by doxorubicin selection using K562 as the parental cell, described upregulation of fructose-biphosphate aldolase A, fructose-biphosphate aldolase C, transaldolase, and alpha-enolase suggesting that the cells need more energy to survive chemical stress (53). FEPS cells, selected with daunorubicin also presented upregulated fructose-biphosphate aldolase A (48).

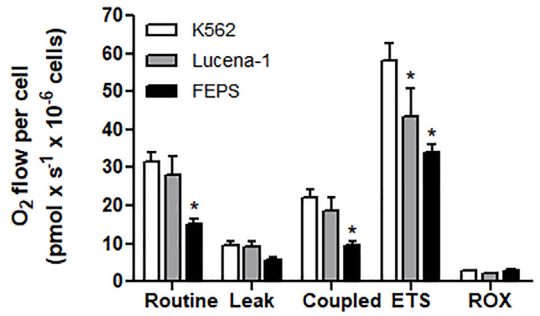


FIGURE 1 | High-resolution respirometry of K562, Lucena-1, and FEPS cells. Respiratory parameters of intact K562, Lucena-1, and FEPS cells. Routine respiration—basal respiration of intact cells; leak respiration—rate of oxygen consumption after the addition of oligomycin, that is, uncoupled respiration; coupled respiration (routine–leak); ETS—maximum respiratory capacity (induced by the addition of FCCP); ROX—rate of oxygen consumption in the presence of rotenone and antimycin A, that is, respiration not associated with electron transport system. All the parameters were corrected by ROX values. The bars marked with asterisks denote the values that are significantly different with reference to K562 cells.

GAPDH, MDR, AND CELL DEATH

Among the differences observed in the present study was the variation found in the levels of GAPDH between K562 and its MDR counterparts. Using a proteomic approach (48), it was possible to verify that in relation to K562, the levels of GAPDH were 2.4 times higher in FEPS and 1.2 times in Lucena-1. These results were validated by western blot (**Figure 2**). Overexpression of GAPDH in Imatinib-resistant cells has been observed by other authors (60, 61). However, these results differ from those found by Cerezo et al. where no difference in GAPDH was observed in daunorubicin resistant cell lines overexpressing ABCB1 (62). Presumably the inconsistency reflects the well-known intrinsic differences encountered in tumor cells of different origin.

Interestingly, a recent report describes a strategy to circumvent the ABCB1 transporter activity by transferring constructs that specifically inhibited GAPDH into target tumor cells by using liposomes. Such treatment was effective *in vitro* and *in vivo* (63).

Therefore, the possibility exists that GAPDH plays a role in the resistance observed in some MDR tumors. Various non-glycolytic roles have been assigned to GAPDH (64). Post-translational modifications of GAPDH may dictate subcellular localization and different functions (31, 65). It has been proposed that Sirtuin1 expression retains GAPDH in the cytosol (66). However, no differences in gene or protein expression of Sirtuin 1, were observed in Lucena-1 or FEPS when compared with their parental cell line K562. As mentioned before, GAPDH translocates to the nucleus where it binds to DNA and participates in a number of DNA-dependent processes (67). However, the lack of differential expression of Sirtuin 1 in Lucena-1 and FEPS does not invalidate the hypothesis of GAPDH mediated action in the nucleus. GAPDH trafficking may occur by a number of mechanisms.

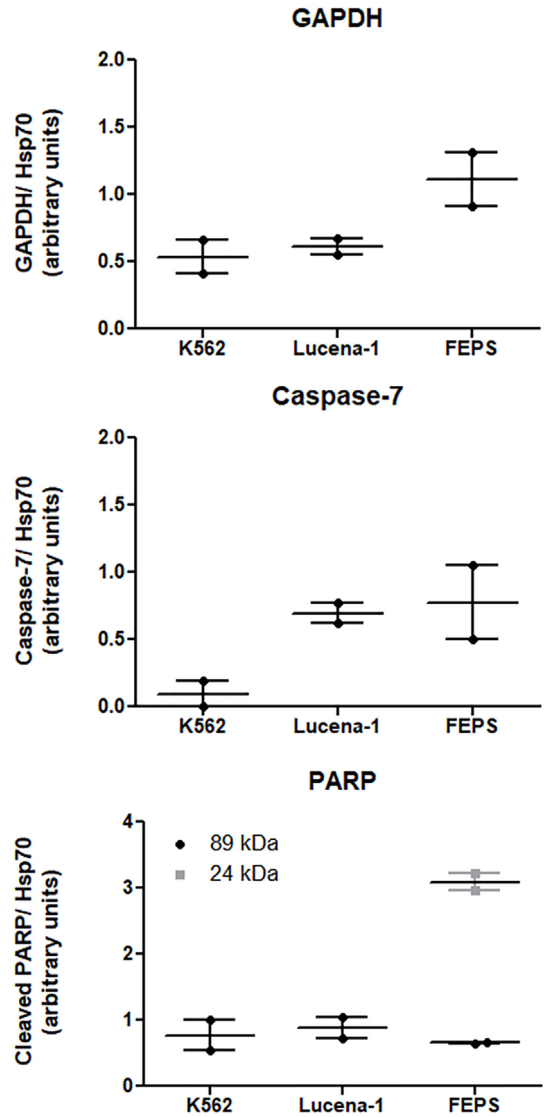


FIGURE 2 | Expression of proteins related to cell death-resistance on K562, Lucena-1, and FEPS cells. Equal amounts of total cellular proteins (100 μg) were loaded in each lane for the detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pro-caspase-7, cleaved PARP, and Hsp70 (loading control) by western blot. Densitometric analysis of each lane was calculated using ImageJ Software. The data are expressed as arbitrary units and represent the mean of two independent experiments.

For example, it is known that GAPDH can bind to microtubules and may thus get access to several intracellular organelles, including the nucleus (68).

It has been suggested that GAPDH is an inhibitor of caspase independent cell death (CID) (61, 69, 70). Death evasion to a number of chemotherapeutic drugs is the characteristic of cells displaying the MDR phenotype. Defects in the apoptotic process involving caspase activation to induce cell death have been observed in a number of multidrug resistant cell lines (62, 71).

Apoptosis is a result of caspase activation as a consequence of mitochondrial permeabilization and cytochrome c release. However, cells might also be killed following mitochondrial permeabilization even when caspase activation is inhibited, in the process known as CID. It has been suggested that, in such cases, cell death might occur as a result of a collapse of mitochondrial function, or the release of other proteins that could mediate the death process such as apoptosis-inducing factor (AIF), Smac/Diablo, HtrA2/Omi, Endonuclease G (72).

Mitochondrial permeabilization leading to cytochrome c release, and subsequent caspase activation, involves Bcl-2 family members having pro and anti-apoptotic properties. No difference in Bcl-2 levels could be detected when Lucena-1 and FEPS were compared with their parental non-MDR cell line K562 (46). On the other hand, the inhibitor of apoptosis survivin is increased in these cells, similarly to what has been described in K562/ADR (73) and other MDR cell lines (74, 75). Survivin suppresses cell death *via* caspase inhibition. Therefore, in a situation where caspases are inhibited, cell death could be a result of CID.

In a study, using CML cells, Lavallard et al. described that the tyrosine kinase inhibitor, Imatinib, was able to induce cell death in Bcr-Abl-positive cells by both caspase-dependent and independent manner (61). To induce CID, Imatinib was added to CML cells treated with caspase-inhibitors. In such situation cells transfected with GAPDH were protected from CID, however, these same transfected cells were not protected from Imatinib-induced apoptosis when no caspase-inhibitors were used. Furthermore, Imatinib-resistant K562 cells spontaneously overexpressed GAPDH compared with parental K562 and were protected from CID (61). This finding is in agreement with the results obtained with both Lucena-1 and FEPS MDR cell lines that also showed resistance to Imatinib and displayed higher levels of GAPDH compared with the parental K562 (**Figure 2**).

In experiments where caspase activity was inhibited but cytochrome c release occurred, it has been suggested that GAPDH acted by increasing the glycolytic metabolism and generating ATP as well as translocating to the nucleus where it is involved in the expression of Atg12 (70). Using GAPDH mutants, that either supported ATP production, but did not translocate to the nucleus or presented nuclear function albeit unable to produce ATP, these authors verified the requirement for the dual role played by GAPDH (70). The induction of Atg12 expression following mitochondrial permeabilization leads to autophagy with the removal of damaged mitochondria and subsequent cell survival (69).

Another death process involves the release of AIF from the mitochondrial intermembrane followed by its translocation to the nucleus. This step is a result of overactivation of the nuclear enzyme poly (ADP-ribose) synthetase 1 (PARP-1) (76). In the mitochondrial intermembrane space, AIF co-localizes with Hsp60. Due to its oxyredutase activity, AIF might act as a scanveger in the mitochondrial electron transport. However, AIF nuclear activity is independent of oxyredutase activity as mutations in the oxyredutase domain do not inhibit death-induction when it translocates to the nucleus (77). Once present in the nucleus AIF induces chromatin condensation and large-scale DNA fragmentation. The use of AIF mutants lacking the DNA-binding property abrogated cell death in spite of the preservation of AIF's nuclear translocation (78).

In response to oxidative/nitrosative stress GAPDH binds to Siah and translocates to the nucleus where it activates PARP-1 (79). In the nucleus PARP-1 functions as a sensor to regulate celular DNA repair. PARP-1 overactivation, leading to AIF translocation to the nucleus, is a result of an attempt to restore damaged DNA. Usually, after repair is completed, the PAR chains are degraded. However, in cells with severely damaged DNA, nuclear PARP-1 is extensively activated and promotes the synthesis of an excess of PAR polymer. This reaches toxic levels and in the cytosol constitutes a death signal inducing AIF nuclear translocation (76, 80). The activation of PARP-1 results in the depletion of the cellular NAD^+ and ATP pools. PARP-1 may be cleaved *in vitro* and *in vivo* by caspase-3 and caspase-7 originating two fragments an 89-kD catalytic fragment and a 24-kD DNA binding domain, capable of arresting the process (81). Similar fragments of 89 and 24 kD were detected in the MDR cell line FEPS, whereas only the 89 was observed in Lucena-1 cells (**Figure 2**). The relationship between PARP-1 activity and drug resistance is unclear but ABCB1 activity occurs at the expense of ATP. It has been described that cells from animals knockout for PARP-1 showed and increased ABCB1 expression and activity (82).

INTRACELLULAR CALCIUM AND MDR

The homeostatic control of cytosolic calcium concentration is of fundamental importance and changes in intracellular microenvironmental calcium levels can impact on cell survival, diverse cell functions, and cell death.

Proteins of the S100 family are calcium sensing proteins important in maintaining the homeostasis of the cell (83). Reports show that the calcium binding protein S100A6, also known as calcyclin or CACYBP, interacts with GAPDH (84). S100A6 is present in tumor cells as well as in normal fibroblasts, smooth, and heart muscle cells (83). This protein, as well as its gene expression, is increased twofold in FEPS compared with K562 (48). However, it is not clear how the interaction of S100A6 and GAPDH might play a role inhibiting CID.

Sorcin is another calcium–protein associated with MDR (85–87). Using K562 cells overexpressing sorcin, it was observed that ABCB1 was upregulated. The opposite was also true. In MDR-K562 cells selected by exposure to doxorubicin it was found that sorcin was upregulated, suggesting that ABCB1 and sorcin may regulate the expression of each other (85). When sorcin was analyzed using the MDR cell line Lucena-1 selected by vincristine, an increased gene and protein expression was also observed compared with the parental K562 (47, 48). This result is in agreement with the proteomics of another doxorubicin-induced K562 where the protein sorcin was also increased (53).

Despite the fact that the relationship between sorcin and ABCB1 has been known for a number of years (88), the meaning of such relation is still unknown. Differences in intracellular calcium levels have been reported in cells presenting the MDR phenotype related to ABCB1 overexpression (89). Some of these differences could be attributed to increased sorcin levels but in most cases, a causal relationship was not established.

Cells transfected with cDNA for *ABCB1* did not mobilize calcium when the SERCA inhibitor thapsigargin was used (90). The explanation proposed by Gutheil et al. was that thapsigargin was being extruded. However, no calcium mobilization could be induced in Lucena-1 cells using thapsigargin in a condition in which the inhibitor could not be extruded from the cell, suggesting that drug efflux could not fully explain the lack of mobilization (91). Considering that the main intracellular calcium store is the endoplasmic reticulum (ER) and that thapsigargin elicits ER-stress inhibiting the ER calcium pump, this is another death-inducing mechanism that is altered in MDR cells. Similarly, other workers using two types of resistant cells, one selected with vincristine and one with a stable transfection with a gene encoding ABCB1, also described the reduced sensitivity of MDR cells to thapsigargin (92, 93). Moreover, using immunofluorescence, they observed differences in the localization of the ER proteins ryanodine receptor (RyR), inositol 1,4,5-trisphosphate receptor and calnexin, between the MDR cells and their parental (92). Despite the fact that no statistical difference was observed in the amount of GAPDH comparing the two types of resistant cells with the parental one, treatment with thapsigargin decreased the protein content of GAPDH as well as ABCB1, suggesting a relationship between the two (93).

CONCLUSION

The MDR phenotype is a very complex phenomenon. There is now a growing awareness that drug resistance transcends the ABCB1 transporters and involves other elements associated to metabolic reprogramming. Accordingly, the enzymes of the glycolytic pathway have been shown to exert several regulatory roles that bear on drug resistance. Therefore, pharmacological interference studies on the MDR phenotype may have a better chance to succeed if they are expanded by include as potential targets GAPDH and other enzymes of the glycolytic pathway. In this review, many ancillary roles of such enzymes have been commented and underlined those that affect, for instance, the supply of ATP for drug extrusion purposes. Furthermore, attention was called to the fact that cells displaying the MDR phenotype also displayed enhanced expression/activity of enzymes involved in the maintenance of redox homeostasis of tumor cells. Thus, chemotherapy aiming at the combined abrogation of drug resistance and careful modulation of the redox equilibrium may open up alternative avenues for the control of tumor growth and metastasis.

AUTHOR CONTRIBUTIONS

VR wrote and reviewed the article. RV and JQ contributed to the writing and data gathering. JQ contributed to the editing and submission of this manuscript. FR contributed to the writing and reviewed the article. MR contributed with the experiments involving the high resolution respirometry (oxygen consumption by the cells) which was included in the paper as **Figure 1.**

FUNDING

This work received financial support from the following Brazilian agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas Filho

de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-PROCAD). RV and JQ were recipients of PhD fellowships from CNPq.

REFERENCES

1. 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
2. Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. *J Natl Cancer Inst* (2000) 92:1295–302. doi:10.1093/jnci/92.16.1295
3. Litman T, Druley TE, Stein WD, Bates SE. From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. *Cell Mol Life Sci* (2001) 58:931–59. doi:10.1007/PL00000912
4. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* (2002) 2:48–58. doi:10.1038/nrc706
5. Tsuruo T, Naito M, Tomida A, Fujita N, Mashima T, Sakamoto H, et al. Molecular targeting therapy of cancer: drug resistance, apoptosis and survival signal. *Cancer Sci* (2003) 94:15–21. doi:10.1111/j.1349-7006.2003.tb01345.x
6. Comerford KM, Wallace TJ, Karhausen J, Louis NA, Montalto MC, Colgan SP. Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. *Cancer Res* (2002) 62:3387–94.
7. Schöning JP, Monteiro M, Gu W. Drug resistance and cancer stem cells: the shared but distinct roles of hypoxia-inducible factors HIF1α and HIF2α. *Clin Exp Pharmacol Physiol* (2017) 44:153–61. doi:10.1111/1440-1681.12693
8. Liu ZH, Dai XM, Du B. Hes1: a key role in stemness, metastasis and multidrug resistance. *Cancer Biol Ther* (2015) 16:353–9. doi:10.1080/15384047.2015.1016662
9. Gustafsson MV, Zheng X, Pereira T, Gradin K, Jin S, Lundkvist J, et al. Hypoxia requires notch signaling to maintain the undifferentiated cell state. *Dev Cell* (2005) 9:617–28. doi:10.1016/j.devcel.2005.09.010
10. Milane L, Duan Z, Amiji M. Role of hypoxia and glycolysis in the development of multi-drug resistance in human tumor cells and the establishment of an orthotopic multi-drug resistant tumor model in nude mice using hypoxic pre-conditioning. *Cancer Cell Int* (2011) 11:3. doi:10.1186/1475-2867-11-3
11. Ganapathy-Kanniappan S, Geschwind JF. Tumor glycolysis as a target for cancer therapy: progress and prospects. *Mol Cancer* (2013) 12:152. doi:10.1186/1476-4598-12-152
12. Warburg O. On respiratory impairment in cancer cells. *Science* (1956) 124:269–70.
13. Folmes CD, Terzic A. Energy metabolism in the acquisition and maintenance of stemness. *Semin Cell Dev Biol* (2016) 52:68–75. doi:10.1016/j.semcdb.2016.02.010
14. Viale A, Corti D, Draetta GF. Tumors and mitochondrial respiration: a neglected connection. *Cancer Res* (2015) 75:3685–6. doi:10.1158/0008-5472.CAN-15-0491
15. Moreno-Sánchez R, Marín-Hernández A, Saavedra E, Pardo JP, Ralph SJ, Rodríguez-Enríquez S. Who controls the ATP supply in cancer cells? Biochemistry lessons to understand cancer energy metabolism. *Int J Biochem Cell Biol* (2014) 50:10–23. doi:10.1016/j.biocel.2014.01.025
16. Porporato PE, Payen VL, Pérez-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
17. Omran Z, Scaife P, Stewart S, Rauch C. Physical and biological characteristics of multi drug resistance (MDR): an integral approach considering pH and drug resistance in cancer. *Semin Cancer Biol* (2017) 43:42–8. doi:10.1016/j.semcancer.2017.01.002
18. Schindler M, Grabski S, Hoff E, Simon SM. Defective pH regulation of acidic compartments in human breast cancer cells (MCF-7) is normalized in adriamycin-resistant cells (MCF-7adr). *Biochemistry* (1996) 35:2811–7. doi:10.1021/bi952234e
19. Altan N, Chen Y, Schindler M, Simon SM. Defective acidification in human breast tumor cells and implications for chemotherapy. *J Exp Med* (1998) 187:1583–98. doi:10.1084/jem.187.10.1583
20. Filomeni G, De Zio D, Cecconi F. Oxidative stress and autophagy: the clash between damage and metabolic needs. *Cell Death Differ* (2015) 22:377–88. doi:10.1038/cdd.2014.150
21. Ricci JE, Muñoz-Pinedo C, Fitzgerald P, Bailly-Maitre B, Perkins GA, Yadava N, et al. Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain. *Cell* (2004) 117:773–86. doi:10.1016/j.cell.2004.05.008
22. Martinvalet D, Dykxhoorn DM, Ferrini R, Lieberman J. Granzyme A cleaves a mitochondrial complex I protein to initiate caspase-independent cell death. *Cell* (2008) 133:681–92. doi:10.1016/j.cell.2008.03.032
23. Jacquemin G, Margiotta D, Kasahara A, Bassoy EY, Walch M, Thiery J, et al. Granzyme B-induced mitochondrial ROS are required for apoptosis. *Cell Death Differ* (2015) 22:862–74. doi:10.1038/cdd.2014.180
24. Cen J, Zhang L, Liu F, Zhang F, Ji BS. Long-term alteration of reactive oxygen species led to multidrug resistance in MCF-7 cells. *Oxid Med Cell Longev* (2016) 2016:7053451. doi:10.1155/2016/7053451
25. Wartenberg M, Richter M, Datchev A, Günther S, Milosevic N, Bekhite MM, et al. Glycolytic pyruvate regulates P-Glycoprotein expression in multicellular tumor spheroids via modulation of the intracellular redox state. *J Cell Biochem* (2010) 109:434–46. doi:10.1002/jcb.22422
26. Cort A, Ozben T, Saso L, De Luca C, Korkina L. Redox control of multidrug resistance and its possible modulation by antioxidants. *Oxid Med Cell Longev* (2016) 2016:4251912. doi:10.1155/2016/4251912
27. Ralser M, Wamelink MM, Kowald A, Gerisch B, Heeren G, Struys EA, et al. Dynamic rerouting of the carbohydrate flux is key to counteracting oxidative stress. *J Biol* (2007) 6:10. doi:10.1186/jbiol61
28. Hildebrandt T, Knuesting J, Berndt C, Morgan B, Scheibe R. Cytosolic thiol switches regulating basic cellular functions: GAPDH as an information hub? *Biol Chem* (2015) 396:523–37. doi:10.1515/hsz-2014-0295
29. Nakajima H, Itakura M, Kubo T, Kaneshige A, Harada N, Izawa T, et al. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) aggregation causes mitochondrial dysfunction during oxidative stress-induced cell death. *J Biol Chem* (2017) 292:4727–42. doi:10.1074/jbc.M116.759084
30. Campanella ME, Chu H, Low PS. Assembly and regulation of a glycolytic enzyme complex on the human erythrocyte membrane. *Proc Natl Acad Sci U S A* (2005) 102:2402–7. doi:10.1073/pnas.0409741102
31. Tristan C, Shahani N, Sedlak TW, Sawa A. The diverse functions of GAPDH: views from different subcellular compartments. *Cell Signal* (2011) 23:317–23. doi:10.1016/j.cellsig.2010.08.003
32. Sawa A, Khan AA, Hester LD, Snyder SH. Glyceraldehyde-3-phosphate dehydrogenase: nuclear translocation participates in neuronal and nonneuronal cell death. *Proc Natl Acad Sci U S A* (1997) 94:11669–74. doi:10.1073/pnas.94.21.11669
33. Brüne B, Lapetina EG. Nitric oxide-induced covalent modification of glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. *Methods Enzymol* (1996) 269:400–7. doi:10.1016/S0076-6879(96)69041-7
34. Lincet H, Icard P. How do glycolytic enzymes favour cancer cell proliferation by nonmetabolic functions? *Oncogene* (2015) 34:3751–9. doi:10.1038/onc.2014.320
35. Bustamante E, Pedersen PL. High aerobic glycolysis of rat hepatoma cells in culture: role of mitochondrial hexokinase. *Proc Natl Acad Sci U S A* (1977) 74:3735–9. doi:10.1073/pnas.74.9.3735
36. Watanabe H, Takehana K, Date M, Shinozaki T, Raz A. Tumor cell autocrine motility factor is the neuroleukin/phosphohexose isomerase polypeptide. *Cancer Res* (1996) 56:2960–3.
37. Jung DW, Kim WH, Williams DR. Chemical genetics and its application to moonlighting in glycolytic enzymes. *Biochem Soc Trans* (2014) 42:1756–61. doi:10.1042/BST20140201
38. Tosato V, Grüning NM, Breitenbach M, Arnak R, Ralser M, Bruschi CV. Warburg effect and translocation-induced genomic instability: two yeast models for cancer cells. *Front Oncol* (2013) 2:212. doi:10.3389/fonc.2012.00212

39. Nikitin DV, Bruschi CV, Sims J, Breitenbach M, Rinnerthaler M, Tosato V. Chromosome translocation may lead to PRK1-dependent anticancer drug resistance in yeast via endocytic actin network deregulation. *Eur J Cell Biol* (2014) 93:145–56. doi:10.1016/j.ejcb.2014.03.003
40. Corrêa S, Pizzatti L, Du Rocher B, Mencalha A, Pinto D, Abdelhay E. A comparative proteomic study identified LRPPRC and MCM7 as putative actors in imatinib mesylate cross-resistance in Lucena cell line. *Proteome Sci* (2012) 10:23. doi:10.1186/1477-5956-10-23
41. De Vos M, Schreiber V, Dantzer F. The diverse roles and clinical relevance of PARPs in DNA damage repair: current state of the art. *Biochem Pharmacol* (2012) 84:137–46. doi:10.1016/j.bcp.2012.03.018
42. Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, et al. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* (1995) 376:37–43. doi:10.1038/376037a0
43. Boulares AH, Yakovlev AG, Ivanova V, Stoica BA, Wang G, Iyer S, et al. Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. *J Biol Chem* (1999) 274:22932–40. doi:10.1074/jbc.274.33.22932
44. Montoni A, Robu M, Pouliot E, Shah GM. Resistance to PARP-inhibitors in cancer therapy. *Front Pharmacol* (2013) 4:18. doi:10.3389/fphar.2013.00018
45. Rumjanek VM, Trindade GS, Wagner-Souza K, de-Oliveira MC, Marques-Santos LF, Maia RC, et al. Multidrug resistance in tumour cells: characterization of the multidrug resistant cell line K562-Lucena 1. *An Acad Bras Cienc* (2001) 73:57–69. doi:10.1590/S0001-37652001000100007
46. Daflon-Yunes N, Pinto-Silva FE, Vidal RS, Novis BF, Berguetti T, Lopes RR, et al. Characterization of a multidrug-resistant chronic myeloid leukemia cell line presenting multiple resistance mechanisms. *Mol Cell Biochem* (2013) 383:123–35. doi:10.1007/s11010-013-1761-0
47. Moreira MA, Bagni C, de Pinho MB, Mac-Cormick TM, dos Santos Mota M, Pinto-Silva FE, et al. Changes in gene expression profile in two multidrug resistant cell lines derived from a same drug sensitive cell line. *Leuk Res* (2014) 38:983–7. doi:10.1016/j.leukres.2014.06.001
48. Vidal SR. *Alvos Terapêuticos no Fenótipo de Resistência a Múltiplas Drogas [Doctoral/PhD's thesis]*. Rio de Janeiro (RJ): Universidade Federal do Rio de Janeiro (2017).
49. da Cunha Vasconcelos F, Mauricio Scheiner MA, Moellman-Coelho A, Mencalha AL, Renault IZ, Rumjanek VM, et al. Low ABCB1 and high OCT1 levels play a favorable role in the molecular response to imatinib in CML patients in the community clinical practice. *Leuk Res* (2016) 51:3–10. doi:10.1016/j.leukres.2016.10.005
50. Maia RC, Vasconcelos FC, Souza PS, Rumjanek VM. Towards comprehension of the ABCB1/P-glycoprotein role in chronic myeloid leukemia. *Molecules* (2018) 23:E119. doi:10.3390/molecules23010119
51. Burger H, van Tol H, Boersma AW, Brok M, Wiemer EA, Stoter G, et al. Imatinib mesylate (STI571) is a substrate for the breast cancer resistance protein (BCRP)/ABCG2 drug pump. *Blood* (2004) 104:2940–2. doi:10.1182/blood-2004-04-1398
52. Ozvegy-Laczka C, Hegedus T, Várady G, Ujhelly O, Schuetz JD, Váradi A, et al. High-affinity interaction of tyrosine kinase inhibitors with the ABCG2 multidrug transporter. *Mol Pharmacol* (2004) 65:1485–95. doi:10.1124/mol.65.6.1485
53. Qinghong S, Shen G, Lina S, Yueming Z, Xiaoou L, Jianlin W, et al. Comparative proteomics analysis of differential proteins in respond to doxorubicin resistance in myelogenous leukemia cell lines. *Proteome Sci* (2015) 13:1. doi:10.1186/s12953-014-0057-y
54. Rutherford T, Clegg JB, Higgs DR, Jones RW, Thompson J, Weatherall DJ. Embryonic erythroid differentiation in the human leukemic cell line K562. *Proc Natl Acad Sci U S A* (1981) 78:348–52. doi:10.1073/pnas.78.1.348
55. Jacquel A, Colosetti P, Grosso S, Belhacene N, Puissant A, Marchetti S, et al. Apoptosis and erythroid differentiation triggered by Bcr-Abl inhibitors in CML cell lines are fully distinguishable processes that exhibit different sensitivity to caspase inhibition. *Oncogene* (2007) 26:2445–58. doi:10.1038/sj.onc.1210034
56. Trindade GS, Capella MA, Capella LS, Affonso-Mitidieri OR, Rumjanek VM. Differences in sensitivity to UVC, UVB and UVA radiation of a multidrug-resistant cell line overexpressing P-glycoprotein. *Photochem Photobiol* (1999) 69:694–9. doi:10.1111/j.1751-1097.1999.tb03348.x
57. Ho HY, Cheng ML, Chiu DT. Glucose-6-phosphate dehydrogenase—beyond the realm of red cell biology. *Free Radic Res* (2014) 48:1028–48. doi:10.3109/10715762.2014.913788
58. Hanschmann EM, Godoy JR, Berndt C, Hudemann C, Lillig CH. Thioredoxins, glutaredoxins, and peroxiredoxins—molecular mechanisms and health significance: from cofactors to antioxidants to redox signaling. *Antioxid Redox Signal* (2013) 19:1539–605. doi:10.1089/ars.2012.4599
59. Milane L, Ganesh S, Shah S, Duan ZF, Amiji M. Multi-modal strategies for overcoming tumor drug resistance: hypoxia, the Warburg effect, stem cells, and multifunctional nanotechnology. *J Control Release* (2011) 155:237–47. doi:10.1016/j.jconrel.2011.03.032
60. Ohmine K, Nagai T, Tarumoto T, Miyoshi T, Muroi K, Mano H, et al. Analysis of gene expression profiles in an imatinib-resistant cell line, KCL22/SR. *Stem Cells* (2003) 21:315–21. doi:10.1634/stemcells.21-3-315
61. Lavallard VJ, Pradelli LA, Paul A, Bénéteau M, Jacquel A, Auberger P, et al. Modulation of caspase-independent cell death leads to resensitization of imatinib mesylate-resistant cells. *Cancer Res* (2009) 69:3013–20. doi:10.1158/0008-5472.CAN-08-2731
62. Cerezo D, Cánovas M, García-Peñarrubia P, Martín-Orozco E. Collateral sensitivity to cold stress and differential Bcl-2 family expression in new daunomycin-resistant lymphoblastoid cell lines. *Exp Cell Res* (2015) 331:11–20. doi:10.1016/j.yexcr.2014.11.017
63. Guan J, Sun J, Sun F, Lou B, Zhang D, Mashayekhi V, et al. Hypoxia-induced tumor cell resistance is overcome by synergistic GAPDH-siRNA and chemotherapy co-delivered by long-circulating and cationic-interior liposomes. *Nanoscale* (2017) 9:9190–201. doi:10.1039/c7nr02663c
64. Nicholls C, Li H, Liu JP. GAPDH: a common enzyme with uncommon functions. *Clin Exp Pharmacol Physiol* (2012) 39:674–9. doi:10.1111/j.1440-1681.2011.05599.x
65. Sirover MA. Subcellular dynamics of multifunctional protein regulation: mechanisms of GAPDH intracellular translocation. *J Cell Biochem* (2012) 113:2193–200. doi:10.1002/jcb.24113
66. Joo HY, Woo SR, Shen YN, Yun MY, Shin HJ, Park ER, et al. SIRT1 interacts with and protects glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from nuclear translocation: implications for cell survival after irradiation. *Biochem Biophys Res Commun* (2012) 424:681–6. doi:10.1016/j.bbrc.2012.07.006
67. Kosova AA, Khodyreva SN, Lavrik OI. Role of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in DNA repair. *Biochemistry (Mosc)* (2017) 82:643–54. doi:10.1134/S0006297917060013
68. Tisdale EJ, Talati NK, Artalejo CR, Shisheva A. GAPDH binds Akt to facilitate cargo transport in the early secretory pathway. *Exp Cell Res* (2016) 349:310–9. doi:10.1016/j.yexcr.2016.10.025
69. Rathmell JC, Kornbluth S. Filling a GAP(DH) in caspase-independent cell death. *Cell* (2007) 129:861–3. doi:10.1016/j.cell.2007.05.030
70. 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
71. Cerezo D, Lencina M, Ruiz-Alcaraz AJ, Ferragut JA, Saceda M, Sanchez M, et al. Acquisition of MDR phenotype by leukemic cells is associated with increased caspase-3 activity and a collateral sensitivity to cold stress. *J Cell Biochem* (2012) 113:1416–25. doi:10.1002/jcb.24016
72. Tait SW, Green DR. Caspase-independent cell death: leaving the set without the final cut. *Oncogene* (2008) 27:6452–61. doi:10.1038/onc.2008.311
73. Wang YJ, Zhao HD, Zhu CF, Li J, Xie HJ, Chen YX. Tuberostemonine reverses multidrug resistance in chronic myelogenous leukemia cells K562/ADR. *J Cancer* (2017) 8:1103–12. doi:10.7150/jca.17688
74. Wang L, Zhang GM, Feng ZH. Down-regulation of survivin expression reversed multidrug resistance in adriamycin-resistant HL-60/ADR cell line. *Acta Pharmacol Sin* (2003) 24:1235–40.
75. Liu F, Xie ZH, Cai GP, Jiang YY. The effect of survivin on multidrug resistance mediated by P-glycoprotein in MCF-7 and its adriamycin resistant cells. *Biol Pharm Bull* (2007) 30:2279–83. doi:10.1248/bpb.30.2279
76. Fatokun AA, Dawson VL, Dawson TM. Parthanatos: mitochondrial-linked mechanisms and therapeutic opportunities. *Br J Pharmacol* (2014) 171:2000–16. doi:10.1111/bph.12416
77. Daugas E, Susin SA, Zamzami N, Ferri KF, Irinopoulou T, Larochette N, et al. Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. *FASEB J* (2000) 14:729–39. doi:10.1096/fasebj.14.5.729
78. Ye H, Cande C, Stephanou NC, Jiang S, Gurbuxani S, Larochette N, et al. DNA binding is required for the apoptogenic action of apoptosis inducing factor. *Nat Struct Biol* (2002) 9:680–4. doi:10.1038/nsb836

79. Nakajima H, Kubo T, Ihara H, Hikida T, Danjo T, Nakatsuji M, et al. Nuclear-translocated glyceraldehyde-3-phosphate dehydrogenase promotes poly(ADP-ribose) polymerase-1 activation during oxidative/nitrosative stress in stroke. *J Biol Chem* (2015) 290:14493–503. doi:10.1074/jbc.M114.635607
80. Sousa FG, Matuo R, Soares DG, Escargueil AE, Henriques JAP, Larsen AK, et al. PARPs and the DNA damage response. *Carcinogenesis* (2012) 33:1433–40. doi:10.1093/carcin/bgs132
81. Chaitanya GV, Steven AJ, Babu PP. PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration. *Cell Commun Signal* (2010) 8:31. doi:10.1186/1478-811X-8-31
82. Wurzer G, Herceg Z, Wesierska-Gadek J. Increased resistance to anticancer therapy of mouse cells lacking the poly(ADP-ribose) polymerase attributable to up-regulation of the multidrug resistance gene product P-glycoprotein. *Cancer Res* (2000) 60:4238–44.
83. Donato R. Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type. *Biochim Biophys Acta* (1999) 1450:191–231. doi:10.1016/S0167-4889(99)00058-0
84. Filipek A, Wojda U, Leśniak W. Interaction of calcyclin and its cyanogen bromide fragments with annexin II and glyceraldehyde 3-phosphate dehydrogenase. *Int J Biochem Cell Biol* (1995) 27:1123–31. doi:10.1016/1357-2725(95)00096-8
85. Qi J, Liu N, Zhou Y, Tan Y, Cheng Y, Yang C, et al. Overexpression of sorcin in multidrug resistant human leukemia cells and its role in regulating cell apoptosis. *Biochem Biophys Res Commun* (2006) 349:303–9. doi:10.1016/j.bbrc.2006.08.042
86. Zhou Y, Xu Y, Tan Y, Qi J, Xiao Y, Yang C, et al. Sorcin, an important gene associated with multidrug-resistance in human leukemia cells. *Leuk Res* (2006) 30:469–76. doi:10.1016/j.leukres.2005.08.024
87. Colotti G, Poser E, Fiorillo A, Genovese I, Chiarini V, Ilari A. Sorcin, a calcium binding protein involved in the multidrug resistance mechanisms in cancer cells. *Molecules* (2014) 19:13976–89. doi:10.3390/molecules190913976
88. Sugawara I, Mizumoto K, Ohkochi E, Hamada H, Tsuruo T, Mori S. Immunocytochemical identification and localization of the Mr 22,000 calcium-binding protein (sorcin) in an adriamycin-resistant myelogenous leukemia cell line. *Jpn J Cancer Res* (1989) 80:469–74. doi:10.1111/j.1349-7006.1989.tb02338.x
89. Sulová Z, Seres M, Barancík M, Gibalová L, Uhrík B, Poleková L, et al. Does any relationship exist between P-glycoprotein-mediated multidrug resistance and intracellular calcium homeostasis. *Gen Physiol Biophys* (2009) 28 Spec No Focus:F89–95.
90. Gutheil JC, Hart SR, Belani CP, Melera PW, Hussain A. Alterations in Ca^{2+} transport ATPase and P-glycoprotein expression can mediate resistance to thapsigargin. *J Biol Chem* (1994) 269:7976–81.
91. Wagner-Souza K, Echevarria-Lima J, Rodrigues LA, Reis M, Rumjanek VM. Resistance to thapsigargin-induced intracellular calcium mobilization in a multidrug resistant tumour cell line. *Mol Cell Biochem* (2003) 252:109–16. doi:10.1023/A:1025586225941
92. Sereš M, Poláková E, Krizanová O, Hudecová S, Klymenko SV, Breier A, et al. Overexpression of P-glycoprotein in L1210/VCR cells is associated with changes in several endoplasmic reticulum proteins that may be partially responsible for the lack of thapsigargin sensitivity. *Gen Physiol Biophys* (2008) 27:211–21.
93. Sereš M, Ditte P, Breier A, Sulová Z. Effect of thapsigargin on P-glycoprotein-negative and P-glycoprotein-positive L1210 mouse leukaemia cells. *Gen Physiol Biophys* (2010) 29:396–401. doi:10.4149/gpb_2010_04_396

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer CVB and the handling Editor declared their past shared affiliation.

frontiers in Oncology

REVIEW
published: 23 March 2018
doi: 10.3389/fonc.2018.00082

Metabolic Reprogramming in Thyroid Carcinoma

*Raquel Guimaraes Coelho[1], Rodrigo S. Fortunato[2] and Denise P. Carvalho[1]**

[1] Laboratório de Fisiologia Endócrina, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [2] Laboratório de Radiobiologia Molecular, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Among all the adaptations of cancer cells, their ability to change metabolism from the oxidative to the glycolytic phenotype is a hallmark called the Warburg effect. Studies on tumor metabolism show that improved glycolysis and glutaminolysis are necessary to maintain rapid cell proliferation, tumor progression, and resistance to cell death. Thyroid neoplasms are common endocrine tumors that are more prevalent in women and elderly individuals. The incidence of thyroid cancer has increased in the Past decades, and recent findings describing the metabolic profiles of thyroid tumors have emerged. Currently, several drugs are in development or clinical trials that target the altered metabolic pathways of tumors are undergoing. We present a review of the metabolic reprogramming in cancerous thyroid tissues with a focus on the factors that promote enhanced glycolysis and the possible identification of promising metabolic targets in thyroid cancer.

Keywords: glycolysis, glutaminolysis, Warburg effect, thyroid cancer, hypoxia-inducible factor, hexokinase, AMP kinase, mammalian target of rapamycin protein

OPEN ACCESS

Edited by:
Simona Pisanti, Università degli Studi di Salerno, Italy

Reviewed by:
Anna Krzeslak, University of Łódź, Poland
Anna Maria Porcelli, Università degli Studi di Bologna, Italy
Sybille Mazurek, Justus Liebig Universität Gießen, Germany

****Correspondence:***
Denise P. Carvalho
dencarv@biof.ufrj.br

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *17 November 2017*
Accepted: *09 March 2018*
Published: *23 March 2018*

Citation:
Coelho RG, Fortunato RS and Carvalho DP (2018) Metabolic Reprogramming in Thyroid Carcinoma. Front. Oncol. 8:82. doi: 10.3389/fonc.2018.00082

INTRODUCTION

Thyroid cancers are the most common endocrine tumors and are more prevalent in women and elderly individuals (1, 2). Although the incidence of thyroid tumors can be high in a population (1), epidemiological studies indicate that only a small fraction of tumors are malignant (1, 3). Some rare thyroid malignancies that derive from the follicular thyroid epithelia are poorly differentiated and frequently metastasize early (4). In contrast, differentiated thyroid carcinomas (DTCs) generally exhibit a good prognosis and excellent outcomes (3, 5, 6).

The therapy for intermediate and high-risk DTCs, includes a combination of surgery, radioiodine ablation, and thyroid stimulating hormone (TSH) suppressive therapy. However, although DTCs are slow-growing tumors, disease recurrence can occur (4–6). In approximately 10% of DTC recurrence cases, tumor progression leads to a more aggressive phenotype, metastatic spread, and further loss of iodide uptake ability (4–6).

In the past several years, targeted therapeutic approaches have been developed as an option to control disease progression. Unfortunately, multikinase inhibitors that target angiogenesis and oncogenic pathways have deleterious side effects and do not result in a cure. Although a significant increase in the progression-free survival rate has been observed with the use of multikinase inhibitors, the diversity of tumor types, and tumor resistance that develops during progression impede this unique therapeutic strategy (5, 6). However, tumor metabolic behavior is known to become quite different as cells to transform into malignant cells. Interestingly, some metabolic feature changes are observed in several tumor types. Although the physiological function of the thyroid gland is very well described, its metabolic control and adaptations remain elusive, especially in thyroid cancer. In this review, we discuss some metabolic adaptations identified in thyroid carcinoma that could be used as future therapeutic targets in this disease.

MAIN MOLECULAR EVENTS RELATED TO THYROID CARCINOGENESIS

The incidence of thyroid cancer has increased in many countries compared to that of other human cancers (7). Approximately 90% of non-medullary thyroid malignancies that originate from thyroid follicular cells are classified as well-DTCs. DTCs are subdivided into follicular thyroid carcinoma (FTC) and papillary thyroid carcinoma (PTC), the latter of which is more prevalent, accounting for approximately 80% of DTCs (8, 9). The oncocytic or Hurthle cell tumors represent approximately 3–5% of follicular thyroid neoplasms (10), and they may be benign (variant of follicular adenoma) or malignant (variant of follicular carcinoma and variant of papillary carcinoma). The main characteristic of Hurthle cell carcinomas is the presence of at least 75% large oxyphilic cells that are characterized by abundant mitochondria (11), and previous exposure to radiation might be a risk factor for the development of Hurthle cell carcinoma and some subtypes of papillary thyroid cancers (8–16).

Undifferentiated thyroid carcinomas represent less than 5% of thyroid malignancies and are frequently associated with disease recurrence and death (4, 8, 9). Finally, medullary thyroid carcinoma, which derives from parafollicular C cells, produces calcitonin and accounts for approximately 5% of thyroid carcinomas (9).

The different morphologic subtypes of DTC are due to specific genetic alterations. RAS is a proto-oncogene that encodes a family of GTPases that are activated through tyrosine kinase receptor pathways involved in the regulation of cell differentiation and proliferation. RAS mutations can be found in 20–25% of all human tumors and in up to 90% of pancreatic cancers (17). Regarding thyroid cancer, RAS mutations are found in approximately 10% of thyroid cancer cases, mainly the follicular variant (18–20). Mutations of the proto-oncogene RAS induce changes in Ras protein, leading to its constitutive activation inside the cell. Although the prevalence of RAS mutations in the thyroid is low, they are associated with aggressive behavior in several other types of cancer (21–23).

Another human gene involved in thyroid carcinogenesis is BRAF. The B-Raf protein is a serine/threonine kinase that is activated downstream of Ras and is involved in cell growth control (24). Mutations in B-Raf induce its constitutive activation, subsequently activating the downstream mitogen-activated protein kinase (MAPK) signaling pathway (20, 24). Although other mutations have been described, $BRAF^{V600E}$ (the substitution of valine for glutamic acid in residue 600) is the most frequent mutation (24). In PTC, BRAF is the predominant mutation (30–40%) and is considered an initiating event in papillary thyroid carcinogenesis (18, 25–27).

Genetic alterations in the RET gene have also been found in several types of cancers (28). RET encodes a transmembrane protein receptor with an intracellular portion containing a tyrosine kinase that triggers its autophosphorylation, initiating intracellular signaling related to the stimulation of the RAS/ERK and PI3 kinase/AKT cascades (25, 28, 29). In addition to BRAF, RET mutations are also responsible for thyroid cancers (9, 16, 25). Somatic point mutations in RET are associated with familiar or sporadic medullary thyroid cancer, since RET is normally expressed in C cells, but not in follicular thyroid cells (29). In PTC, RET translocations (RET/PTC) can be identified in approximately 20% of the cases (9).

In some tumors, PAX-8, which encodes a transcription factor associated with thyroid development, has been implicated in carcinogenesis (30). Tacha et al. (30) found that mutated PAX8 is expressed in some follicular thyroid cancers due to somatic rearrangement leading to the fusion of PAX-8 with PPARγ1 (peroxisome proliferator-activated receptor gamma 1) (30). In FTC, the frequency of PAX8/PPARγ1 rearrangement is estimated to be approximately 30%, but this is not observed in PTCs. RAS mutations are also found in FTC (9, 20, 21).

The therapeutic approach for thyroid cancer may depend not only on the tumor initial mutational status, which leads to different cell biology characteristics, but also to hallmarks related to tumor progression. Some of these molecular changes result in specific metabolic alterations that might contribute to metastasis and a worst prognosis.

CANCER CELL METABOLISM

In the past several years, there has been significant interest in the metabolic reprogramming of cancer cells. In general, non-tumor cells use energy substrates, such as glucose and fatty acids, to generate energy under aerobic conditions. Glucose metabolism is initiated by glycolysis, the pathway that converts one glucose molecule into two molecules of pyruvate, which are transported to the mitochondria for oxidation. The glycolytic pathway is generally coupled with the mitochondrial tricarboxylic acid (TCA) cycle due to the action of the pyruvate dehydrogenase (PDH) protein complex that converts pyruvate into acetyl-CoA. The TCA cycle consists of successive reactions that lead to the transfer of electrons to NAD^+/FAD^+ for the generation of NADH/$FADH_2$, forming a wide range of metabolic intermediaries that are involved in various biosynthetic routes. The TCA cycle allows electron transfer to oxygen and generates a proton gradient across the inner mitochondrial membrane that is necessary for ATP synthesis in a process called oxidative phosphorylation (OXPHOS), a metabolic strategy that enables the cellular production of a greater amount of ATP (**Figure 1**).

Under physiological stress conditions, such as hypoxia or rapid intracellular ATP decreases, the cell increases its anaerobic metabolism, producing higher amounts of lactate from pyruvate. Interestingly, most cancer cells show a constitutive stress metabolic phenotype due to their high proliferation rates, which induces an elevated ATP demand compared to non-tumor cells. Therefore, cancer cells show variable energy substrate selection and a metabolic shift occurs to maintain cell proliferation and survival (31–35). **Figure 1** summarizes the major metabolic modulation on energy flux in tumor cells.

Decades ago, Otto Warburg described the first tumor-specific metabolic characteristic, the so-called Warburg effect or aerobic glycolysis, which is considered the key metabolic hallmark of cancer (31). The Warburg effect is an alteration of cellular metabolism in which the glycolysis pathway is upregulated even in the presence of normal or high oxygen tension, resulting in

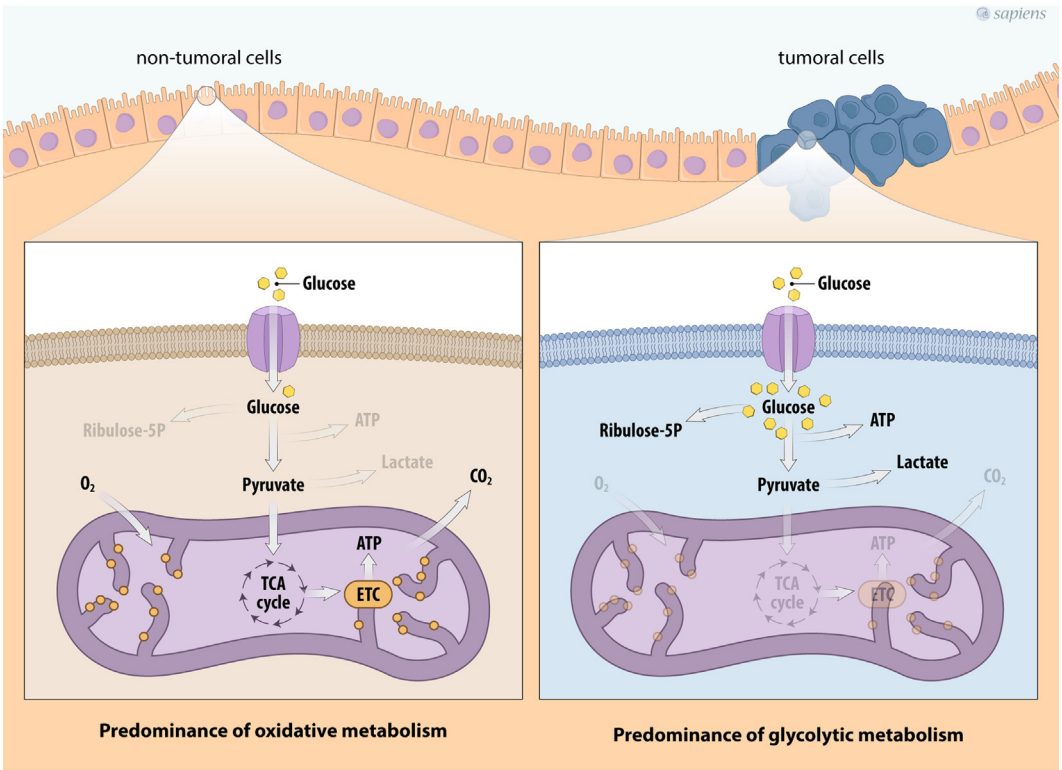


FIGURE 1 | Metabolic profile of cancer cells. Schema describing the metabolic reprogramming of tumor cells with increased glucose uptake and glycolysis that is uncoupled from oxidative phosphorylation. Cancer cell metabolism is characterized by enhanced glycolysis and the phosphate pentose pathway. This aerobic glycolytic phenotype, however, confers the generation of high amounts of lactate. Abbreviations: TCA, tricarboxylic acid; ETC, electron transport chain.

the phenomenon of aerobic glycolysis. However, such metabolic reprogramming in cancer cells represents an energy compensation strategy, since the efficiency of ATP production by glycolysis is much lower than that by OXPHOS (36). Part of this strategy is the upregulation of plasma membrane glucose transporters (GLUTs), a feature that is relatively common in many tumor types and is easily identified by non-invasive imaging positron emission tomography (PET) using fluorodeoxyglucose (34–37). In addition to increased glucose uptake, changes in key enzymes involved in glucose utilization can also be observed (34, 35). Some tumors show increased expression and activity levels of hexokinase (HK) isoforms, phosphofructokinase (PFK1), 6-phophofructo-2-kinase/fructose-2,6-biphosphatase (PFK2), aldolase (ADO), phosphoglycerate kinase (PGK), enolase (ENO), and pyruvate kinase (PK) (37). All these changes can increase pyruvate production from glucose breakdown.

The higher glycolytic flux observed in cancer cells, is not accompanied by increased rates of pyruvate oxidation, but lactate fermentation seems to be higher (**Figure 1**). Although this phenomenon is not fully understood, in recent years, significant progress has been made regarding the underlying molecular mechanisms related to neoplastic transformation and the Warburg effect (32–39). First, the lactate dehydrogenase enzyme (LDH) consumes part of the pyruvate formed by glycolysis to regenerate NAD^+ from the NADH produced by glucose breakdown, allowing a higher rate of glycolysis. Second, the LDH is a reversible enzyme that can generate NADH and pyruvate, thus contributing to mitochondrial OXPHOS. Third, both lactate and pyruvate can be transported from the cytosol to the mitochondria, or they can be secreted out of the cell. Lactate transport is mainly executed by monocarboxylate transporters (MCTs), a family of more than 14 types of transporters (38). Secreted lactate and pyruvate can be taken up by adjacent cancer cells and provide a feedforward mechanism for tumor growth, a phenomenon that is called as the reverse Warburg effect (39–41).

In adverse conditions, such as fluctuating oxygen tension, which is observed in solid tumors in the setting of poor blood vessel irrigation, glycolysis allows cancer cells to live in hypoxic conditions. However, survival at a lower oxygen tension has consequences, such as excessive lactate production and decreased extracellular pH, which leads to a microenvironment that favors the extrusion of tumor cells from primary tissues (33, 36, 40, 41). Therefore, aerobic glycolysis can generate lactate, an important metabolite that favors tumor invasion and progression, which is advantageous for proliferating cells (33, 35, 36, 39). Consequently, the idea that the Warburg effect is due to mitochondrial dysfunction has changed. In many tumor models, OXPHOS changes are important to maintain growth and progression, indicating that OXPHOS may be an important metabolic target in cancer treatment (42–46).

During the process of tumor metabolic reprogramming, many cancer cells show greater glutamine dependence for their survival

and proliferation (47–49). The elevated consumption of glutamine, a non-essential amino acid, has been documented in some tumors by assays that evaluate the uptake of two radionuclides, ^{18}F or ^{11}C (49). High ^{18}F-glutamine uptake was related to increased sodium-dependent neutral amino acid transporter type 2 (SLA1A5) expression and by upregulated glutaminase (GLS) in several tumor models (47–49). GLS initiates glutaminolysis by converting glutamine to glutamate. The destination of glutamate depends on divergent routes. Interestingly, this pathway is involved in the maintenance of the TCA cycle and anabolic processes through the synthesis of non-essential amino acids through transamination, nucleotides (purines and pyrimidines), and fatty acids. Glutamate formed in the cytosol is transported into mitochondria, where it can be converted into α-ketoglutarate by distinct reactions catalyzed by: (a) glutamate–pyruvate transaminase, producing alanine and α-ketoglutarate; (b) glutamate–oxaloacetate transaminase (GOT), which transfers the amino group from glutamate to oxaloacetate producing aspartate and α-ketoglutarate; and finally (c) glutamate dehydrogenase (GLUTD). Together, these reactions represent the major anaplerotic pathways for the synthesis of TCA cycle intermediaries secondary to glutamine metabolism. Glutamine consumption allows the cyclic resynthesis of citrate, which is directed to the formation of fatty acids or the synthesis of amino acids (**Figure 2**). Moreover, glutamine metabolism participates in the generation of antioxidant agents and can also act in cell signaling (47, 49).

All these alterations of glucose and glutamine metabolism observed in cancer cells are synergic. The high glucose uptake linked to energy generation and lactate production reduces oxygen consumption. Furthermore, mitochondrial function is maintained by glutaminolysis and can support biosynthetic processes. Several studies have provided evidence that oncogenic alterations in cancer cells reprogrammed glucose and glutamine metabolism, leading to energy stress that sustains anabolic processes, which are crucial to cancer cell proliferation and progression (31–36, 40, 41, 44, 47, 49).

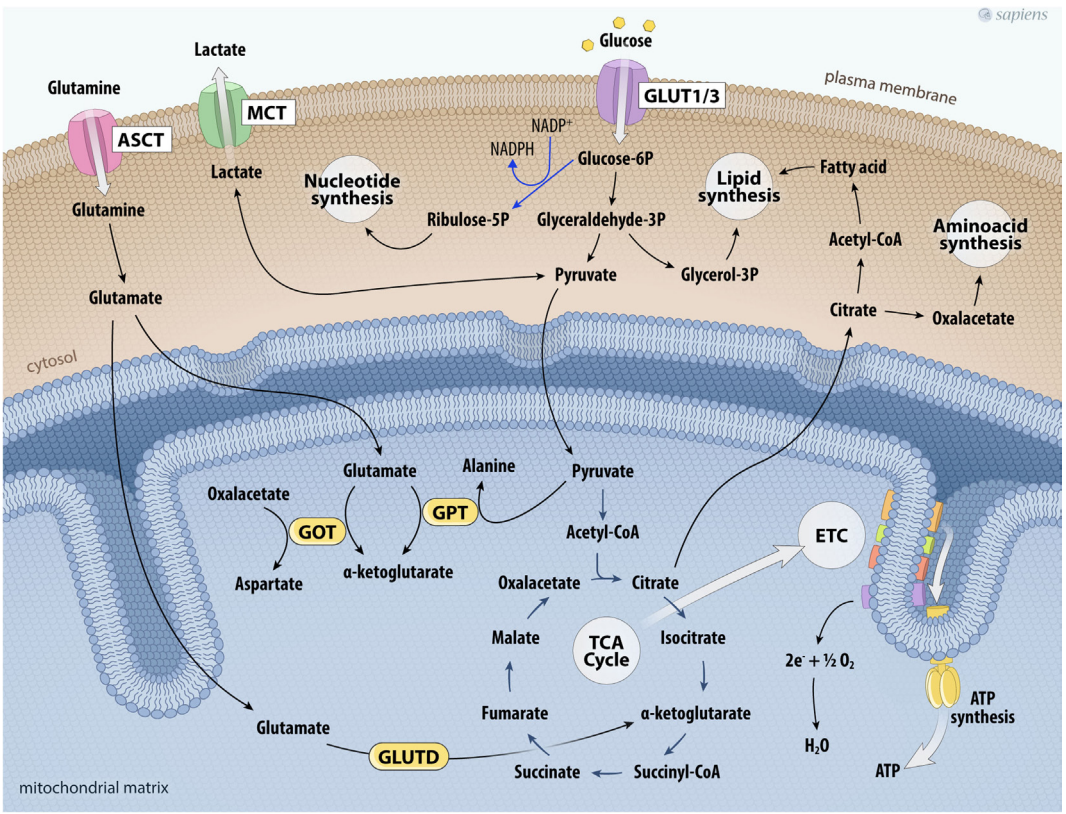


FIGURE 2 | Glutaminolysis and glucose metabolism in cancer cells. The higher glycolytic pathway contributes not only to the production of ATP per glucose consumed, but also feeds other biosynthetic pathways. Deviation of glyceraldehyde-3P to glycerol-3P is important for lipogenesis. Glucose-6P can also shift toward the phosphate pentose pathway that provides ribulose-5-phosphate and NADPH to nucleotide synthesis. On the other hand, glutamine uptake maintains the anaplerotic process in the TCA cycle. Glutamine is taken up *via* the transporter ASCT and is converted into glutamate. Glutamate together with pyruvate can be metabolized by GPT producing α-ketoglutarate and alanine; glutamate is metabolized producing α-ketoglutarate and aspartate by GOT; or glutamate is metabolized by glutamate dehydrogenase (GLUTD) forming α-ketoglutarate. All these reactions contribute to support the TCA cycle. Citrate outside mitochondria contributes to the *de novo* formation of fatty acids and aminoacids. Cancer cell metabolism is also characterized by the upregulation of lactate dehydrogenase to facilitate the conversion of pyruvate to lactate, which is then secreted to the tumor microenvironment *via* the MCT. Abbreviations: ASCT, Asc-type amino acid transporter; ETC, electron transport chain; GLUT1/3, glucose transporter 1 or 3; TCA, tricarboxylic acid; GOT, glutamate-oxaloacetate transaminase; GPT, glutamate–pyruvate transaminase; MCT, monocarboxylate transporter.

THYROID CANCER AND METABOLISM

Extensive documentation is available describing TSH as the main regulator of the function, proliferation, and metabolism of normal thyroid follicular cells, and well-differentiated thyroid cancer (50–56). In thyrocytes, the signaling network of TSH involves intermediates, such as protein kinase A, protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), and MAPK. TSH activation increases glucose metabolism and oxygen consumption to support iodide transport and thyroid hormone (T3 and T4) synthesis (50–54).

Despite the importance of aerobic glycolysis, it is estimated that the ATP content produced by normal thyroid cells is mainly derived from mitochondrial respiration with low glucose consumption (55, 56). Moreover, Mulvey et al. (56) showed that glycolysis seems to be more important to sustain the pentose phosphate pathway (PPP) than ATP production in thyroid cells. The deviation of glycolysis to the PPP in the thyroid could be important to maintain the balance of NADH/NADPH generated, which is crucial for thyroid hormone synthesis.

Regarding thyroid tumors and cellular metabolism, a major aspect is the effect of oncogenes on cell metabolic shift (32). Mutated RAS induces constitutive PI3K/AKT pathway activation independently of TSH stimulation (21, 57). In many tumors, the constitutive PI3K activation results in increased glycolysis flux (58, 59), and the PI3K/AKT pathway is crucial to translocate GLUT1 from the cytoplasm to the plasma membrane in thyroid cells (53). Recently, significant increases in glycolysis, the PPP, glutamine metabolism, and the phosphoserine biosynthetic pathway were identified in colorectal cancers with the KRAS point mutation compared to wild-type cells (59).

Guo et al. (23) showed the impact of RAS mutations on the oxidative profile, which can lead to autophagy induction *in vitro* and *in vivo* in tumors. The autophagy process is characterized by catabolic cellular self-degradation in response to periods of nutrient limitations through macromolecular intracellular recycling (60). According to Guo et al. (23), in addition to providing energy substrates, the autophagy process also preserves the mitochondrial function required for cell growth, especially in models of aggressive cancers. Several years ago, it was demonstrated that in TRβ PV/PV mice, which spontaneously develop well-differentiated FTC, synergism between the $KRAS^{G12D}$ mutation and TRβ PV occurs, leading to MYC oncogene activation and the development of the UTC phenotype (61). Interestingly, a prior study showed that in 40% of all human cancers, deregulated MYC expression could be involved in metabolic reprogramming (62). This gene encodes the Myc transcription factor (c-Myc), a multifunctional protein that plays a role in cell-cycle progression, apoptosis, and cellular transformation (62–64). Recently, Qu et al. (64) showed that $BRAF^{V600E}$ signaling also increases c-Myc expression in the human PTC cell lineage.

In addition to thyroid cancer, c-Myc overexpression has been identified in various cancers (62–64) and it upregulates the expression of genes involved in glucose metabolism (**Figure 3**). The first link found between c-Myc and glycolysis was the positive regulation of lactate dehydrogenase A (LDHA), the enzyme that converts pyruvate from glycolysis to lactate (65). Subsequently, GLUT-1, HK2, PFKM, and ENO1 were also identified as c-MYC targets (66–69).

Furthermore, mutated c-MYC also increases the anaerobic status of tumors, probably due to higher glycolytic flux and down-regulation of OXPHOS (62, 69, 70). Pyruvate dehydrogenase kinase (PDK) is the main regulatory enzyme of mitochondrial pyruvate consumption. This protein can phosphorylate and inactivate PDH, blocking the conversion of pyruvate into acetyl-CoA and its subsequent oxidation in the TCA cycle. In hypoxic conditions, a decrease in oxidative metabolism occurs due to the inhibition of PDH by PDK. This process is modulated by hypoxia-inducible factor-1 (HIF-1), a transcription factor that promotes many cell changes in response to oxygen deprivation (66). Interestingly, cancers that harbor mutated c-Myc also increase the activity of PDK under normoxic conditions (62, 66, 69). Together, these effects contribute to increased glycolysis dependence and the development of the Warburg effect (64, 66–70). **Figure 3** summarizes HIF and MYC targets on glycolytic metabolism.

The ability of tumor cells to maintain growth under hypoxic conditions is crucial to tumor progression, and the crosstalk between HIF-1 and c-Myc has been well documented (63, 66, 69). As a tumor grows, cells that can shift their metabolism in response to differences in blood supply are selected. Low oxygen tension increases HIF-1 expression and stabilization (70). Furthermore, HIF-1 is also activated by inflammatory processes, energy deprivation, and oxidative stress (71–75). Acting together, HIF-1 and c-Myc regulate several adaptations to hypoxic environments (70). As one compelling concept of the Warburg phenotype, HIF-1 enhances glycolysis not only by increasing the transcription of all glycolytic enzymes, but also by increasing their affinity for substrates (73). Moreover, HIF-1 also increases glucose transporter expression and stimulates the inhibitors of mitochondrial metabolism (71–74). In PTC, MTC, and FTC, HIF-1 expression level has been associated with a poor prognosis and metastasis (74).

The upregulation of GLUT1, HK1, HK2, PFK1, PFK2, ENO, PKM2, LDHA, and MCTs is highlighted as the most important action of HIF-1 to increase glycolysis (72, 74–76). Furthermore, HIF-1 also inhibits the PDH complex through PDK 1 overexpression, compromising the synthesis of electron donors (NADH and $FADH_2$) for the respiratory chain complex and promoting the accumulation of TCA cycle intermediates (77). Therefore, HIF-1 impairs OXPHOS, reinforcing the Warburg effect (74–77). Finally, HIF-1 also cooperates with the c-Myc oncogene by reducing mitochondrial biogenesis (77, 78). For this reason, HIF-1 has been described as a central agent that promotes metabolic reprogramming in many cancer cells (73, 75–78).

In addition to its metabolic effects, HIF-1 also stimulates the formation of new blood vessels, a process called angiogenesis (70–74). The steps of tumor angiogenesis induced by HIF-1 are distinct. One possible explanation for tumor HIF-1 overexpression is the loss of von Hippel–Lindau protein activity, which is a tumor-suppressor ubiquitin ligase complex responsible for HIF-1 proteasome degradation (70, 73). In this case, HIF-1 would be able to stimulate anaerobic metabolism even under conditions with minimal fluctuations in oxygen or under normoxia (70). Gatenby and Gilles (34) suggested that the blood vessels recruited

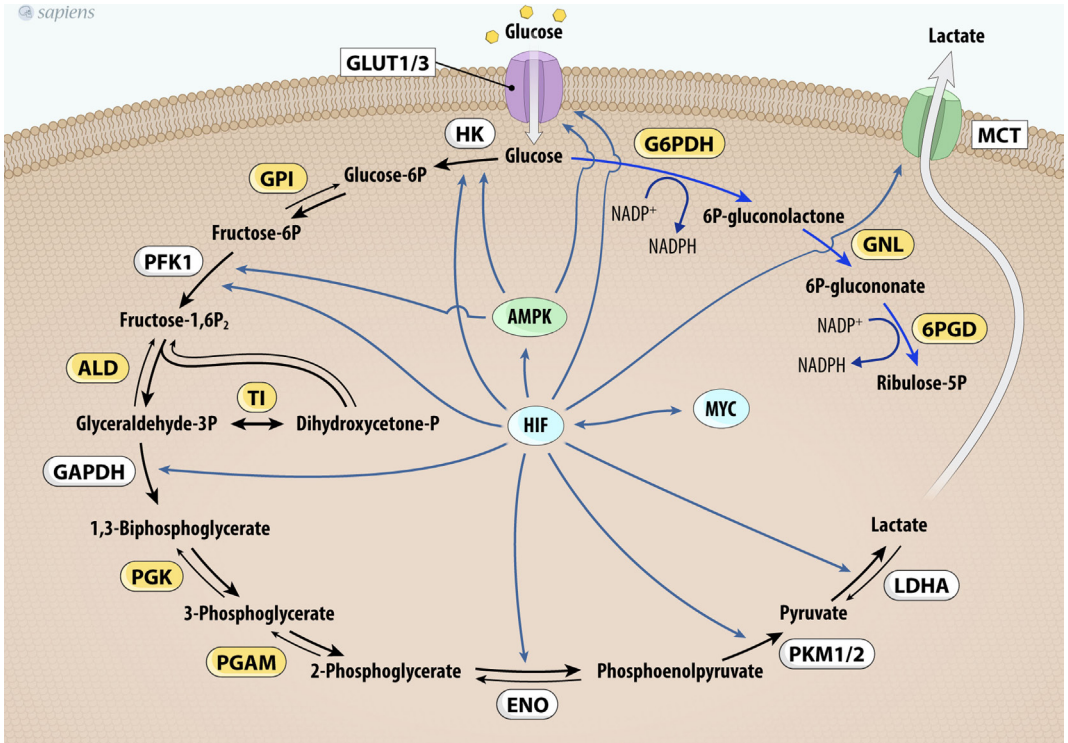


FIGURE 3 | MYC and HIF-1 regulate glucose metabolism. MYC and HIF-1 are described as important regulators of key genes (in white) involved in glucose uptake and glycolysis pathway control. Abbrevations: Glut1/3, glucose transporter 1 or 3; HK, hexokinase; GPI, glucose phosphate isomerase; PFK-1, phosphofructokinase 1; ALD, aldolase; TI, triose phosphate isomerase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGAM, phosphoglycerate mutase; ENO, enolase; PKM1/2, pyruvate kinase isoforms 1 and 2; LDHA, lactate dehydrogenase A; G6PDH, glucose 6-phosphate dehydrogenase; GNL, gluconolactonase; 6PDG, 6-phosphogluconate dehydrogenase; MCT1, monocarboxylate transporter-1; HIF-1, hypoxia-inducible factor-1.

to the tumor microenvironment are disorganized and may not result in efficient cell delivery of oxygen. HIF-1 stimulates angiogenesis predominantly through the increased expression of vascular endothelial growth factor (VEGF). VEGF recruits new microvessels that allow the delivery of nutrients and expansion of the tumor mass. Some previous studies have indicated that VEGF as a risk factor of developing PTC or tumor progression (79, 80).

METABOLIC MARKERS IN THYROID CANCER

GLUT1

The molecular mechanisms related to the upregulation of glucose metabolism in thyroid cancer are not yet completely understood. It has been demonstrated that ^{18}F-FDG uptake can be stimulated by TSH in thyroid cancer tissue *in vivo* (81–84). However, some works have shown that, depending on the thyroid cancer subtype, ^{18}F-FDG uptake can increase through a TSH-independent pathway (85, 86). In a non-tumor thyroid cell model, the action of TSH includes the activation of adenylate cyclase and PI3K (50–55). In the presence of RAS mutations, PI3K is constitutively activated, which may be partially responsible for the increased glucose uptake (57). Haber et al. (86) analyzed GLUT1 protein expression in 38 benign thyroid lesions, including follicular adenomas, Hurthle cell adenomas, nodular goiters, Hashimoto's thyroiditis, Graves' disease, and 28 cases of papillary, follicular, Hurthle cell, anaplastic, and medullary thyroid cancers. The authors showed that GLUT expression is frequently upregulated in thyroid cancers, but it is weakly expressed in benign nodules and in normal thyroid tissue. In addition, the localization of GLUT1 among thyroid cancers shows distinct patterns: (a) a circumferential plasma membrane focally present within the tumor in papillary carcinomas, (b) asymmetric distribution in the basilar membrane of tumor cells adjacent to the stroma and capillary blood supply, or (c) focally in the center of a tumor in metastatic or anaplastic carcinomas. Therefore, the degree and the localization of GLUT1 expression in thyroid cancers may have prognostic significance. Matsuzu et al. (87) studied the differential expression of GLUT genes in normal and pathologic thyroid tissues and demonstrated that the mRNA expression of GLUT 1, 3, 4, and 10 was evident in all thyroid tissues, but no differences were found between normal tissues and those from benign diseases. Recently, we demonstrated that GLUT1 is the predominant glucose transporter expressed in two non-tumor cell lines, PCCL3 (rat origin) and NTHY-ori (human origin) (88). In addition, the PTC cell line showed higher GLUT1 mRNA levels and protein expression compared to non-tumor cells, which may contribute to the elevated glucose uptake found in these cells (88). Recently, Naham et al. (89) analyzed 566 thyroid cancers,

including PTC $BRAF^{V600E}$ and ATC, and showed that PTC not only exhibited higher GLUT1 expression, but also higher GLUT3 expression. Moreover, the highest levels of GLUT1 expression were found in ATC, indicating that GLUT expression levels may be related to tumor aggressiveness.

Hexokinase

In addition to the expression of GLUTs, HK expression and activity are altered in many cancers, contributing to the increased glycolytic flux (32–34, 88–92). HK catalyzes the first irreversible reaction of glycolysis and its enzymatic product is glucose-6-phosphate (G6P), which is a substrate for glycolysis or the PPP. Although there are four HK isoforms, the isoforms HK1 and HK2 seem to be overexpressed in cancer cells (92, 93). Some biochemical characteristics of HK2 are advantageous to cancer cells. First, HK2 does not have a negative regulatory site, which allows greater activity (94). Second, HK2 can bind to outer mitochondrial membrane porins and voltage-gated anion channels (VDAC), facilitating its access to newly synthesized ATP and decreasing the negative feedback of G6P for glucose phosphorylation (94–98). Third, the binding of HK2 to the mitochondria increases its activity, enhancing ATP production through the glycolysis pathway (94–97). Fourth, the binding of HK2 to VDAC improves the stabilization of the mitochondrial membrane, leading to decreased reactive oxygen species (ROS) generation (96, 97). Finally, the binding of HK2/VDAC prevents Bax/Bak unbinding from the mitochondria and apoptosis (97, 98). Therefore, HK2 seems to be important for sustained cancer growth and has been suggested to be a marker of progression and tumor aggressiveness (91–103).

The earliest studies documenting the relationship between HK activity and thyroid carcinogenesis date back to the 1980s (99, 103). Rijksen et al. (103) showed no differences in HK biochemical characteristics when comparing MTC and ATC. Only the affinity of HK for its substrate was higher in ATC than in MTC. Nahm et al. (89) studied 342 PTC samples and found higher HK2 levels in 50% of PTC samples harboring the $BRAF^{V600E}$ mutation. Recently, we demonstrated that HK activity is higher in the BCPAP and TPC1 cell lines than in non-tumor cells (88). Interestingly, HK activity in the cytosolic and mitochondrial fractions was significantly different between the two thyroid cancer cell lines. TPC1 cells that have RET/PTC translocation, showed equally distributed HK activity in the two subcellular fractions, while BCPAP (BRAF mutated) cells had higher HK activity in the mitochondrial fraction (88). According to Hooft et al. (100, 101), HK expression is similar between metastatic and primary DTC tumors, and positive ^{18}FDG uptake on PET is associated with higher HK1 expression, however, mitochondria-bound HK was not evaluated in this study.

Pyruvate Kinase

The PK enzyme catalyzes the last reaction of the glycolytic pathway. It is responsible for the conversion of phosphoenolpyruvate and ADP into pyruvate and ATP, respectively. PK monomer is composed of one active site, three main domains (denominated A, B and C), and a small N-terminal domain (104, 105). The C domain is the dimerization interface of the enzyme, and enzyme dimers can interact in a dimer–dimer configuration forming a tetrameric protein (104).

The PK isoform M1 (PKM1) is a constitutive tetramer exhibiting the highest activity that is expressed in tissues with high metabolic demand, such as brain, heart, and skeletal muscle (106, 107). PK isoform M2 (PKM2) is found in normal proliferating cells, but it is predominantly expressed in tumor cells and seems to be important for cancer cell metabolic adaptation (104, 108–110). The PKM2 isoform, in contrast to PKM1, can occur as dimers or tetramers, depending on the presence or absence of allosteric regulators (104, 105). The main positive allosteric regulator of PKM2 is fructose-1,6-biphosphate (Fructose-1,6-P2) that stabilizes the active tetrameric form of the enzyme (104, 105). PKM2 activity is negatively regulated by acetylation, phosphorylation, and oxidation. The phosphorylation of PKM2 at tyrosine 105 interferes with fructose-1,6-P2 binding and induces transformation from tetrameric to dimeric state. Also, the acetylation of PKM2 at lysine 305, or its oxidation at cysteine 358 decreases PKM2 activity (104, 105, 110). Decreased PKM2 activity leads to the accumulation of upstream glycolysis intermediates and consequently results in the deviation of metabolites to the PPP biosynthetic pathway and improved hexamine formation, nucleotide synthesis, and $NADPH/NADP^+$ formation, contributing to the maintenance of redox homeostasis (110, 111). Therefore, when dimeric PKM2 is present in a tumor, less pyruvate is produced, limiting the mitochondrial substrate, what contributes to the metabolic shift from OXPHOS to aerobic glycolysis (104, 110).

Our group has shown that the human PTC cell lines, BCPAP and TPC1, express higher PKM2 mRNA levels compared to non-tumor cells, but no differences were found in PKM1 mRNA levels. However, the total activity of PK in PTC cells carrying the BRAF mutation (BCPAP) was higher than that in both non-tumor (NTHY-ori) and TPC1 (RET/PTC) cell lines, indicating that PKM2 enzymatic responses depend on the PTC driver mutation (88). It is believed that PKM isoform expression and activity change with tumor progression are linked to an increased tumor growth rate (112, 113). Feng et al. (113) showed that PKM2 expression in human PTC was associated with advanced tumor stages and lymph node metastasis. In addition, more intensive immunostaining of PKM2 was detected in PTCs harboring the BRAF mutation (113). Recently, Bikas et al. (114) showed that some thyroid cancer cells (FTC133 and BCPAP) characterized by glycolysis dependency overexpress PKM2. Although there are few studies in the literature, higher PKM2 expression in thyroid carcinomas appears to be significantly associated with the BRAF mutation, suggesting that this enzyme may be a potential therapeutic target in this type of cancer.

The relationship between dimeric and tetrameric PKM2 states has been described as a key factor for cell proliferation (111, 112, 115, 116). Several studies have shown that dimeric PKM2 can be translocated to the cell nucleus, where it directly interacts with multiple transcriptional factors and acts as a transcriptional coactivator involved in the upregulation of glycolytic genes, cell migration, and adhesion. The STAT3 signaling pathway seems to be involved in these effects of PKM2, which could be responsible for metastatic progression (112, 115, 117–119).

Lactate Dehydrogenase (LDH)

Lactate production plays a critical role in tumor biology. Due to their high glucose consumption, cancer cells display increased lactate production regardless of oxygen availability (31–36). Lactate is formed by the conversion of pyruvate and NADH in a reversible reaction catalyzed by lactate dehydrogenase (LDH). Although the isoforms of LDH are expressed in several tissues, LDHA is upregulated in a wide range of tumor tissues (120, 121). The LDHA converts pyruvate into lactate preferentially, while the lactate dehydrogenase B (LDHB) acts in opposite way (120). When PKM2 activity decreases, a change in the cytosolic NAD^+/NADH ratio occurs (104), what negatively impacts on the pyruvate to lactate conversion by LDHA. The downregulation of LDHA produces energy imbalance and oxidative stress leading to cell death (121, 122). Mirebeau-Prunier et al. (123) showed a lower LDHA/LDHB ratio in thyroid oncocytomas and follicular thyroid tumors. The downregulation of LDHA expression is related to the upregulation of estrogen-related receptor alpha, leading to changes in the oxidative metabolic profile of the tumor (123). In contrast, Kachel et al. (124) showed that LDHA is overexpressed in FTC and PTC compared to non-tumor tissues and its levels were even higher in UTC, suggesting that LDHA could be used as a biomarker of tumor aggressiveness. Comparing two PTC cell lineages, BCPAP and TPC1, we did not find differences in LDHA mRNA expression when compared to non-tumor cells. However, both tumor cell lineages had higher LDH activity and lactate production rates (88).

Monocarboxylate Transporter (MCT)

New evidence has identified MCT as an essential factor in thyroid cancer phenotype (38). MCTs are part of a family of transporters with more than 14 defined members. In the thyroid, MCT10 and MCT8 have been characterized. However, only MCTs 1–4 act in the transport of monocarboxylates, such as lactate, pyruvate, and ketone bodies (38). MCT isoform 1 (MCT1) is a bidirectional lactate transporter present in the plasma membrane that mediates the influx of lactate into the cell. MCT1 is also found in the outer mitochondrial membrane, transporting lactate from the cytosol to the mitochondrial matrix, which increases ATP production *via* OXPHOS (38). MCT isoform 4 (MCT4) is a low-affinity lactate transporter that mediates lactate efflux from cells. In tumor cells, these transporters are important for the maintenance of glycolysis under hypoxic conditions or in normoxia so that tumor cells can utilize lactate and other high-energy substrates produced (38, 121, 124). The lower lactate levels found in the media of some cancer cells in culture suggest a higher lactate uptake *via* MCTs, allowing them to generate large amounts of ATP *via* OXPHOS (121, 124, 125). On the other hand, higher lactate levels outside of cells indicate that MCT4 is responsible for the export of lactate in some cancer cells (38, 125). Therefore, according to Curry et al. (125), MCT1 could be used as an indicator of higher OXPHOS, and MCT4 can be used as a marker of glycolytic metabolism. In head and neck cancers, the expression of MCT4 has been associated with a higher tumor stage and poorer clinical outcomes (125). Curry et al. (125) also described crosstalk between PTC thyrocytes and adjacent fibroblasts with a glycolytic phenotype, resulting in the production of high amounts of lactate, which is transported outside the cell by MCT4. On the other hand, PTC cells showed greater MCT1 staining, which allows lactate intake and consumption by mitochondrial oxidation. From these adjustments, PTC cells may obtain the energy to survive, proliferate, and metastasize (126).

ROLE OF ROS IN THYROID CANCER METABOLISM

During the process of tumor progression, some metabolic changes are associated with high levels of ROS (126, 127). High levels of ROS can generate oxidative stress due to an imbalance between ROS production and antioxidant defenses. The major source of ROS seems to be the mitochondria where they are produced as a consequence of OXPHOS. Therefore, decreased mitochondrial metabolism may be important for decreasing ROS production and protecting cancer cells from death. However, improved antioxidant defenses have been observed as a compensatory mechanism in response to ROS generation, which is often increased in several tumors, including thyroid carcinomas (126, 127).

The NADPH oxidase/dual oxidase enzymes, also called NOXs and DUOXs, are specialized sources of ROS that are widely expressed in a variety of tissues, including the thyroid gland (128, 129). In the thyroid, DUOX1 and DUOX2 are the main producers of H_2O_2, although thyrocytes also express NADPH oxidase 4 (NOX4) that is prominently expressed in PTC and corresponds to an important source of ROS (128–130). In fact, there is a significant positive association between BRAF oncogene activation and NOX4 expression (130).

The oncocytic tumor cells are characterized by the presence of a high number of mitochondria probably due to an imbalance between mitochondria biogenesis and destruction; these cells depend on OXPHOS for energy conservation and produce high ROS levels (131). According to Maximo and Sobrinho-Simoes (132), the increased ROS production of oncocytic cells could be secondary to the decreased activities of complexes I and III of the electron transport chain (133). In fact, the oncocytic phenotype is associated with disruptive mutations in complex I subunits genes encoded by mitochondrial DNA (134), which might be involved in tumor cell death due to inefficient metabolic adaptation, since the induction of the Warburg phenotype through the stabilization of HIF-1 alpha depends on normal complex I function that sustains tumor growth (135).

Interestingly, Paik et al. (136) showed that ROS can also increase glucose metabolism through HIF-1 activation. Using endothelial cells, they showed that increased ROS levels are accompanied by higher glucose uptake and lactate production when these cells are subjected to hypoxia. However, the glycolytic phenotype is blocked by HIF-1 stabilization, suggesting that ROS-driven HIF-1α accumulation accelerates glycolysis in endothelial cells (136).

Reactive oxygen species also change cell metabolism through AMP kinase (AMPK) protein activation (137). Intracellular ROS can stimulate AMPK, a metabolic stress-sensing cytosolic enzyme that regulates energy consumption and production processes (137). Although controversy remains in the literature relative to AMPK pathway involvement in tumorigenesis and cancer progression, several studies have demonstrated that activated

AMPK causes cell-cycle arrest and has a strong antiproliferative effect in different cancer cell lines (137–139). AMPK activation also increases GLUT-1 protein expression, glucose uptake, and utilization of the glycolytic pathway in both non-tumor models and in tumor models, including PTC models (137–139). Some studies have shown that disruption of AMPK activity induces the Warburg effect in tumor cells (137, 140, 141). In an AMPK-deficient mouse model of Peutz–Jeghers syndrome, mammalian target of rapamycin protein (mTOR) is upregulated and HIF-1 promotes higher HK2 and Glut1 expression and increased glucose utilization by tumors (142). According to Bikas et al. (114), AMPK activation seems to be involved in the glucose metabolism dependence that is observed in some PTC cells. We described greater expression of the active phosphorylated form of AMPK in PTC tissue samples and in PTC tumor cell lineages in culture compared to that in non-tumor tissues (139, 143). However, new studies are necessary to understand the role of AMPK in human thyroid cancer, especially in terms of metabolic control, cell growth, apoptosis, and survival.

TARGETING METABOLISM IN THYROID CANCER

Considering the diversity of thyroid tumors and their distinct metabolic requirements, establishing a unique strategy for cancer therapy is not an easy task. However, some drugs for cancer therapy that target the tyrosine kinase receptors signaling cascade are currently being used. These proteins constitute a group of enzymes involved in the control of mitogen signals, energy status, cell survival, and angiogenesis (144, 145). Several tyrosine kinase inhibitors (TKIs) have been developed for thyroid carcinoma treatment, but not all of them have received approval from international health agencies (145). Many TKIs are still in the initial clinical phase of study. Sorafenib, lenvatinib, vandetanib, and cabozantinib are multikinase inhibitors approved by the Food and Drug Administration and the European Medical Agency (EMA) for use in patients with advanced thyroid carcinomas. In fact, these patients had significantly increased progression-free survival rates with the use of these agents (145).

In addition to TKIs, other downstream targets can regulate metabolic pathways (**Figure 4**). PI3K was described as a component of the insulin receptor intracellular signaling pathway and its main substrate is AKT. Once activated, AKT increases phosphorylation events in both the cytoplasm and the nucleus, stimulating glucose uptake; glycolytic flux; inhibition of apoptosis; and activation of mTOR, an important regulator of metabolism and cancer growth (145, 146). PI3K/AKT signaling is inhibited by the tumor suppressor phosphatase and tensin homolog (PTEN), which is often mutated in many types of tumors. Therefore, the loss of PTEN activity allows PI3K/AKT constitutive activation (145, 146). LY294002 is a classic molecule that inhibits PI3K and has already been tested in some carcinomas, including thyroid cancer. Currently, other new drugs targeting PI3K/AKT have been tested in several carcinomas and are in different clinical trial phases of study (144, 145).

Mammalian target of rapamycin protein is the main downstream effector of PI3K/AKT, and its upregulation is involved in tumorigenesis (144–146). Our group demonstrated that the activation of PI3K/AKT/mTOR inhibits iodide uptake by diminishing sodium/iodide symporter transporter (NIS) expression in non-tumor cells (149). Loss of iodide uptake is a negative process in the course of disease evolution, since thyroid cancer treatment depends on the administration of radioactive iodine (145). Several mTOR inhibitors (rapamycin analog), such as everolimus, have been studied in preclinical and clinical trials, including thyroid carcinomas (144, 145). Recently, phase II studies using everolimus in patients with advanced thyroid cancer have reported a partial response and sustained stable disease in a small proportion of patients (5–45%, respectively), with progression-free survival rates of approximately 11–16 months (150). The use of everolimus as monotherapy shows moderate effects, but its clinical relevance mainly derives from its relatively low toxicity profile (144, 151). The combination of another mTOR inhibitor (temsirolimus) and other drugs (MEK inhibitors) has shown synergic effects *in vitro* and is being tested in clinical trials (152, 153).

AMP kinase is a potent physiological mTOR inhibitor. Under low ATP/AMP ratio conditions, AMPK is activated, leading to metabolic adaptations, such as increased catabolism and decreased anabolism that are partially mediated by mTOR inhibition. The crosstalk between mTOR and AMPK has been extensively studied (137, 138, 140). The first evidence associating AMPK with cancer development was the discovery of liver kinase B1 (LKB1). LKB1 is a serine/threonine kinase and the major upstream kinase responsible for AMPK activation through phosphorylation (138). LKB1 is recognized as a tumor suppressor that associates bioenergetics with cell growth control and downregulation of mTOR activity through AMPK activation (138, 142). We have studied the effects of a pharmacological AMPK activator (AICAR) on PTC cell lines and observed decreased cell proliferation and the induction of apoptosis (139). These results suggest that AMPK may be a good target for thyroid cancer therapy (**Figure 4**). Epidemiological studies reported that in thyroid cancer patients who are also diabetic, metformin, an oral anti-diabetic drug, can activate AMPK, resulting in a reduced tumor size and higher remission rates (154). Interestingly, our group showed that the expression and activity of AMPK are increased in human PTC and in PTC cell lines (BCPAP and TPC1) compared to those in non-tumor tissues and non-tumor cell lines (139, 143). As PTCs are well-differentiated and slow-growing carcinomas, it is believed that increased AMPK activation could impair tumor growth.

Although tumor energy metabolism has common characteristics, most molecular targets that may be used for tumor treatment are ubiquitously expressed and function in the entire body. Therefore, it is difficult to produce specific effects only in tumor cells. Glycolytic inhibitors, such as 2-deoxyglucose (2DOG) and 3-bromopyruvate (3-BP), can be used as adjuvant agents to sensitize tumors. Unlike 2DOG, 3-BP acts in many targets and inhibits HK, GAPDH, and MCT activities, thus leading to decreased aerobic glycolysis (155). Furthermore, inhibition of PFK1 activity, a rate-limiting step of glycolysis, is also an interesting strategy in cancer therapy. This protein is activated by fructose-2,6-bisphosphate (F2,6BP), which is produced by PFKFBs. A small molecule, 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one,

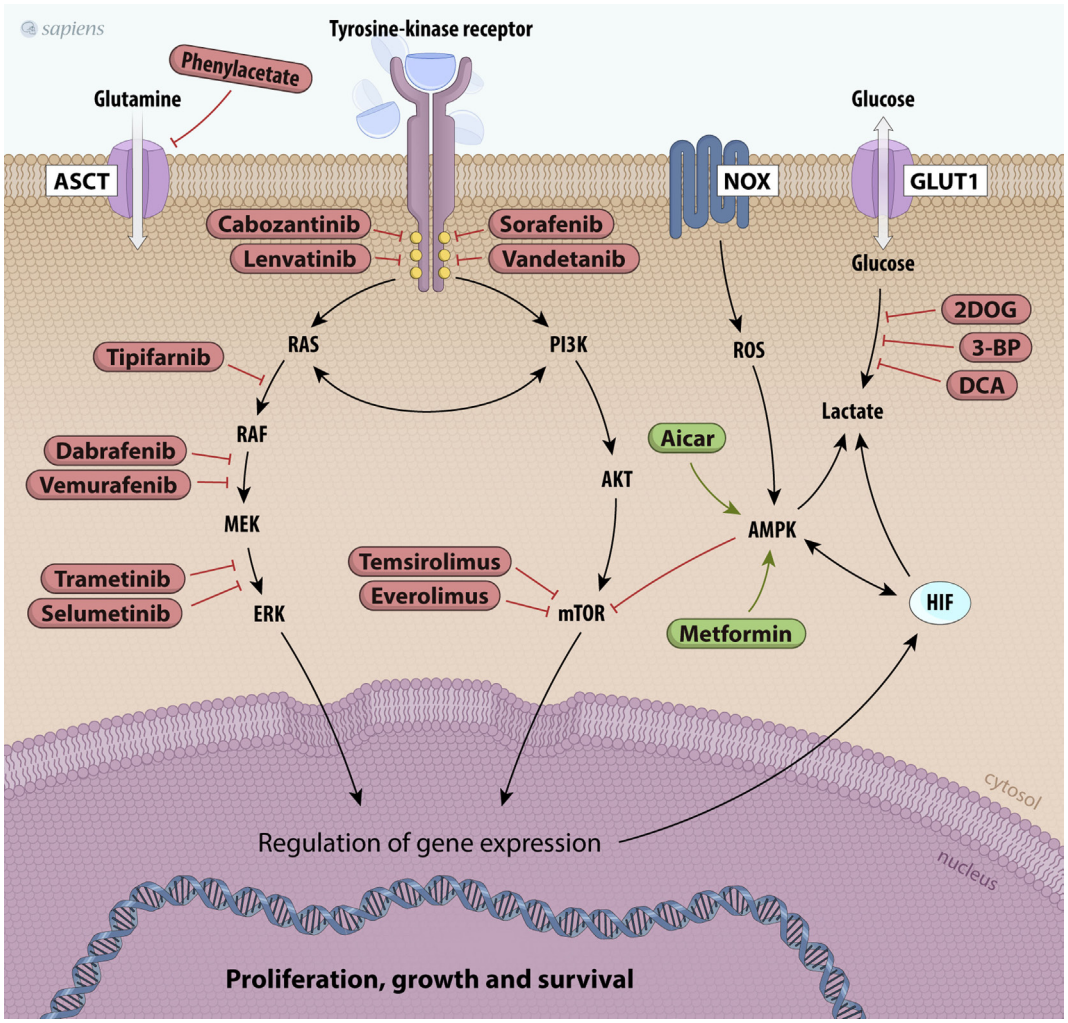


FIGURE 4 | Scheme of principal potential therapeutic targets. The products of mutated genes activated in cancer cells are targets for anticancer drug therapy [Valerio et al. (144), Bible and Ryder (147), and De Falco et al. (148)]. Several kinase inhibitors along the RET/papillary thyroid carcinoma, RAS, PI3K/AKT/mTOR, and MAPK signaling pathways are shown in red. Metabolic alterations in thyroid cancer cells can also be inhibited, such as glutamine uptake (phenylacetate) and glucose metabolism (2DOG, 3-BP, DCA). Finally, other drugs can activate molecules like AMPK, such as metformin and AICAR, which are shown in green. Abbreviations: 2DOG, 2-deoxyglucose; 3-BP, 3-bromopyruvate; DCA, dichloroacetate, AICAR, 5-aminoimidazole-4-carboxamide-ribonucleoside, NOX, NADPH oxidade; MAPK, mitogen-activated protein kinase; AMPK, AMP kinase.

has been found to inhibit PFKFB3, leading to decreased glycolytic flux and slower tumor growth (**Figure 4**) (156).

Other attractive targets for cancer therapy include the inhibition of lactate production. Some previous studies have shown that loss of LDHA function by dichloroacetate (DCA) results in dramatically diminished cellular transformation or xenograft tumor growth in breast cancer (157). Glutaminolysis and amino acid metabolism are very important for tumors. Glutamine is the most abundant amino acid in the plasma and is heavily consumed by tumor cells. Therapies that decrease plasma glutamine concentrations induce tumor regression and prevent muscular catabolism, an endogenous source of glutamine. Phenyl acetate is a promising drug that can reduce the availability of glutamine in the blood and shows low toxicity (158).

Finally, another strategy targeting metabolism in tumors is diet restriction, fasting, or a ketogenic diet (a low-carbohydrate and high-fat diet) (159–162). The hypothesis is based on the glucose dependency of many tumor types. Interestingly, these diets do not increase plasma glucose levels, but produce ketone bodies that can be used as a carbon source for energy production in oxidative processes, altering the Warburg phenotype (159–162).

CONCLUSION

In summary, although thyroid cancer studies are emerging, the mechanism of tumor progression remains unclear. As described previously, metabolic reprogramming is the hallmark of cancer cells. Recent molecular studies in thyroid cancer revealed that oncogenes and tumor suppressor genes not only control growth and apoptotic phenotypes of thyroid carcinomas, but also directly affect cellular energy metabolism and are implicated

in the Warburg phenotype. The higher glucose and glutamine consumption associated with the disruption of mitochondrial OXPHOS create a favorable environment for tumor progression.

AUTHOR CONTRIBUTIONS

RC—writing and elaborating the figures. RF—writing and reviewing the manuscript. DC—writing and reviewing the final version.

ACKNOWLEDGMENTS

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, 472630/2012-1), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, E-26/111.447/2012), Programa de Oncobiologia, and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

REFERENCES

1. Tan GH, Gharib H. Thyroid incidentalomas: management approaches to nonpalpable nodules discovered incidentally on thyroid imaging. *Ann Intern Med* (1997) 126:226–31. doi:10.7326/0003-4819-126-3-199702010-00009
2. Morganti S, Ceda GP, Saccani M, Milli B, Ugolotti D, Prampolini R, et al. Thyroid disease in the elderly: sex-related differences in clinical expression. *J Endocrinol Invest* (2005) 28:101–4.
3. Momesso DP, Vaisman F, Cordeiro de Noronha Pessoa CH, Corbo R, Vaisman M. Small differentiated thyroid cancer: time to reconsider clinical management and treatment. *Surg Oncol* (2012) 21:257–62. doi:10.1016/j.suronc.2012.06.002
4. Pacini F, Cetani F, Miccoli P, Mancusi F, Ceccarelli C, Lippi F, et al. Outcome of 309 patients with metastatic differentiated thyroid carcinoma treated with radioiodine. *World J Surg* (1994) 18:600–4. doi:10.1007/BF00353775
5. Coelho SM, Vaisman M, Carvalho DP. Tumor re-differentiation effect of retinoic acid: a novel therapeutic approach for advanced thyroid cancer. *Curr Pharm Des* (2005) 11:2525–31. doi:10.2174/1381612054367490
6. Haugen BR. Management of the patient with progressive radioiodine non-responsive disease. *Semin Surg Oncol* (1999) 16:34–41. doi:10.1002/(SICI)1098-2388(199901/02)16:1<34::AID-SSU7>3.0.CO;2-2
7. Davis L, Welch HG. Increasing incidence if thyroid cancer in the United States, 1973–2002. *JAMA* (2006) 295:2164–7. doi:10.1001/jama.295.18.2164
8. Parameswaran R, Brooks S, Sadler GP. Molecular pathogenesis of follicular cell derived thyroid cancers. *Int J Surg* (2010) 8:186–93. doi:10.1016/j.ijsu.2010.01.005
9. Nikiforov YE, Nikiforova MN. Molecular genetics and diagnosis of thyroid cancer. *Nat Rev Endocrinol* (2011) 7:569–80. doi:10.1038/nrendo.2011.142
10. Fadare O, Tallini G. Thyroid: oncocytic tumors. *Atlas Genet Cytogenet Oncol Haematol* (2003) 7:202–5. doi:10.4267/2042/37997
11. Gonzales-Campora R, Herrero-Zapatero A, Lerma E, Sanchez F, Galera H. Hurthe cell and mitochondrion-rich cell tumors. A clinicopathologic study. *Cancer* (1986) 57:1154–63. doi:10.1002/1097-0142(19860315)57:6<1154::AID-CNCR2820570616>3.0.CO;2-W
12. Hong JH, Yi HS, Yi S, Kim HW, Lee J, Kim KS. Implications of oncocytic change in papillary thyroid cancer. *Clin Endocrinol* (2016) 85:797–804. doi:10.1111/cen.13115
13. Arganini M, Behar R, Wu TC, Straus F II, McCormick M, DeGroot LJ, et al. Hurthle cell tumors: a twenty-five-year experience. *Surgery* (1986) 100:1108–15.
14. Berho M, Suster S. The oncocytic variant of papillary carcinoma of the thyroid: a clinicopathologic study of 15 cases. *Hum Pathol* (1997) 28:47–53. doi:10.1016/S0046-8177(97)90278-1
15. Beckner ME, Heffess CS, Oertel JE. Oxyphilic papillary thyroid carcinomas. *Am J Clin Pathol* (1995) 103:208–87. doi:10.1093/ajcp/103.3.280
16. de Vries MM, Celestino R, Castro P, Eloy C, Máximo V, van der Wal JE, et al. RET/PTC rearrangement is prevalent in follicular Hürthle cell carcinomas. *Histopathology* (2012) 61:833–43. doi:10.1111/j.1365-2559.2012.04276.x
17. Downward J. Targeting RAS signaling pathways in cancer therapy. *Nat Rev Cancer* (2003) 3:11–2. doi:10.1038/nrc969
18. Bhaijee F, Nikiforov YE. Molecular analysis of thyroid tumors. *Endocr Pathol* (2011) 22:126–33. doi:10.1007/s12022-011-9170-y
19. Peyssonnaux C, Eychene A. The Raf/MEK/ERK pathway: new concepts of activation. *Biol Cell* (2001) 93:53–62. doi:10.1016/S0248-4900(01)01125-X
20. Di Cristofaro J, Marcy M, Vasko V, Sebag F, Fakhry N, Wynford-Thomas D, et al. Molecular genetic study comparing follicular variant versus classic papillary thyroid carcinomas: association of N-ras mutation in codon 61 with follicular variant. *Hum Pathol* (2006) 37:824–30. doi:10.1016/j.humpath.2006.01.030
21. Garcia-Rostan G, Zhao H, Camp RL, Pollan M, Herrero A, Pardo J, et al. Ras mutations are associated with aggressive tumor phenotypes and poor prognosis in thyroid cancer. *J Clin Oncol* (2003) 21(17):3226–35. doi:10.1200/JCO.2003.10.130
22. Sobrinho-Simões M, Máximo V, Rocha AS, Trovisco V, Castro P, Preto A, et al. Intragenic mutations in thyroid cancer. *Endocrinol Metab Clin North Am* (2008) 37:333–62. doi:10.1016/j.ecl.2008.02.004
23. 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* (2001) 25:460–70. doi:10.1101/gad.2016311
24. Ascierto PA, Kirkwood JM, Grob JJ, Simeone E, Grimaldi AM, Maio M, et al. The role of BRAF V600E mutation in melanoma. *J Transl Med* (2012) 10:85–93. doi:10.1186/1479-5876-10-85
25. Kimura ET, Nikiforova MN, Zhu Z, Knauf JA, Nikiforov YE, Fagin JA. High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. *Cancer Res* (2003) 63(7):1454–7.
26. Vasko V, Hu S, Wu G, Xing JC, Larin A, Savchenko V, et al. High prevalence and possible de novo formation of BRAF mutation in metastasized papillary thyroid cancer in lymph nodes. *J Clin Endocrinol Metab* (2005) 90:5265–9. doi:10.1210/jc.2004-2353
27. Salvatore G, De Falco V, Salerno P, Nappi TC, Pepe S, Troncone G, et al. BRAF is a therapeutic target in aggressive thyroid carcinoma. *Clin Cancer Res* (2006) 12:1623–9. doi:10.1158/1078-0432.CCR-05-2378
28. Ciampi R, Nikiforov YE. RET/PTC rearrangements and BRAF mutation in thyroid tumorigenesis. *Endocrinology* (2007) 3:936–41. doi:10.1210/en.2006-0921
29. Salvatore D, Barone MV, Salvatore G, Melillo RM, Chiappetta G, Mineo A, et al. Tyrosines 1015 and 1062 are in vivo autophosphorylation sites in ret and ret-derived oncoproteins. *J Clin Endocrinol Metab* (2000) 85:3898–907. doi:10.1210/jcem.85.10.6882
30. Tacha D, Zhou D, Cheng L. Expression of PAX8 in normal and neoplastic tissues: a comprehensive immunohistochemical study. *Appl Immunohistochem Mol Morphol* (2011) 19:293–9. doi:10.1097/PAI.0b013e3182025f66
31. Warburg O. On respiratory impairment in cancer cells. *Science* (1956) 124:269–70.
32. Kroemer G, Pouyssegur J. Tumor cell metabolism: cancer's Achilles heel. *Cancer Cell* (2008) 13:472–82. doi:10.1016/j.ccr.2008.05.005
33. Vander Heinden 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
34. Gatenby RA, Gillies RJ. Why do cancer have high aerobic glycolysis? *Nat Rev Cancer* (2004) 4:891–857. doi:10.1038/nrc1478
35. Hsu PP, Sabatini DM. Cancer cell metabolism: Warburg and beyond. *Cell* (2008) 134:703–7. doi:10.1016/j.cell.2008.08.021
36. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) 144:646–74. doi:10.1016/j.cell.2011.02.013
37. Marin-Hernández A, Gallardo-Pérez JC, Rodríguez-Enríquez S, Encalada R, Moreno-Sánchez R, Soovedra E. Modeling cancer glycolysis. *Biochim Biophys Acta* (2011) 1807:755–67. doi:10.1016/j.bbabio.2010.11.006
38. Halestrap AP, Wilson MC. The monocarboxylate transporter family – role and regulation. *IUBMB Life* (2012) 64:109–19. doi:10.1002/iub.572
39. Sonveaux P, Végran 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(12):3930–42. doi:10.1172/JCI36843

40. 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
41. Xu XD, Shao SX, Jiang HP, Cao YW, Wang YH, Yang XC, et al. Warburg effect or reverse Warburg effect? A review of cancer metabolism. *Oncol Res Treat* (2015) 38:117–22. doi:10.1159/000375435
42. Skrtić M, Sriskanthadevan S, Jhas B, Gebbia M, Wang X, Wang Z, et al. Inhibition of mitochondrial translation as a therapeutic strategy for human acute myeloid leukemia. *Cancer Cell* (2011) 20:674–88. doi:10.1016/j.ccr.2011.10.015
43. Dakubo GD, Parr RL, Costello LC, Franklin RB, Thayer RE. Altered metabolism and mitochondrial genome in prostate cancer. *J Clin Pathol* (2006) 59:10–6. doi:10.1136/jcp.2005.027664
44. Ho J, de Moura MB, Lin Y, Vincent G, Thorne S, Duncan LM, et al. Importance of glycolysis and oxidative phosphorylation in advanced melanoma. *Mol Cancer* (2012) 11:76. doi:10.1186/1476-4598-11-76
45. Haq R, Fisher DE, Widlund HR. Molecular pathways: BRAF induces bioenergetic adaptation by attenuating oxidative phosphorylation. *Clin Cancer Res* (2015) 20:2257–63. doi:10.1158/1078-0432.CCR-13-0898
46. Tennant DA, Durán RV, Gottlieb E. Targeting metabolic transformation for cancer therapy. *Nat Rev Cancer* (2010) 10:267–77. doi:10.1038/nrc2817
47. Yang L, Moss T, Mangala LS, Marini J, Zhao H, Wahlig S, et al. Metabolic shifts toward glutamine regulate tumor growth, invasion and bioenergetics in ovarian cancer. *Mol Syst Biol* (2014) 10:728. doi:10.1002/msb.20134892
48. Van Geldermalsen M, Wang Q, Nagarajah R, Marshall AD, Thoeng A, Gao D, et al. ASCT2/SLC1A5 controls glutamine uptake and tumor growth in triple-negative basal-like breast cancer. *Oncogene* (2016) 35:3201–8. doi:10.1038/onc.2015.381
49. 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
50. Deichen JT, Schmidt C, Prante O, Maschauer S, Papadopoulos T, Kuwert T. Influence of TSH on uptake of (18F) fluorodeoxyglucose in human thyroid cells in vitro. *Eur J Nucl Med Mol Imaging* (2004) 31:507–12. doi:10.1007/s00259-003-1401-0
51. Filetti S, Damante G, Foti D. Thyrotropin stimulates glucose transport in cultured rat thyroid cells. *Endocrinology* (1987) 120:2576–81. doi:10.1210/endo-120-6-2576
52. Russo D, Damante G, Foti D, Costante G, Filetti S. Different molecular mechanisms are involved in the multihormonal control of glucose transport in FRTL5 rat thyroid cells. *J Endocrinol Invest* (1994) 17:323–7. doi:10.1007/BF03348991
53. Samih N, Hovsepian S, Aouani A, Lombardo D, Fayet G. Glut-1 translocation in FRTL-5 thyroid cells: role of phosphatidylinositol 3-kinase and N-glycosylation. *Endocrinology* (2000) 141:4146–55. doi:10.1210/endo.141.11.7793
54. Blaser D, Maschauer S, Kuwert T, Prante O. In vitro studies on the signal transduction of thyroidal uptake of 18F-FDG and 131I-iodide. *J Nucl Med* (2006) 47:1382–8.
55. Otten J, Dumont JE. Glucose metabolisms in normal human thyroid tissue in vitro. *Eur J Clin Invest* (1972) 2(8):213–9. doi:10.1111/j.1365-2362.1972.tb00646.x
56. Mulvey PF Jr, Kelleher JJ, Slingerland DW. Oxidation of glucose-C14 by human thyroid tissues. *Metabolism* (1963) 12:829.
57. Prante O, Maschauer S, Fremont V, Reinfelder J, Stoehr R, Szkudlinski M, et al. Regulation of uptake of 18F-FDG by a follicular human thyroid cancer cell line with mutation-activated K-Ras. *J Nucl Med* (2009) 50:1364–70. doi:10.2967/jnumed.109.062331
58. Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signalling controls tumor cell growth. *Nature* (2006) 441:424–30. doi:10.1038/nature04869
59. Hutton JE, Wang X, Zimmerman LJ, Slebos RJC, Trenary IA, Young JD, et al. Oncogenic KRAS and BRAF drive metabolic reprogramming in colorectal cancer. *Mol Cell Proteomics* (2016) 15:2924–38. doi:10.1074/mcp.M116.058925
60. Rabinowitz JD, White E. Autophagy and metabolism. *Science* (2010) 33:1344–8. doi:10.1126/science.1193497
61. Zhu X, Zhao L, Park JW, Willingham MC, Cheng S. Synergistic signaling of KRAS and thyroid hormone receptor β mutants promotes undifferentiated thyroid cancer through MYC up-regulation. *Neoplasia* (2014) 16:757–69. doi:10.1016/j.neo.2014.08.003
62. Dang CV, Le A, Gao P. MYC-induced cancer cell energy metabolism and therapeutic opportunities. *Clin Cancer Res* (2009) 5:6479–83. doi:10.1158/1078-0432.CCR-09-0889
63. Dang CV, O'Donnell KA, Zeller KI, Nguyen T, Osthus RC, Li F. The c-Myc target gene network. *Semin Cancer Biol* (2006) 16:253–64. doi:10.1016/j.semcancer.2006.07.014
64. Qu Y, Yang Q, Liu J, Shi B, Ji M, Li G, et al. c-Myc is required for $BRAF^{V600E}$-induced epigenetic silencing by H3K27me3 in tumorigenesis. *Theranostics* (2017) 7:2092–107. doi:10.7150/thno.19884
65. Shim H, Dolde C, Lewis BC, Wu CS, Dang G, Jungmann RA, et al. c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. *Proc Natl Acad Sci U S A* (1997) 94:6658–63. doi:10.1073/pnas.94.13.6658
66. Kim JW, Gao P, Liu YC, 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
67. Kim JW, Zeller KI, Wang Y, Jegga AG, Aronow BJ, O'Donnell KA, et al. Evaluation of myc E-box phylogenetic footprints in glycolytic genes by chromatin immunoprecipitation assays. *Mol Cell Biol* (2004) 24:5923–36. doi:10.1128/MCB.24.13.5923-5936.2004
68. Osthus RC, Shim H, Kim S, Li Q, Reddy R, Mukherjee M, et al. Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. *J Biol Chem* (2000) 275:21797–800. doi:10.1074/jbc.C000023200
69. 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
70. Pouyssegur J, Dayan F, Mazure MM. Hypoxia signaling in cancer and approaches to enforce tumor regression. *Nature* (2006) 441:437–43. doi:10.1038/nature04871
71. Semenza G. Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy. *Trends Pharmacol Sci* (2012) 33:207–14. doi:10.1016/j.tips.2012.01.005
72. Cao D, Hou M, Guan YS, Jiang M, Yang Y, Gou HF. Expression of HIF-1alpha and VEGF in colorectal cancer: association with clinical outcomes and prognostic implications. *BMC Cancer* (2009) 9:432. doi:10.1186/1471-2407-9-432
73. Marin-Hernández A, Gallardo-Pèrez JC, Ralph SJ, Rodríguez-Enríquez S, Moreno-Sánchez R. HIF-1 alpha modulates energy metabolism in cancer by inducing over-expression of specific glycolytic isoforms. *Mini Rev Med Chem* (2009) 9:1084–101. doi:10.2174/138955709788922610
74. Klaus A, Fathi O, Tatjana TW, Bruno N, Oskar K. Expression of hypoxia-associated protein HIF-1α in follicular thyroid cancer is associated with distant metastasis. *Pathol Oncol Res* (2017) 2:1–8. doi:10.1007/s12253-017-0232-4
75. Koukourakis MI, Giatromanolaki A, Harris AL, Sivridis E. Comparison of metabolic pathways between cancer cells and stromal cells in colorectal carcinomas: a metabolic survival role for tumor-associated stroma. *Cancer Res* (2006) 66:632–7. doi:10.1158/0008-5472.CAN-05-3260
76. Kim JM, 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
77. Gottilieb E, Thomlinson IP. Mitochondrial tumor suppressors: a genetic and biochemical update. *Nat Rev Cancer* (2005) 5:857–66. doi:10.1038/nrc1737
78. Zang 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
79. Lv Y, Sun Y, Shi T, Shi C, Qin H, Li Z. Pigment epithelium-derived factor has a role in the progression of papillary thyroid carcinoma by affecting the HIF1α-VEGF signaling pathway. *Oncol Lett* (2016) 12:5217–22. doi:10.3892/ol.2016.5316
80. Bingül I, Vural P, Dogru-Abbasoglu S, Cil E, Uysal M. Vascular endothelial growth factor G+405C polymorphism may contribute to the risk of developing papillary thyroid carcinoma. *J Clin Lab Anal* (2016) 31: 1–6. doi:10.1002/jcla.22110
81. Chin BB, Patel P, Cohade C, Ewertz M, Wahl R, Ladenson P. Recombinant human thyrotropin stimulation of fluoro-D-glucose positron emission

tomography uptake in well-differentiated thyroid carcinoma. *J Clin Endocrinol Metab* (2004) 89:91–5. doi:10.1210/jc.2003-031027
82. Petrich T, Borner AR, Otto D, Hofmann M, Knapp WH. Influence of rhTSH on (18F) fluorodeoxyglucose uptake by differentiated thyroid carcinoma. *Eur J Nucl Med Mol Imaging* (2002) 29:641–7. doi:10.1007/s00259-001-0745-6
83. van Tol KM, Jager PL, Piers DA, Pruim J, de Vries EG, Dullaart RP, et al. Better yield of 18 fluorodeoxyglucose positron emission tomography in patients with metastatic differentiated thyroid carcinoma during thyrotropin stimulation. *Thyroid* (2002) 12:381–7. doi:10.1089/105072502760043459
84. Grünwald F, Kalicke T, Feine U, Lietzenmayer R, Scheidhauer K, Dietlein M, et al. Fluorine-18 fluorodeoxyglucose positron emission tomography in thyroid cancer: results of a multicentre study. *Eur J Nucl Med Mol Imaging* (1999) 26:1547–52. doi:10.1007/s002590050493
85. Wang W, Macapinlac H, Larson SM, Yeh SD, Akhurst T, Finn RD, et al. (18F)-2-fluoro-2-deoxy-D-glucose positron emission tomography localizes residual thyroid cancer in patients with negative diagnostic 131I whole body scans and elevated serum thyroglobulin levels. *J Clin Endocrinol Metab* (1999) 84:2291–302. doi:10.1210/jcem.84.7.5827
86. Haber RS, Weiser KR, Pritsker A, Reder I, Burstein DE. GLUT1 glucose transporter expression in benign and malignant thyroid nodules. *Thyroid* (1997) 7:363–7. doi:10.1089/thy.1997.7.363
87. Matsuzu K, Segade F, Matsuzu U, Carter A, Bowden DW, Perrier ND. Differential expression of glucose transporters in normal and pathologic thyroid tissue. *Thyroid* (2004) 14:806–12. doi:10.1089/thy.2004.14.806
88. Coelho RG, Cazarin JM, Cavalcanti de Albuquerque JP, de Andrade BM, Carvalho DP. Differential glycolytic profile and Warburg effect in papillary thyroid carcinoma cell lines. *Oncol Rep* (2016) 36:3673–81. doi:10.3892/or.2016.5142
89. Nahm JH, Kim HM, Koo JA. Glycolysis-related protein expression in thyroid cancer. *Tumor Biol* (2017) 6:601–6. doi:10.1177/1010428317695922
90. Coelho RG, Calaça IC, Celestrini DM, Correia AH, Costa MA, Sola-Penna M. Clotrimazole disrupts glycolysis in human breast cancer without affecting non-tumoral tissues. *Mol Genet Metab* (2011) 103:394–8. doi:10.1016/j.ymgme.2011.04.003
91. Coelho RG, Calaça IC, Celestrini DM, Correia AH, Costa MA, Zancan P, et al. Hexokinase and phosphofructokinase activity and intracellular distribution correlate with aggressiveness and invasiveness of human breast carcinoma. *Oncotarget* (2015) 6:29375–87. doi:10.18632/oncotarget.4910
92. Soga T. Cancer metabolism: key players in metabolic reprogramming. *Cancer Sci* (2013) 104:275–81. doi:10.1111/cas.12085
93. Wilson JE. Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function. *J Exp Biol* (2003) 206:2049–57. doi:10.1242/jeb.00241
94. Mathupala SP, Ko YH, Pedersen PL. Hexokinase-2 bound to mitochondria: cancer's stygian link to the "Warburg effect" a pivotal target for effective therapy. *Semin Cancer Biol* (2009) 19:17–24. doi:10.1016/j.semcancer.2008.11.006
95. Ros S, Schulze A. Glycolysis back in the limelight: systemic targeting of HK2 block tumor growth. *Cancer Discov* (2013) 3:1105–7. doi:10.1158/2159-8290.CD-13-0565
96. Mathupala SP, Ko YH, Pedersen PL. The pivotal roles of mitochondria in cancer: Warburg and beyond and encouraging prospects for effective therapies. *Biochim Biophys Acta* (2010) 1797:1225–30. doi:10.1016/j.bbabio.2010.03.025
97. Pastorino JG, Hoek JB. Hexokinase II: the integration of energy metabolism and control of apoptosis. *Curr Med Chem* (2003) 10:1535–51. doi:10.2174/0929867033457269
98. Danial NN, Gramm CF, Scorrano L, Zhang CY, Krauss S, Ranger AM, et al. BAD and glucokinase reside in a mitochondrial complex that integrates glycolysis and apoptosis. *Nature* (2003) 424:952–6. doi:10.1038/nature01825
99. Verhagen JN, Van der Heijden MC, Rijksen G, Der Kindersen PJ, Van Unnik JA, Staal GE. Determination and characterization of hexokinase in thyroid cancer and benign neoplasms. *Cancer* (1985) 55:1519–24. doi:10.1002/1097-0142(19850401)55:7<1519::AID-CNCR2820550718>3.0.CO;2-K
100. Hooft L, van der Veldt AAM, van Diest PJ, Hoekstra OS, Berkhof J, Teule GJJ, et al. (18F) fluorodeoxyglucose uptake in recurrent thyroid cancer is related to hexokinase I expression in the primary tumor. *J Clin Endocrinol Metab* (2005) 90:328–34. doi:10.1210/jc.2004-0779
101. Hooft L, van der Veldt AAM, Hoekstra OS, Boers M, Molthoff CFM, van Diest PJ. Hexokinase III, cyclin A and galectin-3 are overexpressed in malignant follicular thyroid nodules. *Clin Endocrinol* (2008) 68:252–7. doi:10.1111/j.1365-2265.2007.03031.x
102. Kim JE, Ahn BC, Hwang MH, Jeon YH, Jeong SY, Lee SW, et al. Combined RNA interference of hexokinase II and 131I-sodium iodide symporter gene therapy for anaplastic thyroid. *J Nucl Med* (2011) 52:1756–63. doi:10.2967/jnumed.111.090266
103. Rijksen G, Oskam R, Molthoff CF, Lee On SJ, Streefkek M, Staal GE. Hexokinase isoenzymes from anaplastic and differentiated medullary thyroid carcinoma in the rat. *Eur J Cancer Clin Oncol* (1984) 20:967–73. doi:10.1016/0277-5379(84)90172-X
104. Dong G, Mao Q, Xia W, Xu Y, Wang J, Xu L, et al. PKM2 and cancer: the function of PKM2 beyond glycolysis. *Oncol Lett* (2016) 11:1980–6. doi:10.3892/ol.2016.4168
105. Dayton TL, Jacks T, Vander Heiden MG. PKM2, cancer metabolism, and the road ahead. *EMBO Rep* (2016) 17:1721–30. doi:10.15252/embr.201643300
106. Osterman J, Fritz PJ. . Pyruvate kinase isoenzymes: a comparative study in tissues of various mammalian species. *Comp Biochem Physiol B* (1973) 44(4):1077–85. doi:10.1016/0305-0491(73)90261-7
107. Kamel R, Schwarzfischer F. Pyruvate kinase isoenzyme patterns of human neoplastic, fetal and adult tissues. *Humangenetik* (1975) 28:65–9.
108. Mazurek S, Grimm H, Boschek CB, Vaupel P, Eigenbrodt E. Pyruvate kinase type M2: a crossroad in the tumor metabolome. *Br J Nutr* (2002) 87:S23–9. doi:10.1079/BJN2001454
109. Mazurek S. Pyruvate kinase type 2: a key regulator within the tumor metabolome and a tool for metabolic profiling of tumors. *Ernst Schering Found Symp Proc* (2007) 4:99–124.
110. Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wei R, et al. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumor growth. *Nature* (2008) 452:230–4. doi:10.1038/nature06734
111. Christofk HR, Vander-Heiden MG, Wu N, Asara JM, Cantley LC. Pyruvate kinase M2 is a phosphotyrosine-binding protein. *Nature* (2008) 452:181–6. doi:10.1038/nature06667
112. Mukherjee J, Phillips JJ, Zheng S, Wiencke J, Ronon SM, Pieper RO. Pyruvate kinase M2 expression, but not pyruvate kinase activity, is up-regulated in a grade-specific manner in human glioma. *PLoS One* (2013) 8:e57610. doi:10.1371/journal.pone.0057610
113. Feng C, Gao Y, Wang C, Yu X, Zhang W, Guan H, et al. Aberrant overexpression of pyruvate kinase M2 is associated with aggressive tumor features and the BRAF mutation in papillary thyroid cancer. *J Clin Endocrinol Metab* (2013) 98:E1524–33. doi:10.1210/jc.2012-4258
114. Bikas A, Jensen K, Patel A, Costello J Jr, McDaniel D, Klubo-Gwiezdzinska J, et al. Glucose-deprivation increase thyroid cancer cells sensitivity to metformin. *Endocr Relat Cancer* (2015) 22:919–32. doi:10.1530/ERC-15-0402
115. Yang W, Zheng Y, Xia Y, Ji H, Chen X, Guo F, et al. ERK1/2-dependent phosphorylation and nuclear translocation of PKM2 promotes the Warburg effect. *Nat Cell Biol* (2012) 14(12):1295–304. doi:10.1038/ncb2629
116. Wang HJ, Hsieh YJ, Cheng WC, Lin CP, Lin YS, Yang SF, et al. JMJD5 regulates PKM2 nuclear translocation and reprograms HIF-1alpha-mediated glucose metabolism. *Proc Natl Acad Sci U S A* (2014) 111:279–84. doi:10.1073/pnas.1311249111
117. Lee J, Kim HK, Han YM, Kim J. Pyruvate kinase isozyme type M2 (PKM2) interacts and cooperates with Oct-4 in regulating transcription. *Int J Biochem Cell Biol* (2008) 40:1043–54. doi:10.1016/j.biocel.2007.11.009
118. Demaria M, Poli V. PKM2, STAT3 and HIF-1alpha: the Warburg's vicious circle. *JAKSTAT* (2012) 1:194–6.
119. Yang P, Li Z, Fu R, Wu H, Li Z. Pyruvate kinase M2 facilitates colon cancer cell migration via the modulation of STAT3 signalling. *Cell Signal* (2014) 26:1853–62. doi:10.1016/j.cellsig.2014.03.020
120. Koukourakis MI, Giatromanolaki A, Sivridis E. Lactate dehydrogenase isoenzymes 1 and 5: differential expression by neoplastic and stromal cells in non-small cell lung cancer and other epithelial malignant tumors. *Tumour Biol* (2003) 24:199–202. doi:10.1159/000074430
121. 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

122. 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 U S A* (2010) 107:2037–42. doi:10.1073/pnas.0914433107
123. Mirebeau-Prunier D, Le Pennec S, Jacques C, Fontaine JF, Gueguen N, Boutet-Bouzamondo N, et al. Estrogen-related receptor alpha modulates lactate dehydrogenase activity in thyroid tumors. *PLoS One* (2013) 8:358683. doi:10.1371/journal.pone.0058683
124. Kachel P, Trojanowicz B, Sekulla C, Prenzel H, Dralle H, Hoang-Vu C. Phosphorylation of pyruvate kinase M2 and lactate dehydrogenase A by fibroblast growth factor receptor 1 in benign and malignant thyroid tissue. *BMC Cancer* (2015) 15:140. doi:10.1186/s12885-015-1135-y
125. Curry JM, Tassone P, Cotzia P, Sprandio J, Luginbuhl A, Cognetti DM, et al. Multicompartment metabolism in papillary thyroid cancer. *Laryngoscope* (2016) 10:2410–8. doi:10.1002/lary.25799
126. Gill KS, Tassone P, Hamilton J, Hjelm N, Luginbuhl A, Cognetti D, et al. Thyroid cancer metabolism: a review. *J Thyroid Disord Ther* (2016) 5:1–15. doi:10.4172/2167-7948.1000200
127. Yi JW, Park JY, Sung JY, Kwak SH, Yu J, Chang JH, et al. Genomic evidence of reactive oxygen species elevation in papillary thyroid carcinoma with Hashimoto thyroiditis. *Endocr J* (2015) 62:857–77. doi:10.1507/endocrj.EJ15-0234
128. Weyemi U, Caillou B, Talbot M, Ameziane-El-Hassani R, Lacroix L, Lagent-Chevallier O, et al. Intracellular expression of reactive oxygen species-generating NADPH oxidase NOX4 in normal and cancer thyroid tissues. *Endocr Relat Cancer* (2010) 17:27–37. doi:10.1677/ERC-09-0175
129. Weyemi U, Lagente-Chevallier O, Boufraqech M, Prenois F, Courtin F, Caillou B, et al. ROS-generating NADPH oxidase NOX4 is a critical mediator in oncogenic H-Ras-induced DNA damage and subsequent senescence. *Oncogene* (2012) 31:1117–29. doi:10.1038/onc.2011.327
130. Azouzi N, Cailloux J, Cazarin JM, Knauf JA, Cracchiolo J, AlGhuzlan A, et al. NADPH oxidase NOX4 is a critical mediator of $BRAF^{V600E}$-induced downregulation of the sodium/iodide symporter in papillary thyroid carcinomas. *Antioxid Redox Signal* (2017) 20:864–77. doi:10.1089/ars.2015.6616
131. Stankov K, Biondi A, D'Aurelio M, Gasparre G, Falasca A, Romero G, et al. Mitochondrial activities of a cell line derived from thyroid Hurthle cell tumors. *Thyroid* (2006) 16:325–31. doi:10.1089/thy.2006.16.325
132. Maximo V, Sobrinho-Simoes M. Mitochondrial DNA "common" deletion in Hurthle cell lesions of the thyroid. *J Pathol* (2000) 192:561–2. doi:10.1002/1096-9896(200012)192:4<561::AID-PATH790>3.0.CO;2-3
133. Bonora E, Porcelli AM, Gasparre G, Biondi A, Ghelli A, Carelli V, et al. Defective oxidative phosphorylation in thyroid oncocytic carcinoma is associated with pathogenic mitochondrial DNA mutations affecting complexes I and III. *Cancer Res* (2006) 66:6087–96. doi:10.1158/0008-5472.CAN-06-0171
134. Gasparre G, Porcelli AM, Bonora E, Pennisi LF, Toller M, Iommarini L, et al. Disruptive mitochondrial DNA mutations in complex I subunits are markers of oncocytic phenotype in thyroid tumors. *Proc Natl Acad Sci U S A* (2007) 104:9001–6. doi:10.1073/pnas.0703056104
135. Calabrese C, Iommarini L, Kurelac I, Calvaruso MA, Capristo M, Lollini PL, et al. Respiratory complex I is essential to induce a Warburg profile in mitochondria-defective tumor cells. *Cancer Metab* (2013) 1:11. doi:10.1186/2049-3002-1-11
136. Paik JY, Jung k-H, Lee JH, Parl JW, Lee KH. Reactive oxygen species-driven HIF1a triggers accelerated glycolysis in endothelial cells exposed to low oxygen tension. *Nucl Med Biol* (2015) 45:8–14. doi:10.1016/j.nucmedbio.2016.10.006
137. Wu CA, Chao Y, Shiah SG, Lin WW. Nutrient deprivation induces the Warburg effect through ROS/AMPK-dependent activation of pyruvate dehydrogenase kinase. *Biochim Biophys Acta* (2013) 1833:1147–56. doi:10.1016/j.bbamcr.2013.01.025
138. Hardie DG, Lin SC. AMP-activated protein kinase – not just an energy sensor. *F1000Res* (2017) 22:6–1724. doi:10.12688/f1000research.11960.1
139. Cazarin JM, Coelho RG, Hecht F, Andrade BM, Carvalho DP. 5'-AMP-activated protein kinase regulates papillary (TPC1 and BCPAP) thyroid cancer cell survival, migration, invasion and epithelial-to-mesenchymal transition. *Thyroid* (2016) 26:933–42. doi:10.1089/thy.2015.0440
140. Luo Z, Zang M, Guo W. AMPK as a metabolic tumor suppressor: control of metabolism and cell growth. *Future Oncol* (2010) 6:457–70. doi:10.2217/fon.09.174
141. Faubert B, Boily G, Izreig S, Griss T, Samborska B, Dong Z, et al. AMPK is a negative regulator of the Warburg effect and suppresses tumor growth in vivo. *Cell Metab* (2013) 17:113–24. doi:10.1016/j.cmet.2012.12.001
142. Shackelford DB, Vasquez DS, Corbeil J, Wu S, Leblanc M, Wu CL, et al. mTOR and HIF-1alpha-mediated tumor metabolism in an LKB1 mouse model of Peutz-Jeghers syndrome. *Proc Natl Acad Sci U S A* (2009) 106:11137–42. doi:10.1073/pnas.0900465106
143. Vidal AP, Andrade BM, Vaisman F, Cazarin J, Pinto LF, Breitenbach MM, et al. AMP-activated protein kinase signaling is upregulated in papillary thyroid cancer. *Eur J Endocrinol* (2013) 169:521–8. doi:10.1530/EJE-13-0284
144. Valerio L, Pieruzzi L, Giani C, Agate L, Bottici V, Lorusso L, et al. Targeted therapy in thyroid cancer: state of the art. *Clin Oncol* (2017) 29(5):316–24. doi:10.1016/j.clon.2017.02.009
145. Schmidbauer B, Menhart K, Hellwig D, Grosse J. Differentiated thyroid cancer-treatment: state of the art. *Int J Mol Sci* (2017) 18(6):1292. doi:10.3390/ijms18061292
146. Martini M, De Santis MC, Braccini L, Gulluni F, Hirsch E. PI3K/AKT signaling pathway and cancer: an updated review. *Ann Med* (2014) 46(6):372–83. doi:10.3109/07853890.2014.912836
147. Bible KC, Ryder M. Evolving molecularly targeted therapies for advanced-stage thyroid cancers. *Nat Rev Clin Oncol* (2016) 3:403–16. doi:10.1038/nrclinonc.2016.19
148. De Falco V, Carlomagno F, Li Hy, Santoro M. The molecular basis for RET tyrosine-kinase inhibitors in thyroid cancer. *Best Pract Res Clin Endocrinol Metab* (2017) 31:307–18. doi:10.1016/j.beem.2017.04.013
149. de Souza EC, Padrón AS, Braga WM, de Andrade BM, Vaisman M, Nasciutti LE, et al. mTOR downregulates iodide uptake in thyrocytes. *J Endocrinol* (2010) 206(1):113–20. doi:10.1677/JOE-09-0436
150. Lim SM, Chang H, Yoon MJ, Hong YK, Kim H, Chung WY, et al. A multicenter phase II trial of everolimus in locally advanced or metastatic thyroid cancer of all histologic subtypes. *Ann Oncol* (2013) 24:3089–94. doi:10.1093/annonc/mdt379
151. Lorch JH, Busaidy N, Ruan DT, Janne PA, Limaye SA, Wirth LJ, et al. A phase II study of everolimus in patients with aggressive RAI refractory (RAIR) thyroid cancer (TC). *J Clin Oncol* (2013) 31(Suppl):Abstract 6023.
152. Wagle N, Grabiner BC, Van Allen EM, Amin-Mansour A, TaylorWeiner A, Rosenberg M, et al. Response and acquired resistance to everolimus in anaplastic thyroid cancer. *N Engl J Med* (2014) 371(15):1426–33. doi:10.1056/NEJMoa1403352
153. Schneider TC, de Wit D, Links TP, van Erp NP, van der Hoeven JJM, Gelderblom H, et al. Everolimus in patients with advanced follicular-derived thyroid cancer: results of a phase II clinical trial. *J Clin Endocrinol Metab* (2017) 102(2):698–707. doi:10.1210/jc.2016-2525
154. Klubo-Gwiezdzinska J, Costello J, Patel A, Bauer A, Jensen K, Mete M, et al. Treatment with metformin is associated with higher remission rate in diabetic patients with thyroid cancer. *J Clin Endocrinol Metab* (2013) 98:3269–79. doi:10.1210/jc.2012-3799
155. Yadav S, Pandey SK, Kumar A, Kujur PK, Singh RP, Singh SM. Antitumor and chemosensitizing action of 3-bromopyruvate: implication of deregulated metabolism. *Chem Biol Interact* (2017) 270:73–89. doi:10.1016/j.cbi.2017.04.015
156. 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
157. Sun RC, Fadia M, Dahlstrom JE, Parish CR, Board PG, Blackburn AC. Reversal of the glycolytic phenotype by dichloroacetate inhibits metastatic breast cancer cell growth in vitro and in vivo. *Breast Cancer Res Treat* (2009) 120:253–60. doi:10.1007/s10549-009-0435-9
158. Samid D, Ram Z, Hudgins WR, Shack S, Liu L, Walbridge S, et al. Selective activity of phenylacetate against malignant gliomas: resemblance to fetal brain damage in phenylketonuria. *Cancer Res* (1994) 54:891–5.
159. Nebeling LC, Miraldi F, Shurin SB, Lerner E. Effects of a ketogenic diet on tumor metabolism and nutritional status in pediatric oncology patients: two

case reports. *J Am Coll Nutr* (1995) 14:202–8. doi:10.1080/07315724.1995.10718495

160. Otto C, Kaemmer U, Illert B, Muehling B, Pfetzer N, Wittig R, et al. Growth of human gastric cancer cells in nude mice is delayed by a ketogenic diet supplemented with omega-3 fatty acids and medium-chain triglycerides. *BMC Cancer* (2008) 8:122. doi:10.1186/1471-2407-8-122
161. Seyfried TN, Kiebish M, Mukherjee P, Marsh J. Targeting energy metabolism in brain cancer with calorically restricted ketogenic diets. *Epilepsia* (2008) 49(Suppl 8):114–6. doi:10.1111/j.1528-1167.2008.01853.x
162. Zhou W, Mukherjee P, Kiebish MA, Markis WT, Mantis JG, Seyfried TN. The calorically restricted ketogenic diet, an effective alternative therapy for malignant brain cancer. *Nutr Metab (Lond.)* (2007) 4:5. doi:10.1186/1743-7075-4-5

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

MINI REVIEW
published: 23 March 2018
doi: 10.3389/fonc.2018.00081

Metabolic Symbiosis and Immunomodulation: How Tumor Cell-Derived Lactate May Disturb Innate and Adaptive Immune Responses

Alexandre Morrot[1,2†], Leonardo Marques da Fonseca[3†], Eduardo J. Salustiano[3†], Luciana Boffoni Gentile[3], Luciana Conde[4], Alessandra Almeida Filardy[4], Tatiany Nunes Franklim[3], Kelli Monteiro da Costa[3], Celio Geraldo Freire-de-Lima[3] and Leonardo Freire-de-Lima[3*]*

[1] Faculdade de Medicina, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [2] Laboratório de Imunoparasitologia, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brazil, [3] Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [4] Instituto de Microbiologia, Departamento de Imunologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

OPEN ACCESS

Edited by:
Stephen G. Maher,
Trinity College, Dublin, Ireland

Reviewed by:
Paolo E. Porporato,
Università degli Studi di Torino, Italy
Prashant Trikha,
Nationwide Children's Hospital, United States

***Correspondence:**
Alexandre Morrot
alexandre.morrot@ioc.fiocruz.br,
morrot@micro.ufrj.br;
Leonardo Freire-de-Lima
leolima@biof.ufrj.br

[†]These authors have contributed equally to this work.

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: 15 December 2017
Accepted: 09 March 2018
Published: 23 March 2018

Citation:
Morrot A, Fonseca LM, Salustiano EJ, Gentile LB, Conde L, Filardy AA, Franklim TN, da Costa KM, Freire-de-Lima CG and Freire-de-Lima L (2018) Metabolic Symbiosis and Immunomodulation: How Tumor Cell-Derived Lactate May Disturb Innate and Adaptive Immune Responses. Front. Oncol. 8:81. doi: 10.3389/fonc.2018.00081

The tumor microenvironment (TME) is composed by cellular and non-cellular components. Examples include the following: (i) bone marrow-derived inflammatory cells, (ii) fibroblasts, (iii) blood vessels, (iv) immune cells, and (v) extracellular matrix components. In most cases, this combination of components may result in an inhospitable environment, in which a significant retrenchment in nutrients and oxygen considerably disturbs cell metabolism. Cancer cells are characterized by an enhanced uptake and utilization of glucose, a phenomenon described by Otto Warburg over 90 years ago. One of the main products of this reprogrammed cell metabolism is lactate. "Lactagenic" or lactate-producing cancer cells are characterized by their immunomodulatory properties, since lactate, the end product of the aerobic glycolysis, besides acting as an inducer of cellular signaling phenomena to influence cellular fate, might also play a role as an immunosuppressive metabolite. Over the last 10 years, it has been well accepted that in the TME, the lactate secreted by transformed cells is able to compromise the function and/or assembly of an effective immune response against tumors. Herein, we will discuss recent advances regarding the deleterious effect of high concentrations of lactate on the tumor-infiltrating immune cells, which might characterize an innovative way of understanding the tumor-immune privilege.

Keywords: cancer, metabolism, lactate, immune evasion, cytokines

INTRODUCTION

Cancer as a Metabolic Disease

Historically, cancer has been considered a product of multiple pathologies. In second century AD, the philosopher and physician Claudius Galenus was the first to employ the Greek word *onco* (swelling) for all types of tumors, leaving Hippocrates' term *karkinos* exclusively for malignant tumors. During his time, Galenus asserted that tumors were the result of "black bile" accumulation. It was only during the nineteenth century that this theory was revisited and cancer begun to be perceived as the result of acquired metabolic abnormalities (1). Nowadays, it is well accepted that cancer development and progression is modulated by the disordered growth of cells featuring self-sufficiency of

growth signals, evasion of apoptosis, angiogenesis, invasiveness, and metastasis (2, 3). When cells break free from the restraints on cell division, they start assuming inappropriate proliferation rates and distinct metabolic profiles, becoming abnormal in their own way (4, 5). Cells originating from solid tumors may gain the ability to dissolve the extracellular matrix (ECM), invade nearby tissues, reaching the bloodstream or lymphatic vessels, or remain within the boundaries of the original tissue, being characterized as malignant or benign tumors, respectively. Several genomic changes lead normal cells through malignant transformation. These changes can be anything from point mutations and deletions to chromosome rearrangements, as long as they result in irreversible changes affecting cell cycle (6). Any individual suffers several mutations in various cell types during its lifetime, due to diverse exogenous or endogenous factors. Most of these mutations are promptly corrected or lead to apoptosis. The accumulation of uncorrected mutations leads to the development of benign or malignant tumors (3). Loss of tumor suppressor factors, germline mutations, and overexpression of oncogenes are some of the changes that may collaborate for the occurrence of somatic mutations that escape DNA repair processes (7, 8).

The tumor microenvironment (TME) comprises both cellular and non-cellular components (9–11). The acellular components include the ECM, as well as soluble signals secreted by transformed and tumor-associated cancer cells. Several cell types associate with tumors, including fibroblasts, endothelial cells, and immune cells. Together, all components form an organ-like structure capable of interacting with the organism as a whole (12–14). To maintain tumor growth, several adaptations may be driven by neoplastic cells. A well-known mechanism is the formation of immature and abnormal vessels, in a phenomenon named neoangiogenesis (15, 16). In this context, both the platelet-derived growth factor and the vascular endothelial growth factor (VEGF) are recognized as the main proangiogenic signals upregulated by cancer cells during tumor growth (15, 17, 18).

The incredible proliferation rate of tumor cells can make a single mutated cell generate a tumor of ≈1 cm in diameter containing over 10^9 cells. Such a high proliferation ratio demands effective metabolic pathways, capable of meeting the steep energy requirements while supplying the biosynthetic precursors that maintain cell anabolism and redox balance in the neoplastic cell (19). Reprogramming of cellular metabolism has been observed in several types of cancer and is considered a hallmark of this disease (3, 20). The elucidation of these atypical metabolic activities is a lively field in the study of cancer biology, showing great potential for the development of novel therapeutic approaches. Several studies have shown that inhibition of some metabolic pathways of cancer cells is able to prevent tumor growth and metastasis (21, 22).

METABOLIC SYMBIOSIS: A PROPOSED CONCEPT OF ENERGY MANAGEMENT BETWEEN CANCER CELLS IN THE TME

The impact of the acidosis induced by lactate and protons in the TME is a hot topic in cancer research (23–25). It is a well-established fact that a high enough lactate production can overcome the cellular proton buffering capability, resulting in a decrease of the cellular pH. Such condition, besides influencing the dynamics of waste and reuse of energy by cancer cells, modulates the function of distinct tumor-associated cell types as well (26–29). Several papers published over the last 10 years, demonstrated that when cancer cells experience low tension of oxygen, the hypoxia-inducible factor-1α (HIF-1α) transcription factor is stabilized, increasing glucose (Glc) uptake and secretion of substantial levels of lactate and protons out of cytoplasm by the monocarboxylated transporter 4 (MCT4) (**Figure 1B**), promoting a biochemical event termed lactic acidosis. By contrast, when cancer cells are adjacent to blood vessels and oxygen availability is sufficient, the transformed cells preferably use lactate as energy source (29–33). For this reason, lactate should not be considered a waste metabolite. In fact, it is reused by different cell subpopulations in a tumor (28, 29). Recently, Lee and colleagues (34) showed that an oxygen-regulated protein (NDGR3), which is usually degraded under normoxia *via* the prolyl hydroxylase 2/Von Hippel–Lindau (PDH2/VHL) system, becomes protected from degradation when bound to lactate. The authors demonstrated that when stable, NDRG3 is able to bind the proto-oncogene c-Raf, a serine/threonine-protein kinase, and induce activation of the Raf–ERK pathway, thus promoting cell growth and angiogenesis. However, inhibition of lactate production compromises NDRG3-mediated hypoxia responses (34).

This metabolic model of lactate reuse in the TME has been described as a metabolic symbiosis, where lactate works as a medium to convey energy from highly glycolytic/hypoxic transformed cells to more oxidative cancer cells (35, 36). In the TME, the uptake of lactate and protons by oxygenated cancer cells occurs in a dynamic way through the monocarboxylated transporter 1 (MCT1) (**Figure 1B**), which has been previously identified as gatekeeper of this metabolic symbiosis (29). In the same study, the authors demonstrated that cells with inhibited or silenced MCT1 became more sensitive to cell death, which may support lactate management within the TME as a valid therapeutic strategy. Therefore, it would plausible to speculate that the high-lactate concentration at the intercellular space might affect the functionality of diverse tumor-associated cells, including those of the innate and adaptive immune system (see sections below).

THE EFFECT OF LACTATE IN THE TME

In normal cell metabolism, the consumed Glc is catabolized into pyruvate, which is then transported to the mitochondria to fuel the tricarboxylic acid cycle in a series of redox reactions. The resulting free electrons go through the electron transport chain (ETC), beginning the oxidative phosphorylation (OXPHOS) and ultimately leading to a high production of ATP (37) (**Figure 1A**). In the early 1920s, Otto Warburg observed that tumor cells remain in glycolytic state, constitutively absorbing Glc and converting pyruvate to lactate, in the presence of oxygen (**Figure 1A**). Lactate production is 40-fold greater in tumor cells, so the transport of lactate to the ECM by MCTs (38–41) is essential for the glycolytic switch. This metabolic behavior is named "Warburg Effect," or aerobic glycolysis, one of the main characteristics studied in

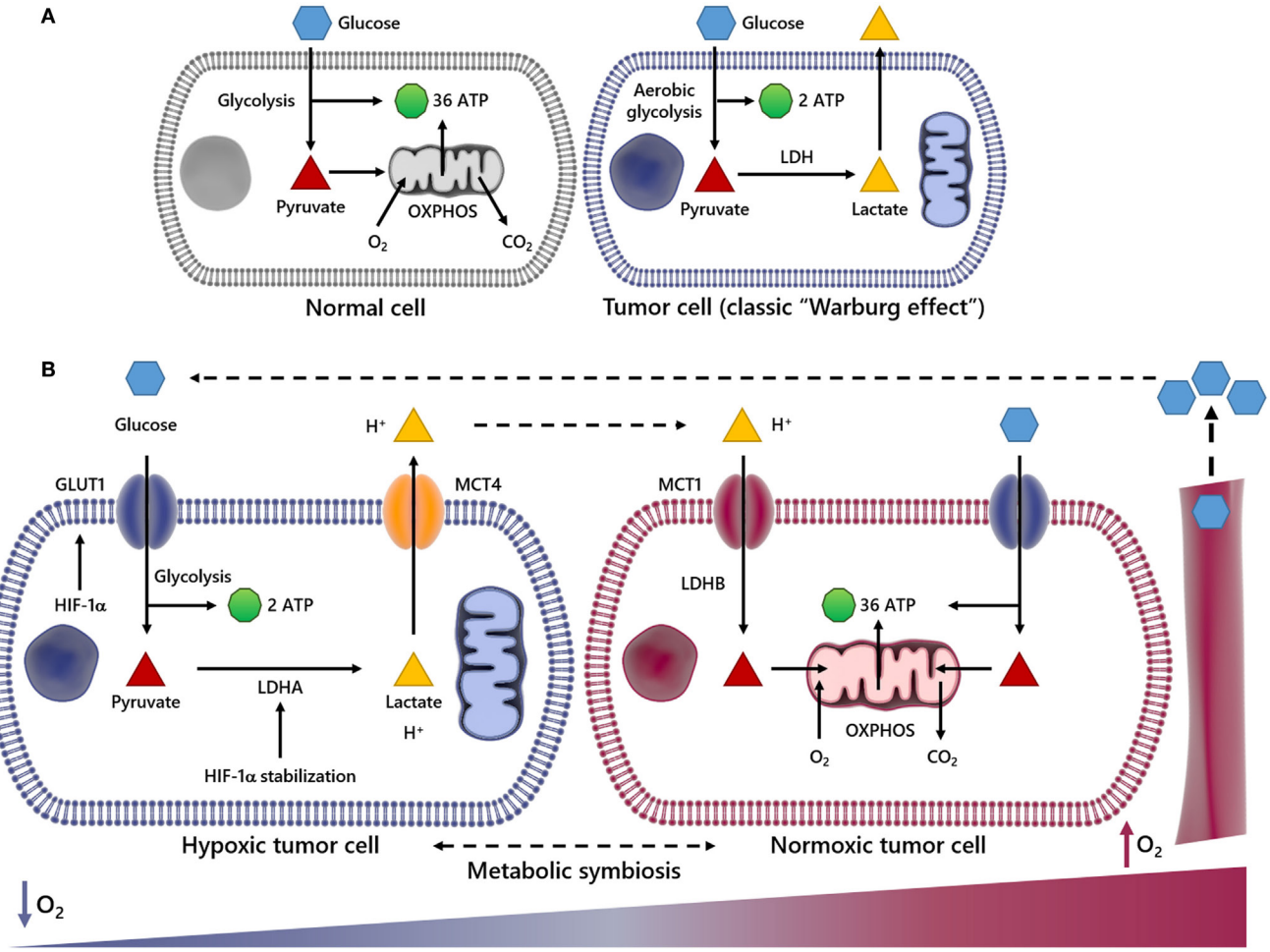


FIGURE 1 | Scheme summarizing metabolic differences between normal and cancer cells and metabolic symbiosis. In normal cells, glucose (Glc) is initially metabolized to pyruvate and further to carbon dioxide (CO_2) through tricarboxylic acid cycle and oxidative phosphorylation (OXPHOS) processes in the mitochondria, generating 36 ATP molecules per Glc molecule consumed **(A)**. In this process, O_2 is indispensable, since it is used as the final electron acceptor **(A)**. However, in cancer cells undergoing aerobic glycolysis (Warburg effect), Glc is broken down into pyruvate and finally converted into lactate, deviating Glc metabolites from energy production to anabolic process. This event generates two ATP molecules per Glc molecule. The panel **(B)** illustrates an event named metabolic symbiosis. It has been well documented that when cancer cells are near or distant of blood vessels, they are well or poorly oxygenated, respectively. It is also known that when cancer cells are subject to low oxygen tension (↓O_2) hypoxia-inducible factor-1α (HIF-1α) is stabilized, increasing the transcriptional activation of genes encoding glucose transporters (GLUTs), lactate dehydrogenase A (LDHA), as well as the uptake of Glc and secretion of lactate and protons out of cytoplasm through the monocarboxylated transporter 4 (MCT4). However, when transformed cells are close to blood vessels and the availability of O_2 is enough, lactate is taken by monocarboxylated transporter 1 (MCT1) and utilized as energy source after conversion into pyruvate by lactate dehydrogenase B (LDHB). In this way, lactate may not be pointed out as a waste metabolite, since it is reused by different cell subpopulations in a tumor.

cancer metabolism (19) (**Figure 1B**). Glycolysis produces ATP faster yet less efficiently than OXPHOS, forcing the tumor cell to consume much more Glc than a normal cell to produce enough energy to maintain its high proliferative status. Therefore, the glycolysis is an advantage for the tumor cell only when Glc supply is not limited. The importance of Glc for the metabolism of cancer cells is so evident that low-carbohydrate diet as a therapeutic approach for cancer patients, aiming to starve tumor cells, was described to limit the growth of incurable cancers in a pilot trial with 10 patients (42). In that regard, the uptake of a radioactive Glc analog, [^{18}F]fluorodeoxyGlc, is used as a diagnostic tool for the positron emission tomography (FDG-PET) imaging of highly proliferative tumor regions (43, 44). Currently we know that tumor cells primarily fulfill their energetic needs by the oxidation of Glc, glutamine and other nutrients coupled to the ETC, using oxygen as the final acceptor of electrons (45, 46). In cancer cells, the anaerobic respiration is optional, and there is no mitochondrial defect (47); in fact, tumor cells still retain OXPHOS and mitochondrial activity (39). The reduced mitochondrial activity is a direct result either of oxygen deprivation or activation of HIF-1α (48, 49), which is able to promote the transcriptional activation of genes encoding glucose transporters (GLUTs), as well as glycolytic enzymes, such as lactate dehydrogenase A (LDHA) (50). When the supply of oxygen is low, LDHA is essential to sustain glycolysis and the production of ATP by regenerating NAD^+ form NADH. In this way, HIF-1α regulates the production

of lactate, the end by-product of this reaction, which consumes two ATP but generates four ATP, generating two net ATP per Glc molecule as seen in (**Figures 1A,B**) (50). Upstream of HIF-1α and the previously discussed Raf–ERK, the Ras oncogenic pathway seems to be critical for the metabolic reprogramming observed during carcinogenesis. Overexpression of oncogenic H-Ras^{V12} was able to drive immortalized fibroblasts to consume more Glc and to release more lactate (51). Conversely, Ras inhibition in a model of glioblastoma (GBM) effectively shuts down Glc uptake and glycolysis itself, leading to the downregulation of 12 genes from the glycolytic pathway and increased extracellular pH due to reduced lactate efflux (52). The role of PDH2 is prominent in this, since oncogenic H-Ras signaling, as well as hypoxia, triggers oxidative stress, PDH2 dimerization, and inactivation, leading to HIF-1α stabilization and ultimately the OXPHOS to glycolysis shift (53).

An immediate consequence of the Warburg effect is the accumulation of lactate and protons in the TME (23, 54). It has been shown that in patients diagnosed with different stages of cervical cancer, primary tumors exhibiting high-lactate levels often lead to the manifestation of metastatic foci (55). The same group, using human larynx squamous carcinoma cells, showed that increased lactate concentration can augment cell motility and migration, corroborating the data observed in patients (56). In addition, the use of siRNA to inhibit the expression of LDHA, whose expression can be induced by lactate itself, is able to inhibit the migration of glioma cells as well as downregulate active matrix metalloproteinase-2 (57). Increased lactic acid is also responsible for the overexpression of factors related to tumor progression, such as CD44, hyaluronic acid and transforming growth factor-beta (TGF-β) (58) (**Figure 2**), a pro-carcinogenic cytokine able to activate the epithelial–mesenchymal transition process, an event that permits dissemination of tumor cells from the primary site into the surrounding stroma, setting the stage for metastatic spread (59–61). In addition, due to its antioxidant properties, increased lactate concentrations may offer a degree or resistance against any therapy relying on the production of oxygen reactive species, such as radiotherapy (62). As it stands, further studies on the production of lactate by solid tumors represent an important step toward the understanding of tumor progression and malignancy, as well as for therapy development.

The TME is characterized by acidity and low oxygen tension (63, 64), events capable of modulating not only the growth and survival of tumor cells but also the recruitment of inflammatory cells that are reeducated in the microenvironment to favor tumor spread and metastasis. In this scenario, various inflammatory cells, such as T lymphocytes, dendritic cells (DCs), natural killer (NK) cells, and macrophages (Mϕ), acquire pro-carcinogenic

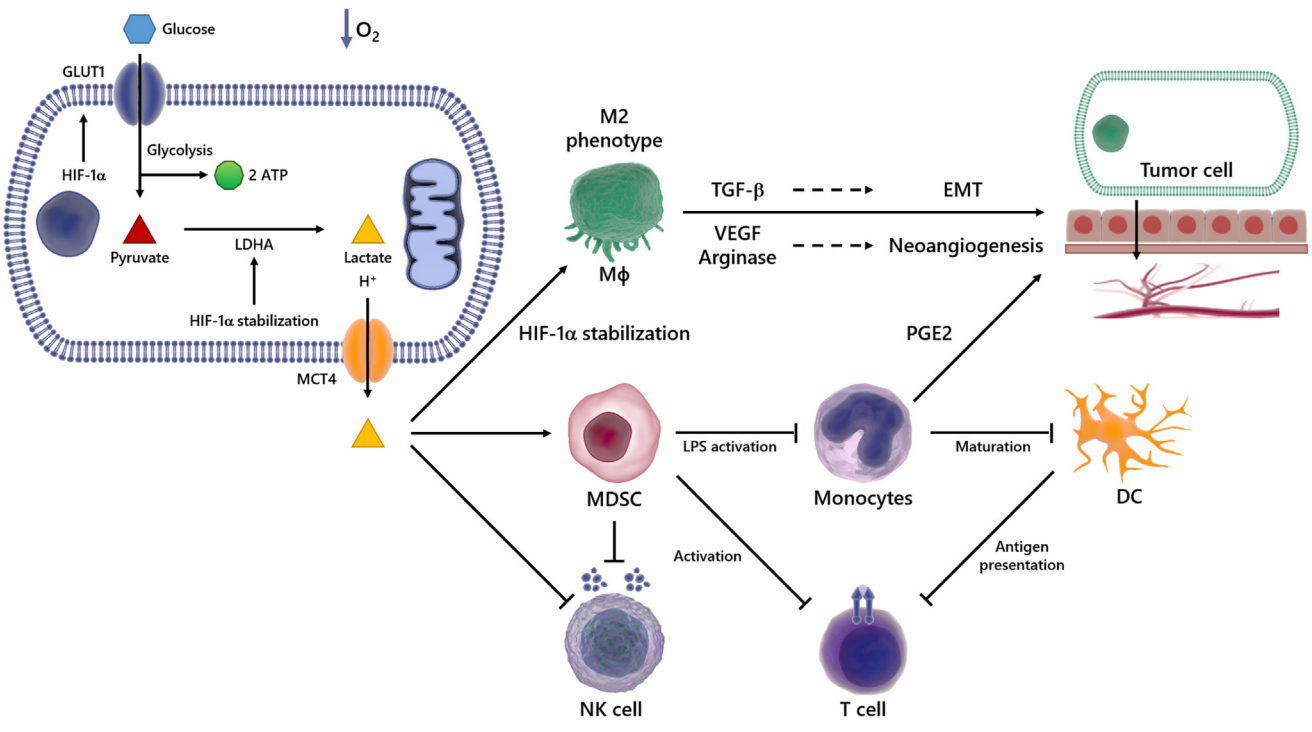


FIGURE 2 | Overview of immunosuppressive effects of lactate in the tumor microenvironment (TME). In a hypoxic environment, Glc enters the cell *via* glucose transporter (GLUT) 1 and is broken down into pyruvate and then in lactate, which is transported out of the cell *via* monocarboxylated transporter 4 (MCT4). The lactate produced by transformed cells culminates in an acidified TME. This phenomenon is able to suppress the anticancer immune responses, particularly through impaired T and natural killer (NK) cells activation, reduced antigen presentation, compromised dendritic cell (DC) differentiation and maturation. It also promotes the emergence of the M2 Mϕ, which secretes high levels of pro-carcinogenic cytokines, such as transforming growth factor-beta (TGF-β) and vascular endothelial growth factor (VEGF), involved in processes such as epithelial–mesenchymal transition (EMT) and angiogenesis, events implicated in metastasis and cancer progression.

properties (**Figure 2**) (65–69). Recruitment and accumulation of those cells in the TME is an essential phenomenon to sustain the tumor growth (70). The immune system's role in the first phases of tumor establishment is well described, being capable of detecting and destroying cancer cells, halting their growth and spread, in a phenomenon termed immunosurveillance (71, 72), which was initially proposed by Paul Ehrlich and later developed by Sir Frank Macfarlane Burnet and Lewis Thomas (13, 73). Defects in this event might favor tumor progression and, consequently, the acquisition of a malignant phenotype. Any cancer cell that manages to escape death triggered by immune response could still have their proliferation hindered by immune mechanisms, reaching an equilibrium. On the other hand, the immunogenicity is molded through selective pressure exerted by the immune system, in an event termed immunoediting (74–77). Consequently, novel tumor variants emerge, bearing more mutations, making them more likely to evade detection and elimination by immune effector cells like NK and $CD8^+$ T cells (74, 78). The immunoediting stage is the longest phase, and it is characterized by the dynamic coevolution of cancer and immune cells (78–80). Ultimately, cells reach an escape phase, where the accumulation of edited cells drives tumor growth and the manifestation of clinical symptoms (74). The presence of the immune system in the TME undoubtedly compromises tumor growth and, in fact, correlates with favorable prognosis in some cancer types, such as renal, ovarian, colorectal, and breast cancers (74, 81). The expression of molecules able to compromise cell-to-cell interaction (3, 82–84), as well as soluble factors such as VEGF (85), cytokines (86–88), prostaglandin E2 (PGE2) (89), soluble Fas and FasL (90), and soluble MICA (91), all contribute to the appearance of multifaceted local and regional immunosuppressive networks (92–94). For example, the occurrence of the IL-4, TGF-β, IL-13, and IL-10 cytokines in the TME induces the emergence of M2 instead of M1 Mϕ (86, 87). In addition, secretion of nitric oxide, IL-10, arginase-1, IL-6, and VEGF promotes cell death and avoids the antitumor function of immune cells (95–99).

As stated earlier, the TME is rich in lactate (38–41), an immunosuppressive soluble factor that promotes cancer development (54, 74, 100). Particularly, several studies have shown that tumor-derived lactate is capable of inhibiting the activation of immune cells such as monocytes, Mϕ and T lymphocytes (101–103). It has been demonstrated that high LDHA levels are deeply correlated with tumor size, as well as with the clinical stages of the disease (104, 105). Accordingly, the infiltration of immune cells in the TME correlates with high LDHA expression and/or activity (106). Besides lactate accumulation in the primary tumor site, its immunosuppressive properties can outspread to distant sites, thus stimulating invasion and metastasis in a paracrine fashion (107) (**Figure 2**).

Despite being mainly produced by skeletal muscle, various tissues generate lactate, and its elimination in healthy conditions is handled primarily through the liver and secondarily through the kidneys (108). The citric acid cycle is also a source of lactate, as pyruvate can be diverted to lactate and NAD^+ generation through LDH activity (109, 110). The high amounts of lactate in the extracellular microenvironment contribute to lowering the extracellular pH, which can be as low as 6.0–6.5 (111), producing acidosis and inducing angiogenesis and a reduction in efficacy of the immune system (101, 112, 113). Tumor-associated immune cells from myeloid and lymphoid origin can be modulated by hypoxic conditions as well as high levels of lactate, then favoring the acquisition of malignant phenotypes (23, 64, 114–116).

LACTATE AND MYELOID CELLS

Over the last 15 years, several studies demonstrated that tumor-derived lactate modulates the functionality of immune cells, contributing to the establishment of an immunosuppressive microenvironment, which favors the developing of tumors (106, 117–119). Lactate promotes the development of myeloid-derived suppressor cells (MDSCs), the most prominent bone marrow-derived cell population that exerts broadly immunosuppressive functions (106). In the TME, MDSCs potently suppress both innate and adaptive immunity by preventing the maturation of DCs, suppressing NK-cell cytotoxicity, inhibiting T-cell activation, and favoring the differentiation of regulatory T cells (**Figure 2**) (117).

In addition, lactate suppresses monocytes' LPS-induced activation by influencing their gene expression. Particularly, the expression of most LPS-induced genes was significantly delayed in the presence of lactate, including TNF, IL-23, CCL2, and CCL17. These effects are mediated by delayed LPS-induced phosphorylation of protein kinase B (AKT) and degradation of IkB, with reduced nuclear accumulation of NFκB (119). Furthermore, lactate stabilizes the transcription factor HIF-1α in monocytes, which ultimately promote the expression of PGE2 and the growth of human colon cancer HCT116 cells (120).

Another suppressive function of lactate is to impair the differentiation of monocytes into Mϕ or DCs in the TME (118, 121–123) and in non-tumor conditions (103, 124) (**Figure 2**). It was reported that lactate blocks LPS activation of bone marrow-derived Mϕ (BMDMs) (123), and also, in hypoxia or normoxia, lactate drives tumor-associated Mϕ polarization to the "tumor friendly" M2 profile (**Figure 2**) (125, 126). Mechanistically, lactate stabilizes HIF-1α, which leads to the transcription of a broader set of M2-associated genes, including VEGF, TGF-β and arginase-1, as well as Fizz1, Mgl1, and Mgl2 (**Figure 2**) (98, 125–128). The role of PDH2 as a regulator of the metabolic reprogramming in Mϕ was observed in both RAW264 cells and in primary BMDM, since transfection with shRNA targeting PDH2 or conditional PDH2 knocking out led to decreased ATP levels along and increased lactate release into the medium (129). M2 Mϕ and their products favor tumor growth and metastasis by suppressing antitumor immune responses, activating and enhancing angiogenesis. Particularly, VEGF triggers the development of neovascularization of the tumor. Similarly, arginase-1 plays an indirect role in angiogenesis through reorganization of the tumor ECM and contributes for the generation of essential metabolites during cell division, such as polyamines, supporting cancer cells growth (130–133). The importance of arginase-1 in tumor development was demonstrated by the use of arginase-1 KO mice, which presented tumors 50% smaller than tumors from wild type mice (125, 134). Finally, distinct studies have shown that when present in high levels, lactate inhibits antigen presentation and

IL-12 production by DCs (54, 135) (**Figure 2**) and enhances IL-10 production as well, generating an immunosuppressive profile in the TME (136, 137).

LACTATE AND LYMPHOID CELLS

The immunobiological effects of lactate on immune cells from lymphoid origin have been mainly investigated in NK and T cells. The cytotoxic effect mediated by both cell types is of fundamental importance in immunological surveillance against the emergence and spread of malignant disease. NK cells represent large granular lymphocytes that induce their antitumor responses through the ligation of particular cell-surface receptors (138), such as the natural killer group 2, member D receptor (NKG2D), which induces the release of cytotoxic granules that promote the lysis of cancer cells (139, 140).

An elegant study developed by Husain and colleagues (106) revealed that the low production of lactate in LDHA-depleted tumors was able to improve the cytolytic functions of NK cells. However, when NK cells where pretreated with lactate *in vitro*, its cytolytic property was compromised and/or abrogated. The molecular mechanism responsible for such effect was investigated, and the authors demonstrated that the decline of NK cytotoxic activity was promoted by the lower expression of granzyme and perforin in lactate-treated cells (106). Furthermore, it was described that lactate works as a potent inhibitor of histone deacetylases, suggesting that lactate might be able to regulate (at transcriptional level) several genes involved not only in cell metabolism but also in immune responses, such as NCR1/NKp46, an activating NK cell receptor (141, 142).

In 2014, Crane and coworkers demonstrated that GBM cells secrete LDH-5, an enzymatically active isoform of LDH (143), that besides catalyzing the conversion of pyruvate to lactate in an efficient way (144), is also capable to upregulate HIF-related pathways (145) and induce the expression of NKG2D ligands on healthy monocytes, thus subverting antitumor immune responses (143). In a previous study realized by the same group, it was demonstrated that TGF-β downregulates NKG2D expression in NK cells *in vitro* (146), supporting the idea that the elevated production/concentration of TGF-β in acidic TME is one of the main evasion mechanisms adopted by cancer cells (146).

It is well accepted that a robust presence of T cells in the TME is associated with good clinical outcome in distinct types of cancers (147–149). It is important to notice that new progresses in cancer immunotherapy are allied to the use of monoclonal antibodies directed against T cell-immune checkpoints. Examples include CTLA4 (149–151) and PD-1 (152, 153). These outstanding findings undoubtedly confirm the necessity of an effective T cell activation to control tumor growth and spread (147). As with other types of immune cells, cancer cells limit T cell immunity by distinct ways. In this regard, the acidification of the TME is a clear example, and several papers have demonstrated that lactate plays a pivotal role in this process (54, 154–156). As with transformed cells, activated T cells may generate energy through aerobic glycolysis (157–159). The upregulation of glycolytic enzymes intensifies the uptake of Glc and glycolytic rate, favoring the secretion of lactate into the microenvironment (157). It is possible to imagine that when together in the TME, both cancer cells and activated T lymphocytes significantly increase the production/secretion of lactate. As it stands, it has been demonstrated that the very acidic microenvironment suppresses the proliferation and cytokine production by activated T cells (101). A recent study developed by Brand and colleagues revealed that pathophysiological concentrations of lactic acid repeal the upregulation of the nuclear factor of activated T cells in both NK and T cells, which significantly reduced the production of IFN-γ (160). These results corroborate previous findings that lactic acid is able to downmodulate the function of cells from lymphoid origin, then contributing to tumor escape from immune attack.

CONCLUDING REMARKS AND PERSPECTIVES

This review presents a snapshot of metabolic changes in cancer cells, describing how, even in aerobic conditions, transformed cells opt for glycolysis instead of OXPHOS to sustain their energy demand, high proliferation and biosynthesis rates, a process named "Warburg effect." This metabolic reprogramming culminates in a high-lactate and protons output, which is also exported to the extracellular environment by MCT4, generating acidosis, neoangiogenesis, and immunosuppression, directly modulating the TME. Although several genetic, biochemical, and pathophysiologic mechanisms have been identified as causes of malignancy in high-lactate tumors, it remains unclear why seemingly identical tumors may exhibit extreme differences in their lactate levels. Although it is certainly another challenge for future research in this field, several reports point out that high-lactate amounts help in generating a hostile microenvironment for normal cells, affecting the activation and differentiation of effector immune cells as well as antigen presentation and the production of cytokines. Future studies, particularly in solid tumors characterized by highly acidic environments, are needed to better understand the effect of lactate and other "waste" metabolites on cancer progression. The participation of lactate in TME and its immunosuppressive actions not only make it crucial for tumor survival and growth but also turns it into an interesting and promising candidate to therapeutic target in cancer chemotherapy. Following this reasoning, classic and novel drugs that modulate TME pH might be useful as potential immunomodulatory tools in cancer patients, particularly in combination with immunotherapeutic strategies.

AUTHOR CONTRIBUTIONS

Wrote the paper: AM, LF, LG, LC, AF, TF, KC, ES, CF-d-L, and LF-d-L. All the authors read and approved the final version of the manuscript.

FUNDING

This work was supported by grants from Fundação do Câncer, Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq).

REFERENCES

1. Hajdu SI, Vadmal M, Tang P. A note from history: landmarks in history of cancer, part 7. *Cancer* (2015) 121(15):2480–513. doi:10.1002/cncr.29365
2. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* (2000) 100(1):57–70. doi:10.1016/S0092-8674(00)81683-9
3. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) 144(5):646–74. doi:10.1016/j.cell.2011.02.013
4. Zhang YS, Duchamp M, Oklu R, Ellisen LW, Langer R, Khademhosseini A. Bioprinting the cancer microenvironment. *ACS Biomater Sci Eng* (2016) 2(10):1710–21. doi:10.1021/acsbiomaterials.6b00246
5. Zhang J, Pavlova NN, Thompson CB. Cancer cell metabolism: the essential role of the nonessential amino acid, glutamine. *EMBO J* (2017) 36(10):1302–15. doi:10.15252/embj.201696151
6. Rakoff-Nahoum S. Why cancer and inflammation? *Yale J Biol Med* (2006) 79(3–4):123–30.
7. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* (1990) 61(5):759–67. doi:10.1016/0092-8674(90)90186-I
8. Solomon E, Borrow J, Goddard AD. Chromosome aberrations and cancer. *Science* (1991) 254(5035):1153–60. doi:10.1126/science.1957167
9. Novikova MV, Khromova NV, Kopnin PB. Components of the hepatocellular carcinoma microenvironment and their role in tumor progression. *Biochemistry (Mosc)* (2017) 82(8):861–73. doi:10.1134/S0006297917080016
10. Timaner M, Beyar-Katz O, Shaked Y. Analysis of the stromal cellular components of the solid tumor microenvironment using flow cytometry. *Curr Protoc Cell Biol* (2016) 70:1981–82. doi:10.1002/0471143030.cb1918s70
11. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nat Med* (2013) 19(11):1423–37. doi:10.1038/nm.3394
12. Egeblad M, Nakasone ES, Werb Z. Tumors as organs: complex tissues that interface with the entire organism. *Dev Cell* (2010) 18(6):884–901. doi:10.1016/j.devcel.2010.05.012
13. Burnet FM. The concept of immunological surveillance. *Prog Exp Tumor Res* (1970) 13:1–27. doi:10.1159/000386035
14. Andon FT, Digifico E, Maeda A, Erreni M, Mantovani A, Alonso MJ, et al. Targeting tumor associated macrophages: the new challenge for nanomedicine. *Semin Immunol* (2017) 34:103–13. doi:10.1016/j.smim.2017.09.004
15. Kim R, Emi M, Tanabe K, Arihiro K. Tumor-driven evolution of immunosuppressive networks during malignant progression. *Cancer Res* (2006) 66(11):5527–36. doi:10.1158/0008-5472.CAN-05-4128
16. Nishida N, Yano H, Nishida T, Kamura T, Kojiro M. Angiogenesis in cancer. *Vasc Health Risk Manag* (2006) 2(3):213–9. doi:10.2147/vhrm.2006.2.3.213
17. Jain RK. Transport of molecules across tumor vasculature. *Cancer Metastasis Rev* (1987) 6(4):559–93. doi:10.1007/BF00047468
18. Lin EY, Li JF, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, et al. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Res* (2006) 66(23):11238–46. doi:10.1158/0008-5472.CAN-06-1278
19. 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
20. Pavlova NN, Thompson CB. The emerging hallmarks of cancer metabolism. *Cell Metab* (2016) 23(1):27–47. doi:10.1016/j.cmet.2015.12.006
21. Patra KC, Wang Q, Bhaskar PT, Miller L, Wang Z, Wheaton W, et al. Hexokinase 2 is required for tumor initiation and maintenance and its systemic deletion is therapeutic in mouse models of cancer. *Cancer Cell* (2013) 24(2):213–28. doi:10.1016/j.ccr.2013.06.014
22. Shroff EH, Eberlin LS, Dang VM, Gouw AM, Gabay M, Adam SJ, et al. MYC oncogene overexpression drives renal cell carcinoma in a mouse model through glutamine metabolism. *Proc Natl Acad Sci U S A* (2015) 112(21):6539–44. doi:10.1073/pnas.1507228112
23. Corbet C, Feron O. Tumour acidosis: from the passenger to the driver's seat. *Nat Rev Cancer* (2017) 17(10):577–93. doi:10.1038/nrc.2017.77
24. Dang CV, Kim JW. Convergence of cancer metabolism and immunity: an overview. *Biomol Ther (Seoul)* (2018) 26(1):4–9. doi:10.4062/biomolther.2017.194
25. Porporato PE, Dhup S, Dadhich RK, Copetti T, Sonveaux P. Anticancer targets in the glycolytic metabolism of tumors: a comprehensive review. *Front Pharmacol* (2011) 2:49. doi:10.3389/fphar.2011.00049
26. Danhier P, Banski P, Payen VL, Grasso D, Ippolito L, Sonveaux P, et al. Cancer metabolism in space and time: beyond the Warburg effect. *Biochim Biophys Acta* (2017) 1858(8):556–72. doi:10.1016/j.bbabio.2017.02.001
27. Kobliakov VA. Role of proton pumps in tumorigenesis. *Biochemistry (Mosc)* (2017) 82(4):401–12. doi:10.1134/S0006297917040010
28. Semenza GL. Tumor metabolism: cancer cells give and take lactate. *J Clin Invest* (2008) 118(12):3835–7. doi:10.1172/JCI37373
29. 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(12):3930–42. doi:10.1172/JCI36843
30. Strickaert A, Saiselet M, Dom G, De Deken X, Dumont JE, Feron O, et al. Cancer heterogeneity is not compatible with one unique cancer cell metabolic map. *Oncogene* (2017) 36(19):2637–42. doi:10.1038/onc.2016.411
31. Payen VL, Porporato PE, Baselet B, Sonveaux P. Metabolic changes associated with tumor metastasis, part 1: tumor pH, glycolysis and the pentose phosphate pathway. *Cell Mol Life Sci* (2016) 73(7):1333–48. doi:10.1007/s00018-015-2098-5
32. Spugnini EP, Sonveaux P, Stock C, Perez-Sayans M, De Milito A, Avnet S, et al. Proton channels and exchangers in cancer. *Biochim Biophys Acta* (2015) 1848(10 Pt B):2715–26. doi:10.1016/j.bbamem.2014.10.015
33. Perez-Escuredo J, Van Hee VF, Sboarina M, Falces J, Payen VL, Pellerin L, et al. Monocarboxylate transporters in the brain and in cancer. *Biochim Biophys Acta* (2016) 1863(10):2481–97. doi:10.1016/j.bbamcr.2016.03.013
34. Lee DC, Sohn HA, Park ZY, Oh S, Kang YK, Lee KM, et al. A lactate-induced response to hypoxia. *Cell* (2015) 161(3):595–609. doi:10.1016/j.cell.2015.03.011
35. Kim SY. Cancer energy metabolism: shutting power off cancer factory. *Biomol Ther (Seoul)* (2018) 26(1):39–44. doi:10.4062/biomolther.2017.184
36. Yoshida GJ. Metabolic reprogramming: the emerging concept and associated therapeutic strategies. *J Exp Clin Cancer Res* (2015) 34:111. doi:10.1186/s13046-015-0221-y
37. Vazquez A, Kamphorst JJ, Markert EK, Schug ZT, Tardito S, Gottlieb E. Cancer metabolism at a glance. *J Cell Sci* (2016) 129(18):3367–73. doi:10.1242/jcs.181016
38. Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. *J Gen Physiol* (1927) 8(6):519–30. doi:10.1085/jgp.8.6.519
39. Koppenol WH, Bounds PL, Dang CV. Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer* (2011) 11(5):325–37. doi:10.1038/nrc3038
40. Holm E, Hagmuller E, Staedt U, Schlickeiser G, Gunther HJ, Leweling H, et al. Substrate balances across colonic carcinomas in humans. *Cancer Res* (1995) 55(6):1373–8.
41. Doherty JR, Cleveland JL. Targeting lactate metabolism for cancer therapeutics. *J Clin Invest* (2013) 123(9):3685–92. doi:10.1172/JCI69741
42. Fine EJ, Segal-Isaacson CJ, Feinman RD, Herszkopf S, Romano MC, Tomuta N, et al. Targeting insulin inhibition as a metabolic therapy in advanced cancer: a pilot safety and feasibility dietary trial in 10 patients. *Nutrition* (2012) 28(10):1028–35. doi:10.1016/j.nut.2012.05.001
43. Jadvar H, Alavi A, Gambhir SS. 18F-FDG uptake in lung, breast, and colon cancers: molecular biology correlates and disease characterization. *J Nucl Med* (2009) 50(11):1820–7. doi:10.2967/jnumed.108.054098
44. Gambhir SS. Molecular imaging of cancer with positron emission tomography. *Nat Rev Cancer* (2002) 2(9):683–93. doi:10.1038/nrc882
45. Fan J, Kamphorst JJ, Mathew R, Chung MK, White E, Shlomi T, et al. Glutamine-driven oxidative phosphorylation is a major ATP source in transformed mammalian cells in both normoxia and hypoxia. *Mol Syst Biol* (2013) 9:712. doi:10.1038/msb.2013.65
46. Hensley CT, Faubert B, Yuan Q, Lev-Cohain N, Jin E, Kim J, et al. Metabolic heterogeneity in human lung tumors. *Cell* (2016) 164(4):681–94. doi:10.1016/j.cell.2015.12.034
47. Frezza C, Gottlieb E. Mitochondria in cancer: not just innocent bystanders. *Semin Cancer Biol* (2009) 19(1):4–11. doi:10.1016/j.semcancer.2008.11.008
48. 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(3):177–85. doi:10.1016/j.cmet.2006.02.002
49. 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(3):187–97. doi:10.1016/j.cmet.2006.01.012
50. Valvona CJ, Fillmore HL, Nunn PB, Pilkington GJ. The regulation and function of lactate dehydrogenase a: therapeutic potential in brain tumor. *Brain Pathol* (2016) 26(1):3–17. doi:10.1111/bpa.12299

51. Mazure NM, Chen EY, Yeh P, Laderoute KR, Giaccia AJ. Oncogenic transformation and hypoxia synergistically act to modulate vascular endothelial growth factor expression. *Cancer Res* (1996) 56(15):3436–40.
52. Blum R, Jacob-Hirsch J, Amariglio N, Rechavi G, Kloog Y. Ras inhibition in glioblastoma down-regulates hypoxia-inducible factor-1alpha, causing glycolysis shutdown and cell death. *Cancer Res* (2005) 65(3):999–1006.
53. Lee G, Won HS, Lee YM, Choi JW, Oh TI, Jang JH, et al. Oxidative dimerization of PHD2 is responsible for its inactivation and contributes to metabolic reprogramming via HIF-1alpha activation. *Sci Rep* (2016) 6:18928. doi:10.1038/srep18928
54. 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
55. 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(2):409–15.
56. Goetze K, Walenta S, Ksiazkiewicz M, Kunz-Schughart LA, Mueller-Klieser W. Lactate enhances motility of tumor cells and inhibits monocyte migration and cytokine release. *Int J Oncol* (2011) 39(2):453–63. doi:10.3892/ijo.2011.1055
57. Baumann F, Leukel P, Doerfelt A, Beier CP, Dettmer K, Oefner PJ, et al. Lactate promotes glioma migration by TGF-beta2-dependent regulation of matrix metalloproteinase-2. *Neuro Oncol* (2009) 11(4):368–80. doi:10.1215/15228517-2008-106
58. Rudrabhatla SR, Mahaffey CL, Mummert ME. Tumor microenvironment modulates hyaluronan expression: the lactate effect. *J Invest Dermatol* (2006) 126(6):1378–87. doi:10.1038/sj.jid.5700255
59. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* (2009) 119(6):1420–8. doi:10.1172/JCI39104
60. Micalizzi DS, Ford HL. Epithelial-mesenchymal transition in development and cancer. *Future Oncol* (2009) 5(8):1129–43. doi:10.2217/fon.09.94
61. Freire-de-Lima L. Sweet and sour: the impact of differential glycosylation in cancer cells undergoing epithelial-mesenchymal transition. *Front Oncol* (2014) 4:59. doi:10.3389/fonc.2014.00059
62. Sattler UG, Meyer SS, Quennet V, Hoerner C, Knoerzer H, Fabian C, et al. Glycolytic metabolism and tumour response to fractionated irradiation. *Radiother Oncol* (2010) 94(1):102–9. doi:10.1016/j.radonc.2009.11.007
63. Goel S, Duda DG, Xu L, Munn LL, Boucher Y, Fukumura D, et al. Normalization of the vasculature for treatment of cancer and other diseases. *Physiol Rev* (2011) 91(3):1071–121. doi:10.1152/physrev.00038.2010
64. Jun JC, Rathore A, Younas H, Gilkes D, Polotsky VY. Hypoxia-inducible factors and cancer. *Curr Sleep Med Rep* (2017) 3(1):1–10. doi:10.1007/s40675-017-0062-7
65. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* (2002) 3(11):991–8. doi:10.1038/ni1102-991
66. Landskron G, De la Fuente M, Thuwajit P, Thuwajit C, Hermoso MA. Chronic inflammation and cytokines in the tumor microenvironment. *J Immunol Res* (2014) 2014:149185. doi:10.1155/2014/149185
67. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* (2012) 21(3):309–22. doi:10.1016/j.ccr.2012.02.022
68. 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(11):2746–56. doi:10.1158/0008-5472.CAN-11-1272
69. Palazon A, Aragones J, Morales-Kastresana A, de Landazuri MO, Melero I. Molecular pathways: hypoxia response in immune cells fighting or promoting cancer. *Clin Cancer Res* (2012) 18(5):1207–13. doi:10.1158/1078-0432.CCR-11-1591
70. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* (2011) 331(6024):1565–70. doi:10.1126/science.1203486
71. Porta C, Larghi P, Rimoldi M, Totaro MG, Allavena P, Mantovani A, et al. Cellular and molecular pathways linking inflammation and cancer. *Immunobiology* (2009) 214(9–10):761–77. doi:10.1016/j.imbio.2009.06.014
72. Malmberg KJ, Carlsten M, Bjorklund A, Sohlberg E, Bryceson YT, Ljunggren HG. Natural killer cell-mediated immunosurveillance of human cancer. *Semin Immunol* (2017) 31:20–9. doi:10.1016/j.smim.2017.08.002
73. Manjili MH. Revisiting cancer immunoediting by understanding cancer immune complexity. *J Pathol* (2011) 224(1):5–9. doi:10.1002/path.2865
74. Teng MW, Galon J, Fridman WH, Smyth MJ. From mice to humans: developments in cancer immunoediting. *J Clin Invest* (2015) 125(9):3338–46. doi:10.1172/JCI80004
75. Sistigu A, Di Modugno F, Manic G, Nistico P. Deciphering the loop of epithelial-mesenchymal transition, inflammatory cytokines and cancer immunoediting. *Cytokine Growth Factor Rev* (2017) 36:67–77. doi:10.1016/j.cytogfr.2017.05.008
76. Wang M, Zhang C, Song Y, Wang Z, Wang Y, Luo F, et al. Mechanism of immune evasion in breast cancer. *Onco Targets Ther* (2017) 10:1561–73. doi:10.2147/OTT.S126424
77. O'Reilly E, Tirincsi A, Logue SE, Szegezdi E. The Janus face of death receptor signaling during tumor immunoediting. *Front Immunol* (2016) 7:446. doi:10.3389/fimmu.2016.00446
78. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* (2004) 21(2):137–48. doi:10.1016/j.immuni.2004.07.017
79. Schreiber RD. Cancer vaccines 2004 opening address: the molecular and cellular basis of cancer immunosurveillance and immunoediting. *Cancer Immun* (2005) 5(Suppl 1):1.
80. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. *Annu Rev Immunol* (2004) 22:329–60. doi:10.1146/annurev.immunol.22.012703.104803
81. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* (2003) 348(3):203–13. doi:10.1056/NEJMoa020177
82. van Kempen LC, Ruiter DJ, van Muijen GN, Coussens LM. The tumor microenvironment: a critical determinant of neoplastic evolution. *Eur J Cell Biol* (2003) 82(11):539–48. doi:10.1078/0171-9335-00346
83. Kerkar SP, Restifo NP. Cellular constituents of immune escape within the tumor microenvironment. *Cancer Res* (2012) 72(13):3125–30. doi:10.1158/0008-5472.CAN-11-4094
84. Yu P, Rowley DA, Fu YX, Schreiber H. The role of stroma in immune recognition and destruction of well-established solid tumors. *Curr Opin Immunol* (2006) 18(2):226–31. doi:10.1016/j.coi.2006.01.004
85. Gabrilovich D, Ishida T, Oyama T, Ran S, Kravtsov V, Nadaf S, et al. Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo. *Blood* (1998) 92(11):4150–66.
86. Siveen KS, Kuttan G. Role of macrophages in tumour progression. *Immunol Lett* (2009) 123(2):97–102. doi:10.1016/j.imlet.2009.02.011
87. Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG, et al. Macrophage polarization in tumour progression. *Semin Cancer Biol* (2008) 18(5):349–55. doi:10.1016/j.semcancer.2008.03.004
88. Beck C, Schreiber H, Rowley D. Role of TGF-beta in immune-evasion of cancer. *Microsc Res Tech* (2001) 52(4):387–95. doi:10.1002/1097-0029(20010215)52:4<387:AID-JEMT1023>3.0.CO;2-W
89. He X, Stuart JM. Prostaglandin E2 selectively inhibits human CD4+ T cells secreting low amounts of both IL-2 and IL-4. *J Immunol* (1999) 163(11):6173–9.
90. Erdogan B, Uzaslan E, Budak F, Karadag M, Ediger D, Oral B, et al. The evaluation of soluble Fas and soluble Fas ligand levels of bronchoalveolar lavage fluid in lung cancer patients. *Tuberk Toraks* (2005) 53(2):127–31.
91. Holdenrieder S, Stieber P, Peterfi A, Nagel D, Steinle A, Salih HR. Soluble MICB in malignant diseases: analysis of diagnostic significance and correlation with soluble MICA. *Cancer Immunol Immunother* (2006) 55(12):1584–9. doi:10.1007/s00262-006-0167-1
92. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. *Immunology* (2007) 121(1):1–14. doi:10.1111/j.1365-2567.2007.02587.x
93. Santoni M, Berardi R, Amantini C, Burattini L, Santini D, Santoni G, et al. Role of natural and adaptive immunity in renal cell carcinoma response to VEGFR-TKIs and mTOR inhibitor. *Int J Cancer* (2014) 134(12):2772–7. doi:10.1002/ijc.28503
94. Bernal M, Concha A, Saenz-Lopez P, Rodriguez AI, Cabrera T, Garrido F, et al. Leukocyte infiltrate in gastrointestinal adenocarcinomas is strongly associated with tumor microsatellite instability but not with tumor immunogenicity. *Cancer Immunol Immunother* (2011) 60(6):869–82. doi:10.1007/s00262-011-0999-1

95. Botti C, Seregni E, Ferrari L, Martinetti A, Bombardieri E. Immunosuppressive factors: role in cancer development and progression. *Int J Biol Markers* (1998) 13(2):51–69.
96. Gastman BR, Johnson DE, Whiteside TL, Rabinowich H. Tumor-induced apoptosis of T lymphocytes: elucidation of intracellular apoptotic events. *Blood* (2000) 95(6):2015–23.
97. Mocellin S, Wang E, Marincola FM. Cytokines and immune response in the tumor microenvironment. *J Immunother* (2001) 24(5):392–407. doi:10.1097/00002371-200109000-00002
98. Rodriguez PC, Quiceno DG, Zabaleta J, Ortiz B, Zea AH, Piazuelo MB, et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res* (2004) 64(16):5839–49. doi:10.1158/0008-5472.CAN-04-0465
99. Whiteside TL. Immune suppression in cancer: effects on immune cells, mechanisms and future therapeutic intervention. *Semin Cancer Biol* (2006) 16(1):3–15. doi:10.1016/j.semcancer.2005.07.008
100. Spranger S, Spaapen RM, Zha Y, Williams J, Meng Y, Ha TT, et al. Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by CD8(+) T cells. *Sci Transl Med* (2013) 5(200):200ra116. doi:10.1126/scitranslmed.3006504
101. 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(9):3812–9. doi:10.1182/blood-2006-07-035972
102. Dietl K, Renner K, Dettmer K, Timischl B, Eberhart K, Dorn C, et al. Lactic acid and acidification inhibit TNF secretion and glycolysis of human monocytes. *J Immunol* (2010) 184(3):1200–9. doi:10.4049/jimmunol.0902584
103. Puig-Kroger A, Pello OM, Selgas R, Criado G, Bajo MA, Sanchez-Tomero JA, et al. Peritoneal dialysis solutions inhibit the differentiation and maturation of human monocyte-derived dendritic cells: effect of lactate and glucose-degradation products. *J Leukoc Biol* (2003) 73(4):482–92. doi:10.1189/jlb.0902451
104. Girgis H, Masui O, White NM, Scorilas A, Rotondo F, Seivwright A, et al. Lactate dehydrogenase A is a potential prognostic marker in clear cell renal cell carcinoma. *Mol Cancer* (2014) 13:101. doi:10.1186/1476-4598-13-101
105. Sun X, Sun Z, Zhu Z, Guan H, Zhang J, Zhang Y, et al. Clinicopathological significance and prognostic value of lactate dehydrogenase A expression in gastric cancer patients. *PLoS One* (2014) 9(3):e91068. doi:10.1371/journal.pone.0091068
106. 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(3):1486–95. doi:10.4049/jimmunol.1202702
107. Dart A. Tumour metabolism: lactic acid: not just a waste product? *Nat Rev Cancer* (2016) 16(11):676–7. doi:10.1038/nrc.2016.109
108. Sola-Penna M. Metabolic regulation by lactate. *IUBMB Life* (2008) 60(9):605–8. doi:10.1002/iub.97
109. 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 U S A* (2007) 104(49):19345–50. doi:10.1073/pnas.0709747104
110. Feron O. Pyruvate into lactate and back: from the Warburg effect to symbiotic energy fuel exchange in cancer cells. *Radiother Oncol* (2009) 92(3):329–33. doi:10.1016/j.radonc.2009.06.025
111. Xie H, Hanai J, Ren JG, Kats L, Burgess K, Bhargava P, et al. Targeting lactate dehydrogenase – a inhibits tumorigenesis and tumor progression in mouse models of lung cancer and impacts tumor-initiating cells. *Cell Metab* (2014) 19(5):795–809. doi:10.1016/j.cmet.2014.03.003
112. Mendler AN, Hu B, Prinz PU, Kreutz M, Gottfried E, Noessner E. Tumor lactic acidosis suppresses CTL function by inhibition of p38 and JNK/c-Jun activation. *Int J Cancer* (2012) 131(3):633–40. doi:10.1002/ijc.26410
113. Husain Z, Seth P, Sukhatme VP. Tumor-derived lactate and myeloid-derived suppressor cells: linking metabolism to cancer immunology. *Oncoimmunology* (2013) 2(11):e26383. doi:10.4161/onci.26383
114. Kato Y, Ozawa S, Tsukuda M, Kubota E, Miyazaki K, St-Pierre Y, et al. Acidic extracellular pH increases calcium influx-triggered phospholipase D activity along with acidic sphingomyelinase activation to induce matrix metalloproteinase-9 expression in mouse metastatic melanoma. *FEBS J* (2007) 274(12):3171–83. doi:10.1111/j.1742-4658.2007.05848.x
115. Wilson WR, Hay MP. Targeting hypoxia in cancer therapy. *Nat Rev Cancer* (2011) 11(6):393–410. doi:10.1038/nrc3064
116. Walenta S, Wetterling M, Lehrke M, Schwickert G, Sundfor 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(4):916–21.
117. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol* (2012) 12(4):253–68. doi:10.1038/nri3175
118. Gottfried E, Kunz-Schughart LA, Ebner S, Mueller-Klieser W, Hoves S, Andreesen R, et al. Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. *Blood* (2006) 107(5):2013–21. doi:10.1182/blood-2005-05-1795
119. Peter K, Rehli M, Singer K, Renner-Sattler K, Kreutz M. Lactic acid delays the inflammatory response of human monocytes. *Biochem Biophys Res Commun* (2015) 457(3):412–8. doi:10.1016/j.bbrc.2015.01.005
120. Wei L, Zhou Y, Yao J, Qiao C, Ni T, Guo R, et al. Lactate promotes PGE2 synthesis and gluconeogenesis in monocytes to benefit the growth of inflammation-associated colorectal tumor. *Oncotarget* (2015) 6(18):16198–214. doi:10.18632/oncotarget.3838
121. Gottfried E, Kreutz M, Haffner S, Holler E, Iacobelli M, Andreesen R, et al. Differentiation of human tumour-associated dendritic cells into endothelial-like cells: an alternative pathway of tumour angiogenesis. *Scand J Immunol* (2007) 65(4):329–35. doi:10.1111/j.1365-3083.2007.01903.x
122. Hoque R, Farooq A, Ghani A, Gorelick F, Mehal WZ. Lactate reduces liver and pancreatic injury in toll-like receptor- and inflammasome-mediated inflammation via GPR81-mediated suppression of innate immunity. *Gastroenterology* (2014) 146(7):1763–74. doi:10.1053/j.gastro.2014.03.014
123. Errea A, Cayet D, Marchetti P, Tang C, Kluza J, Offermanns S, et al. Lactate inhibits the pro-inflammatory response and metabolic reprogramming in murine macrophages in a GPR81-independent manner. *PLoS One* (2016) 11(11):e0163694. doi:10.1371/journal.pone.0163694
124. Selleri S, Bifsha P, Civini S, Pacelli C, Dieng MM, Lemieux W, et al. Human mesenchymal stromal cell-secreted lactate induces M2-macrophage differentiation by metabolic reprogramming. *Oncotarget* (2016) 7(21):30193–210. doi:10.18632/oncotarget.8623
125. 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(7519):559–63. doi:10.1038/nature13490
126. Colegio OR. Lactic acid polarizes macrophages to a tumor-promoting state. *Oncoimmunology* (2016) 5(3):e1014774. doi:10.1080/2162402X.2015.1014774
127. Yang Z, Ming XF. Functions of arginase isoforms in macrophage inflammatory responses: impact on cardiovascular diseases and metabolic disorders. *Front Immunol* (2014) 5:533. doi:10.3389/fimmu.2014.00533
128. Jung YJ, Isaacs JS, Lee S, Trepel J, Neckers L. IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. *FASEB J* (2003) 17(14):2115–7. doi:10.1096/fj.03-0329fje
129. Guentsch A, Beneke A, Swain L, Farhat K, Nagarajan S, Wielockx B, et al. PHD2 is a regulator for glycolytic reprogramming in macrophages. *Mol Cell Biol* (2017) 37(1):e00236-16. doi:10.1128/MCB.00236-16
130. Chang CI, Liao JC, Kuo L. Macrophage arginase promotes tumor cell growth and suppresses nitric oxide-mediated tumor cytotoxicity. *Cancer Res* (2001) 61(3):1100–6.
131. Wang N, Liang H, Zen K. Molecular mechanisms that influence the macrophage m1-m2 polarization balance. *Front Immunol* (2014) 5:614. doi:10.3389/fimmu.2014.00614
132. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. *Nat Rev Immunol* (2005) 5(8):641–54. doi:10.1038/nri1668
133. Crowther M, Brown NJ, Bishop ET, Lewis CE. Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors. *J Leukoc Biol* (2001) 70(4):478–90.
134. Korrer MJ, Zhang Y, Routes JM. Possible role of arginase-1 in concomitant tumor immunity. *PLoS One* (2014) 9(3):e91370. doi:10.1371/journal.pone.0091370
135. Dong H, Bullock TN. Metabolic influences that regulate dendritic cell function in tumors. *Front Immunol* (2014) 5:24. doi:10.3389/fimmu.2014.00024
136. Kelly B, O'Neill LA. Metabolic reprogramming in macrophages and dendritic cells in innate immunity. *Cell Res* (2015) 25(7):771–84. doi:10.1038/cr.2015.68

137. O'Neill LA, Pearce EJ. Immunometabolism governs dendritic cell and macrophage function. *J Exp Med* (2016) 213(1):15–23. doi:10.1084/jem.20151570
138. Campbell AR, Duggan MC, Suarez-Kelly LP, Bhave N, Opheim KS, McMichael EL, et al. MICA-expressing monocytes enhance natural killer cell Fc receptor-mediated antitumor functions. *Cancer Immunol Res* (2017) 5(9):778–89. doi:10.1158/2326-6066.CIR-16-0005
139. Prajapati K, Perez C, Rojas LBP, Burke B, Guevara-Patino JA. Functions of NKG2D in CD8(+) T cells: an opportunity for immunotherapy. *Cell Mol Immunol* (2018) 14:1–10. doi:10.1038/cmi.2017.161
140. Ding H, Yang X, Wei Y. Fusion proteins of NKG2D/NKG2DL in cancer immunotherapy. *Int J Mol Sci* (2018) 19(1):E177. doi:10.3390/ijms19010177
141. Latham T, Mackay L, Sproul D, Karim M, Culley J, Harrison DJ, et al. Lactate, a product of glycolytic metabolism, inhibits histone deacetylase activity and promotes changes in gene expression. *Nucleic Acids Res* (2012) 40(11):4794–803. doi:10.1093/nar/gks066
142. Lai CB, Mager DL. Role of runt-related transcription factor 3 (RUNX3) in transcription regulation of natural cytotoxicity receptor 1 (NCR1/NKp46), an activating natural killer (NK) cell receptor. *J Biol Chem* (2012) 287(10):7324–34. doi:10.1074/jbc.M111.306936
143. Crane CA, Austgen K, Haberthur K, Hofmann C, Moyes KW, Avanesyan L, et al. Immune evasion mediated by tumor-derived lactate dehydrogenase induction of NKG2D ligands on myeloid cells in glioblastoma patients. *Proc Natl Acad Sci U S A* (2014) 111(35):12823–8. doi:10.1073/pnas.1413933111
144. Kayser G, Kassem A, Sienel W, Schulte-Uentrop L, Mattern D, Aumann K, et al. Lactate-dehydrogenase 5 is overexpressed in non-small cell lung cancer and correlates with the expression of the transketolase-like protein 1. *Diagn Pathol* (2010) 5:22. doi:10.1186/1746-1596-5-22
145. Koukourakis MI, Giatromanolaki A, Simopoulos C, Polychronidis A, Sivridis E. Lactate dehydrogenase 5 (LDH5) relates to up-regulated hypoxia inducible factor pathway and metastasis in colorectal cancer. *Clin Exp Metastasis* (2005) 22(1):25–30. doi:10.1007/s10585-005-2343-7
146. Crane CA, Han SJ, Barry JJ, Ahn BJ, Lanier LL, Parsa AT. TGF-beta downregulates the activating receptor NKG2D on NK cells and CD8+ T cells in glioma patients. *Neuro Oncol* (2010) 12(1):7–13. doi:10.1093/neuonc/nop009
147. Molon B, Cali B, Viola A. T cells and cancer: how metabolism shapes immunity. *Front Immunol* (2016) 7:20. doi:10.3389/fimmu.2016.00020
148. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* (2006) 313(5795):1960–4. doi:10.1126/science.1129139
149. Jain P, Jain C, Velcheti V. Role of immune-checkpoint inhibitors in lung cancer. *Ther Adv Respir Dis* (2018) 12:1753465817750075. doi:10.1177/1753465817750075
150. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* (2010) 363(8):711–23. doi:10.1056/NEJMoa1003466
151. Alaia C, Boccellino M, Zappavigna S, Amler E, Quagliuolo L, Rossetti S, et al. Ipilimumab for the treatment of metastatic prostate cancer. *Expert Opin Biol Ther* (2018) 18(2):205–13. doi:10.1080/14712598.2018.1420777
152. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* (2012) 366(26):2443–54. doi:10.1056/NEJMoa1200690
153. Medina PJ, Adams VR. PD-1 pathway inhibitors: immuno-oncology agents for restoring antitumor immune responses. *Pharmacotherapy* (2016) 36(3):317–34. doi:10.1002/phar.1714
154. Ganapathy-Kanniappan S. Linking tumor glycolysis and immune evasion in cancer: emerging concepts and therapeutic opportunities. *Biochim Biophys Acta* (2017) 1868(1):212–20. doi:10.1016/j.bbcan.2017.04.002
155. 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
156. Kareva I, Hahnfeldt P. The emerging "hallmarks" of metabolic reprogramming and immune evasion: distinct or linked? *Cancer Res* (2013) 73(9):2737–42. doi:10.1158/0008-5472.CAN-12-3696
157. Chang CH, Curtis JD, Maggi LB Jr, Faubert B, Villarino AV, O'Sullivan D, et al. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* (2013) 153(6):1239–51. doi:10.1016/j.cell.2013.05.016
158. Jones RG, Thompson CB. Revving the engine: signal transduction fuels T cell activation. *Immunity* (2007) 27(2):173–8. doi:10.1016/j.immuni.2007.07.008
159. Gerriets VA, Rathmell JC. Metabolic pathways in T cell fate and function. *Trends Immunol* (2012) 33(4):168–73. doi:10.1016/j.it.2012.01.010
160. 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(5):657–71. doi:10.1016/j.cmet.2016.08.011

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 07 February 2018
doi: 10.3389/fonc.2018.00022

Posttranslational Modifications of Pyruvate Kinase M2: Tweaks that Benefit Cancer

Gopinath Prakasam[1†], Mohammad Askandar Iqbal[2†], Rameshwar N. K. Bamezai[1] and Sybille Mazurek[3*]*

[1] School of Life Sciences, Jawaharlal Nehru University, New Delhi, India, [2] Department of Biotechnology, Faculty of Natural Sciences, Jamia Millia Islamia, New Delhi, India, [3] Institute of Veterinary Physiology and Biochemistry, University of Giessen, Giessen, Germany

OPEN ACCESS

Edited by:
Leonardo Freire-de-Lima, Universidade Federal do Rio de Janeiro, Brazil

Reviewed by:
Daniele Vergara, University of Salento, Italy
Andrew M. Chan, The Chinese University of Hong Kong, Hong Kong
Pankaj Seth, Harvard University, United States
Leonardo Marques da Fonseca, Universidade Federal do Rio de Janeiro, Brazil

***Correspondence:**
Rameshwar N. K. Bamezai
bamezai@hotmail.com;
Sybille Mazurek
sybille.mazurek@vetmed.uni-giessen.de

[†]These authors have contributed equally to this work.

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *29 November 2017*
Accepted: *22 January 2018*
Published: *07 February 2018*

Citation:
Prakasam G, Iqbal MA, Bamezai RNK and Mazurek S (2018) Posttranslational Modifications of Pyruvate Kinase M2: Tweaks that Benefit Cancer. Front. Oncol. 8:22. doi: 10.3389/fonc.2018.00022

Cancer cells rewire metabolism to meet biosynthetic and energetic demands. The characteristic increase in glycolysis, i.e., Warburg effect, now considered as a hallmark, supports cancer in various ways. To attain such metabolic reshuffle, cancer cells preferentially re-express the M2 isoform of pyruvate kinase (PKM2, M2-PK) and alter its quaternary structure to generate less-active PKM2 dimers. The relatively inactive dimers cause the accumulation of glycolytic intermediates that are redirected into anabolic pathways. In addition, dimeric PKM2 also benefits cancer cells through various non-glycolytic moonlight functions, such as gene transcription, protein kinase activity, and redox balance. A large body of data have shown that several distinct posttranslation modifications (PTMs) regulate PKM2 in a way that benefits cancer growth, e.g., formation of PKM2 dimers. This review discusses the recent advancements in our understanding of various PTMs and the benefits they impart to the sustenance of cancer. Understanding the PTMs in PKM2 is crucial to assess their therapeutic potential and to design novel anticancer strategies.

Keywords: M2 isoform of pyruvate kinase, posttranslational modifications, cancer metabolism, glycolysis, Warburg effect, cancer therapy

INTRODUCTION

The key metabolic anomaly associated with tumor cells in contrast to their non-transformed counterparts is rewiring of metabolism to escalate glucose uptake rate and to break down glucose largely into lactate, regardless of the presence of oxygen, a phenomenon known as aerobic glycolysis or Warburg effect (1). The preferential expression of M2 isoform of pyruvate kinase (PKM2) by the cancer cells is required for metabolic reprogramming, a nearly universal phenomenon in cancer (2–6). To further ripe benefits out of the PKM2 expression, cancer cells posttranslationally modify it into an enzymatically less active dimeric state. Thus, retarding the PKM2 catalyzed conversion of phosphoenol pyruvate (PEP) into pyruvate, causing piling up of the glycolytic intermediates and their channeling into pentose phosphate pathway (PPP) as well as phospholipid and amino acid synthesis to facilitate biosynthesis of macromolecules and reducing equivalent [nicotinamide adenine dinucleotide phosphate (NADPH + H+)], eventually supporting cancer cell proliferation (4). The dimer and tetramer ratio of PKM2 is regulated by numerous factors including metabolic intermediates [fructose 1, 6-bisphosphate (FBP), succinylaminoimidazolecarboxamide ribose 5-phosphate

(SAICAR) and serine], physical interaction with oncoproteins, and importantly by various posttranslation modifications (PTMs) (7–9). Notably, the PTMs contribute to expansion of functional proteome in the mammalian cells (10). Recent advancements in PTMs research have uncovered many intricate regulatory mechanisms through which PTMs regulate the cell signaling and metabolic pathways. PKM2 protein sequence consists of many conserved amino acid sites that are modified covalently by PTMs such as phosphorylation, acetylation, hydroxylation, oxidation, ubiquitination, and sumoylation. This review deals with investigating the extrinsic and intrinsic stimuli responsible for PTMs of PKM2 and scrutinizes the impact on PKM2 biology along with the associated canonical and non-canonical functional implications that benefit cancer.

MAMMALIAN PK GENE, TRANSCRIPT VARIANTS, AND PROTEINS

Pyruvate kinase (ATP-pyruvate 2-O-phosphotransferase, EC 2.7.1.40), a terminal glycolytic pathway enzyme, catalyzes the irreversible transphosphorylation reaction between PEP and ADP to yield a molecule of pyruvate and ATP in an oxygen-independent manner. All living entities ubiquitously express enzyme PK, thereby conserving at least one form of PK. The human genome consists of two PK genes; the *PKLR* gene on chromosome 1 and the *PKM* gene on chromosome 15, which code for four PK isoforms designated as PKL, PKR, PKM1, and PKM2 (11). The expression of PK isoforms is highly regulated and is tissue-specific, suggesting that different kinetic properties of PK isoforms satisfy different metabolic needs of different tissues. For instance, PKM2 is predominantly expressed in the tissues with high anabolic biosynthesis such as proliferating embryonic cells and tumor cells. Likewise, tissues bearing a high rate of gluconeogenesis, such as liver, possess accordingly compatible PKL isoform. The *PKLR* gene, by using tissue-specific alternate promoters, codes for the full-length PKR isoform in erythrocytes and a shorter variant PKL isoform (lacking 1 exon) in the liver as well as to a smaller extent in intestine and kidney (12). The *PKM* gene codes for PKM1 and PKM2 isoforms, through alternative splicing of the mutually exclusive exons 9 and 10. Out of 12 exons of PKM pre-mRNA, the mature transcript that includes exon 9 and excludes exon 10 is designated as PKM1. The mature mRNA that includes exon 10 and excludes exon 9 is termed PKM2 (M2-PK). The proteins coded by these mature mRNAs' are of identical size (531 amino acids); however, out of 56 amino acids that are coded by the exon 9 and exon 10, 22 amino acids are different (13). The expression of the constitutively active PKM1 isoform is ideal for adult differentiated tissues that demand a large supply of ATP such as brain, adult skeletal muscle, and heart. The PKM2 isoform is mainly expressed in the dividing cells with growing anabolic demands, e.g., embryonic cells and cancer cells (5, 13).

The prototypic PKM2 isoform is highly expressed in embryonic tissues and is steadily replaced by other isoforms during differentiation. There are several studies substantiating the isoform shift back to PKM2 during tumorigenesis. For instance, L-M2 shift in hepatocarcinogenesis (14, 15), M1–M2 shift during metabolic transformation, i.e., aerobic glycolysis (16). Although M2 is tightly associated with tumor growth and metabolism (as suggested by the reduction in tumor growth on replacing M2 by M1), it is also present in some differentiated tissues such as lung, adipose, pancreatic islets, retina, distal renal tubules, and so on (17, 18).

REGULATION OF PKM2 RE-EXPRESSION

The molecular mechanism that regulates the alternative splicing of PKM isoforms remained ambiguous until recently. The study by David et al. unraveled a potential mechanistic link by which cancer cells could re-express PKM2. In this study, the authors revealed the role of c-Myc (the most frequent deregulated oncogene)-mediated transcriptional activation of heterogeneous nuclear ribonucleoproteins [hnRNPA1, hnRNPA2, and polypyrimidine tract binding protein (PTB)] in regulating alternative splicing of the PKM isoforms (19). The hnRNPs' selectively bind the sequence flanking exon 9 in PKM pre-mRNA to repress its inclusion to the mature mRNA, and thus, indirectly facilitating the inclusion of exon 10 into the mature mRNA, resulting in the expression of PKM2 mRNA in cancer cells.

The expression of PKM2 has been shown to be influenced by a spectrum of signaling pathways that are stimulated by tumor microenvironment (hypoxia and nutrient status), aberrant oncogenic mutations, growth factors, and hormones. These signaling pathways, through the network of transcription factors, regulate PKM2 expression. Transcription factors that directly bind to the consensus sequence in the *PKM* gene include hypoxia-inducible factor-1α (HIF-1α), nuclear factor kappa B (NF-κB), peroxisome proliferator-activated receptor gamma (PPARγ), and specificity protein 1 (Sp1) (8, 20). Under hypoxic conditions, HIF-1 stimulates the expression of PKM2 isoform by binding to the hypoxic response element present in the first intron of PKM gene. Notably, PKM2 interacts with HIF-1 transcription complex to co-regulate its own transcription through a positive feedback loop (21). In addition to hypoxia, aberrant oncogenic signaling in cancer cells has been shown to facilitate the normoxic stabilization of HIF-1α to rewire the metabolism by inducing the expression of PKM2. For example, mammalian target of rapamycin (mTOR), downstream to the axis of receptor tyrosine kinase/PI3K/protein kinase B (AKT), which is frequently deregulated in cancer cells, is known to stimulate the expression of c-Myc and normoxic stabilization of HIF-1. Both transcription factors increase the transcriptional activation of many glycolytic enzymes including PKM2 to stimulate aerobic glycolysis (22). Accordingly, the stimulation of the PI3K/AKT/mTOR pathway by insulin facilitated the normoxic stabilization of hypoxic inducible factor 1 (HIF1), thereby increasing PKM2 expression to promote cancer type metabolism (23). Following EGFR stimulation, NF-κB (p65) induces the expression of PKM2 by recruiting HIF-1α to co-regulate the transcriptional activation of the *PKM* gene (24). In PTEN null mouse liver cells, hyperactivated AKT2 induced the expression of peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear hormone receptor and a transcription factor. PPARγ specifically binds to the promoter of the *PKM* and *HK* (hexokinase) genes to augment their expression, thus causing

the metabolic reprogramming which contributes to the liver pathophysiology (25). Hormones, such as insulin (23, 26, 27), triiodothyronine-T3 (28), and glucocorticoid (29) also regulate PKM2 gene expression. The Sp1 transcription factor constitutively activates the transcription of the *PKM* gene by binding to the consensus DNA binding sites (GC boxes) in the PKM gene promoter. The Sp3 transcription factor synergies Sp1 to enhance the abovementioned function (30). Glucose stimulation facilitates dephosphorylation of Sp1 by protein phosphatase 1, which enhances its DNA binding activity (GC boxes). As a result demonstrates an increased PKM transcription (31).

In addition to the abovementioned mechanisms, PKM2 gene expression is also enhanced by epigenetic hypomethylation of intron 1 within the PKM gene (32). miRNAs including miR-122, miR-133a, miR-133b, and miR-326 also affect the expression of PKM2 and thus metabolic reprogramming in cancer (33–36).

In conclusion, the plasticity in the regulation of the PKM gene expression is exploited by cancer cells to promote their growth and metabolism.

STRUCTURAL BIOLOGY OF PKM2

Human PKM2 protein comprises 531 amino acids and each monomeric subunit is subdivided as A-, B- and C domains based on their characteristic functional features (**Figure 1A**). The adjoining region between the A- and B domains together forms the catalytic active site. The C domain near the carboxyl terminus harbors the binding site for the allosteric activator (FBP) as well as the intersubunit contact domain (ISCD) and the nuclear localization signal sequence. Notably, the 22 amino acids that differ between PKM1 and PKM2 (coded by the alternatively spliced exons) span across the ISCD domain (37). This difference allows PKM2 to

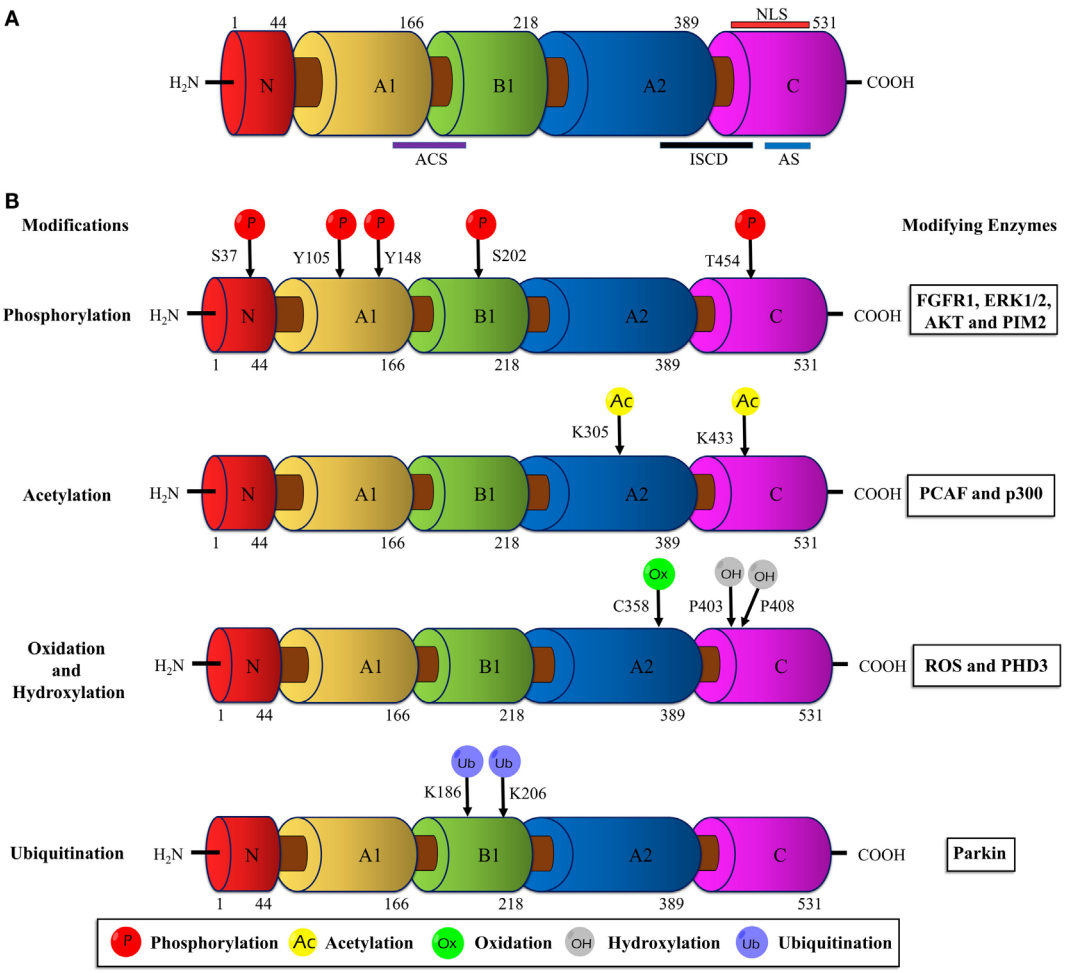


FIGURE 1 | Schematic illustration of the PKM2 domain structures and major sites of posttranslational modifications (PTMs). **(A)** The interface between the A1 and B domains of PKM2 forms the catalytic active site (ACS); the flanking region between the A2 and C domains, known as intersubunit contact domain (ISCD), is a key component that enables the formation of tetrameric oligomers; the C domain accommodates the allosteric activator (FBP) binding site and a nuclear localization signal sequence (NLS). **(B)** The major sites of PKM2 that are subjected to phosphorylation, acetylation, oxidation, hydroxylation, and ubiquitination are marked and the modifying enzymes or factors that dictate the PTMs are added on the right. Abbreviations: PK, pyruvate kinase; p, phosphorylation; Ac, acetylation; Ox, oxidation; OH, hydroxylation; Ub, ubiquitination; FGFR1, fibroblast growth factor receptor-1; ERK1/2, extracellular signal-regulated kinase 1/2; AKT, protein kinase B; p300, histone acetyltransferase; ROS, reactive oxygen species; PHD3, prolyl hydroxylase 3; PKM2, M2 isoform of pyruvate kinase; FBP, fructose 1, 6-bisphosphate.

have unique functional properties such as allosteric regulation by FBP and the ability to exist in tetrameric and dimeric forms. In contrast, PKM1 is not allosterically regulated and exists only in a tetrameric form.

The dimeric PKM2 emerges as the result of an interaction between two monomers at their A domain. Two PKM2 dimers associate at their C domain (ISCD) to form the tetrameric PKM2. In addition, PKM2 can form hybrids with PKL in liver, jejunum, colon, and rectum. Likewise, heterotetrameric hybrids between PKM2 and PKM1 were reported in esophagus, stomach, and also recently in lung and breast cancer cell lines (29, 38–40).

DYNAMIC PKM2 ACTIVITY REGULATION AND CANCER METABOLISM

Although the different isozymes of PK catalyze the identical biochemical reaction, they differ in their kinetic properties such as varied affinity to its substrate PEP, allosteric regulation, and ability to exist in more than one oligomeric state. Unlike PKM1 isoform, a constitutively active tetrameric enzyme, the activity of PKM2 is influenced by its oligomeric state, i.e., dimer or tetramer. Whereas the tetrameric form of PKM2 has a high affinity for PEP and is highly active the dimeric form is characterized by a low PEP affinity and is nearly inactive under physiological conditions (3). The less active dimeric form of PKM2 supports the channeling of glucose carbons into synthetic processes which debranch from glycolytic intermediates, while the active tetrameric PKM2 is relatively less supportive to anabolic synthesis. The tetramer:dimer ratio of PKM2 is regulated by different mechanisms, i.e., metabolic intermediates, binding of oncoproteins, and posttranslational modifications (5).

The glycolytic intermediate FBP and the amino acid serine synthesized from glycolytic glycerate 3-P induce the subunit association from dimeric PKM2 to the enzymatically highly active tetramer (41, 42). In the same way, binding of SAICAR (an intermediate from the *de novo* purine biosynthesis pathway) to PKM2 stimulates PK activity under glucose-deprived conditions (43). PKM2 activity is allosterically inhibited by the amino acids—alanine, tryptophan, and phenylalanine (6, 8, 9). Besides the metabolic intermediates, numerous proteins are known to physically interact with PKM2 to modulate its enzymatic activity. The E7 oncoprotein encoded by the high-risk type human papillomavirus 16 binds to PKM2 to facilitate its subunit dissociation from tetramer to inactive dimer even in the presence of the allosteric activator FBP (44). Interaction of PKM2 with cytosolic promyelocytic leukemia (cPML), Jumonji C domain containing dioxygenase (JMJD5), and Tyr-46-phosphorylated MUC1-C (mediated by EGFR) inhibit PK activity through different mechanisms (45–47). cPML selectively binds the tetrameric PKM2 and inhibits its activity without dissociating it into PKM2 dimers. JMJD5 decreases PKM2 activity by inhibiting the formation of active tetramers. However, the underlying mechanism of inhibition of PK activity by Tyr-46-phosphorylated MUC1-C is not known. Interestingly, the interaction between PKM2 and death-associated protein kinase, a serine/threonine kinase enhances the enzyme activity of PKM2 (48). Thus, PKM2 acts as a molecular switch in regulating the metabolic fate of glycolytic intermediates (energy generation or anabolism) *via* modulation of its activity and structural configuration by the abovementioned mechanisms.

POSTTRANSLATIONAL MODIFICATIONS OF PKM2 AND IMPLICATIONS IN CANCER

Unlike other isoforms of PK, PKM2 harbors numerous conserved PTM sites, often modified by phosphorylation, acetylation, prolyl-hydroxylation, oxidation, ubiquitination, and sumoylation in response to various stimuli in cancer cells (**Figure 1B**). These PTMs modulate structural and functional properties of PKM2, such as oligomeric state, catalytic activity, binding of allosteric activators, protein stability, conditional protein interaction, and subcellular localization. Eventually, the posttranslationally modified PKM2 provides metabolic and non-metabolic benefits to cancer cells.

PHOSPHORYLATION

Tyrosine Phosphorylation

The pioneering study from the laboratory of Eigenbrodt et al. demonstrated that Src—non-receptor tyrosine kinase—phosphorylates PKM2 to inhibit its catalytic activity (49). Further studies by his team suggested that tyrosine phosphorylation results in an inhibition of PKM2 thereby supporting the diversion of the glycolytic flux into synthetic routes of cell building blocks (2, 50). Nearly two decades later, Hitosugi et al. confirmed the findings and demonstrated that aberrant oncogenic tyrosine kinases (e.g., FGFR1, BCR-ABL, and JAK2) phosphorylate tyrosine (Y) 105 residue of PKM2 to inhibit its activity. They further showed that Y105 phosphorylation hinders the allosteric activation of PKM2 by FBP, resulting in PKM2 activity inhibition and channeling of glycolytic intermediates into biosynthetic metabolism to support tumor growth (4) (**Figure 2**). Hitosugi et al., using a phosphoproteomic screen, also identified additional phosphorylated tyrosine residues in PKM2 including, Y83, Y148, Y175, Y370, and Y390. However, subsequent characterization using mutation analysis revealed that only Y105 phosphorylation is significant in inhibiting PKM2 activity and in supporting tumor metabolism and growth. The dynamic regulation of PKM2 Y105 phosphorylation/dephosphorylation may result in a fine-tuning between aerobic glycolysis and oxidative phosphorylation to support distinct metabolic needs of the proliferating and quiescent cells. The role of tyrosine phosphatases that dephosphorylate PKM2 to rescue its activity remained unclear. Recent studies on adipocytes, using substrate trapping and mutagenesis assay, revealed an interaction between protein-tyrosine phosphatase 1B and the tyrosine 105 domain of PKM2 thereby inducing its dephosphorylation (51) (**Figure 2**).

Apart from tyrosine phosphorylation, PKM2 has been shown to specifically bind tyrosine-phosphorylated peptides or proteins, which in turn force PKM2 to release its allosteric activator FBP, leading to an inhibition of PKM2 activity, and thus, supporting anabolic metabolism in dividing tumor cells (52). Yang et al. reported that the epidermal growth factor (EGF) stimulated nuclear translocation and association of PKM2 with Y333

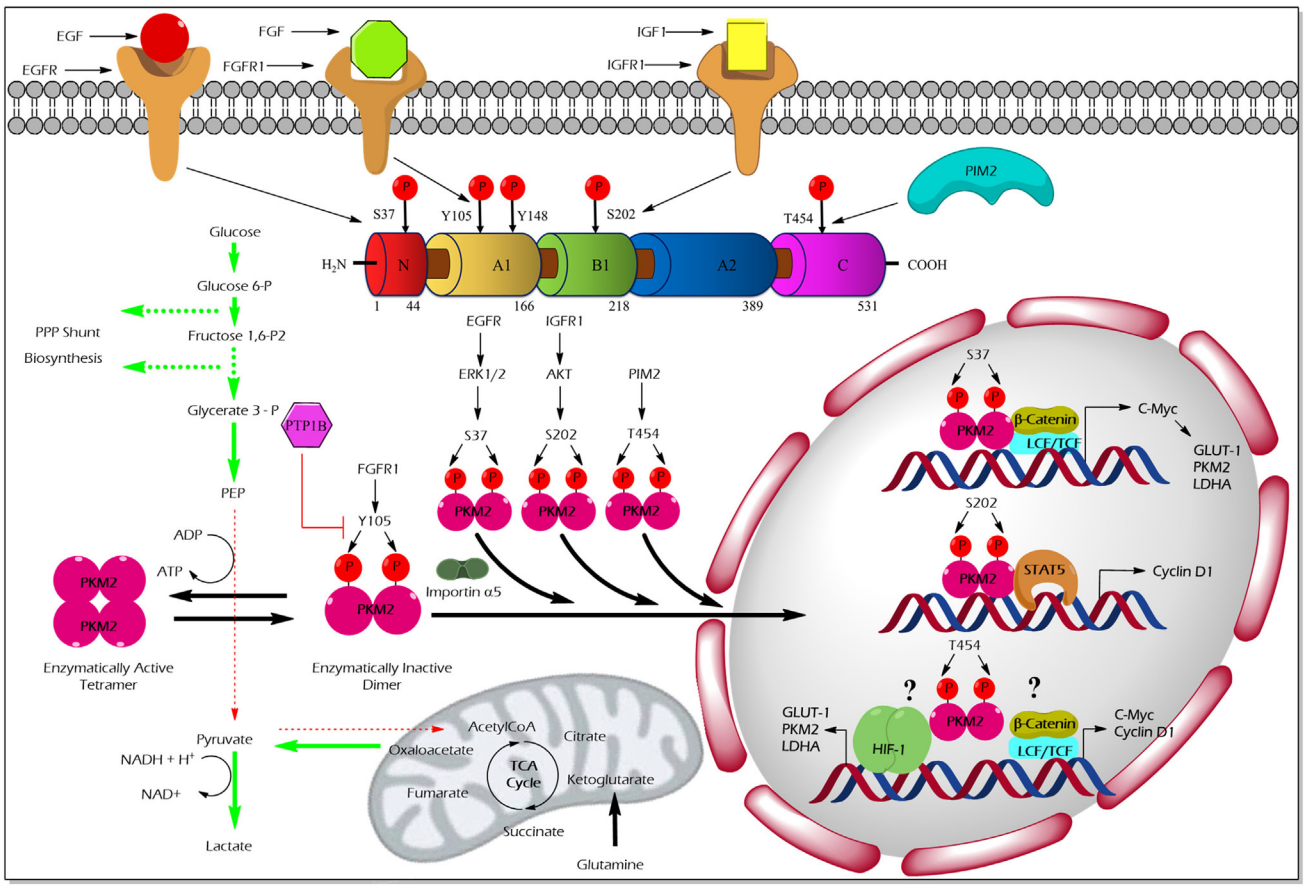


FIGURE 2 | Regulation of M2 isoform of pyruvate kinase (PKM2) enzyme activity and non-glycolytic nuclear functions by phosphorylation. Aberrant oncogenic FGFR1-mediated tyrosine (Y) phosphorylation of PKM2 at Y105 residue facilitates the formation of the enzymatically inactive dimeric form of PKM2 which promotes aerobic glycolysis and biosynthesis of macromolecules by causing accumulation and diversion of the glycolytic intermediates into synthetic pathways of cell building blocks. Conversely, protein-tyrosine phosphatase 1B (PTP1B), a tyrosine protein phosphatase, dephosphorylates tyrosine phosphorylated PKM2 and reverses the above-stated features to establish metabolic homeostasis under physiological conditions. Unlike the Y105 phosphorylation, serine (S) or threonine (T) phosphorylation of PKM2 at S37, S202, and T454 by growth factor-stimulated serine/threonine protein kinases [extracellular signal-regulated kinase (ERK) 1/2, protein kinase B (AKT), and proviral insertion in murine lymphomas 2 (PIM2)], direct the nuclear translocation of PKM2 with the aid of nuclear importin α5. The nuclear-localized PKM2 assists the transcriptional activation of β-catenin and signal transducer and activator of transcription 5 (STAT5) to facilitate the expression of different genes such as cyclin D1, c-Myc, glucose transporter 1 (GLUT1), lactate dehydrogenase A (LDHA), and PKM2, all essential for tumor cell metabolic reprogramming and division. Green arrows in the illustration indicate the augmented glycolytic flux. Red arrows indicate the restricted flux of glycolysis and the fate of the glycolytic intermediates as a result of the dimeric state of PKM2.

phosphorylated β-catenin. This interaction mediates the binding of the β-catenin/TCF transcription complex to the CCDN1 promoter responsible for the co-activation of cyclin D1 transcription, thus, promoting brain tumor development (53).

Serine/Threonine Phosphorylation

Unlike tyrosine phosphorylation of PKM2 that principally modulates its glycolytic function, serine/threonine phosphorylation of PKM2 supports its nuclear localization and opens up new vistas of non-glycolytic moonlight functions, i.e., gene transcriptional regulation and protein kinase activity (**Figure 2**).

Cancer cells stimulated with EGF triggers extracellular signal-regulated kinase 2 (ERK2), serine/threonine protein kinase, to phosphorylate PKM2 at the serine (S) 37 residue. PIN and importin α5 selectively bind the S37-phosphorylated PKM2 and induce its translocation into the nucleus where PKM2 serves as a transcriptional co-activator of β-catenin to express its target gene, c-Myc. The transcription factor c-Myc, in turn, enhances the expression of glucose transporter 1 (GLUT1), lactate dehydrogenase A, and remarkably PKM2, through a positive feedback loop. Together, EGF-stimulated S37 phosphorylation of PKM2 facilitates metabolic reprogramming in cancer cells (54). Likewise, an extracellular matrix protein 1 (a secretory glycoprotein) has been shown to stimulate PKM2 S37 phosphorylation, to enhance the transcriptional activation of glycolytic enzymes that support aerobic glycolysis in tumor cells (55). AKT stimulated by insulin-like growth factor (IGF-1) in cancer cells has been shown to interact and phosphorylate PKM2 at the S202 residue to promote its entry into the nucleus to facilitate the transcriptional activation of signal transducer and activator of transcription (STAT)5A

targets gene, cyclin D1 (56). This study proposes PKM2 as a co-activator of the transcription factor, STAT5A, which functions downstream to the IGF/PI3K/AKT signaling pathway to promote tumor growth (56). The effect of PKM2 serine phosphorylation by A-Raf is dependent upon the metabolism of serine, alanine, and glutamine. For instance, primary fibroblasts which are characterized by serine consumption and glutamine production, expression of wild-type A-Raf induces PKM2 dimerization which leads to inhibition of the metabolism of glucose to lactate. On the other hand, NIH 3T3 cells, characterized by glutamine consumption and serine production, expression of gag-A-Raf favors the metabolism of glucose to lactate by increasing highly active tetrameric form of PKM2 (57). Oncogenic proviral insertion in murine lymphomas 2 (a serine/threonine protein kinase) has been shown to phosphorylate the threonine (T) 454 residue of PKM2 to stimulate its non-glycolytic nuclear function. The non-glycolytic function of T454 phosphorylated PKM2, primarily involves the transcriptional co-activation of HIF-1α and β-catenin transcription factors to support cancer growth and drug resistance (58).

ACETYLATION

Acetylation is another important PTM where the class of enzymes termed N-terminal- or lysine-acetyltransferase catalyzes the transfer of the acetyl group from acetyl-CoA to the alpha- or epsilon-amino group of the lysine residue. Many basic cellular functions, such as chromatin remodeling, gene expression as well as several protein functions, and to maintain cellular homeostasis, depend upon an exact regulation of the dynamic process between acetylation and deacetylation.

Acetylation of PKM2 lysine residues 305 and 433 alter its activity, protein stability, and non-glycolytic protein kinase function (**Figure 3**). High glucose concentration induces lysine (K) 305 acetylation, mediated by the PCAF (P300/CBP-associated factor) acetyltransferase, in cancer cells to reduce PK activity. Lysine 305 acetylation targets PKM2 for lysosomal degradation through a chaperon-mediated autophagy. Hence, the state of reduced PKM2 expression and activity stockpiles the upper glycolytic intermediates and redirects the flux through the PPP shunt for biosynthesis of building blocks (nucleotides and amino acids), which eventually promotes tumor growth (59). Unlike the molecular mechanism described earlier, a recent study by Lv et al. revealed a remarkable non-glycolytic protein kinase function of PKM2 following its K433 acetylation mediated by p300 acetyltransferase in response to a spectrum of mitogenic and oncogenic stimuli (60). In detail, K433 acetylation of PKM2 promotes nuclear localization and protein kinase activity of the enzymatically inactive dimeric PKM2 to phosphorylate STAT3 at the Y705 residue and histone H3 at the T11 residue. The latter activates a transcriptional program that supports cell proliferation and tumorigenesis (60). Different deacetylases specifically remove the acetylation mark from PKM2 and limit the ability of PKM2 to promote tumorigenesis. For example, deacetylation of nuclear PKM2 by SIRT6 (sirtuin 6) at the K433 residue results in the export of PKM2 to the cytoplasm, thereby restricting its non-glycolytic functions (i.e., transcriptional co-activator and protein kinase) that support cancer (61). SIRT2 averts the metabolic benefits of PKM2 to cancer by deacetylation of PKM2 at the K305 residue which prevents the lysosomal degradation of PKM2 (62).

HYDROXYLATION

In hydroxylation, proline or lysine amino acid residues of mammalian proteins are covalently modified by the addition of a hydroxyl group. Proline hydroxylation of mammalian proteins is primarily catalyzed by the prolyl hydroxylases family of enzymes in the presence of molecular oxygen, Fe^{2+} (iron), 2-oxoglutarate, and ascorbate. Proline hydroxylation has an important role in numerous cellular mechanisms, including cancer. A well-known example is the regulatory role of proline hydroxylation of the transcription factor HIF1 alpha depending upon oxygen supply.

Proline (p) hydroxylation of PKM2 at 403 and 408 by the PHD3 enzyme (**Figure 4**) favors the interaction of PKM2 with the HIF1 transcription complex, which results in recruitment of p300-acetyltransferase to facilitate the transactivation of HIF target genes (21). Another study by Wang et al. revealed that under hypoxic conditions, JMJD5 interacts with PKM2 and facilitates its nuclear localization to promote HIF1 mediated transcriptional activation of glycolytic enzymes that support cancer cell metabolism and proliferation (47) (**Figure 4**).

OXIDATION

High reactive oxygen species (ROS) concentrations induce an oxidation of PKM2 at Cysteine 358 which results in a dissociation of PKM2 to the less active dimeric form. The functional characterization of PKM2 oxidation revealed an essential role in the conservation of the redox homeostasis in cancer cells. The enzymatically inert C358 oxidized PKM2 causes an accumulation of glycolytic intermediates which are required to channel glycolytic carbons into PPP thereby generating NADPH + H^+ for detoxification of ROS (63) (**Figure 5**). Besides hypoxia and addition of either hydrogen peroxide or thiol oxidizing diamide into the cultivation medium of tumor cells also insulin is described to increase intracellular ROS production and cysteine oxidation of PKM2 (23).

UBIQUITINATION AND SUMOYLATION

M2 isoform of pyruvate kinase has been shown to interact with the HECT domain (a protein domain found in ubiquitin-protein ligases) of HERC1, a giant protein with a molecular weight of 532 kDa, which acts as both an E3 ubiquitin ligase and a guanine nucleotide exchange factor. The interaction between PKM2 and HERC1 neither altered PKM2 activity nor induced its proteasomal degradation (64). Since PKM2 also phosphorylates GDP, a possible role of PKM2 as a local GTP producer for the guanine nucleotide exchange function of the RLD1 (RCC1-like domain) of HERC 1 is discussed. Another E3-ligase which interacts with PKM2 is Parkin. Monoubiquitination of PKM2 and PKM1 at lysine (K) 186 and 206 residues by Parkin resulted in a reduction of PKM activity but did not affect its stability. The interaction between PKM2 and Parkin was favored by glucose starvation. Parkin silencing rescued the activity of PK and supported tumor growth (65).

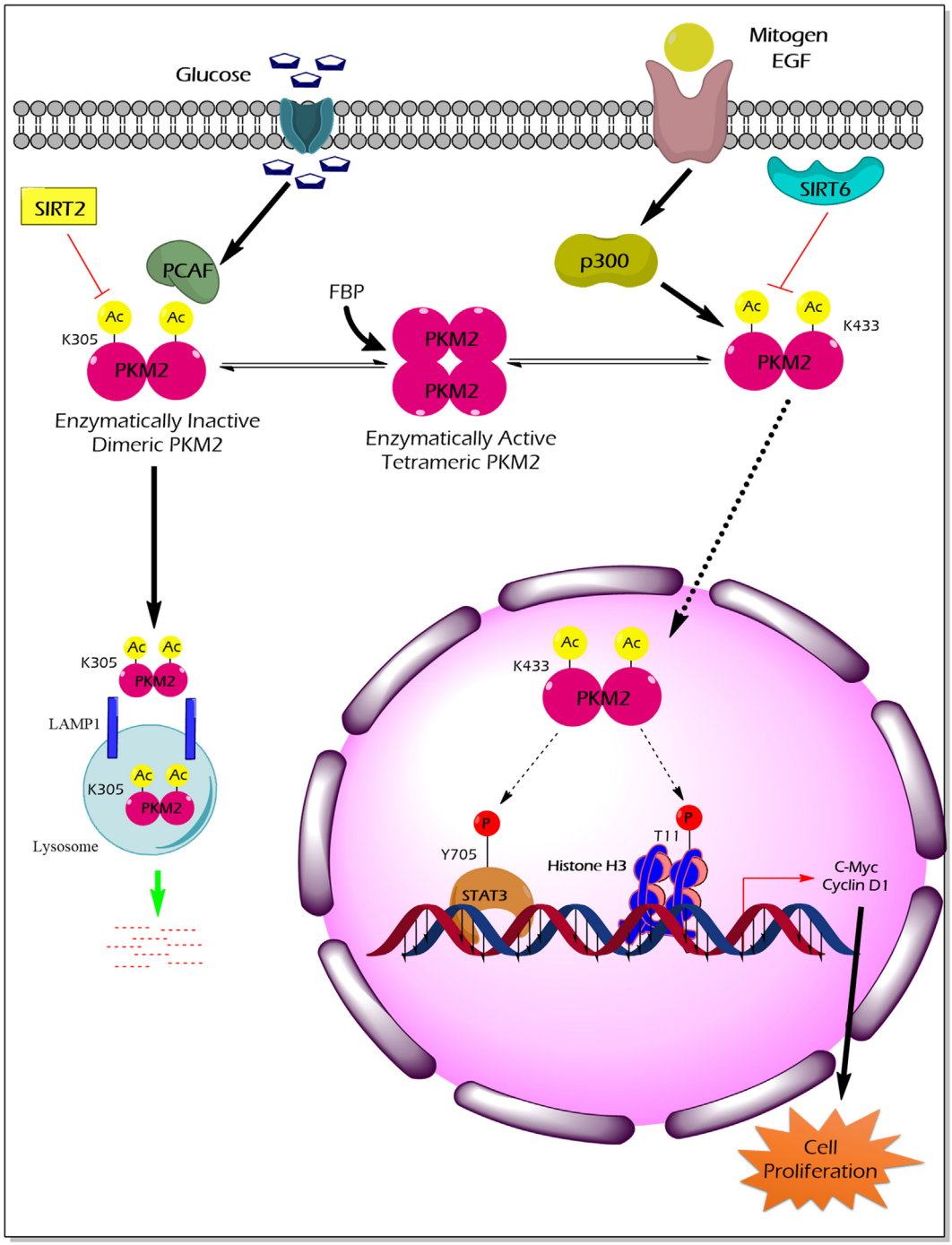


FIGURE 3 | The impact of PKM2 acetylation on its enzymatic activity, protein stability, nuclear localization, and non-glycolytic functions. High glucose concentration facilitates the acetylation of PKM2 lysine (K) 305 residue by PCAF acetyltransferase. K305-acetylated PKM2 exhibits reduced enzyme activity and undergoes lysosomal degradation through chaperon-mediated autophagy. Low PKM2 expression and reduced enzyme activity support the anabolic metabolism and rapid division of tumor cells. Mitogens and oncogenic signals elicit acetyltransferase-p300 to acetylate K433 residues of PKM2, resulting in the release of the allosteric activator FBP from the PKM2 protein, translocation of PKM2 into the nucleus, and activation of the protein kinase activity of PKM2. Inside the nucleus, K433-acetylated PKM2 protein kinase phosphorylates STAT3 at the Y705 residue and histone H3 at T11, thereby activating the transcriptional program that the supports cell proliferation and tumorigenesis. SIRT2 deacetylates K305 residue, and SIRT6 deacetylates K433 residue of PKM2, thereby impairing the tumor-promoting property of PKM2. Abbreviations: EGF, epidermal growth factor; PCAF, P300/CBP-associated factor; p300, histone acetyltransferase; Ac, acetylation; SIRT2, NAD-dependent deacetylase sirtuin-2; SIRT6, NAD-dependent deacetylase sirtuin-6; FBP, fructose 1, 6-bisphosphate; STAT3, signal transducer and activator of transcription 3; LAMP1, lysosomal-associated membrane protein 1; PKM2, M2 isoform of pyruvate kinase.

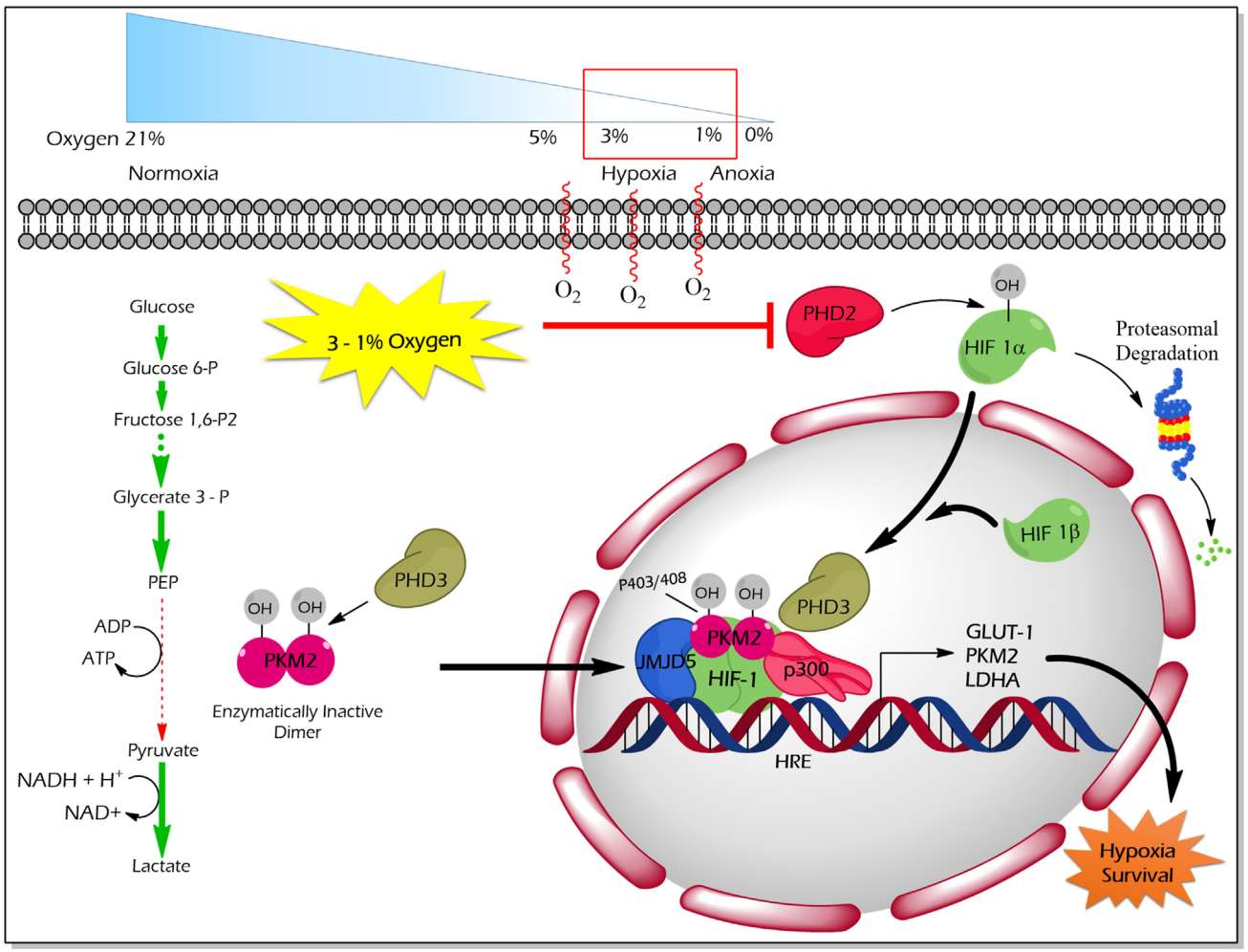


FIGURE 4 | Regulation of non-glycolytic nuclear functions of PKM2 by proline hydroxylation. PHD3-dependent proline hydroxylated PKM2 (p402 and p408 residues) interacts with HIF-1 in the nucleus. PKM2 recruits p300 to HIF1 transcription complex and facilitate the transcription of target genes, which govern the metabolic reprogramming as well as the tolerance versus hypoxic condition. JMJD5 interaction with PKM2 assists the latter to localize into the nucleus and to promote HIF1-mediated transcriptional activation of glycolytic enzymes under hypoxic conditions. Abbreviations: OH, hydroxylation; HIF1, hypoxic inducible factor 1; PHD3, prolyl hydroxylase 3; JMJD5, Jumonji C domain-containing dioxygenase; p300, histone acetyltransferase; HRE, hypoxic response element; GLUT1, glucose transporter 1; LDHA, lactate dehydrogenase A; PKM2, M2 isoform of pyruvate kinase.

In addition, the laforin/malin E3-ubiquitin ligase complex, which is mutated in neurodegenerative Lafora disease, interacts and polyubiquitinates both M-isoforms of PK. When accessed for the biochemical properties, the enzyme activity of the PKM isoforms remained unaffected. Interestingly, this posttranslational modification impairs the nuclear localization of PKM2 following UV treatment. In the case of PKM1 neither UV treatment nor laforin/malin-mediated polyubiquitination affects its nuclear localization (66). Apart from interacting with E3 ubiquitin ligases, PKM2 does also bind the deubiquitin enzyme USP20 (ubiquitin-specific protease, a deubiquitin enzyme). USP20 interaction with PKM2 was suggested to apparently enhance its protein stability (67). The interaction of PKM2 with SUMO-E3 ligase PIAS3 (protein inhibitor of activated STAT3) induces sumoylation of PKM2 and translocation of the PKM2–PIAS3 complex into the nucleus. The functional significance of the interaction between PKM2 and PIAS3 as well as of the nuclear localization of the complex remains ambiguous (68). In summary, the functional relevance of the interaction of PKM2 with E3-ligases and deubiquitin enzymes as well as with SUMO E3-ligases is still not completely understood.

GLYCOSYLATION

Threonine 405 and serine 406 residues of PKM2 were recently identified to be O-GlcNAcylated (*O*-linked β-*N*-acetylglucosamine) by O-GlcNAc transferase and this modification is dynamically regulated by the fluctuations in nutrient supply. Investigation of various human cancer cells and sporadic breast tumor tissue samples revealed PKM2 *O*-GlcNAcylation across the tumor cell types and patient samples. PKM2 T405 and S406 O-GlcNAc modification reduces PK activity by acting as a barrier that

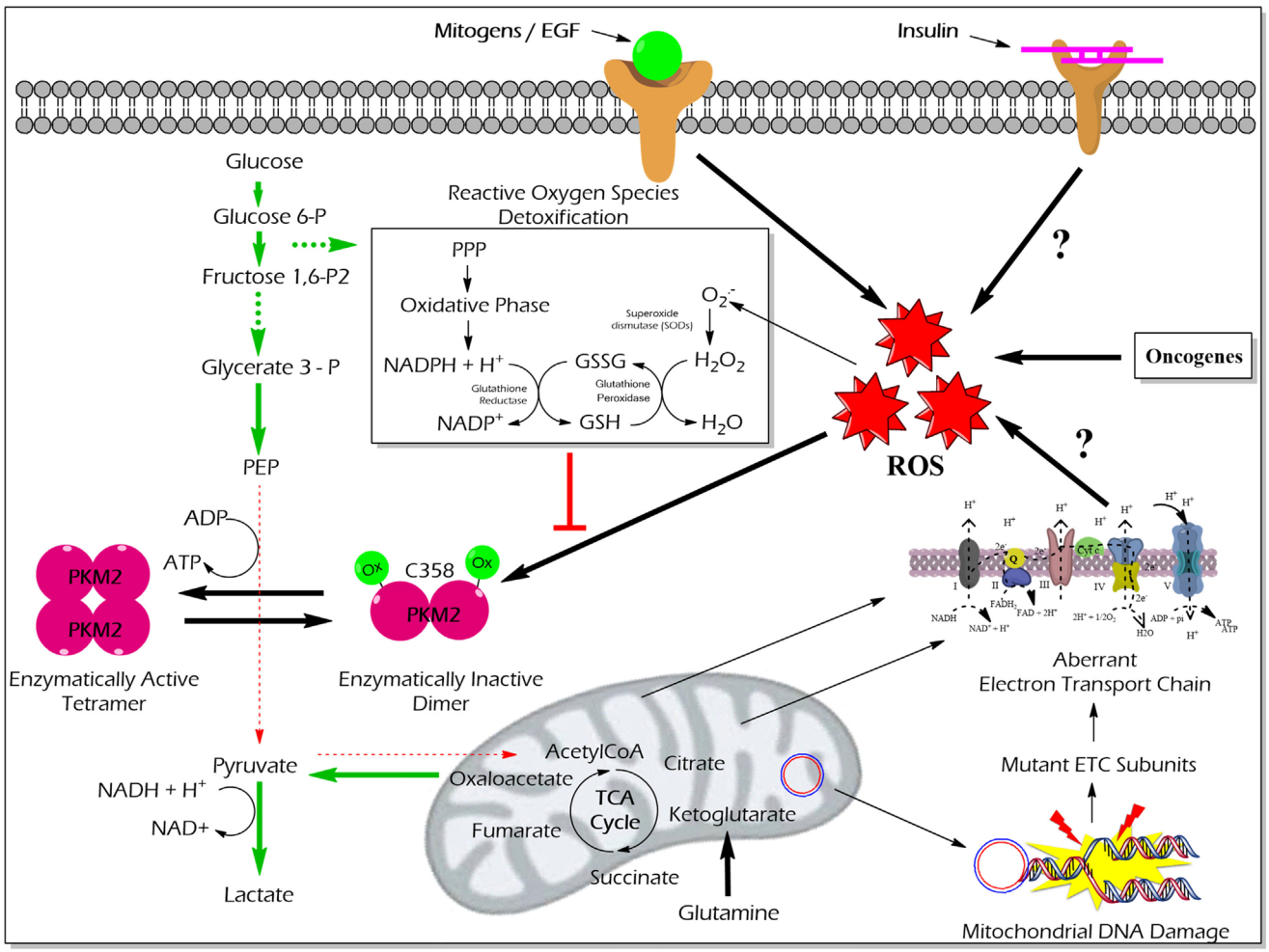


FIGURE 5 | Effect of ROS-mediated oxidization of PKM2 on cellular metabolism and redox balance. Cancer cells produce large amounts of ROS that oxidize the cysteine (C) 358 residue of PKM2, which leads to the inhibition of PKM2 as well as a piling up of glycolytic intermediate glucose 6-phosphate which is then diverted into the PPP. The oxidative PPP facilitates the synthesis of NADPH + H^+ which regenerates GSH thereby minimizing ROS-induced damages of the cancer cells. Abbreviations: ROS, reactive oxygen species; PPP, pentose phosphate pathway; NADPH, nicotinamide adenine dinucleotide phosphate; GSH, glutathione; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; ETC, electron transport chain; PKM2, M2 isoform of pyruvate kinase; PPP, pentose phosphate pathway.

impedes the formation of stabilizing H-bonds at the C–C dimerization interface, thereby, destabilizing PKM2 active tetramer into enzymatically less active dimer. The state of reduced PK activity redirects the glycolytic metabolic intermediates to the anabolic metabolic pathway to support the synthesis of biomass and tumor proliferation. In addition, *O*-GlcNAcylation of PKM2 facilitates its translocation into the nucleus and the latter requires additional Ser37 phosphorylation of PKM2, mediated by EGF-stimulated ERK1/2 protein kinase and importin α5. O-GlcNAcylated, nuclear PKM2 activates transcription factor c-Myc to express GLUT1 and LHDA to further fine-tune aerobic glycolysis. The expression of *O*-GlcNAcylation-ablated PKM2 (T405A/S406A) reverses the pro-cancer metabolism and abrogates the tumor progression (69). Notably, this study highlights the crosstalk between PKM2 PTMs Thr405/Ser406 *O*-GlcNAcylation, and Ser37 phosphorylation is mutually inclusive to translocate PKM2 into the nucleus for executing non-glycolytic nuclear function (69).

METHYLATION

Co-activator-associated arginine methyltransferase 1 also known as PRMT4 methylates specifically the dimeric form of PKM2 at Arg445/447/455 residues in the C domain. TEPP-46 and FBP, the allosteric activators that induce PKM2 tetramerization limits PKM2 methylation. Importantly, PKM2 activity remains unaltered by methylation; however, methylated PKM2 reprograms the metabolic phenotype toward aerobic glycolysis from oxidative phosphorylation to support tumor cell proliferation, migration, and metastasis. Mechanistically, methylated PKM2 localizes to the mitochondrial-associated endoplasmic reticulum membrane to interact with inositol 1, 4, 5-trisphosphate receptors ($InsP_3Rs$) and to reduce $InsP_3R$ expression, thus, decreasing the mitochondrial membrane potential and Ca^{2+} uptake. This is essential to support TCA cycle and oxidative phosphorylation by activating the pyruvate dehydrogenase in Ca^{2+}-dependent processes. The tumor supportive features of methylated

TABLE 1 | The effect of various posttranslation modifications (PTM) of M2 isoform of pyruvate kinase (PKM2) on tumor metabolism and growth.

PTM	Modifying enzymes	Site of PTM	Effect on tumor metabolism and growth	Non-metabolic nuclear function	PKM2 knockdown (or) PTM-mimetic/ ablative mutations	Reference
Tyrosine phosphorylation	pp60 v-src	Not characterized	Promotes aerobic glycolysis and tumor growth	Not characterized	Not characterized	(49, 50)
Tyrosine phosphorylation	FGFR1, BCR-ABL, and JAK-2	Tyr105	Promotes aerobic glycolysis and tumor growth	Not characterized	Regressed tumor growth	(4)
Serine phosphorylation	Extracellular signal-regulated kinase 2 and extracellular matrix protein 1	Ser37	Promotes aerobic glycolysis and tumor growth	Stimulates expression of c-Myc target genes [glucose transporter 1 (GLUT1) and lactate dehydrogenase A (LDHA)]	Regressed tumor growth	(54, 55)
Serine phosphorylation	AKT	Ser202	Promotes aerobic glycolysis and tumor growth	Stimulates expression of cyclin D1	Regressed tumor cell growth in culture	(56)
Threonine phosphorylation	Proviral insertion in murine lymphomas 2	Thr 454	Promotes aerobic glycolysis and tumor growth	Stimulates expression of hypoxia-inducible factor-1α (HIF-1α) and β-catenin target genes	Regressed tumor cell growth in culture	(58)
Lysine acetylation	PCAF	Lys305	Promotes aerobic glycolysis and tumor growth	Not characterized	PTM mimetic mutation (K305Q) promoted tumor growth	(59)
Lysine acetylation	p300 acetyl transferase	Lys433	Promotes aerobic glycolysis and tumor growth	Phosphorylates signal transducer and activator of transcription 3 and H3	PTM mimetic mutation (K433Q) promoted tumor growth and non-acetylated PKM2 (K433R) regressed tumor growth	(60)
Proline hydroxylation	Prolyl hydroxylases 3	Pro 403 and 408	Promotes aerobic glycolysis	Stimulates expression of HIF-1α target genes (GLUT1, PKM2 and LDHA)	No such assays were carried out	(21)
Cysteine oxidation	Reactive oxygen species	Cys358	Promotes aerobic glycolysis and tumor growth	Not characterized	Cysteine oxidation—ablative mutations (C358S) regressed tumor growth	(63)
Ubiquitination	Parkin	Lys186 and 206	Promotes aerobic glycolysis	Not characterized	Not elucidated	(65)
Serine/threonine O-linked glycosylation (*O*-GlcNAcylation)	O-GlcNAc transferase	Thr405 and Ser403	Promotes aerobic glycolysis and tumor growth	Stimulates expression of GLUT1 and LDHA	PTM-ablative mutations (T405A and S406A) regressed tumor growth	(69)
Arginine methylation	Co-activator-associated arginine methyltransferase 1	Arg 445/447/455	Promotes aerobic glycolysis and tumor growth	Not characterized	Regressed tumor growth by delivering competitive non-methylated PKM2 peptide using nanoparticles	(70)

PKM2 were averted by PKM2 knockdown or by introducing PKM2 methylation defective mutant or by delivering competitive non-methylated PKM2 peptide using nanoparticles (70).

CONCLUDING REMARKS

The broad spectrum of PTMs in PKM2 which impart both metabolic and non-metabolic benefits to cancer underlines the significance of this isoenzyme in cancer cells. While PKM2 is expressed not only in tumor tissues but also in some differentiated cells and tissues, the posttranslational modifications of PKM2 are very specific for tumor cells. Accordingly, targeting the different posttranslational modifications of PKM2 is a promising strategy in cancer treatment (**Table 1**). Owing to the heterogeneous nature of tumors, it is important to expand our understanding of the roles of PTMs in PKM2, warranting further investigation and high-throughput PTM data from different tumors in order to refine the clinical targeting of PKM2 PTMs.

AUTHOR CONTRIBUTIONS

GP, MI, RB, and SM equally participated in drafting the review article; RB and SM critically reviewed for important intellectual content; and GP designed all the graphics.

REFERENCES

1. Warburg O. On the origin of cancer cells. *Science* (1956) 123(3191):309–14. doi:10.1126/science.123.3191.309
2. Eigenbrodt E, Reinacher M, Scheefers-Borchel U, Scheefers H, Friis R. Double role for pyruvate kinase type M2 in the expansion of phosphometabolite pools found in tumor cells. *Crit Rev Oncog* (1992) 3(1–2):91–115.
3. Mazurek S, Boschek CB, Hugo F, Eigenbrodt E. Pyruvate kinase type M2 and its role in tumor growth and spreading. *Semin Cancer Biol* (2005) 15(4):300–8. doi:10.1016/j.semcancer.2005.04.009
4. Hitosugi T, Kang S, Vander Heiden MG, Chung TW, Elf S, Lythgoe K, et al. Tyrosine phosphorylation inhibits PKM2 to promote the Warburg effect and tumor growth. *Sci Signal* (2009) 2(97):ra73. doi:10.1126/scisignal.2000431
5. Mazurek S. Pyruvate kinase type M2: a key regulator of the metabolic budget system in tumor cells. *Int J Biochem Cell Biol* (2011) 43(7):969–80. doi:10.1016/j.biocel.2010.02.005
6. Chaneton B, Gottlieb E. Rocking cell metabolism: revised functions of the key glycolytic regulator PKM2 in cancer. *Trends Biochem Sci* (2012) 37(8):309–16. doi:10.1016/j.tibs.2012.04.003
7. Gupta V, Bamezai RN. Human pyruvate kinase M2: a multifunctional protein. *Protein Sci* (2010) 19(11):2031–44. doi:10.1002/pro.505
8. Iqbal MA, Gupta V, Gopinath P, Mazurek S, Bamezai RN. Pyruvate kinase M2 and cancer: an updated assessment. *FEBS Lett* (2014) 588(16):2685–92. doi:10.1016/j.febslet.2014.04.011
9. Prakasam G, Iqbal MA, Gupta V, Kumar B, Bamezai RNK. Pyruvate Kinase M2. In: Choi S, editor. *Encyclopedia of Signaling Molecules*. Cham: Springer International Publishing (2018). p. 4323–33. doi:10.1007/978-3-319-67199-4
10. Torres MP, Dewhurst H, Sundararaman N. Proteome-wide structural analysis of PTM hotspots reveals regulatory elements predicted to impact biological function and disease. *Mol Cell Proteomics* (2016) 15(11):3513–28. doi:10.1074/mcp.M116.062331
11. Harada K, Saheki S, Wada K, Tanaka T. Purification of four pyruvate kinase isozymes of rats by affinity elution chromatography. *Biochim Biophys Acta* (1978) 524(2):327–39. doi:10.1016/0005-2744(78)90169-9
12. Noguchi T, Yamada K, Inoue H, Matsuda T, Tanaka T. The L- and R-type isozymes of rat pyruvate kinase are produced from a single gene by use of different promoters. *J Biol Chem* (1987) 262(29):14366–71.
13. Noguchi T, Inoue H, Tanaka T. The M1- and M2-type isozymes of rat pyruvate kinase are produced from the same gene by alternative RNA splicing. *J Biol Chem* (1986) 261(29):13807–12.
14. Hacker HJ, Steinberg P, Bannasch P. Pyruvate kinase isoenzyme shift from L-type to M2-type is a late event in hepatocarcinogenesis induced in rats by a choline-deficient/DL-ethionine-supplemented diet. *Carcinogenesis* (1998) 19(1):99–107. doi:10.1093/carcin/19.1.99
15. Steinberg P, Klingelhoffer A, Schafer A, Wust G, Weisse G, Oesch F, et al. Expression of pyruvate kinase M2 in preneoplastic hepatic foci of N-nitrosomorpholine-treated rats. *Virchows Arch* (1999) 434(3):213–20. doi:10.1007/s004280050330
16. Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wei R, et al. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature* (2008) 452(7184):230–3. doi:10.1038/nature06734
17. Brinck U, Eigenbrodt E, Oehmke M, Mazurek S, Fischer G. L- and M2-pyruvate kinase expression in renal cell carcinomas and their metastases. *Virchows Arch* (1994) 424(2):177–85. doi:10.1007/BF00193498
18. Bluemlein K, Gruning NM, Feichtinger RG, Lehrach H, Kofler B, Ralser M. No evidence for a shift in pyruvate kinase PKM1 to PKM2 expression during tumorigenesis. *Oncotarget* (2011) 2(5):393–400. doi:10.18632/oncotarget.278
19. David CJ, Chen M, Assanah M, Canoll P, Manley JL. HnRNP proteins controlled by c-Myc deregulate pyruvate kinase mRNA splicing in cancer. *Nature* (2010) 463(7279):364–8. doi:10.1038/nature08697
20. Li Z, Yang P, Li Z. The multifaceted regulation and functions of PKM2 in tumor progression. *Biochim Biophys Acta* (2014) 1846(2):285–96. doi:10.1016/j.bbcan.2014.07.008
21. Luo W, Hu H, Chang R, Zhong J, Knabel M, O'Meally R, et al. Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. *Cell* (2011) 145(5):732–44. doi:10.1016/j.cell.2011.03.054
22. Sun Q, Chen X, Ma J, Peng H, Wang F, Zha X, et al. Mammalian target of rapamycin up-regulation of pyruvate kinase isoenzyme type M2 is critical for aerobic glycolysis and tumor growth. *Proc Natl Acad Sci U S A* (2011) 108(10):4129–34. doi:10.1073/pnas.1014769108
23. Iqbal MA, Siddiqui FA, Gupta V, Chattopadhyay S, Gopinath P, Kumar B, et al. Insulin enhances metabolic capacities of cancer cells by dual regulation of glycolytic enzyme pyruvate kinase M2. *Mol Cancer* (2013) 12:72. doi:10.1186/1476-4598-12-72
24. Yang W, Xia Y, Cao Y, Zheng Y, Bu W, Zhang L, et al. EGFR-induced and PKCepsilon monoubiquitylation-dependent NF-kappaB activation upregulates PKM2 expression and promotes tumorigenesis. *Mol Cell* (2012) 48(5):771–84. doi:10.1016/j.molcel.2012.09.028
25. Panasyuk G, Espeillac C, Chauvin C, Pradelli LA, Horie Y, Suzuki A, et al. PPARgamma contributes to PKM2 and HK2 expression in fatty liver. *Nat Commun* (2012) 3:672. doi:10.1038/ncomms1667
26. Asai Y, Yamada K, Watanabe T, Keng VW, Noguchi T. Insulin stimulates expression of the pyruvate kinase M gene in 3T3-L1 adipocytes. *Biosci Biotechnol Biochem* (2003) 67(6):1272–7. doi:10.1271/bbb.67.1272
27. Li Q, Liu X, Yin Y, Zheng JT, Jiang CF, Wang J, et al. Insulin regulates glucose consumption and lactate production through reactive oxygen species and pyruvate kinase M2. *Oxid Med Cell Longev* (2014) 2014:504953. doi:10.1155/2014/504953
28. Ashizawa K, Fukuda T, Cheng SY. Transcriptional stimulation by thyroid hormone of a cytosolic thyroid hormone binding protein which is homologous to a subunit of pyruvate kinase M1. *Biochemistry* (1992) 31(10):2774–8. doi:10.1021/bi00125a018
29. Saheki S, Saheki K, Tanaka T. Changes in pyruvate kinase isozymes of rat small intestine during development and the synergistic effect on them of thyroid and glucocorticoid hormones. *Enzyme* (1979) 24(1):8–17. doi:10.1159/000458623
30. Netzker R, Weigert C, Brand K. Role of the stimulatory proteins Sp1 and Sp3 in the regulation of transcription of the rat pyruvate kinase M gene. *Eur J Biochem* (1997) 245(1):174–81. doi:10.1111/j.1432-1033.1997.00174.x
31. Schafer D, Hamm-Kunzelmann B, Brand K. Glucose regulates the promoter activity of aldolase A and pyruvate kinase M2 via dephosphorylation of Sp1. *FEBS Lett* (1997) 417(3):325–8. doi:10.1016/S0014-5793(97)01314-8
32. Desai S, Ding M, Wang B, Lu Z, Zhao Q, Shaw K, et al. Tissue-specific isoform switch and DNA hypomethylation of the pyruvate kinase PKM gene in human cancers. *Oncotarget* (2014) 5(18):8202–10. doi:10.18632/oncotarget.1159

33. Wong TS, Liu XB, 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(2):251–7. doi:10.1002/ijc.23583
34. 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(11):1102–12. doi:10.1093/neuonc/noq080
35. Liu AM, Xu Z, Shek FH, Wong KF, Lee NP, Poon RT, et al. miR-122 targets pyruvate kinase M2 and affects metabolism of hepatocellular carcinoma. *PLoS One* (2014) 9(1):e86872. doi:10.1371/journal.pone.0086872
36. Liu G, Li YI, Gao X. Overexpression of microRNA-133b sensitizes non-small cell lung cancer cells to irradiation through the inhibition of glycolysis. *Oncol Lett* (2016) 11(4):2903–8. doi:10.3892/ol.2016.4316
37. Dombrauckas JD, Santarsiero BD, Mesecar AD. Structural basis for tumor pyruvate kinase M2 allosteric regulation and catalysis. *Biochemistry* (2005) 44(27):9417–29. doi:10.1021/bi0474923
38. Saheki S, Harada K, Sanno Y, Tanaka T. Hybrid isozymes of rat pyruvate kinase. Their subunit structure and developmental changes in the liver. *Biochim Biophys Acta* (1978) 526(1):116–28. doi:10.1016/0005-2744(78)90296-6
39. Staal GEJ, Rijksen G. Pyruvate kinase in selected human tumors. In: Pretlow TG, Pretlow TP. *Biochemical and Molecular Aspects of Selected Cancers*, (Chap. 10). San Diego: Academic Press (1991). p. 313–37.
40. Prakasam G, Singh RK, Iqbal MA, Saini SK, Tiku AB, Bamezai RNK. Pyruvate kinase M knockdown-induced signaling via AMP-activated protein kinase promotes mitochondrial biogenesis, autophagy, and cancer cell survival. *J Biol Chem* (2017) 292(37):15561–76. doi:10.1074/jbc.M117.791343
41. Ashizawa K, Willingham MC, Liang CM, Cheng SY. In vivo regulation of monomer-tetramer conversion of pyruvate kinase subtype M2 by glucose is mediated via fructose 1,6-bisphosphate. *J Biol Chem* (1991) 266(25):16842–6.
42. Chaneton B, Hillmann P, Zheng L, Martin ACL, Maddocks ODK, Chokkathukalam A, et al. Serine is a natural ligand and allosteric activator of pyruvate kinase M2. *Nature* (2012) 491(7424):458–62. doi:10.1038/nature11540
43. Keller KE, Tan IS, Lee YS. SAICAR stimulates pyruvate kinase isoform M2 and promotes cancer cell survival in glucose-limited conditions. *Science* (2012) 338(6110):1069–72. doi:10.1126/science.1224409
44. Zwerschke W, Mazurek S, Massimi P, Banks L, Eigenbrodt E, Jansen-Durr P. Modulation of type M2 pyruvate kinase activity by the human papillomavirus type 16 E7 oncoprotein. *Proc Natl Acad Sci U S A* (1999) 96(4):1291–6. doi:10.1073/pnas.96.4.1291
45. Shimada N, Shinagawa T, Ishii S. Modulation of M2-type pyruvate kinase activity by the cytoplasmic PML tumor suppressor protein. *Genes Cells* (2008) 13(3):245–54. doi:10.1111/j.1365-2443.2008.01165.x
46. Kosugi M, Ahmad R, Alam M, Uchida Y, Kufe D. MUC1-C oncoprotein regulates glycolysis and pyruvate kinase M2 activity in cancer cells. *PLoS One* (2011) 6(11):e28234. doi:10.1371/journal.pone.0028234
47. Wang HJ, Hsieh YJ, Cheng WC, Lin CP, Lin YS, Yang SF, et al. JMJD5 regulates PKM2 nuclear translocation and reprograms HIF-1alpha-mediated glucose metabolism. *Proc Natl Acad Sci U S A* (2014) 111(1):279–84. doi:10.1073/pnas.1311249111
48. Mor I, Carlessi R, Ast T, Feinstein E, Kimchi A. Death-associated protein kinase increases glycolytic rate through binding and activation of pyruvate kinase. *Oncogene* (2012) 31(6):683–93. doi:10.1038/onc.2011.264
49. Presek P, Reinacher M, Eigenbrodt E. Pyruvate kinase type M2 is phosphorylated at tyrosine residues in cells transformed by Rous sarcoma virus. *FEBS Lett* (1988) 242(1):194–8. doi:10.1016/0014-5793(88)81014-7
50. Eigenbrodt E, Mazurek S, Friis RR. Double role of pyruvate kinase type M2 in the regulation of phosphometabolite pools. In: Bannasch P, Kanduc D, Papa S, Tager JM, editors. *Cell Growth and Oncogenesis*. Basel: Birkhäuser (1998). p. 15–30.
51. Bettaieb A, Bakke J, Nagata N, Matsuo K, Xi Y, Liu S, et al. Protein tyrosine phosphatase 1B regulates pyruvate kinase M2 tyrosine phosphorylation. *J Biol Chem* (2013) 288(24):17360–71. doi:10.1074/jbc.M112.441469
52. Christofk HR, Vander Heiden MG, Wu N, Asara JM, Cantley LC. Pyruvate kinase M2 is a phosphotyrosine-binding protein. *Nature* (2008) 452(7184):181–6. doi:10.1038/nature06667
53. Yang W, Xia Y, Ji H, Zheng Y, Liang J, Huang W, et al. Nuclear PKM2 regulates beta-catenin transactivation upon EGFR activation. *Nature* (2011) 480(7375):118–22. doi:10.1038/nature10598
54. Yang W, Zheng Y, Xia Y, Ji H, Chen X, Guo F, et al. ERK1/2-dependent phosphorylation and nuclear translocation of PKM2 promotes the Warburg effect. *Nat Cell Biol* (2012) 14(12):1295–304. doi:10.1038/ncb2629
55. Lee KM, Nam K, Oh S, Lim J, Lee T, Shin I. ECM1 promotes the Warburg effect through EGF-mediated activation of PKM2. *Cell Signal* (2015) 27(2):228–35. doi:10.1016/j.cellsig.2014.11.004
56. Park YS, Kim DJ, Koo H, Jang SH, You YM, Cho JH, et al. AKT-induced PKM2 phosphorylation signals for IGF-1-stimulated cancer cell growth. *Oncotarget* (2016) 7(30):48155–67. doi:10.18632/oncotarget.10179
57. Mazurek S, Drexler HC, Troppmair J, Eigenbrodt E, Rapp UR. Regulation of pyruvate kinase type M2 by A-Raf: a possible glycolytic stop or go mechanism. *Anticancer Res* (2007) 27(6B):3963–71.
58. Yu Z, Zhao X, Huang L, Zhang T, Yang F, Xie L, et al. Proviral insertion in murine lymphomas 2 (PIM2) oncogene phosphorylates pyruvate kinase M2 (PKM2) and promotes glycolysis in cancer cells. *J Biol Chem* (2013) 288(49):35406–16. doi:10.1074/jbc.M113.508226
59. Lv L, Li D, Zhao D, Lin R, Chu Y, Zhang H, et al. Acetylation targets the M2 isoform of pyruvate kinase for degradation through chaperone-mediated autophagy and promotes tumor growth. *Mol Cell* (2011) 42(6):719–30. doi:10.1016/j.molcel.2011.04.025
60. Lv L, Xu Y-PP, Zhao D, Li F-LL, Wang W, Sasaki N, et al. Mitogenic and oncogenic stimulation of K433 acetylation promotes PKM2 protein kinase activity and nuclear localization. *Mol Cell* (2013) 52(3):340–52. doi:10.1016/j.molcel.2013.09.004
61. Bhardwaj A, Das S. SIRT6 deacetylates PKM2 to suppress its nuclear localization and oncogenic functions. *Proc Natl Acad Sci U S A* (2016) 113(5):E538–47. doi:10.1073/pnas.1520045113
62. Park SH, Ozden O, Liu G, Song HY, Zhu Y, Yan Y, et al. SIRT2-mediated deacetylation and tetramerization of pyruvate kinase directs glycolysis and tumor growth. *Cancer Res* (2016) 76(13):3802–12. doi:10.1158/0008-5472.CAN-15-2498
63. Anastasiou D, Poulogiannis G, Asara JM, Boxer MB, Jiang J-KK, Shen M, et al. Inhibition of pyruvate kinase M2 by reactive oxygen species contributes to cellular antioxidant responses. *Science* (2011) 334(6060):1278–83. doi:10.1126/science.1211485
64. Garcia-Gonzalo FR, Cruz C, Munoz P, Mazurek S, Eigenbrodt E, Ventura F, et al. Interaction between HERC1 and M2-type pyruvate kinase. *FEBS Lett* (2003) 539(1–3):78–84. doi:10.1016/S0014-5793(03)00205-9
65. Liu K, Li F, Han H, Chen Y, Mao Z, Luo J, et al. Parkin regulates the activity of pyruvate kinase M2. *J Biol Chem* (2016) 291(19):10307–17. doi:10.1074/jbc.M115.703066
66. Viana R, Lujan P, Sanz P. The laforin/malin E3-ubiquitin ligase complex ubiquitinates pyruvate kinase M1/M2. *BMC Biochem* (2015) 16:24. doi:10.1186/s12858-015-0053-6
67. Kim SR, Kim JO, Lim KH, Yun JH, Han I, Baek KH. Regulation of pyruvate kinase isozyme M2 is mediated by the ubiquitin-specific protease 20. *Int J Oncol* (2015) 46(5):2116–24. doi:10.3892/ijo.2015.2901
68. Spoden GA, Morandell D, Ehehalt D, Fiedler M, Jansen-Durr P, Hermann M, et al. The SUMO-E3 ligase PIAS3 targets pyruvate kinase M2. *J Cell Biochem* (2009) 107(2):293–302. doi:10.1002/jcb.22125
69. Wang Y, Liu J, Jin X, Zhang D, Li D, Hao F, et al. O-GlcNAcylation destabilizes the active tetrameric PKM2 to promote the Warburg effect. *Proc Natl Acad Sci U S A* (2017) 114(52):13732–7. doi:10.1073/pnas.1704145115
70. Liu F, Ma F, Wang Y, Hao L, Zeng H, Jia C, et al. PKM2 methylation by CARM1 activates aerobic glycolysis to promote tumorigenesis. *Nat Cell Biol* (2017) 19(11):1358–70. doi:10.1038/ncb3630

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer LF and the handling editor declared their shared affiliation.

frontiers in Oncology

MINI REVIEW
published: 05 February 2018
doi: 10.3389/fonc.2018.00015

DNA Damage, Repair, and Cancer Metabolism

*Marc-Olivier Turgeon[1], Nicholas J. S. Perry[1] and George Poulogiannis[1,2]**

[1] Department of Cancer Biology, Institute of Cancer Research, London, United Kingdom, [2] Division of Computational and Systems Medicine, Department of Surgery and Cancer, Imperial College London, London, United Kingdom

Although there has been a renewed interest in the field of cancer metabolism in the last decade, the link between metabolism and DNA damage/DNA repair in cancer has yet to be appreciably explored. In this review, we examine the evidence connecting DNA damage and repair mechanisms with cell metabolism through three principal links. (1) Regulation of methyl- and acetyl-group donors through different metabolic pathways can impact DNA folding and remodeling, an essential part of accurate double strand break repair. (2) Glutamine, aspartate, and other nutrients are essential for *de novo* nucleotide synthesis, which dictates the availability of the nucleotide pool, and thereby influences DNA repair and replication. (3) Reactive oxygen species, which can increase oxidative DNA damage and hence the load of the DNA-repair machinery, are regulated through different metabolic pathways. Interestingly, while metabolism affects DNA repair, DNA damage can also induce metabolic rewiring. Activation of the DNA damage response (DDR) triggers an increase in nucleotide synthesis and anabolic glucose metabolism, while also reducing glutamine anaplerosis. Furthermore, mutations in genes involved in the DDR and DNA repair also lead to metabolic rewiring. Links between cancer metabolism and DNA damage/DNA repair are increasingly apparent, yielding opportunities to investigate the mechanistic basis behind potential metabolic vulnerabilities of a substantial fraction of tumors.

Keywords: metabolism, DNA repair, DNA damage, cancer, reactive oxygen species

OPEN ACCESS

Edited by:
Leonardo Freire-de-Lima, Universidade Federal do Rio de Janeiro, Brazil

Reviewed by:
Daniele Vergara, University of Salento, Italy
Bernd Groner, Georg Speyer Haus, Germany
Vandna Kukshal, Washington University in St. Louis, United States

****Correspondence:***
George Poulogiannis
george.poulogiannis@icr.ac.uk

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *15 December 2017*
Accepted: *17 January 2018*
Published: *05 February 2018*

Citation:
Turgeon MO, Perry NJ and Poulogiannis G (2018) DNA Damage, Repair, and Cancer Metabolism. Front. Oncol. 8:15. doi: 10.3389/fonc.2018.00015

INTRODUCTION

Over the past decade, a renewed interest in cancer metabolism has emerged. The idea originated from the work of Otto Warburg and colleagues—first published in the 1920s—who noticed the propensity for cancer cells to consume increased quantities of glucose (1, 2). We now understand that there is extensive metabolic rewiring in cancer cells, and we are starting to decipher how cancer cells reprogram their metabolism to adapt to changes in their microenvironment and support their high metabolic needs (3–5). Cancer metabolism is a broad topic and our current understanding of how metabolic reprogramming is linked to malignant transformation has been extensively reviewed elsewhere (6–8). Therefore, in this review, we decided to focus on the connections between cell metabolism and another major aspect of cancer biology, DNA-repair/DNA-damage pathways.

The major hallmarks, as described by Pavlova and Thompson (7), include: deregulated glucose and amino acid uptake, opportunistic ways of acquiring nutrients, use of metabolic intermediates for biomass and nicotinamide adenine dinucleotide phosphate (NADPH) synthesis, increased demand for nitrogen, and alterations in metabolite-driven gene expression and interaction with the microenvironment (7, 9). Importantly, the wide differences in metabolic dependencies across cancer types, or stage of progression, as well as the dynamic shifts between metabolic pathways, make the study of cancer metabolism and the development of new therapies targeting these pathways very challenging.

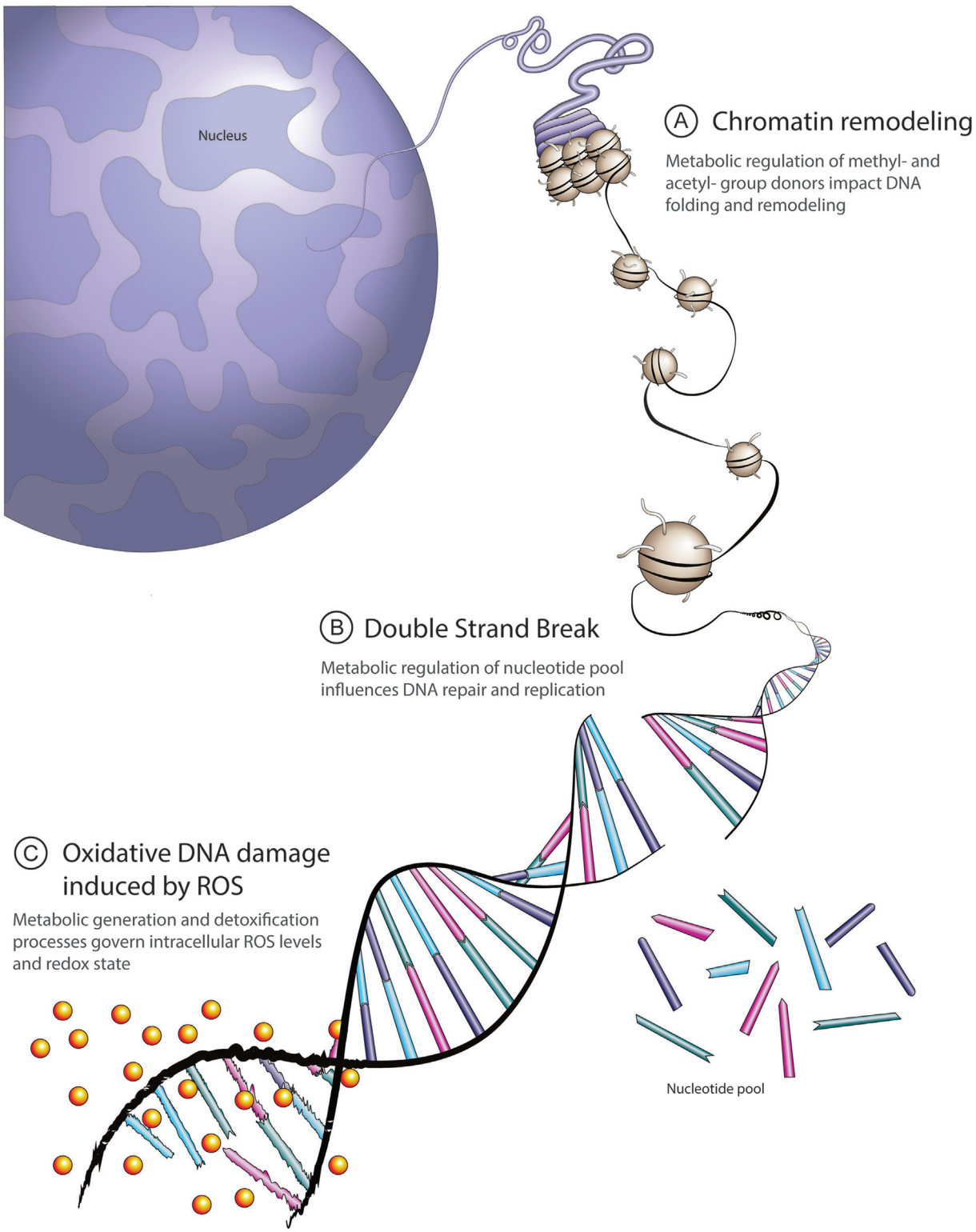


FIGURE 1 | Overview of principal links between cell metabolism and DNA repair. **(A)** Methyl-group donors from the S-adenosylmethionine pathway and acetyl-donors from citrate cycle-derived acetyl coenzyme A contribute to dynamic chromatin packaging and remodeling essential to DNA double-strand repair. **(B)** Metabolic intermediates derived from glucose, glutamine, and aspartate are required for *de novo* nucleotide synthesis. The ready availability of a pool of nucleotides facilitates appropriate DNA repair and replication. **(C)** Intracellular reactive oxygen species (ROS) levels reflect a balance between generation and detoxification. A principal ROS detoxification mechanism involves reduced glutathione (GSH), determined by glutamine and cysteine availability, as well as NADPH levels. High ROS-induced DNA damage leads to excessive burden on the DNA repair machinery.

In addition to the accepted role for cell metabolism in cancer, it is well established that DNA-repair/DNA-damage pathways are important in cancer progression because dysregulation leads to higher levels of genomic instability, increased mutation rate, and enhanced intra-tumor heterogeneity (10–13). There are currently three principal mechanisms through which changes in cell metabolic status are thought to have an influence on DNA-damage/DNA-repair pathways: chromatin remodeling, double-strand break (DSB) repair, and redox homeostasis (**Figure 1**). Uncovering new links between these important aspects of cancer biology might lead to the development of new targeted therapies in DNA-repair deficient cancers or even improving the efficacy of existing therapies, such as PARP inhibitors, anthracyclines, and platinum salts. In this review, we examine work seeking to uncover the links between DNA-repair/DNA-damage and cell metabolism.

Metabolic Status Affects DNA Folding and Repair Pathways

The first link between DNA repair and cell metabolism involves DNA folding and organization. Chromatin packaging and remodeling, through different histone posttranslational modifications—including acetylation, methylation, phosphorylation, and ubiquitination, as well as through DNA modifications such as methylation—can regulate gene expression levels by modulating the access to DNA of different protein complexes (14, 15). Interestingly, similar mechanisms can also regulate access of DNA-repair proteins to the DNA double-helix (16). When repairing DSBs, the first step involves unfolding DNA to allow access of the repair complexes to the DSB (17). There is a growing body of evidence suggesting that these mechanisms can in fact regulate the choice of DNA-repair pathway (i.e., homologous recombination), or non-homologous end-joining used to repair the DSB (16). Substrates added to histones or DNA are derived from metabolic intermediates (18–20) (**Figure 2**). For example, methyl-group donors mostly come from the S-adenosylmethionine (SAM) pathway (18), while the sole acetyl-group donor is acetyl coenzyme A (acetyl-CoA), known for the transfer of its acetyl group to oxaloacetate to form citrate and start the tricarboxylic acid (TCA) cycle (21). It has been shown that expression levels of the enzyme, ATP citrate lyase, can regulate the availability of acetyl-CoA in the cell (22). Restricting the amount of acetyl-group donors can disrupt proper DNA organization and have an impact on DNA folding and DNA remodeling essential to successful DNA DSB repair (22, 23). Acetyl-CoA can also be generated from acetate and CoA from the acetyl-CoA synthetase 2 enzyme. In fact, under metabolic stress conditions in which oxygen and lipids are deprived, acetate-generated acetyl-CoA is enhanced and acetate-derived carbons are incorporated in lipid synthesis (24, 25). Acetate-derived acetyl-CoA has also been shown to be involved in histone acetylation, suggesting that acetate availability could influence histone acetylation in cancer cells (26).

Additionally, changes in the activity of the SAM pathway due to availability of necessary nutrients or substrates also influence DNA or histone methylation by regulating the pool of methyl-donors (27). The SAM pathway is interconnected with methionine, tetrahydrofolate (THF), one-carbon metabolism, and choline pathways. These pathways tend to compensate for one another; however, depletion of choline, folate, or methionine can still have an impact on the final SAM concentration (18). Such changes in the SAM pathway not only influence gene expression of cancer-associated genes through epigenetic modifications but can also impact DNA folding during DNA repair processes. The latter can also be influenced directly by metabolic intermediates such as fumarate. DNA-PK dependent activation of fumarase contributes to enhanced local generation of fumarate, which in turn promotes DNA repair *via* inhibition of KDM2B-mediated histone demethylase activity (28). Accumulation of fumarate can also lead to epithelial-to-mesenchymal transition and poor clinical outcome (29). Since modifications in histone and DNA are important for proper repair of DNA DSBs (30), changes in nutrient and substrate levels that disrupt these modifications are bound to affect DNA-repair pathways.

Nucleotide Levels Affect DNA-Repair Potential of Cancer Cells

Another major mechanism upon which cell metabolism can regulate DNA-repair/DNA-damage is through the regulation of the pool of nucleotides used for DNA replication and repair (31, 32). Many different metabolic pathways are involved in *de novo* nucleotide synthesis and can have an impact on the levels of intracellular nucleotides available (**Figure 2**) (31, 33, 34). An important precursor in the synthesis of the ribose backbone, essential for both purines and pyrimidines, comes from ribose-5-phosphate, an intermediate of the pentose-phosphate pathway (PPP) (31). Briefly, the PPP utilizes glucose-6-phosphate (G6P), an intermediate of glycolysis, which can be redirected to generate metabolic intermediates necessary for nucleotide and protein synthesis, as well as generation of NADPH. The PPP is a major focus in cancer metabolism, and it has been carefully reviewed elsewhere (31, 35, 36). The increased glucose consumption observed in cancer cells has been shown, at least in some cancers, to be used to fuel the PPP, where it can potentially be used to generate reducing power in the form of NADPH and nucleotide precursors (32). Additionally, other metabolic pathways are involved more specifically in purine or pyrimidine ring synthesis. The amide group of glutamine is essential in two steps of inosine monophosphate synthesis, an intermediate in *de novo* purine synthesis, and in one step of uridine monophosphate synthesis, an intermediate in *de novo* pyrimidine synthesis (37, 38). In glioblastoma, it has been shown that α-ketoglutarate is diverted out of the TCA cycle to synthesize more glutamine through the glutamine synthetase enzyme. The resulting glutamine is then used for *de novo* purine synthesis (39). Furthermore, aspartate is essential for the synthesis of pyrimidines, and glycine is also essential for purine synthesis (38). Loss-of-function mutations in arginosuccinate synthase 1, which leads to reduced arginine production in the urea cycle, promotes the use of aspartate for pyrimidine synthesis (33). Aspartate is mostly synthesized from glutamate and oxaloacetate. Therefore, the availability of glutamine and/or other amino acids/metabolic substrates can influence the amount and ratio of nucleotides produced in a cell and might provide a regulatory mechanism for DNA-repair.

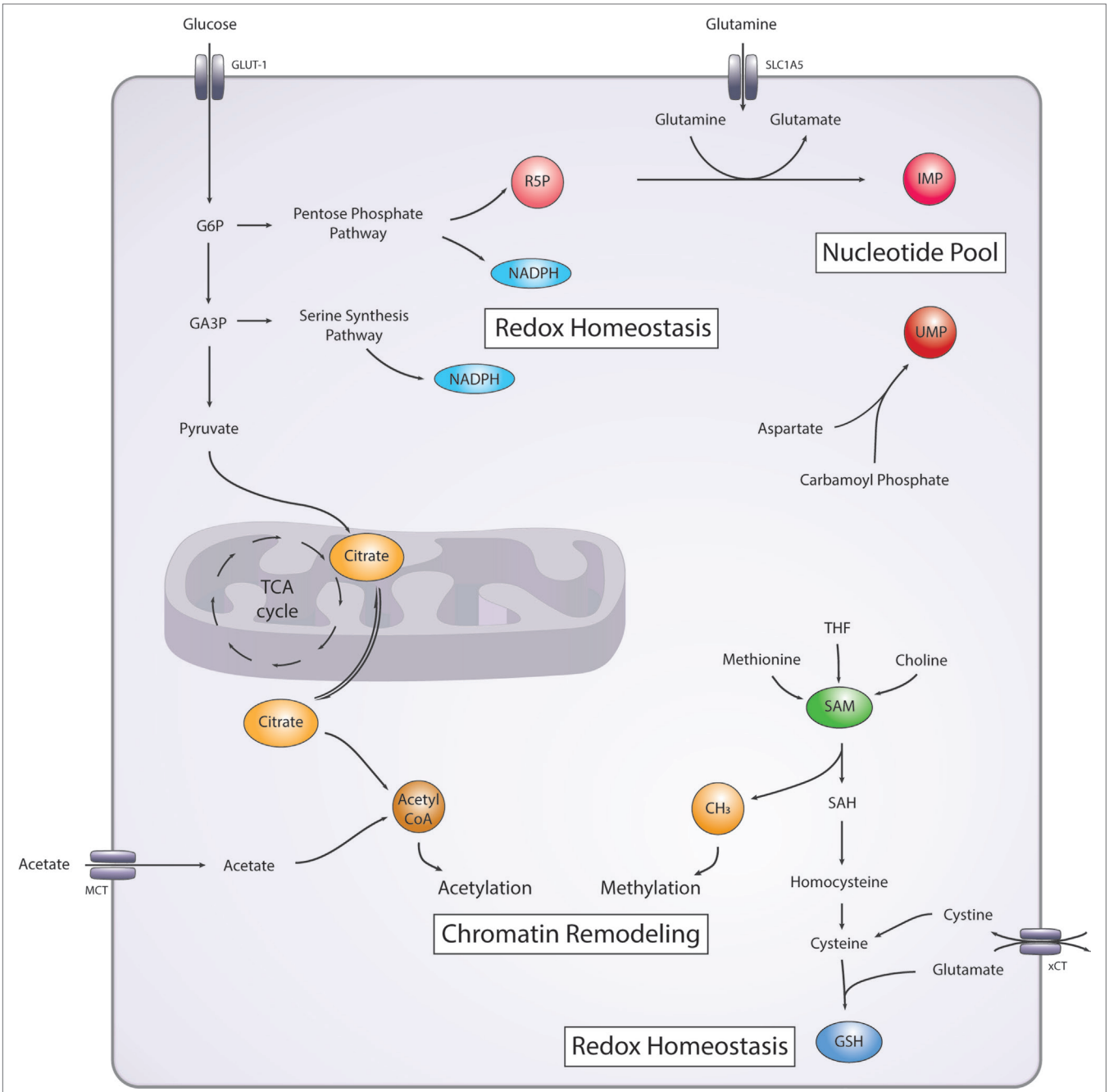


FIGURE 2 | Simplified diagram of the main metabolic pathways involved in DNA damage/repair. **Nucleotide pool:** nucleotide precursor ribose-5-phosphate (R5P) is generated from the pentose-phosphate pathway (PPP). Purines and pyrimidines precursors, inosine monophosphate (IMP), and uridine monophosphate (UMP), respectively are synthesized from glutamine, and aspartate and carbamoyl phosphate, respectively. **Redox homeostasis:** nicotinamide adenine dinucleotide phosphate (NADPH) is generated from the PPP and the serine synthesis pathway. Glutathione (GSH) is generated from cysteine and glutamate. **Chromatin remodeling:** methyl-group donors (CH_3) are generated from the S-adenosyl methionine (SAM) pathway and acetyl-group donors (acetyl coenzyme A) are generated from the citrate cycle or from acetate. GLUT-1, glucose transporter 1; SCL1A5, alanine, serine, cysteine-preferring transporter 2 (ASCT2); G6P, glucose 6-phosphate; GA3P, glyceraldehyde 3-phosphate; SAH, S-adenosyl homocysteine; xCT, cystine-glutamate antiporter; MCT, monocarboxylate transporter family.

Deregulated Redox Homeostasis Promotes DNA Damage

The metabolic regulation of reactive oxygen species (ROS) levels is the third and final link that we address here between DNA-repair/DNA-damage and cell metabolism. High ROS levels affect many aspects of tumor biology and, here, we focus on their role in inducing DNA damage and genomic instability. Most of the DNA lesions that are formed by ROS-induced damage are

single strand breaks (SSBs) that can be repaired through nucleotide or base excision repair (NER/BER) (40). However, these SSBs can lead to stalling of the replication fork or error in replication, ultimately leading to DSBs (40). The accumulation of oxidative DNA damage increases the burden on the DNA-repair machinery. Tight regulation of cellular redox stress is essential, since high ROS levels can lead to oxidative stress and oxidative damage of proteins, DNA, and lipids, while a certain level of ROS is essential for activating signaling pathways involved in multiple biological processes (41–43). Cells have evolved a number of ways to balance ROS levels (**Figure 2**). Glutathione (GSH) is one of the major ROS-scavenging molecules (44), and it occurs in two versions; the reduced form, sulfhydryl GSH, and the oxidized, glutathione disulfide (GSSG). The enzyme GSH peroxidase catalyzes the reduction of H_2O_2 to water and lipid hydroperoxides to their corresponding lipid alcohols and in turn oxidizes GSH to GSSG. The reverse reaction is catalyzed by the enzyme glutathione reductase, also known as glutathione-disulfide reductase using the reducing potential of NADPH (45).

Glutathione is synthesized in the cytoplasm from cysteine and glutamate, and cysteine availability is the rate-limiting step of the synthesis reaction (46, 47). Cysteine can be synthesized from serine through the transsulfuration pathway (47), or it can be transported into the cells as cystine in exchange for glutamate using the cystine-glutamate antiporter xCT (39). Interestingly, mTORC2 can phosphorylate and inhibit the xCT antiporter, therefore providing a mechanism to regulate intracellular cysteine levels (48). Furthermore, it has been shown that, in some cancer types, growing cells in DMEM containing cystine leads to xCT dependent glutamine dependency *in vitro*, providing evidence for context dependent changes in metabolism (5). The main source of NADPH comes from the PPP and a significant amount also comes from the serine synthesis pathway through the THF pathway. Both harness the energy from the catabolism of metabolic intermediates to generate NADPH (31, 47). Interestingly, an important regulator of ROS levels is the transcription factor nuclear factor E2-related factor 2 (NRF2) that has been shown to directly regulate the key serine synthesis enzymes (PHGDH, PSAT1, ATF4) (49). This provides a potential mechanism through which NRF2 can regulate ROS levels by influencing NADPH and GSH levels. Importantly, the DNA-repair protein, BRCA1, regulates NRF2, providing an additional link between DNA repair pathways and ROS levels (50). Overall, there are still many open questions with regards to how cancer cells can rewire their metabolism based on different nutrient availability, but there is growing evidence to suggest that maintaining cellular redox homeostasis plays a major role on DNA-damage/DNA-repair pathways.

DNA Damage Response (DDR) Triggers Metabolic Rewiring

While DNA-repair pathways can be influenced by cellular metabolic status and nutrient availability in the tumor microenvironment, accumulation of DNA damage due to extrinsic and intrinsic genotoxic stress, or deficient DNA repair can also cause abrupt rewiring of cell metabolism. Cells have evolved the DDR pathway (DDR) to monitor this genotoxic stress and maintain accurate transmission of genetic information to subsequent generations. The DDR can, therefore, halt cell-cycle progression, induce DNA-repair mechanisms, or trigger programmed cell death when DNA damage is irreparable (51). Ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) kinases are two key enzymes in the recognition of DNA damage and implementation of the DDR (51, 52). Upon activation by DNA-damage, ATM and ATR generate a second wave of phosphorylation that impacts many downstream effector proteins. An important aspect of DDR, driven by ATM and ATR activation, is the induction of metabolic rewiring to promote the resolution of genotoxic stress. ATM has been shown to activate the PPP through induction of the rate-limiting enzyme glucose-6-phosphate dehydrogenase (G6PD), to support the synthesis of reducing power in the form of NADPH, and generate ribose-5-phosphate for nucleotide synthesis (53, 54).

Increased carbon flux to the PPP from glucose derivatives is driven not only by increased glucose consumption often observed in cancer cells (55, 56) but also through ROS-mediated inactivation of many glycolytic enzymes, notably PKM2 that promotes flux into the oxidative arm of the PPP as well as serine biosynthesis (57). Many studies have shown increased glucose dependency following DDR (58), and these data demonstrate the challenge posed to cancer cells under genotoxic stress. They need to contain ROS levels, enhance nucleotide synthesis to repair DNA damage, and inhibit cell-cycle progression until resolution. To this end, SIRT4 activation upon DDR has been shown to inhibit glutamine consumption in HepG2, HeLa, HEK293T cells, and in lung tissue (58). The reduced glutamine consumption or, more specifically, reduced anaplerosis of glutamine into the TCA cycle, confers improved cell survival compared to SIRT4 knockout cells that cannot activate this response (58). While this study suggests that DDR undoubtedly causes changes in metabolism, conflict in the literature indicates that the nature of these changes is context- and cell type-dependent. For example, the concept that cells undergoing genotoxic stress need to synthesize more nucleotides to repair the DNA damage contradicts the reduced glutamine consumption. As mentioned previously, the amide group of glutamine is essential for purine and pyrimidine *de novo* synthesis. Other interesting points to note are that in non-cancerous tissues/cells, there are increases in fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS) in response to acute or chronic genotoxic stress (59). Increased FAO/OXPHOS is driven by AMP kinase (AMPK) activation resulting from the depletion of ATP. In the context of that study, cells are depleted of ATP because of the utilization of NAD+ by the DNA repair enzyme PARP-1. PARP-1 generates PAR chains from NAD+ and PARylates DNA to induce repair. This leads to depletion of NAD+, reduction of ATP synthesis, and activation of AMPK (59). This study further supports that DNA damage impacts cell metabolism. However, given the current literature, it is still unclear how a cell's nutrient availability can dictate the nature of its metabolic rewiring.

Metabolic Changes Driven by DDR Gene Mutations

The p53 protein encoded by the *TP53* gene is another key player in DDR through its role in DNA repair and cell-cycle regulation (60–62). While p53 levels are kept low under normal conditions,

upon DNA damage or other cellular stress, p53 is activated by phosphorylation events that prevent its degradation (60). Wild-type p53 activation regulates cell-cycle entry by transcriptionally repressing cyclin B and CDC25B, and it also plays an important role in DNA-repair. More specifically, it determines whether or not to repair the DNA or trigger senescence or apoptosis. p53 also plays many modulatory roles in the metabolic rewiring of cells. Among these, is its regulation of glycolysis through transcriptional activation of the *TP53*-induced glycolysis and apoptosis regulator (TIGAR) protein (63). TIGAR inhibits glycolysis by dephosphorylating fructose-2,6-biphosphate (F2,6B) leading to degradation of F2,6P and inhibition of phosphofructo kinase 1, an enzyme integral to glycolysis. p53 has also been shown to inhibit other enzymes of glycolysis as well as directly repress the expression of glucose transporters, GLUT1 and GLUT4 (61, 64). One might surmise that this impediment in glycolysis potentially leads to a surplus in glycolytic intermediates, leading to redirection toward the PPP. However, p53 has also been shown to inhibit the flux of glucose to the PPP by directly binding to G6PD and preventing its active dimer formation (61).

In addition, p53 has been shown to transcriptionally regulate proteins involved in the electron transport chain and mitochondrial stability, therefore, providing a mechanism to regulate OXPHOS (61, 65). Together, these data suggest that the role of p53 in metabolic regulation is context and tissue dependent. Importantly, *TP53* is one of the most mutated genes in cancer and many gain-of-function mutations have been described, leading to different changes in p53 depending on the mutation and the context. For example, mutant $p53^{3KR}$ cannot induce cell-cycle-arrest, senescence, or apoptosis, but it can still regulate metabolic target genes resulting in decreased ROS levels and reduced glycolytic flux (61, 66). Therefore, mutations in DDR or DNA-repair genes may result from the application of selective pressures established by the metabolic niches of cancer cells.

Other genes involved in DNA-repair are also commonly mutated in cancer (13). Some of the most well-known DNA-repair genes associated with cancer are *BRCA1* and *BRCA2* but mutations in other genes such as *ATM*, *ATR*, and *CDK12* [for a more extensive list, see Ref. (13)] also lead to DNA-repair deficient cancers (13, 67–70). Some cancers with a similar phenotype to DNA-repair mutant cancer do not harbor any mutations in those pathways. Together, these DNA-repair deficient cancers have been described as having a "BRCAness" phenotype (13, 71). Some metabolic changes have been described in the context of *BRCA*1 or *BRCA2* mutations (50, 72–74); however, it is still unclear whether these are due to their DNA-repair function or some alternative role. Since connections have been drawn between metabolism and DNA-repair, it seems likely that the "BRCAness" phenotype and *BRCA1/2* mutations would be associated with changes in metabolism. Again, as with most other mutations found in cancer, the metabolic changes associated with these DNA repair defects might reflect the context and tissue of origin.

CONCLUSION

Historically, cancer has been thought of as a genetic disease driven by the accumulation of multiple mutation "hits" (75–77). However, in recent years, this paradigm has begun to shift, and cancer is often regarded as a "metabolic disease" (78, 79), which is influenced by complex interactions between the tumor and its microenvironment. Therefore, when examining data suggesting that mutations lead to the rewiring of cancer metabolism, in fact, it may be more pertinent to question whether these mutations arise in the first place from selective pressures applied by extrinsic metabolic factors in the tumor microenvironment. In a noteworthy example, while KRAS-mutant cancers exhibit resistance to low-glucose growth conditions, glucose deprivation has been shown to drive the accumulation of novel KRAS mutations in KRAS wild-type cancers (80). This indicates that extrinsic factors, such as the availability of essential nutrients, directly influence the fate of resulting genetic alterations that confer growth and survival advantages of cancer cells in their given microenvironment.

With regards to mutations in DNA-repair genes, it is possible that the highly dynamic and fluctuating conditions encountered in the tumor microenvironment provide an evolutionary pressure to select for cancer cells that have heightened genomic instability and are more proficient at adapting to these changes as a result. Consistently, it has been shown that tumors with increased heterogeneity are less responsive to therapy and behave more aggressively than tumors arising from single clonal populations (81–83). As we have discussed, a multitude of extrinsic metabolic factors affects DNA repair and, subsequently, genomic stability (**Figures 1** and **2**). Therefore, when considering future strategies to refine existing or develop novel cancer therapies, it will be essential at all stages of research and drug design to take account of the dynamic interplay between microenvironment and metabolic factors that are proving to influence treatment efficacy so substantially.

AUTHOR CONTRIBUTIONS

MOT and GP wrote the manuscript. MOT and NP designed the figure. NP drew the figure and contributed to the revision of the manuscript, and bibliography.

FUNDING

MOT and NP are supported by Cancer Research UK Ph.D. studentships. Work in the GP lab is supported by the Institute of Cancer Research and a Cancer Research UK Grand Challenge award (C59824/A25044).

REFERENCES

1. Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. *J Gen Physiol* (1927) 8(6):519–30. doi:10.1085/jgp.8.6.519
2. Warburg O. On the origin of cancer cells. *Science* (1956) 123(3191):309–14. doi:10.1126/science.123.3191.309
3. Wellen KE, Thompson CB. Cellular metabolic stress: considering how cells respond to nutrient excess. *Mol Cell* (2010) 40(2):323–32. doi:10.1016/j.molcel.2010.10.004
4. Anastasiou D. Tumour microenvironment factors shaping the cancer metabolism landscape. *Br J Cancer* (2017) 116(3):277–86. doi:10.1038/bjc.2016.412

5. Muir A, Danai LV, Gui DY, Waingarten CY, Lewis CA, Vander Heiden MG. Environmental cystine drives glutamine anaplerosis and sensitizes cancer cells to glutaminase inhibition. *Elife* (2017) 6:e27713. doi:10.7554/eLife.27713
6. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. *Nat Rev Cancer* (2011) 11(2):85–95. doi:10.1038/nrc2981
7. Pavlova NN, Thompson CB. The emerging hallmarks of cancer metabolism. *Cell Metab* (2016) 23(1):27–47. doi:10.1016/j.cmet.2015.12.006
8. Martinez-Outschoorn UE, Peiris-Pages M, Pestell RG, Sotgia F, Lisanti MP. Cancer metabolism: a therapeutic perspective. *Nat Rev Clin Oncol* (2017) 14(2):113. doi:10.1038/nrclinonc.2017.1
9. DeBerardinis RJ, Chandel NS. Fundamentals of cancer metabolism. *Sci Adv* (2016) 2(5):e1600200. doi:10.1126/sciadv.1600200
10. Burrell RA, McGranahan N, Bartek J, Swanton C. The causes and consequences of genetic heterogeneity in cancer evolution. *Nature* (2013) 501(7467):338–45. doi:10.1038/nature12625
11. Chae YK, Anker JF, Carneiro BA, Chandra S, Kaplan J, Kalyan A, et al. Genomic landscape of DNA repair genes in cancer. *Oncotarget* (2016) 7(17):23312–21. doi:10.18632/oncotarget.8196
12. Gavande NS, VanderVere-Carozza PS, Hinshaw HD, Jalal SI, Sears CR, Pawelczak KS, et al. DNA repair targeted therapy: the past or future of cancer treatment? *Pharmacol Ther* (2016) 160:65–83. doi:10.1016/j.pharmthera.2016.02.003
13. Lord CJ, Ashworth A. BRCAness revisited. *Nat Rev Cancer* (2016) 16(2):110–20. doi:10.1038/nrc.2015.21
14. Attwood JT, Yung RL, Richardson BC. DNA methylation and the regulation of gene transcription. *Cell Mol Life Sci* (2002) 59(2):241–57. doi:10.1007/s00018-002-8420-z
15. Miller JL, Grant PA. The role of DNA methylation and histone modifications in transcriptional regulation in humans. *Subcell Biochem* (2013) 61:289–317. doi:10.1007/978-94-007-4525-4_13
16. Jeggo PA, Downs JA. Roles of chromatin remodellers in DNA double strand break repair. *Exp Cell Res* (2014) 329(1):69–77. doi:10.1016/j.yexcr.2014.09.023
17. Sinha M, Peterson CL. Chromatin dynamics during repair of chromosomal DNA double-strand breaks. *Epigenomics* (2009) 1(2):371–85. doi:10.2217/epi.09.22
18. Niculescu MD, Zeisel SH. Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline. *J Nutr* (2002) 132(8 Suppl):2333S–5S.
19. Pietrocola F, Galluzzi L, Bravo-San Pedro JM, Madeo F, Kroemer G. Acetyl coenzyme A: a central metabolite and second messenger. *Cell Metab* (2015) 21(6):805–21. doi:10.1016/j.cmet.2015.05.014
20. Reid MA, Dai Z, Locasale JW. The impact of cellular metabolism on chromatin dynamics and epigenetics. *Nat Cell Biol* (2017) 19(11):1298–306. doi:10.1038/ncb3629
21. Choudhary C, Weinert BT, Nishida Y, Verdin E, Mann M. The growing landscape of lysine acetylation links metabolism and cell signalling. *Nat Rev Mol Cell Biol* (2014) 15(8):536–50. doi:10.1038/nrm3841
22. Sivanand S, Rhoades S, Jiang Q, Lee JV, Benci J, Zhang J, et al. Nuclear acetyl-CoA production by ACLY promotes homologous recombination. *Mol Cell* (2017) 67(2):252–65.e256. doi:10.1016/j.molcel.2017.06.008
23. Zaidi N, Swinnen JV, Smans K. ATP-citrate lyase: a key player in cancer metabolism. *Cancer Res* (2012) 72(15):3709–14. doi:10.1158/0008-5472.CAN-11-4112
24. Kamphorst JJ, Chung MK, Fan J, Rabinowitz JD. Quantitative analysis of acetyl-CoA production in hypoxic cancer cells reveals substantial contribution from acetate. *Cancer Metab* (2014) 2:23. doi:10.1186/2049-3002-2-23
25. Schug ZT, Peck B, Jones DT, Zhang Q, Grosskurth S, Alam IS, et al. Acetyl-CoA synthetase 2 promotes acetate utilization and maintains cancer cell growth under metabolic stress. *Cancer Cell* (2015) 27(1):57–71. doi:10.1016/j.ccell.2014.12.002
26. Mews P, Donahue G, Drake AM, Luczak V, Abel T, Berger SL. Acetyl-CoA synthetase regulates histone acetylation and hippocampal memory. *Nature* (2017) 546(7658):381–6. doi:10.1038/nature22405
27. Mehrmohamadi M, Mentch LK, Clark AG, Locasale JW. Integrative modelling of tumour DNA methylation quantifies the contribution of metabolism. *Nat Commun* (2016) 7:13666. doi:10.1038/ncomms13666
28. Jiang Y, Qian X, Shen J, Wang Y, Li X, Liu R, et al. Local generation of fumarate promotes DNA repair through inhibition of histone H3 demethylation. *Nat Cell Biol* (2015) 17(9):1158–68. doi:10.1038/ncb3209
29. Sciacovelli M, Goncalves E, Johnson TI, Zecchini VR, da Costa AS, Gaude E, et al. Fumarate is an epigenetic modifier that elicits epithelial-to-mesenchymal transition. *Nature* (2016) 537(7621):544–7. doi:10.1038/nature19353
30. Russo G, Landi R, Pezone A, Morano A, Zuchegna C, Romano A, et al. DNA damage and repair modify DNA methylation and chromatin domain of the targeted locus: mechanism of allele methylation polymorphism. *Sci Rep* (2016) 6:33222. doi:10.1038/srep33222
31. Patra KC, Hay N. The pentose phosphate pathway and cancer. *Trends Biochem Sci* (2014) 39(8):347–54. doi:10.1016/j.tibs.2014.06.005
32. Rao X, Duan X, Mao W, Li X, Li Z, Li Q, et al. O-GlcNAcylation of G6PD promotes the pentose phosphate pathway and tumor growth. *Nat Commun* (2015) 6:8468. doi:10.1038/ncomms9468
33. Rabinovich S, Adler L, Yizhak K, Sarver A, Silberman A, Agron S, et al. Diversion of aspartate in ASS1-deficient tumours fosters de novo pyrimidine synthesis. *Nature* (2015) 527(7578):379–83. doi:10.1038/nature15529
34. Kim J, Hu Z, Cai L, Li K, Choi E, Faubert B, et al. CPS1 maintains pyrimidine pools and DNA synthesis in KRAS/LKB1-mutant lung cancer cells. *Nature* (2017) 546(7656):168–72. doi:10.1038/nature22359
35. Jiang P, Du W, Wu M. Regulation of the pentose phosphate pathway in cancer. *Protein Cell* (2014) 5(8):592–602. doi:10.1007/s13238-014-0082-8
36. Kowalik MA, Columbano A, Perra A. Emerging role of the pentose phosphate pathway in hepatocellular carcinoma. *Front Oncol* (2017) 7:87. doi:10.3389/fonc.2017.00087
37. Cory JG, Cory AH. Critical roles of glutamine as nitrogen donors in purine and pyrimidine nucleotide synthesis: asparaginase treatment in childhood acute lymphoblastic leukemia. *In Vivo* (2006) 20(5):587–9.
38. Lane AN, Fan TW. Regulation of mammalian nucleotide metabolism and biosynthesis. *Nucleic Acids Res* (2015) 43(4):2466–85. doi:10.1093/nar/gkv047
39. Tardito S, Oudin A, Ahmed SU, Fack F, Keunen O, Zheng L, et al. Glutamine synthetase activity fuels nucleotide biosynthesis and supports growth of glutamine-restricted glioblastoma. *Nat Cell Biol* (2015) 17(12):1556–68. doi:10.1038/ncb3272
40. Lindahl T. Instability and decay of the primary structure of DNA. *Nature* (1993) 362(6422):709–15. doi:10.1038/362709a0
41. Alleman RJ, Katunga LA, Nelson MA, Brown DA, Anderson EJ. The "Goldilocks Zone" from a redox perspective-adaptive vs. deleterious responses to oxidative stress in striated muscle. *Front Physiol* (2014) 5:358. doi:10.3389/fphys.2014.00358
42. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. *Curr Biol* (2014) 24(10):R453–62. doi:10.1016/j.cub.2014.03.034
43. Panieri E, Santoro MM. ROS homeostasis and metabolism: a dangerous liason in cancer cells. *Cell Death Dis* (2016) 7(6):e2253. doi:10.1038/cddis.2016.105
44. Shanware NP, Mullen AR, DeBerardinis RJ, Abraham RT. Glutamine: pleiotropic roles in tumor growth and stress resistance. *J Mol Med (Berl)* (2011) 89(3):229–36. doi:10.1007/s00109-011-0731-9
45. Lu SC. Regulation of glutathione synthesis. *Mol Aspects Med* (2009) 30(1–2):42–59. doi:10.1016/j.mam.2008.05.005
46. Hultberg B, Hultberg M. High glutathione turnover in human cell lines revealed by acivicin inhibition of gamma-glutamyltranspeptidase and the effects of thiol-reactive metals during acivicin inhibition. *Clin Chim Acta* (2004) 349(1–2):45–52. doi:10.1016/j.cccn.2004.05.024
47. Yang M, Vousden KH. Serine and one-carbon metabolism in cancer. *Nat Rev Cancer* (2016) 16(10):650–62. doi:10.1038/nrc.2016.81
48. Gu Y, Albuquerque CP, Braas D, Zhang W, Villa GR, Bi J, et al. mTORC2 regulates amino acid metabolism in cancer by phosphorylation of the cystine-glutamate antiporter xCT. *Mol Cell* (2017) 67(1):128–38.e127. doi:10.1016/j.molcel.2017.05.030
49. DeNicola GM, Chen PH, Mullarky E, Sudderth JA, Hu Z, Wu D, et al. NRF2 regulates serine biosynthesis in non-small cell lung cancer. *Nat Genet* (2015) 47(12):1475–81. doi:10.1038/ng.3421
50. Gorrini C, Baniasadi PS, Harris IS, Silvester J, Inoue S, Snow B, et al. BRCA1 interacts with Nrf2 to regulate antioxidant signaling and cell survival. *J Exp Med* (2013) 210(8):1529–44. doi:10.1084/jem.20121337
51. Marechal A, Zou L. DNA damage sensing by the ATM and ATR kinases. *Cold Spring Harb Perspect Biol* (2013) 5(9):a012716. doi:10.1101/cshperspect.a012716
52. Mirzayans R, Severin D, Murray D. Relationship between DNA double-strand break rejoining and cell survival after exposure to ionizing radiation in human fibroblast strains with differing ATM/p53 status: implications for evaluation

of clinical radiosensitivity. *Int J Radiat Oncol Biol Phys* (2006) 66(5):1498–505. doi:10.1016/j.ijrobp.2006.08.064
53. Cosentino C, Grieco D, Costanzo V. ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair. *EMBO J* (2011) 30(3):546–55. doi:10.1038/emboj.2010.330
54. Aird KM, Worth AJ, Snyder NW, Lee JV, Sivanand S, Liu Q, et al. ATM couples replication stress and metabolic reprogramming during cellular senescence. *Cell Rep* (2015) 11(6):893–901. doi:10.1016/j.celrep.2015.04.014
55. Jones RG, Thompson CB. Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes Dev* (2009) 23(5):537–48. doi:10.1101/gad.1756509
56. Koppenol WH, Bounds PL, Dang CV. Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer* (2011) 11(5):325–37. doi:10.1038/nrc3038
57. 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(6060):1278–83. doi:10.1126/science.1211485
58. Jeong SM, Xiao C, Finley LW, Lahusen T, Souza AL, Pierce K, et al. SIRT4 has tumor-suppressive activity and regulates the cellular metabolic response to DNA damage by inhibiting mitochondrial glutamine metabolism. *Cancer Cell* (2013) 23(4):450–63. doi:10.1016/j.ccr.2013.02.024
59. Brace LE, Vose SC, Stanya K, Gathungu RM, Marur VR, Longchamp A, et al. Increased oxidative phosphorylation in response to acute and chronic DNA damage. *NPJ Aging Mech Dis* (2016) 2:16022. doi:10.1038/npjamd.2016.22
60. Lakin ND, Jackson SP. Regulation of p53 in response to DNA damage. *Oncogene* (1999) 18(53):7644–55. doi:10.1038/sj.onc.1203015
61. Berkers CR, Maddocks OD, Cheung EC, Mor I, Vousden KH. Metabolic regulation by p53 family members. *Cell Metab* (2013) 18(5):617–33. doi:10.1016/j.cmet.2013.06.019
62. Broustas CG, Lieberman HB. DNA damage response genes and the development of cancer metastasis. *Radiat Res* (2014) 181(2):111–30. doi:10.1667/RR13515.1
63. Li H, Jogl G. Structural and biochemical studies of TIGAR (TP53-induced glycolysis and apoptosis regulator). *J Biol Chem* (2009) 284(3):1748–54. doi:10.1074/jbc.M807821200
64. Schwartzenberg-Bar-Yoseph F, Armoni M, Karnieli E. The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression. *Cancer Res* (2004) 64(7):2627–33. doi:10.1158/0008-5472.CAN-03-0846
65. Matoba S, Kang JG, Patino WD, Wragg A, Boehm M, Gavrilova O, et al. p53 regulates mitochondrial respiration. *Science* (2006) 312(5780):1650–3. doi:10.1126/science.1126863
66. Li T, Kon N, Jiang L, Tan M, Ludwig T, Zhao Y, et al. Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence. *Cell* (2012) 149(6):1269–83. doi:10.1016/j.cell.2012.04.026
67. Foulkes WD, Stefansson IM, Chappuis PO, Begin LR, Goffin JR, Wong N, et al. Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. *J Natl Cancer Inst* (2003) 95(19):1482–5. doi:10.1093/jnci/djg050
68. Thull DL, Vogel VG. Recognition and management of hereditary breast cancer syndromes. *Oncologist* (2004) 9(1):13–24. doi:10.1634/theoncologist.9-1-13
69. Atchley DP, Albarracin CT, Lopez A, Valero V, Amos CI, Gonzalez-Angulo AM, et al. Clinical and pathologic characteristics of patients with BRCA-positive and BRCA-negative breast cancer. *J Clin Oncol* (2008) 26(26):4282–8. doi:10.1200/JCO.2008.16.6231
70. O'Donovan PJ, Livingston DM. BRCA1 and BRCA2: breast/ovarian cancer susceptibility gene products and participants in DNA double-strand break repair. *Carcinogenesis* (2010) 31(6):961–7. doi:10.1093/carcin/bgq069
71. Turner N, Tutt A, Ashworth A. Hallmarks of 'BRCAness' in sporadic cancers. *Nat Rev Cancer* (2004) 4(10):814–9. doi:10.1038/nrc1457
72. Privat M, Radosevic-Robin N, Aubel C, Cayre A, Penault-Llorca F, Marceau G, et al. BRCA1 induces major energetic metabolism reprogramming in breast cancer cells. *PLoS One* (2014) 9(7):e102438. doi:10.1371/journal.pone.0102438
73. Rytelewski M, Tong JG, Buensuceso A, Leong HS, Maleki Vareki S, Figueredo R, et al. BRCA2 inhibition enhances cisplatin-mediated alterations in tumor cell proliferation, metabolism, and metastasis. *Mol Oncol* (2014) 8(8):1429–40. doi:10.1016/j.molonc.2014.05.017
74. Cuyas E, Fernandez-Arroyo S, Alarcon T, Lupu R, Joven J, Menendez JA. Germline BRCA1 mutation reprograms breast epithelial cell metabolism towards mitochondrial-dependent biosynthesis: evidence for metformin-based "starvation" strategies in BRCA1 carriers. *Oncotarget* (2016) 7(33):52974–92. doi:10.18632/oncotarget.9732
75. Nordling CO. A new theory on cancer-inducing mechanism. *Br J Cancer* (1953) 7(1):68–72. doi:10.1038/bjc.1953.8
76. Ashley DJ. The two "hit" and multiple "hit" theories of carcinogenesis. *Br J Cancer* (1969) 23(2):313–28. doi:10.1038/bjc.1969.41
77. Knudson AG Jr. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* (1971) 68(4):820–3. doi:10.1073/pnas.68.4.820
78. Coller HA. Is cancer a metabolic disease? *Am J Pathol* (2014) 184(1):4–17. doi:10.1016/j.ajpath.2013.07.035
79. Seyfried TN, Flores RE, Poff AM, D'Agostino DP. Cancer as a metabolic disease: implications for novel therapeutics. *Carcinogenesis* (2014) 35(3):515–27. doi:10.1093/carcin/bgt480
80. Yun J, Rago C, Cheong I, Pagliarini R, Angenendt P, Rajagopalan H, et al. Glucose deprivation contributes to the development of KRAS pathway mutations in tumor cells. *Science* (2009) 325(5947):1555–9. doi:10.1126/science.1174229
81. Cahill DP, Kinzler KW, Vogelstein B, Lengauer C. Genetic instability and darwinian selection in tumours. *Trends Cell Biol* (1999) 9(12):M57–60. doi:10.1016/S0962-8924(99)01661-X
82. Jogi A, Vaapil M, Johansson M, Pahlman S. Cancer cell differentiation heterogeneity and aggressive behavior in solid tumors. *Ups J Med Sci* (2012) 117(2):217–24. doi:10.3109/03009734.2012.659294
83. Meacham CE, Morrison SJ. Tumour heterogeneity and cancer cell plasticity. *Nature* (2013) 501(7467):328–37. doi:10.1038/nature12624

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 02 February 2018
doi: 10.3389/fonc.2018.00013

Metabolic Profile of Oral Squamous Carcinoma Cell Lines Relies on a Higher Demand of Lipid Metabolism in Metastatic Cells

*Ana Carolina B. Sant'Anna-Silva[1], Gilson C. Santos[1,2], Samir P. Costa Campos[1], André Marco Oliveira Gomes[1], Juan Alberto Pérez-Valencia[1] and Franklin David Rumjanek[1]**

[1] Instituto de Bioquímica Médica Leopoldo de Meis, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [2] Centro Nacional de Biologia Estrutural e Bioimagem I (CENABIO I)/Centro Nacional de Ressonância Magnética Nuclear (CNRMN), Laboratório de Ressonância Magnética Nuclear de Biomoléculas (bioNMR), Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

OPEN ACCESS

Edited by:
Simona Pisanti, Università degli Studi di Salerno, Italy

Reviewed by:
Daniele Vergara, University of Salento, Italy
Tone Frost Bathen, Norwegian University of Science and Technology, Norway

***Correspondence:**
Franklin David Rumjanek
franklin@bioqmed.ufrj.br

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *30 October 2017*
Accepted: *16 January 2018*
Published: *02 February 2018*

Citation:
Sant'Anna-Silva ACB, Santos GC, Campos SPC, Oliveira Gomes AM, Pérez-Valencia JA and Rumjanek FD (2018) Metabolic Profile of Oral Squamous Carcinoma Cell Lines Relies on a Higher Demand of Lipid Metabolism in Metastatic Cells. Front. Oncol. 8:13. doi: 10.3389/fonc.2018.00013

Tumor cells are subjected to a broad range of selective pressures. As a result of the imposed stress, subpopulations of surviving cells exhibit individual biochemical phenotypes that reflect metabolic reprograming. The present work aimed at investigating metabolic parameters of cells displaying increasing degrees of metastatic potential. The metabolites present in cell extracts fraction of tongue fibroblasts and of cell lines derived from human tongue squamous cell carcinoma lineages displaying increasing metastatic potential (SCC9 ZsG, LN1 and LN2) were analyzed by ^{1}H NMR (nuclear magnetic resonance) spectroscopy. Living, intact cells were also examined by the non-invasive method of fluorescence lifetime imaging microscopy (FLIM) based on the auto fluorescence of endogenous NADH. The cell lines reproducibly exhibited distinct metabolic profiles confirmed by Partial Least-Square Discriminant Analysis (PLS-DA) of the spectra. Measurement of endogenous free and bound NAD(P)H relative concentrations in the intact cell lines showed that ZsG and LN1 cells displayed high heterogeneity in the energy metabolism, indicating that the cells would oscillate between glycolysis and oxidative metabolism depending on the microenvironment's composition. However, LN2 cells appeared to have more contributions to the oxidative status, displaying a lower NAD(P)H free/bound ratio. Functional experiments of energy metabolism, mitochondrial physiology, and proliferation assays revealed that all lineages exhibited similar energy features, although resorting to different bioenergetics strategies to face metabolic demands. These differentiated functions may also promote metastasis. We propose that lipid metabolism is related to the increased invasiveness as a result of the accumulation of malonate, methyl malonic acid, n-acetyl and unsaturated fatty acids $(CH_2)_n$ in parallel with the metastatic potential progression, thus suggesting that the NAD(P)H reflected the lipid catabolic/anabolic pathways.

Keywords: oral squamous cell carcinoma, metastasis, cancer progression, nuclear magnetic resonance, metabolomics, metabolic reprogramming

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most prevalent type of head and neck squamous cell carcinoma frequently leading to metastasis to the neck lymph nodes (1). OSCC evolves from high proliferation rates of the oral squamous epithelium ultimately forming an *in situ* carcinoma (2, 3) followed by metastasis and a high lethality rate (4, 5).

Compared to normal cells, cancer cells have been shown to display a reprogrammed metabolism resulting from the specific energy demands imposed by growth factor signaling (6, 7). Furthermore, in the case of metastatic cells, migration and colonization of distant tissues also contribute to the extra energy burden. Thus, we envision metastatic cells as a subpopulation of cells that were selected in terms of a fine-tuned coordination that integrates nutrient uptake, anabolic, and catabolic processes. In addition, the microenvironment is variable insofar as the tumor anatomy is concerned. Whereas glucose, glutamine, and oxygen are freely available for those cells located on the surface of the tumor mass, the inner layers of cells are confronted by a radically different milieu characterized by paucity of nutrients and by hypoxia (8, 9). Consequently, these constraints introduce selective pressures that will reward metabolic plasticity. Those cells that can adjust to the different environments in the tumors will either thrive locally or eventually become detached and give rise to potentially metastatic cells. Successful adjustment can be achieved by gain of function through the concerted activation of expression of key enzymes that affect the metabolic flux and proliferative pathways as well as genes involved in the acquisition of resistance to anoikis through suppression of apoptotic programs. However, it is important to bear in mind that the metastatic phenotype probably results from non-adaptive innovation, that is, through the integration of pre-existing signaling pathways. By becoming manifest, these pathways confer different properties that enable cells to survive in an otherwise incompatible microenvironment (10–12).

Recently, the metabolomic approach using nuclear magnetic resonance (NMR) has become increasingly more informative. The availability of metabolomic data has been very useful for unraveling the metabolic pathways of several types of cancer as well as the biochemical features pertaining to metastasis (13–15). The main advantage of metabolomics rests on its ability to instantly and globally analyze metabolites quantitatively and qualitatively so that not only the involved pathways can be highlighted, but also their fluxes could be deduced (16, 17). Likewise, two-photon fluorescence lifetime imaging microscopy (FLIM), a non-invasive technique, has been successfully used to probe intact living cells in order to investigate their metabolism, thus affording a snapshot of their energy status. Experimentally, the auto fluorescence generated by both NADH and NADPH has been used to investigate the mitochondrial redox state and hence the energy producing pathways (18–20).

In the present study, we performed ^{1}H NMR and FLIM determinations combined with functional experiments in order to evaluate the metabolic alterations that may be relevant to the metastatic phenotypes of tongue squamous cells carcinoma (SCC) cells.

MATERIAL AND METHODS

Cell Lines

In the present study, cell lines developed and isolated from squamous cellular carcinoma SCC-9 (ATCC CRL-1629) by Agostini et al. (21) were used. The first cell line produced named SCC-9 ZsGreen stably expresses a green fluorescent zebrafish plasmid (ZsG). The paper describes how SCC-9 cells were inoculated into the footpads of BALB/c nude mice and were recovered as LN1 cells, the first metastatic generation. Another round of inoculation of LN1 cells produced LN2 cells, the second metastatic generation. Normal fibroblasts isolated from biopsies (3) were a gift by Dr. Ricardo Colleta from Department of Oral Diagnosis (School of Dentistry of Piracicaba, State University of Campinas, Brazil).

Cell Culture

For SCC-9 derived cells, Dulbecco's Modified Eagle Medium: nutrient Mixture F12 (DMEM/F12; Gibco®, Life Technologies™, USA) was used. Media were supplemented with 10% fetal bovine serum (FBS—Cultilab, Brasil) and hydrocortisone 400 ng/ml (Sigma-Aldrich, USA). For fibroblasts, we used Dulbecco's Modified Eagle Medium (DMEM *low*; Gibco®, Life Technologies™, USA) supplemented with 10% *donor bovine serum* (DBS; Gibco®, Life Technologies™, USA) and 1% penicillin–streptomycin. 1.1×10^6 cells of ZsG, LN1, LN2, and fibroblasts were transferred to 60.1 cm^2 Petri dishes and maintained for 48 h in an incubator series 8000 water-jacketed CO_2 (Thermo Scientific), with 5% of CO_2 humidity atmosphere. At least four independent biological replicates of each cell line were used for experimental analysis. All cell lines were genotyped and tested free for *Mycoplasma* sp. infection using polymerase chain reaction.

NMR Metabolomics

ZsG, LN1, and LN2 cells were cultivated in DMEM/F12 and fibroblasts in DMEM low until 80% confluence. Then, ~1.2×10^7 cells from each replicate were trypsinized, centrifuged, and all the cell pellets were normalized to ~30 mg for metabolite extraction. We adapted the protocol for polar phase extraction according to adaptations of the method of Bligh and Dyer biphasic extraction previously described (22). Briefly, cells were extracted with methanol/chloroform/water (2:1:0.8), vortexed for 2 min after each addition, and homogenized during 30 min in a shaker in an ice bath. The polar aqueous phase (supernatant) was centrifuged at 4,600 *g* for 20 min at 15°C, the supernatant was dried overnight under vacuum (SpeedVac) and stored at −80°C until used. The samples were suspended in 50 mM phosphate buffer, pH 7.4, containing 10% of D_2O and 0.1 mM of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) for chemical shift reference.

All spectra were acquired in a Bruker Avance III HD spectrometer running at 500.13 MHz for ^{1}H at 298 K. One dimensional spectra were acquired with excitation sculpting for water saturation, 20 ppm spectral width, 1.74 s relaxation delay, 64 K points, and 3 K accumulations. For assignments, we used ^{1}H-^{1}H TOCSY and pJRES. Spectra were aligned and processed in TOPSPIN 3.5 (Bruker-Biospin) and exported to AMIX (Bruker-Biospin),

normalized by DSS intensity as internal reference, 0.02 ppm binned, and finally water and DSS signal were excluded. The peak intensities were used for the relative metabolite quantification in each sample.

For statistical analysis, datasets of at least four independent samples of each ZsG, LN1, LN2, and fibroblasts cell culture were collected. For the univariate analysis, we used multiple *t*-test with two-stage setup method of Benjamini–Krieger–Yekutieli, with false discovery rate (FDR) of 5%, with approach, assuming consistent SD, on GraphPad Prism 6.0. For multivariate analysis, we used Metaboanalyst 3.0 (23) with default practice (skip missing value imputation), filtered data with median intensity value (due to large amount of data), and normalized by sum of intensities. In addition, we applied the pareto scaling to reduce the impact of the buckets with high prevalence in each comparison [signaled as threonine, threitol, and $(CH_2)n$] in the statistical analysis and to keep the data structure intact. Multivariate methods were used to compare all clusters (four groups) or paired groups (fibroblasts vs. ZsG; ZsG vs. LN1; LN1 vs. LN2) by means of principal component analysis and partial least-square discriminant analysis (PLS-DA). To validate class discrimination and avoid overfitting, we used a permutation test (1,000 permutations) based on separation distance, B/W-ratio, and cross-validation by the leave-one-out method (24, 25). We also calculated the variable importance in the projection (VIP-score) to analyze the ranking of the most important metabolites in separation groups and the PLS-regression coefficients for components 1, 2, and 3.

REACTOME free software (26) was used to determine the related metabolic pathways to metabolites accumulated in each comparison. Briefly, we listed all the related enzymes that produce each metabolite by manual curation, and it was analyzed into REACTOME using *Homo sapiens* as background, showing the corresponding metabolic pathways. FDR correction was used with significance of 0.05.

Fluorescence Lifetime Imaging

The autofluorescence lifetime images of isolate cultured cells were acquired using a laser scanning confocal fluorescence microscope Nikon Eclipse TE2000-U equipped with a Spectra-Physics MaiTai HP Laser (Spectra-Physics). The microscope is coupled to a fluorescence correlation spectrometer Alba Flim (ISS, Inc.) and data were collected by VistaVision software (ISS, Inc.) and analyzed by SimFCS 4 software (27). The samples were excited by two photons at 740 nm using a 60×/1.20 Plan-Apochromat water immersion objective lens (Nikon). The emission was detected on an Avalanche Photodiode detector, through a bypass filter 450 nm. A total of 100 frames from five different fields of each sample were collected. The image size acquired was 256 × 256 and the pixel dwell time was 40 µs. The calibration was done using a solution of 250 µM of NADH in water. The fluorescence lifetime was calculated by the phasor approach. Briefly, through Fast Fourier Transformation, the fluorescence decay in each pixel is plotted on the phasor plot by the coordinates G and S. The distribution of phasor points reveals areas of different lifetimes within the universal semicircle (28). Free and bound NADH have lifetimes of 0.38 and 3.4 ns, respectively, and their fluorescence intensity decays are plotted in the universal semicircle (29, 30).

High-Resolution Respirometry

High-resolution respirometry (Oroboros Oxygraph-2k) was performed to evaluate the oxygen consumption of intact cells, as previously described (31). 10^6 cells/ml of ZsG, LN1 and LN2 cells were cultivated separately and suspended in culture medium (DMEM/F12) without fetal bovine serum and phenol red. Then, cells were placed into a respiration chamber until the steady-state respiratory flux was attained (~10 min). Subsequently, the following parameters were measured: ATP synthase independent respiration in the presence of oligomycin (oligo) allowing the observation of oxygen consumption uncoupled to ATP synthesis ("leak"); content of oxygen consumption inhibited by the presence of oligomycin ("coupled"); titration with carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone (FCCP) to assess the maximal oxygen consumption rates once it stimulates the electron transfer system efficiency ("ETS"); residual oxygen consumption ("ROX") in the presence of rotenone (NADH dehydrogenase inhibitor) and antimycin A (AA) (cytochrome bc_1 inhibitor). Data acquisition and analysis were carried out with DatLab 5.1 software (Oroboros Instruments, Innsbruck, Austria).

Lactate Release in Culture Media

After 24 h of incubation with completed DMEM/F12 + 10% FBS and hydrocortisone 400 ng/ml, the culture medium was replaced by fresh DMEM/F12 without phenol red and FBS. Aliquots from the culture medium were collected at 0 and 60 min of incubation to evaluate lactate release through enzymatic assay. The lactate assay was performed in hydrazine/glycine buffer, pH 9.2, containing 5 mg/ml β-NAD^+ and 15 U/ml lactate dehydrogenase (LDH; Sigma-Aldrich). NADH absorbance was monitored in a microplate reader (SpectraMax M5, Molecular Devices) at 340 nm.

Proliferation Assay

Proliferation assays were performed using Sulforhodamine B (SRB) colorimetric assays, as previously described (32). Briefly, cells were placed into 96-well plates. After each endpoint (0, 24, 48, and 72 h), the culture medium was removed and cells were fixed with 10% trichloroacetic acid for 1 h at 25°C. The plates were then washed with distilled water and incubated with SRB solution (1% in acetic acid) for 15 min. After rinsing with 1% acetic acid, the cell monolayers were dried and the proteins were solubilized with Tris pH 10.4. Absorbance was measured at 490 nm using a spectrophotometer (SpectraMax Plus 384, Molecular Devices).

Statistical Analysis

Besides metabolomics, all the experiments were plotted as means ± SD for *n* independent experiments. Statistical significance to evaluate two groups was determined by the unpaired *t*-test, one-way ANOVA, and Dunnett posttest. Univariate analysis was done by multiple *t*-test, two-way ANOVA, and posttest Holm–Sidak. All set at alpha = 0.05. The graphs were generated by GraphPad Prism version 6.0 for Windows (GraphPad Software,

La Jolla, CA, USA) and Excel (Microsoft Corporation®) with 95% of confidence level.

RESULTS

Metabolic Profiles of Normal, Tumor, and Metastatic Cells

In order to gather insights into the intracellular metabolism of cells representing different stages of tumor progression, ^{1}H NMR spectra of cellular extracts were compared. Tongue normal fibroblasts were also investigated. High quality spectra of at least four replicates from each cell line (ZsG, LN1, and LN2) as well as fibroblasts samples were obtained. All determinations revealed similar spectral characteristics within each cluster of cell line, indicating that metabolic profiles of individual biological replicates were reproducible. Representative 1D ^{1}H NMR of the polar phase of the cellular extracts are shown in **Figures 1A,B** within the range of 0.8 and 8.0 ppm. Assignments of previously identified metabolites were obtained by comparing chemical shifts and spectral peak multiplicities with data from the literature and BMRB (33), HMDB (34), and COLMAR (35) data bases.

The major differences between each step of the metastasis progression, i.e., fibroblasts vs. ZsG, ZsG vs. LN1, and LN1 vs. LN2 were associated to the accumulation or decrease of threonine, threitol, n-acetyl glutamate, methyl malonate, malonate, n-acetyl, creatine, ribose, lactate, ethanolamine, phosphoethanolamine, and unsaturated lipids (**Table 1**).

Metabolomic profiles comparing the isolated groups of cells showed clearly the discrimination between normal and cancer cells by multivariate analysis PLS-DA score plot and VIP scores (**Figures 1C,D**). These analyses were validated using cross validation by the leave-one-out method (23–25), revealing an accuracy of −0.58 R^2 (clear variation) of 0.90 and Q^2 (predictive capability) of 0.57 for two components, representing a reliable classification model. In both measures, the value 1 indicates absolute fitting and high predictive power (36).

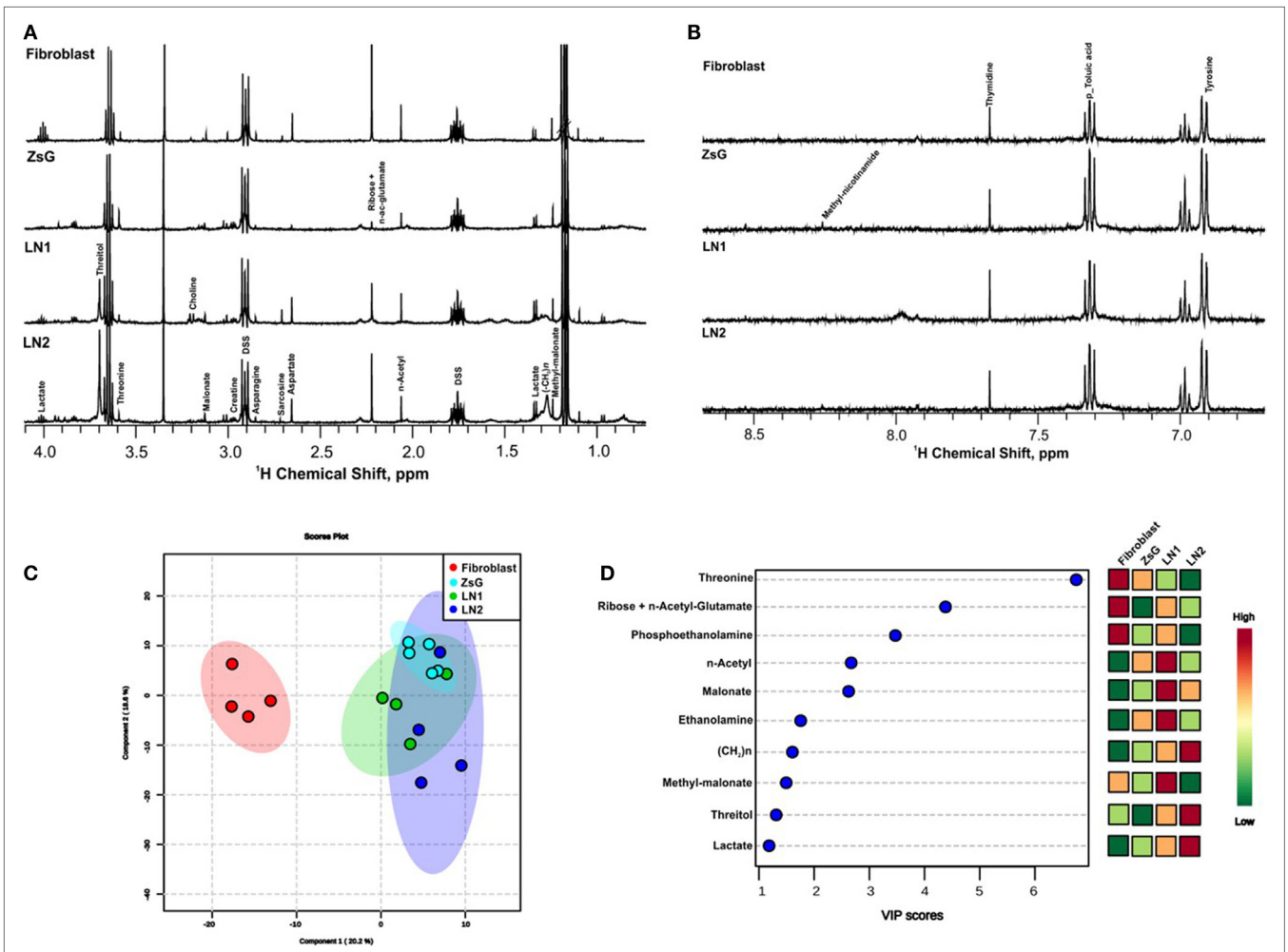


FIGURE 1 | Metabolic profiles of normal, tumor, and metastatic cells. 1D ^{1}H nuclear magnetic resonance representative spectra from fibroblast, ZsG, LN1, and LN2 cells polar extract of aliphatic region **(A)**, and aromatic region **(B)**. **(C)** 2D Score plot of partial least-square discriminant analysis, with components 1 and 2 of multivariate analysis comparing all cell lines. **(D)** VIP scores of the most significantly altered metabolites important for multivariate analysis (p-value <0.05).

Several metabolic changes accompany tumorigenesis progression toward metastasis. To evaluate these alterations during the transformation and over tumor development, we previously defined paired comparisons replicating parental and derived cells. Then, we compared the metabolic profiling between each group and according to multivariate analysis PLS-DA, many metabolites were informative to distinguish the clusters. Among these values, we gathered the buckets with the highest VIP scores (>1; $p < 0.05$ from Student's *t*-test and FDR correction $q < 0.05$) and loading factors, considering the most discriminating power in each the comparison (**Table 1**; Figure S1 in Supplementary Material).

Metabolite Accumulation Is due Mainly to Lipid and Nitrogen Metabolism, through Aminoacid Transformation

In order to understand the origin and the biochemical implications of those metabolites, all the enzymes related to their formation were analyzed using the free software REACTOME (26), thus revealing the most representative biochemical pathways involved. The summary of the major findings is shown in **Table 2** and Table S1 in Supplementary Material. It is interesting to mention that there is only one metabolic pathway, related to metabolism of amino acids through aldehyde dehydrogenase 6 family member A1 (ALDH6A1), which use NAD^+ as cofactor, in fibroblasts and ZsG comparison. ZsG vs. LN1 comparison shows that β-oxidation and lipid metabolism are the most important processes in LN1 cells. In the same way, lipid metabolism is highly dependent on NAD^+, through hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase [trifunctional protein], alpha subunit (HADHA). A similar pattern is observed in LN1 vs. LN2 comparison. However, the fatty acid synthase (FASN) uses NADPH as cofactor. Also, interactomes of the related enzymes were performed using STRING software (37), showing clusters of acetylase activity and amino acid metabolism for fibroblast vs. ZsG and ZsG vs. LN1 comparisons, connecting lipid metabolism to acetylation in this last comparison. Between LN1 and LN2, there is a single cluster which is related to beta-oxidation (Figure S2 in Supplementary Material). Lipid metabolism appears to be prominently linked to metastatic phenotypes, while cell cycle regulation seems to be a feature of the less invasive cells.

NAD(P)H FLIM Reveals that LN2 Cells Displays Longer Lifetimes

Most of biochemical pathways depend on reactions promoted by the availability of NAD(P)H, including fatty acids metabolism. Thus, the measurements of NAD(P)H free/bound ratios afford clues about enzymatic activities and enhanced processes (38). In this regard, we performed FLIM to evaluate the NADH free to bound ratio in ZsG, LN1 and LN2 cells. To simplify the analysis, we used the phasor plot approach, in which the fluorescence decay in each pixel is plotted on the phasor plot universe (29).

TABLE 1 | Multivariate analysis data and the highest VIP scores for each comparison (fibroblasts vs. ZsG; ZsG vs. LN1; LN1 vs. LN2).

	Metabolite	δH (ppm)	Status	False discovery rate (FDR) (*q*-value)
Fibroblast vs. ZsG	Threonine	3.59	↓	<1E−15
	Creatine	2.97	↑	1.48E−03
	n-acetyl	2.03	↑	1.86E−03
	Malonate	3.15	↑	1.80E−02
	Ribose + n-acetyl-glutamate	2.21	↑	3.93E−02
	Malonate	3.13	↑	3.93E−02
ZsG vs. LN1	Ribose + n-acetyl-glutamate	2.21	↑	<1E−15
	Threitol	3.69	↑	<1E−15
	Phosphoethanolamine	3.99	↑	8.60E−06
	Ethanolamine	3.83	↑	1.39E−05
	(CH2)n	1.27	↑	1.02E−02
	n-acetyl	2.03	↑	4.35E−02
LN1 vs. LN2	(CH2)n	1.27	↑	4.90E−13
	Ribose + n-acetyl-glutamate	2.21	↓	4.86E−07
	Methyl-malonate	1.21	↓	4.17E−02

Table shows the metabolites identified, ^{1}H chemical shift values in ppm scale (δH) and status represented by arrows ↑↓ indicating increased or decreased metabolites, respectively, based on VIP scores (>1; $p < 0.05$ from Student's t-test and FDR correction $q < 0.05$).

TABLE 2 | Enzymes related to metabolite accumulation.

Metabolite	Related enzymes	Cell comparison
Threonine	THNSL1 THNSL2	Fibroblast vs. ZsG
Creatine	GAMT ASL	Fibroblast vs. ZsG
n-acetyl	NAT1 NAT2 NAT8 NAT8B GNPNAT1 AANAT HGSNAT ESCO1 ESCO2	Fibroblast vs. ZsG; ZsG vs. LN1
Malonate	ALDH6A1	Fibroblast vs. ZsG
Ribose + n-acetyl-glutamate	RBKS NAGS	Fibroblast vs. ZsG; ZsG vs. LN1; LN1 vs. LN2
Phosphoethanolamine	ETNK1 ETNK2	ZsG vs. LN1
Ethanolamine	PHOSPHO1	ZsG vs. LN1
Threitol	FGGY	ZsG vs. LN1
(CH2)n	Fatty acid synthase HADHA HADHB	ZsG vs. LN1; LN1 vs. LN2
Methyl-malonate	ALDH6A1	LN1 vs. LN2

Table shows the enzymes related to the accumulation of metabolites and the corresponding cell comparisons.

With this approach, it is possible to observe that when cells are undergoing glycolysis there is a higher free/bound NAD(P)H ratio. In contrast, when cells have an increment in oxidative phosphorylation (OXPHOS), a lower free/bound NAD(P)H ratio is observed (39).

In **Figure 2A,** the intensity map of NADH fluorescence shows a different distribution of these molecules. To analyze the classical metabolic states (glycolytic and oxidative states), we divided the lifetime distribution of the cells in two cursors, named short and long lifetime cursor (**Figures 2B,C**). The short lifetime represents a higher NAD(P)H free/bound ratio (colored in red), which means lower binding to dehydrogenases, which is classically associated to a glycolytic profile. The long lifetime represents a lower NAD(P)H free/bound ratio (colored in green). This is related to an increased proportion of binding of NAD(P)H molecules to dehydrogenases, hence associated to OXPHOS (39) (**Figure 2E**). Moreover, we quantified the percentage of pixels in each image within the cursors and observed that ZsG and LN1 cell lines showed the same pattern of NAD(P)H free/bound ratio whereas LN2 has a lower ratio, indicating that these cell line exhibited a more oxidative profile (**Figure 2D**).

To better understand and compare the cell lines regarding the lifetime distribution, we analyzed the FLIM maps of each replicate pictured (40). Because the cell lines were shown to be heterogeneous and their phasor plots covered a wide range of different lifetimes, we evaluated the mean phasor plot of each image acquired taking into account all cells present in a certain field in order to obtain the phasor fingerprint of each cell population. **Figure 3** shows the distribution regarding means of G and S coordinates of ZsG, LN1, and LN2 cells. Analyzing the metabolic trajectory, we did not observe significant differences between the cell lines regarding the color-coded scale (**Figure 3B**), the phasor fingerprint (**Figure 3C**), and also the comparison of the mean G position of each cell line (**Figure 3D**, left). Notwithstanding, LN1 cells seem to have a dislocated dispersion of lifetimes when compared to ZsG and LN2. This can be seen by analyzing the mean S position of each cell line (**Figure 3D**, right), in which the pixels of LN1 images have a lower position on the phasor plot. Thus, we observed that although there were no dramatic differences among the cell lines regarding the metabolic pathways (glycolytic vs. oxidative states), they were different when the lifetime distributions were considered.

Mitochondrial Respiration and Lactate Exportation Does Not Impair the Malignant Transformation in SCC Derived Cell Lines

To further analyze the contributions of oxidative metabolism accompanying the FLIM experiments, we performed

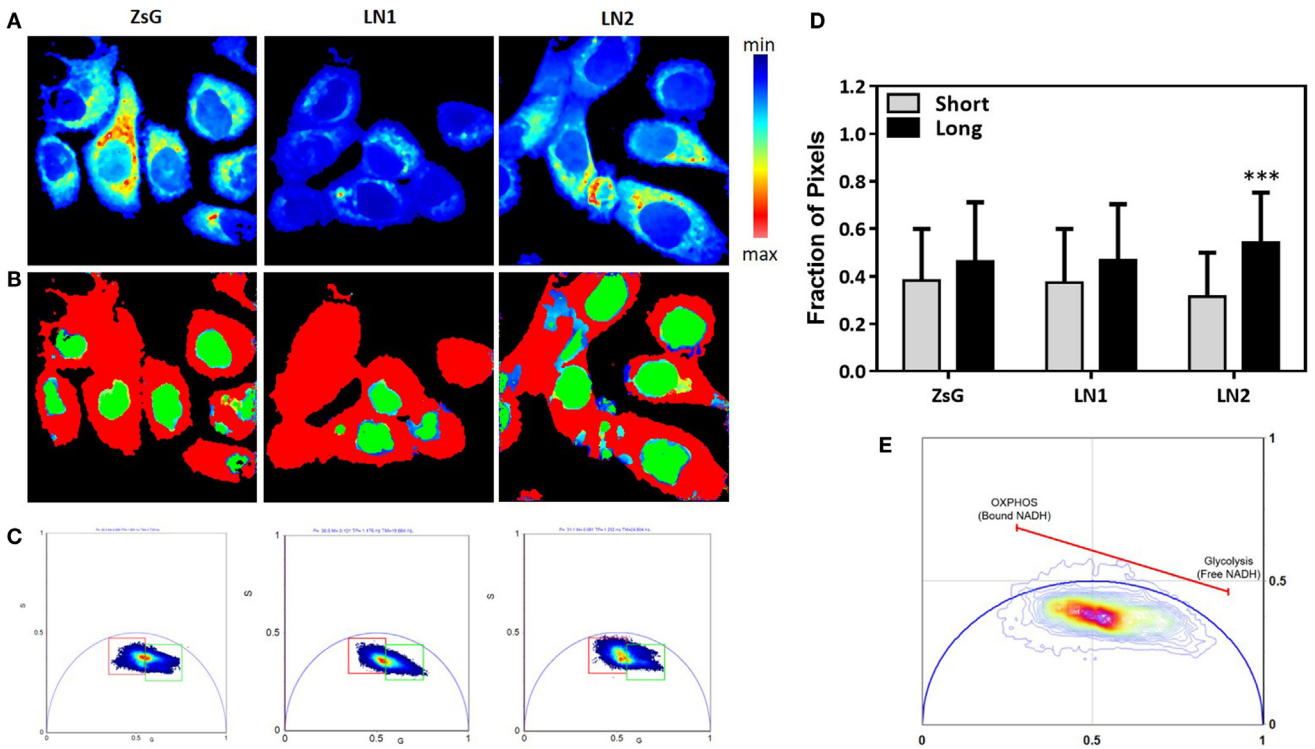


FIGURE 2 | NADH fluorescence lifetime distribution in ZsG, LN1, and LN2 cells. **(A)** Color-coded images generated by the cursor selection according to the phasor plot (colored squares). **(B)** Two-photon fluorescence microscopy intensity images showing NADH fluorescence of ZsG, LN1 and LN2 cells excited at 740 nm. **(C)** Phasor plot illustrating the metabolic trajectories or the fraction of free/bound NADH in FLIM images. **(D)** Fraction of pixels in the phasor plot. **(E)** Metabolic trajectory on the phasor plot. All the images were obtained on a two-photon microscope (Nikon Eclipse TE2000-U) and Plan-Apochromat 60×/1.20 water immersion objective lens (Nikon). Data were acquired on VistaVision software and Alba FLIM instrument (ISS) and analyzed on SimFCS software. Values represent mean ± SD; $N = 4$; ***$P < 0.0001$; ns compared within each group.

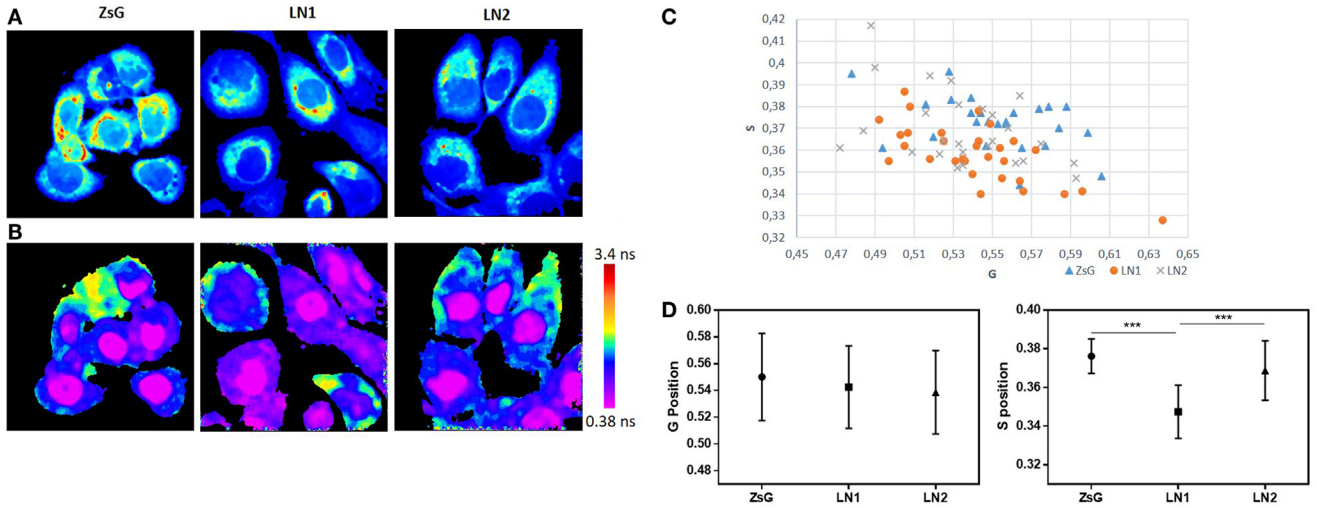


FIGURE 3 | NADH fluorescence lifetime distribution in ZsG, LN1, and LN2 cells. **(A)** Two-photon fluorescence microscopy intensity images showing NADH fluorescence of ZsG, LN1, and LN2 cells excited at 740 nm. **(B)** FLIM map of ZsG, LN1, and LN2 represented by a color-coded scale, by which pixels with a high free/bound NADH ratio are in purple, and pixels with a low free/bound NADH ratio are in red. All the lifetimes ratios are related to a specific color of the scale. **(C)** Phasor fingerprint, representing the mean S and G position of each image phasor distribution. **(D)** Mean S (left) and mean G (right) position of each cell line lifetimes distribution on phasor plot. Values represent mean ± SD; $N = 5$, with five images each; $^{***}P < 0.0001$. All the images were obtained on a two-photon microscope (Nikon Eclipse TE2000-U) and Plan-Apochromat 60×/1.20 water immersion objective lens (Nikon). Data were acquired on VistaVision software and Alba FLIM instrument (ISS) and analyzed on SimFCS software.

high-resolution respirometry assays using OXPHOS modulators in intact cells. **Figure 4A** displays a representative trace of oxygen consumption rate in each isolated cell line in the presence of culture medium containing the essential substrates (glucose and glutamine). In order to evaluate the dependence of respiratory complexes on oxygen consumption, we used membrane permeable compounds that affect mitochondrial function. **Figure 4B** shows the absolute values of oxygen consumption showing that it is not possible to distinguish ZsG, LN1, and LN2 by examining their oxidative status.

Metabolomic data showed that cells accumulate lactate during their malignant process (**Figure 1D**). Moreover, the proliferative phenotype is associated to higher lactate production and transportation to extracellular matrix in keeping with an enhanced glycolytic activity. In order to investigate this feature, we carried out a dynamic lactate liberation assay in presence or absence of AA, which stimulates glycolytic activity. Our results showed that even in the presence of AA there were no differences between all three cell lines (**Figure 4C**). Additionally, proliferation essays showed that LN1 and LN2 are less proliferative than ZsG cell line (**Figure 4D**).

DISCUSSION

The ability of cancer cells to detach from the primary tumor migrate through the blood/lymphatic system and eventually colonize distant tissues must be accompanied by metabolic reprograming. Probing those changes can help to understand the mechanisms of the metastatic processes. Accordingly, we first identified the major metabolites accumulated as the product of metabolic pathways in tongue SCC progression model used here. Taking a holistic approach, we performed ^{1}H RMN spectroscopy of ZsG, LN1, and LN2 cell lines as well as normal fibroblasts. We found that the major variations that could be associated to metastasis involved pathways of amino acids and fatty acid metabolism. These were revealed by alterations in the amounts of threonine, ribose, n-acetyl, malonate, methylmalonate, malonate, threitol, n-acetyl glutamate, ethanolamine, phosphoetanolamine, unsaturated lipids (CH_2)n, and lactate (**Figures 1A–D**). In order to understand the metabolic pathways related to the principal metabolites accumulated in cells, we carried out paired comparisons considering parental (or one step before in malignant process) and derived cell lines having observed that therein lay the highest differences. Thus, the fibroblasts vs. ZsG, ZsG vs. LN1, and LN1 vs. LN2 cell lines were compared (**Table 1**; Figure S1 in Supplementary Material). The results in **Table 1** show the main differences encountered for metabolites present in the cell lines. The individual occurrence and concentrations of the compounds listed at any given moment could arise from either upstream activation of enzymes participating in the catabolism of various precursors, or from inhibition of downstream enzymes participating in their breakdown. To make a mechanistic sense of these findings, one would have to take into account the fluxes of all involved pathways and then propose models that would fit into coherent biochemical patterns compatible with the phenotypes of the cells displaying different metastatic potentials. Despite the unknown kinetics following the dynamic interplay that contributed toward the pattern observed, the results reflect in reproducible manner instant snapshots of the metabolic status of each cell line. That is, the differences detected are definitely informative in terms of the metabolic reprograming that took place for each cell line. In other words, the results emphasize that

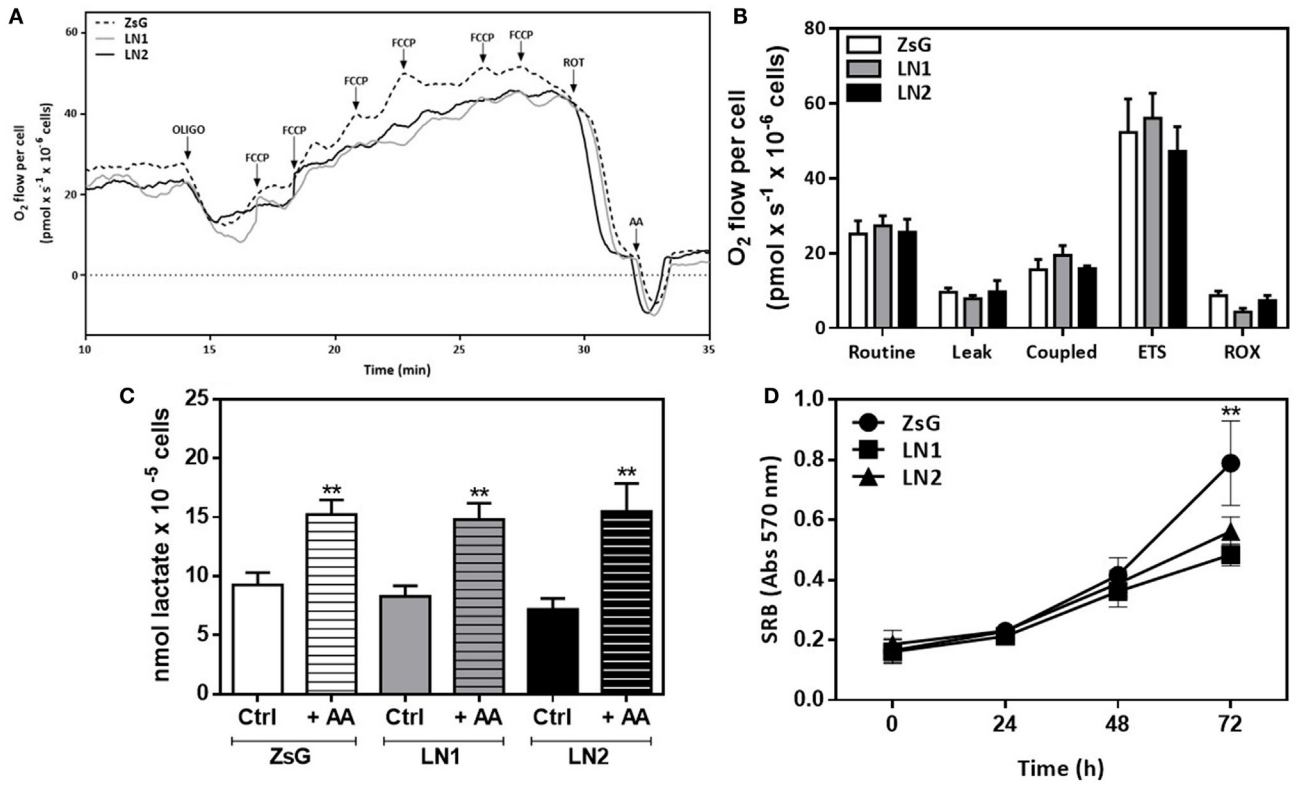


FIGURE 4 | Mitochondrial respiration and lactate exportation does not impair the malignant transformation in squamous cells carcinoma-derived cell lines. High-resolution respirometry assay using Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria) in intact cells. Representative traces of oxygen consumption rate in intact cells **(A)** and absolute results of O_2 flow per cell (pmol × s^{-1} × 10^{-6} cells) **(B)**. During the assay, cells were maintained in DMEM F12 medium without FBS. Oxygen consumption was evaluated after the addition of modulators of mitochondrial activity [oligomycin, FCCP, rotenone and antimycin A (AA)]. Routine respiration corresponds to endogenous oxygen consumption of cells. All parameters were corrected to ROX values. **(C)** Lactate release after 60 min of incubation with fresh DMEM F12 medium without FBS in the absence (Ctrl) or presence (+AA) of AA. **(D)** Proliferation using sulforhodamine B (SRB) colorimetric assays. Values represent means ± SD; $n = 4$; $^{**}P < 0.005$ comparing among cell lines (oxygen consumption and proliferation) or within cell line (lactate release).

the cell lines have individual profiles that can suggest specific ontologies and functional meanings. Nevertheless, it is possible to speculate that some of the compounds listed in **Table 1** may actually have roles as intermediaries. This means that in their own right the metabolites could act as effectors participating in regulatory networks, altering the expression patterns and, therefore, impacting cellular plasticity. Among the compounds listed in **Table 1**, some of the molecules, such as creatine, threitol, threonine, phosphoethanolamine, ethanolamine, and ribose, irrespective of their potential additional roles in other metabolic pathways, could be grouped under the functional class of osmolytes. These are compounds that interchangeably stabilize the intracellular milieu by buffering osmotic perturbations, for instance. The notion that the osmolytes and compatible solutes listed in **Table 1** may play such a role opens up the possibility that they could act as effectors of a much broader regulatory network, given their ability to affect flexible and random coil conformations of transcription factors (41–43). In this way, the compounds detected in our metabolomic analyses may have a dual role. On the one hand, they may arise from differentially activated metabolic pathways responding to energy demands and supplying precursors such as building blocks for membranes, DNA, etc. Alternatively, the metabolites could act as bona fide regulators by altering the conformations of members of the signaling pathways and thus generate phenotypes such as the metastatic ones. This model is attractive since one could invoke new levels of regulation that are independent of gain or loss of function induced by those regulations and to grow and survive, cancer cells display alternate metabolic pathways toward tumorigenesis and progression. This hypothesis is currently being investigated in our laboratory.

Further information extracted from the metabolome involved the manual search for enzymes related to metabolite accumulation in their synthesis/degradation using biochemical pathways found in REACTOME free software (Table S1 in Supplementary Material). The results obtained pointed clearly to the involvement of the pathways connected to lipid metabolism. The importance of lipid metabolism becomes apparent when considering that lipid metabolism byproducts can influence growth promoting activity and hence tumorigenesis (44). However, the less invasive cells exhibit enzymes, which although could be broadly included in the lipid metabolism category are associated to specialized reactions pertaining to lipid biochemistry, or perhaps more to the point, catabolism of hydrophobic compounds.

Upon comparison between the metabolomic data between fibroblasts and ZsG, we found that threonine accumulated in fibroblasts. The metabolism of threonine involves the reaction catalyzed by threonine synthase (THNSL1 and THNSL2) forming L-threonine from O-phospho-L-homoserine (45). Threonine biosynthesis initiates from the precursor aspartate leading to a chain of reactions dependent of ATP and NADPH (46). In contrast, ZsG cells accumulated metabolites that promote biosynthesis relying in the reduction of $NADP^+$ producing NADPH, such as malonate and ribose. It is worth mentioning that methylmalonate (accumulated in LN1 cells) and malonate are both regulated by ALDH6A1, and that this enzyme is related to fatty acid degradation and amino acid metabolism (**Table 2**) (47).

Creatine pathway is not related to oxidation/reduction of NAD(P)H despite playing important roles in maintaining storage of ATP in skeletal muscle cells. During muscular contraction, the creatine-phosphate molecules can be decomposed forming ATP before fermentation reestablishes ATP content (48). Creatine may thus be adjuvant in maintaining the demand of ATP (49) in ZsG cell lines. Our data also shows enhanced activity of cell cycle regulation in ZsG and LN1 cells. This has been observed in several cancer cell lines with chromatin cohesion defects, which lead to a poor response to paclitaxel (50). This could mean that ZsG and LN1 cells may be more sensitive to spindle poisons than LN2 cells. Again, the catabolism of some fatty acids such as ω-fatty acids play a major role in the metabolism and biotransformation of exogenous compounds such as xenobiotics. This is achieved for example, by P450 (CYP) mixed function oxidase system that mediates by hydroxylation reactions that in general increase the solubility of these compounds, thus facilitating their excretion. The cytochrome P450 mixed function oxidase enzymes are also known to be involved in the biosynthesis of endogenous substrates such as cholesterol (51).

Phosphoethanolamine and ethanolamine are accumulated in LN1 cells when compared to ZsG. Phospholipids are required to build bilayer membranes in cells, and many studies show that its content increases as long as malignant process arises (52, 53). The membrane components including lipids and proteins allow the formation of ion channels, receptors, and signal transducers important to the interaction of extracellular matrix and cytosol (50), suggesting that those metabolites play important roles in proliferation and growth signaling in LN1 cells.

Comparison between LN1 and LN2 cell lines show that the unsaturated lipids (CH2)n are the only class accumulated in LN2 cells. (CH2)n metabolism shares pathways with valine, leucine, and isoleucine degradation (amino acid metabolism) and fatty acid metabolism. All of them are related to NAD(P)H dynamics. FLIM data confirm these observations (**Figure 2D**), showing that LN2 cells displays lower NAD(P)H free/bound ratio.

Ribose metabolism is associated to the pentose phosphate pathway (PPP), an important pathway that provides cancer cells with NADPH, ribose-5-phosphate by oxidative pathway. NADPH is essential in the antioxidant defense by glutathione production, while ribose-5-phosphate is an important element for nucleotide biosynthesis. Upregulation of PPP promotes cancer cell survival, proliferation, angiogenesis, invasion and metastasis, and resistance to radiotherapy and chemotherapy. On the other hand, the non-oxidative moiety of PPP reenters fructose-6-phosphate and glyceraldehyde-3-phosphate of the glycolytic pathway, fueling proliferation (54, 55). In the context of our model, the accumulation of ribose was observed in ZsG and LN1 cells. Interestingly, respirometry assays did not show any differences between the cell lines. Plausibly, this could be due to the non-oxidative PPP, fueling the bioenergetic pathways. Furthermore, *N*-acetylglutamate is an obligatory allosteric activator of carbamoyl phosphate synthetase I (CPS-1) (56). CPS-1 is related to cell growth and metabolite levels associated with nucleic acid biosynthesis pathway, as shown by CPS-1 knockdown in lung adenocarcinoma (57). Our analysis revealed that *N*-acetylglutamate is increased in ZsG and LN1 cells, which is compatible with amino acid metabolism and its contributions to nucleotide biosynthesis.

We did not detect differences in lactate exported from cells (**Figure 4C**) when the three cell lines were compared. The balance between accumulation and exportation lactate in tumor cells has been described as an important factor in regulating glucose metabolism and NAD^+/NADH availability (58, 59). Much is known about lactate regulation in cancer cells and metabolic switch bearing glycolysis and tumorigenesis (60). Indeed, ZsG cells displaying higher proliferative rates were shown to incorporate less lactate when compared to LN1 and LN2 cells.

The interaction processes between NAD(P)H and proteins was also investigated here. Because these coenzymes play important roles in energy metabolism and, therefore, impact on tumor transformation and progression (20, 38, 61), their participation in the establishment of the metastatic phenotypes was determined, reflecting the importance of measuring NAD(P)H lifetime as an informative biomarker for understanding metabolic reprograming, mitochondrial physiology, oxidative stress, and apoptosis (39). Real-time NAD(P)H lifetime imaging by two-photon fluorescence microscopy allowed prospection into the dynamics of NAD(P)H, by analyzing its binding to specific dehydrogenases, representing an increased NAD(P)H free/bound ratio in glycolytic cells (39). As observed in FLIM experiments, LN2 cells exhibit a relatively smaller free/bound NAD(P)H ratio. However, there were no significant differences when comparing free/bound ratios of ZsG to LN1 cells.

Taken together, the results of the metabolomic analysis and FLIM analysis allowed us to conclude that several pathways connected to lipid metabolism appear to be prominently linked to metastatic phenotypes, while cell cycle regulation and amino acid metabolism are most related to less invasive cells.

AUTHOR CONTRIBUTIONS

AS-S and GS contributed to N.M.R. metabolomics performance and analysis; AS-S, AG, and SC contributed to FLIM microscopy performance and interpretation of data; AS-S contributed to respirometry, lactate release, and proliferation assays; AS-S and JP-V contributed to the analyses of metabolic pathways; AS-S, JP-V, GS, SC, and FR discussed the data; FR coordinated the project. All authors participated in the writing and revision process.

ACKNOWLEDGMENTS

We would like to thank to Prof. Dr. Fabio Almeida for kindly providing support with N.M.R. operation, data acquisition, and brilliantly contributions to scientific discussions.

FUNDING

This project was supported by Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro—FAPERJ (E-26/201.251/2014), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—CAPES (AUXPE-COFFECUB 2528/2014 Process 23038.001758/2014-47), and Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq (403310/2016-4).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fonc.2018.00013/full#supplementary-material.

REFERENCES

1. Sano D, Myers JN. Metastasis of squamous cell carcinoma of the oral tongue. *Cancer Metastasis Rev* (2007) 26:645–62. doi:10.1007/s10555-007-9082-y
2. Scully C, Bagan J. Oral squamous cell carcinoma: overview of current understanding of aetiopathogenesis and clinical implications. *Oral Dis* (2009) 15(6):388–99. doi:10.1111/j.1601-0825.2009.01563.x
3. Kelner N, Rodrigues PC, Bufalino A, Fonseca FP, Santos-Silva AR, Miguel MC, et al. Activin A immunoexpression as predictor of occult lymph node metastasis and overall survival in oral tongue squamous cell carcinoma. *Head Neck* (2015) 37(4):479–86. doi:10.1002/hed.23627
4. Argiris A, Karamouzis MV, Raben D, Ferris RL. Head and neck cancer. *Lancet* (2008) 371(9625):1695–709. doi:10.1016/S0140-6736(08)60728-X
5. Sinevici N, O'sullivan J. Oral cancer: deregulated molecular events and their use as biomarkers. *Oral Oncol* (2016) 61:12–8. doi:10.1016/j.oraloncology.2016.07.013
6. Hsu PP, Sabatini DM. Cancer cell metabolism: warburg and beyond. *Cell* (2008) 134(5):703–7. doi:10.1016/j.cell.2008.08.021
7. 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
8. Moreno-Sánchez R, Rodríguez-Enríquez S, Marín-Hernández A, Saavedra E. Energy metabolism in tumor cells. *FEBS J* (2007) 274(6):1393–418. doi:10.1111/j.1742-4658.2007.05686.x
9. Pavlova NN, Thompson CB. The emerging hallmarks of cancer metabolism. *Cell Metab* (2016) 23(1):27–47. doi:10.1016/j.cmet.2015.12.006
10. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metab* (2008) 7(1):11–20. doi:10.1016/j.cmet.2007.10.002
11. Cairns R, Harris I, Mak T. Regulation of cancer cell metabolism. *Nat Rev Cancer* (2011) 11(2):85–95. doi:10.1038/nrc2981
12. Sullivan LB, Gui DY, Hosios AM, Bush LN, Freinkman E, Vander Heiden MG. Supporting aspartate biosynthesis is an essential function of respiration in proliferating cells. *Cell* (2015) 162(3):552–63. doi:10.1016/j.cell.2015.07.017
13. Shao W, Gu J, Huang C, Liu D, Huang H, Huang Z, et al. Malignancy-associated metabolic profiling of human glioma cell lines using 1H NMR spectroscopy. *Mol Cancer* (2014) 13(1):197. doi:10.1186/1476-4598-13-197
14. Wang H, Zhang H, Deng P, Liu C, Li D, Jie H, et al. Tissue metabolic profiling of human gastric cancer assessed by 1H NMR. *BMC Cancer* (2016) 16(1):371. doi:10.1186/s12885-016-2356-4
15. Battini S, Faitot F, Imperiale A, Cicek AE, Heimburger C, Averous G, et al. Metabolomics approaches in pancreatic adenocarcinoma: tumor metabolism profiling predicts clinical outcome of patients. *BMC Med* (2017) 15(1):56. doi:10.1186/s12916-017-0810-z
16. Choi H, Pavelka N. When one and one gives more than two: challenges and opportunities of integrative omics. *Front Genet* (2012) 2:105. doi:10.3389/fgene.2011.00105
17. Barallobre-Barreiro J, Chung Y-L, Mayr M. Proteomics and metabolomics for mechanistic insights and biomarker discovery in cardiovascular disease. *Rev Esp Cardiol (Engl Ed)* (2013) 66(8):657–61. doi:10.1016/j.rec.2013.04.009
18. Skala MC, Riching KM, Gendron-Fitzpatrick A, Eickhoff J, Eliceiri KW, White JG, et al. In vivo multiphoton microscopy of {NADH} and {FAD} redox states, fluorescence lifetimes, and cellular morphology in precancerous epithelia. *Proc Natl Acad Sci U S A* (2007) 104(49):19494–9. doi:10.1073/pnas.0708425104
19. Stringari C, Cinquin A, Cinquin O, Digman MA, Donovan PJ, Gratton E. Phasor approach to fluorescence lifetime microscopy distinguishes different metabolic states of germ cells in a live tissue. *Proc Natl Acad Sci U S A* (2011) 108(33):13582–7. doi:10.1073/pnas.1108161108
20. Blacker TS, Duchen MR. Investigating mitochondrial redox state using NADH and NADPH autofluorescence. *Free Radic Biol Med* (2016) 100:53–65. doi:10.1016/j.freeradbiomed.2016.08.010
21. Agostini M, Almeida LY, Bastos DC, Ortega RM, Moreira FS, Seguin F, et al. The fatty acid synthase inhibitor orlistat reduces the growth and metastasis of orthotopic tongue oral squamous cell carcinomas. *Mol Cancer Ther* (2014) 13(3):585–95. doi:10.1158/1535-7163.MCT-12-1136
22. Santos GC, Zeidler JD, Pérez-Valencia JA, Sant'Anna-Silva ACB, Da Poian AT, El-Bacha T, et al. Metabolomic analysis reveals vitamin D-induced decrease in polyol pathway and subtle modulation of glycolysis in HEK293T cells. *Sci Rep* (2017) 7(1):9510. doi:10.1038/s41598-017-10006-9
23. Xia J, Sinelnikov IV, Han B, Wishart DS. MetaboAnalyst 3.0-making metabolomics more meaningful. *Nucleic Acids Res* (2015) 43(W1):W251–7. doi:10.1093/nar/gkv380
24. Westerhuis JA, Huub CJ, Hoefsloot HCJ, Smit S, Vis DJ, Smilde AK, et al. Assessment of PLSDA cross validation. *Metabolomics* (2008) 4(1):81–9. doi:10.1007/s11306-007-0099-6
25. Szymańska E, Saccenti E, Smilde AK, Westerhuis JA. Double-check: validation of diagnostic statistics for PLS-DA models in metabolomics studies. *Metabolomics* (2012) 8:3–16. doi:10.1007/s11306-011-0330-3
26. Fabregat A, Sidiropoulos K, Garapati P, Gillespie M, Hausmann K, Haw R, et al. The reactome pathway knowledgebase. *Nucleic Acids Res* (2016) 44(D1):D481–7. doi:10.1093/nar/gkv1351
27. Digman MA, Gratton E. Analysis of diffusion and binding in cells using the RICS approach. *Microsc Res Tech* (2009) 72(4):323–32. doi:10.1002/jemt.20655
28. Marcu L. Fluorescence lifetime techniques in medical applications. *Ann Biomed Eng* (2012) 40(2):304–31. doi:10.1007/s10439-011-0495-y
29. Digman MA, Caiolfa VR, Zamai M, Gratton E. The phasor approach to fluorescence lifetime imaging analysis. *Biophys J* (2008) 94(2):L14–6. doi:10.1529/biophysj.107.120154
30. Aguilar-Arnal L, Ranjit S, Stringari C, Orozco-Solis R, Gratton E, Sassone-Corsi P. Spatial dynamics of SIRT1 and the subnuclear distribution of NADH species. *Proc Natl Acad Sci U S A* (2016) 113(45):12715–20. doi:10.1073/pnas.1609227113
31. Hutter E, Renner K, Pfister G, Stöckl P, Jansen-Dürr P, Gnaiger E. Senescence-associated changes in respiration and oxidative phosphorylation in primary human fibroblasts. *Biochem J* (2004) 380:919–28. doi:10.1042/BJ20040095
32. Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat Protoc* (2006) 1(3):1112–6. doi:10.1038/nprot.2006.179
33. Ulrich EL, Akutsu H, Doreleijers JF, Harano Y, Ioannidis YE, Lin J, et al. BioMagResBank. *Nucleic Acids Res* (2007) 36(Database):D402–8. doi:10.1093/nar/gkm957
34. Wishart DS, Jewison T, Guo AC, Wilson M, Knox C, Liu Y, et al. HMDB 3.0—The human metabolome database in 2013. *Nucleic Acids Res* (2013) 41(D1):D801–7. doi:10.1093/nar/gks1065
35. Robinette SL, Zhang F, Brüschweiler-Li L, Brüschweiler R. Web server based complex mixture analysis by NMR. *Anal Chem* (2008) 80(10):3606–11. doi:10.1021/ac702530t
36. Triba MN, Le Moyec L, Amathieu R, Goossens C, Bouchemal N, Nahon P, et al. {PLS}/{OPLS} models in metabolomics: the impact of permutation of

dataset rows on the {K}-fold cross-validation quality parameters. *Mol Biosyst* (2014) 11(1):13–9. doi:10.1039/C4MB00414K
37. Szklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, et al. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res* (2017) 45(D1):D362–8. doi:10.1093/nar/gkw937
38. Chiarugi A, Dölle C, Felici R, Ziegler M. The NAD metabolome – a key determinant of cancer cell biology. *Nat Rev Cancer* (2012) 12(11):741–52. doi:10.1038/nrc3340
39. Stringari C, Edwards RA, Pate KT, Waterman ML, Donovan PJ, Gratton E. Metabolic trajectory of cellular differentiation in small intestine by phasor fluorescence lifetime microscopy of NADH. *Sci Rep* (2012) 2(1):568. doi:10.1038/srep00568
40. Lakowicz JR, Szmacinski H, Nowaczyk K, Johnson ML. Fluorescence lifetime imaging of free and protein-bound NADH. *Proc Natl Acad Sci U S A* (1992) 89(4):1271–5. doi:10.1073/pnas.89.4.1271
41. Mansouri AL, Grese LN, Rowe EL, Pino JC, Chennubhotla SC, Ramanathan A, et al. Folding propensity of intrinsically disordered proteins by osmotic stress. *Mol Biosyst* (2016) 12:3695–701. doi:10.1039/C6MB00512H
42. Strifler G, Tuboly E, Görbe A, Boros M, Pécz D, Hartmann P. Targeting mitochondrial dysfunction with L-alpha glycerylphosphorylcholine. *PLoS One* (2016) 11(11):e0166682. doi:10.1371/journal.pone.0166682
43. Staby L, O'Shea C, Willemoës M, Theisen F, Kragelund BB, Skriver K. Eukaryotic transcription factors: paradigms of protein intrinsic disorder. *Biochem J* (2017) 474(15):2509–32. doi:10.1042/BCJ20160631
44. Ray U, Roy SS. Aberrant lipid metabolism in cancer cells – the role of oncolipid-activated signaling. *FEBS J* (2017). doi:10.1111/febs.14281
45. Laber B, Gerbling KP, Harde C, Neff KH, Nordhoff E, Pohlenz HD. Mechanisms of interaction of *Escherichia coli* threonine synthase with substrates and inhibitors. *Biochemistry* (1994) 33(11):3413–23. doi:10.1021/bi00177a035
46. Zhang Y, Meng Q, Ma H, Liu Y, Cao G, Zhang X, et al. Determination of key enzymes for threonine synthesis through in vitro metabolic pathway analysis. *Microb Cell Fact* (2015) 14(1):86. doi:10.1186/s12934-015-0275-8
47. Bröer S, Bröer A. Amino acid homeostasis and signalling in mammalian cells and organisms. *Biochem J* (2017) 474(12):1935–63. doi:10.1042/BCJ20160822
48. Wyss M, Wallimann T. Creatine metabolism and the consequences of creatine depletion in muscle. *Mol Cell Biochem* (1994) 133–134(1):51–66. doi:10.1007/BF01267947
49. Jacobus WE, Moreadith RW, Vandegaer KM. Control of heart oxidative phosphorylation by creatine kinase in mitochondrial membranes. *Ann N Y Acad Sci* (1983) 414:73–89. doi:10.1111/j.1749-6632.1983.tb31676.x
50. Szachowicz-Petelska B, Dobrzyńska I, Skrodzka M, Darewicz B, Figaszewski ZA, Kudelski J. Phospholipid composition and electric charge in healthy and cancerous parts of human kidneys. *J Membr Biol* (2013) 246(5):421–5. doi:10.1007/s00232-013-9554-7
51. Cederbaum AI. Molecular mechanisms of the microsomal mixed function oxidases and biological and pathological implications. *Redox Biol* (2015) 4:60–73. doi:10.1016/j.redox.2014.11.008
52. Dobrzyńska I, Szachowicz-Petelska B, Darewicz B, Figaszewski ZA. Characterization of human bladder cell membrane during cancer transformation. *J Membr Biol* (2015) 248(2):301–7. doi:10.1007/s00232-015-9770-4
53. Cheng M, Bhujwalla ZM, Glunde K. Targeting phospholipid metabolism in cancer. *Front Oncol* (2016) 6:266. doi:10.3389/fonc.2016.00266
54. Herling A, König M, Bulik S, Holzhütter HG. Enzymatic features of the glucose metabolism in tumor cells. *FEBS J* (2011) 278(14):2436–59. doi:10.1111/j.1742-4658.2011.08174.x
55. Phan LM, Yeung S-CJ, Lee M-H. Cancer metabolic reprogramming: importance, main features, and potentials for precise targeted anti-cancer therapies. *Cancer Biol Med* (2014) 11(1):1–19. doi:10.7497/j.issn.2095-3941.2014.01.001
56. Liu H, Dong H, Robertson K, Liu C. DNA methylation suppresses expression of the urea cycle enzyme carbamoyl phosphate synthetase 1 (CPS1) in human hepatocellular carcinoma. *Am J Pathol* (2011) 178(2):652–61. doi:10.1016/j.ajpath.2010.10.023
57. Çeliktas M, Tanaka I, Tripathi SC, Fahrmann JF, Aguilar-Bonavides C, Villalobos P, et al. Role of CPS1 in cell growth, metabolism, and prognosis in LKB1-inactivated lung adenocarcinoma. *J Natl Cancer Inst* (2017) 109(3):1–9. doi:10.1093/jnci/djw231
58. Schafer ZT, Grassian AR, Song L, Jiang Z, Gerhart-Hines Z, Irie HY, et al. Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. *Nature* (2009) 461(7260):109–13. doi:10.1038/nature08268
59. Hirschhaeuser F, Sattler UGA, Mueller-Klieser W. Lactate: a metabolic key player in cancer. *Cancer Res* (2011) 71(22):6921–5. doi:10.1158/0008-5472.CAN-11-1457
60. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the warburg effect: the metabolic requirements of cell proliferation. *Science* (2009) 324(5930):1029–33. doi:10.1126/science.1160809
61. Blacker TS, Mann ZF, Gale JE, Ziegler M, Bain AJ, Szabadkai G, et al. Separating NADH and NADPH fluorescence in live cells and tissues using FLIM. *Nat Commun* (2014) 5:3936. doi:10.1038/ncomms4936

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 19 May 2017
doi: 10.3389/fonc.2017.00098

Targeting Ongoing DNA Damage in Multiple Myeloma: Effects of DNA Damage Response Inhibitors on Plasma Cell Survival

Ana Belén Herrero[1,2] *and Norma Carmen Gutiérrez*[1,2,3]*

[1] Cancer Research Center-IBMCC (USAL-CSIC), Salamanca, Spain, [2] Institute of Biomedical Research of Salamanca (IBSAL), Salamanca, Spain, [3] Hematology Department, University Hospital of Salamanca, Salamanca, Spain

Human myeloma cell lines (HMCLs) and a subset of myeloma patients with poor prognosis exhibit high levels of replication stress (RS), leading to DNA damage. In this study, we confirmed the presence of DNA double-strand breaks (DSBs) in several HMCLs by measuring γH2AX and RAD51 foci and analyzed the effect of various inhibitors of the DNA damage response on MM cell survival. Inhibition of ataxia telangiectasia and Rad3-related protein (ATR), the main kinase mediating the response to RS, using the specific inhibitor VE-821 induced more cell death in HMCLs than in control lymphoblastoid cells and U266, an HMCL with a low level of DNA damage. The absence of ATR was partially compensated by ataxia telangiectasia-mutated protein (ATM), since chemical inhibition of both kinases using VE-821 and KU-55933 significantly increased the death of MM cells with DNA damage. We found that ATM and ATR are involved in DSB repair by homologous recombination (HR) in MM. Inhibition of both kinases resulted in a stronger inhibition that may underlie cell death induction, since abolition of HR using two different inhibitors severely reduced survival of HMCLs that exhibit DNA damage. On the other hand, inhibition of the other route involved in DSB repair, non-homologous end joining (NHEJ), using the DNA-PK inhibitor NU7441, did not affect MM cell viability. Interestingly, we found that NHEJ inhibition did not increase cell death when HR was simultaneously inhibited with the RAD51 inhibitor B02, but it clearly increased the level of cell death when HR was inhibited with the MRE11 inhibitor mirin, which interferes with recombination before DNA resection takes place. Taken together, our results demonstrate for the first time that MM cells with ongoing DNA damage rely on an intact HR pathway, which thereby suggests therapeutic opportunities. We also show that inhibition of HR after the initial step of end resection might be more appropriate for inducing MM cell death, since it prevents the occurrence of a compensatory NHEJ repair mechanism. These preclinical observations provide the rationale for its clinical evaluation.

Keywords: DNA damage response, DNA damage, ataxia telangiectasia-mutated protein, ataxia telangiectasia and Rad3-related protein, double-strand break repair, homologous recombination, non-homologous end joining

OPEN ACCESS

Edited by:
Luciana Boffoni Gentile, Federal University of Rio de Janeiro, Brazil

Reviewed by:
Valerio Donato, New York University, USA
Gavin P. McStay, New York Institute of Technology, USA
Eduardo J. Salustiano, Federal University of Rio de Janeiro, Brazil

***Correspondence:**
Norma Carmen Gutiérrez
normagu@usal.es

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *08 March 2017*
Accepted: *01 May 2017*
Published: *19 May 2017*

Citation:
Herrero AB and Gutiérrez NC (2017) Targeting Ongoing DNA Damage in Multiple Myeloma: Effects of DNA Damage Response Inhibitors on Plasma Cell Survival. Front. Oncol. 7:98. doi: 10.3389/fonc.2017.00098

INTRODUCTION

Multiple myeloma (MM), the second most common hematological malignancy, arises from the abnormal clonal proliferation of malignant plasma cells (1, 2). Current therapies have significantly improved survival of MM patients. On the one hand, high-dose melphalan followed by autologous hematopoietic cell transplant has become a standard of care for young patients after bortezomib-based induction regimens. On the other hand, the introduction of novel agents, particularly bortezomib combined with alkylating drugs and prednisone, or lenalidomide plus dexamethasone has also improved the outcome of patients who are ineligible for high-dose therapy (3, 4). Nevertheless, MM remains an incurable disease, and new therapeutic strategies are still needed. A prominent feature of MM cells is their genome instability, whose underlying molecular basis is not fully understood. Recently, it has been proposed that high levels of ongoing intrinsic DNA damage and deregulated double-strand break (DSB) repair influence the acquisition of genomic changes (5–8).

Double-strand breaks pose a serious threat to cell viability and genome stability if left unrepaired or if they are repaired incorrectly (9). These DNA lesions can be generated exogenously, by exposure to a variety of genotoxic agents, or endogenously, due to various factors that slow down or stall replication forks, a phenomenon known as replication stress (RS). Sources of RS include fragile sites, replication–transcription complex collision, secondary DNA structures, and oncogenic stress (10, 11). To limit the impact of DSBs, cells have evolved the DNA damage response (DDR), a signal transduction cascade that coordinates the signaling and repair of these genomic lesions (9). Ataxia telangiectasia-mutated protein (ATM) and ataxia telangiectasia and Rad3-related protein (ATR) are central components of the DDR. ATM is a serine–threonine kinase that phosphorylates several key proteins, leading to cell cycle arrest through phosphorylation of CHK2, DNA repair, or apoptosis (12–14). It is mainly activated by DSBs, such as those induced by ionizing radiation (IR). In contrast, ATR is the key kinase in signaling the response to single-strand DNA (ssDNA), which can occur at persistent DSBs and on stalled replication forks. It is considered to be the main kinase mediating the response to RS (10).

Once DNA damage has been detected, DSBs are mainly repaired by two pathways: non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is based on a direct ligation of the two ends of damaged DNA molecules and repairs DSBs mainly in G1 phase, although it is active throughout the cell cycle (15–17). HR promotes the recovery or repair of lesions that arise during replication and has a less important role in the repair of non-replication-associated DSBs (18). The pathway starts with the 5′-to-3′ resection of DNA ends, which is initiated by the endonuclease MRE11. The generated 3′-ssDNA overhangs bind to replication protein A, which is then exchanged for RAD51, the recombinase involved in the search for homology in the sister chromatid, thereby allowing subsequent repair (19).

High levels of RS leading to DNA damage have recently been described in a subset of MM (8). These patients, who overexpressed genes belonging to a chromosomal instability and DNA damage signature, displayed poor prognosis. On the other hand, several inhibitors of proteins involved in the DDR have been developed and some of them are either close to or are already being clinically trialed (20, 21). However, the effect of these types of compounds on MM cell viability has not been investigated in depth. In this study, we confirmed the presence of DSBs in several human myeloma cell lines (HMCLs) and evaluated cell survival after inhibition of the DDR. Our results show that MM with ongoing endogenous DNA damage depends on ATR over ATM and on HR over NHEJ, providing evidence of the presence of RS in these cells. However, the absence of ATR was partially compensated by ATM, and NHEJ was activated when HR was inhibited before DNA resection. These findings suggest that multiple inhibition of the DDR or inhibition of HR after DNA resection could extend the therapeutic opportunities. The implications of our findings for the treatment of the disease are discussed.

MATERIALS AND METHODS

Cells and Culture Conditions

The HMCL MM1S was acquired from American Type Culture Collection, and JJN3, RPMI-8226, U266, and OPM2 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen. LINF903, an Epstein–Barr virus-transformed B-cell line established from a healthy individual, was obtained from the National DNA Bank of the University of Salamanca, Spain. MM and LINF903 cell lines were cultured in RPMI 1640–L-glutamine medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% of fetal bovine serum (Sigma-Aldrich) and antibiotics (Gibco Life Technologies, Grand Island, NY, USA). All cells were incubated at 37°C in a 5% CO_2 atmosphere. The presence of mycoplasma was routinely checked with a MycoAlert kit (Lonza, Basel, Switzerland) and only mycoplasma-free cells were used in the experiments.

Immunofluorescence Staining

Cells (50,000) were mounted on glass slides by cytospinning for 10 min at 1,000 rpm. Cells were fixed in 2% paraformaldehyde for 20 min, permeabilized in 0.2% Triton X-100 in PBS for 10 min, blocked in 3% BSA in PBS for 30 min and incubated with anti-γ-H2AX (mouse, clone JBW301, Millipore) or anti-Rad51 (Ab-1, rabbit, Millipore) at 1:1,000 dilution for 2 h. After washing, slides were incubated with fluorescent secondary antibodies (1:1,000, Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 568 anti-rabbit) for 1 h. Slides were mounted with ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA, USA), and images were acquired using a DeltaVision system made up of a Olympus IX71 microscope, a Photometrics Coolsnap camera, and SOFWORX software. A 60× oil immersion objective was used.

Immunoblotting

Cells were washed with PBS and resuspended in RIPA lysis buffer (Santa Cruz Biotechnology) containing protease inhibitors (Complete, Roche Applied Science, Indianapolis, IN, USA) and phosphatase inhibitors (Santa Cruz Biotechnology). Protein concentration was measured using the Bradford assay (BioRad,

Hercules, CA, USA). Protein samples (20 μg/lane) were subjected to SDS-PAGE and transferred to PVDF membrane (BioRad). After blocking, membranes were incubated with anti-human antibodies. The following primary antibodies were used: anti-p-ATM (pSer[1981], mouse, clone 10H11.E12, Santa Cruz Biotechnology), anti-ATM (rabbit, clone D2E2, Cell Signaling, Danvers, MA, USA), anti-p-ATR (Ser428, rabbit, Cell Signaling), anti-ATR (goat, Santa Cruz Biotechnology) anti-tubulin (rabbit, Abcam, Cambridge, UK). Horseradish peroxidase-linked donkey anti-rabbit, anti-mouse, or anti-goat antibodies (Santa Cruz Biotechnology) were used as secondary antibodies at 1:10,000 dilution. Immunoblots were incubated for 1 h at RT and developed using enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA).

Reagents

KU-55933 and VE-821 were obtained from MedChemtronica (Stockholm, Sweden). Mirin was purchased from Sigma and NU7471 from Santa Cruz Biotechnology.

Cell Apoptosis Assays

Apoptosis was measured using Annexin V–fluorescein isothiocyanate/propidium iodide (Annexin V-FITC/PI) double staining (Immunostep, Spain) according to the manufacturer's guidelines.

Cell Cycle Analysis

Cells were washed in PBS and fixed in 70% ethanol for later use. Cells were rehydrated with PBS, resuspended in 500 μl of PI/RNase staining solution (Immunostep), and incubated for 20 min at RT in the dark. Samples were analyzed using a FACSCalibur flow cytometer.

HR Functional Assay

To determine the *in vivo* levels of HR, a reporter plasmid (22) was integrated into the genome of JJN3 and U266 cell lines as follows: cell lines were transfected with 1 μg of the HR reporter plasmid linearized by digestion with *Nhe*I. Amaxa Cell Line Nucleofector Kit V and an Amaxa Nucleofector device (Lonza, Allendale, NJ, USA) were used with programs X-005, for the U266 cell line, and T-016 for the JJN3 cell line. Three days after transfection G418 was added at 500 μg/ml. Medium containing G418 was changed every 3 days. Stable pools were obtained after 3 weeks of selection and were named U266-HR and JJN3-HR. In the chromosomally integrated reporter cassette, a unique DSB can be introduced by the rare-cutting endonuclease *I-Sce*I. Upon induction of a DSB, a functional GFP gene can be reconstituted by gene conversion, the predominant HR repair pathway in mammalian cells (22). To evaluate HR efficiency, 10^6 cells per transfection were cotransfected with 5 μg of an *I-Sce*I-expressing plasmid together with 0.5 μg of pDsRed-N1

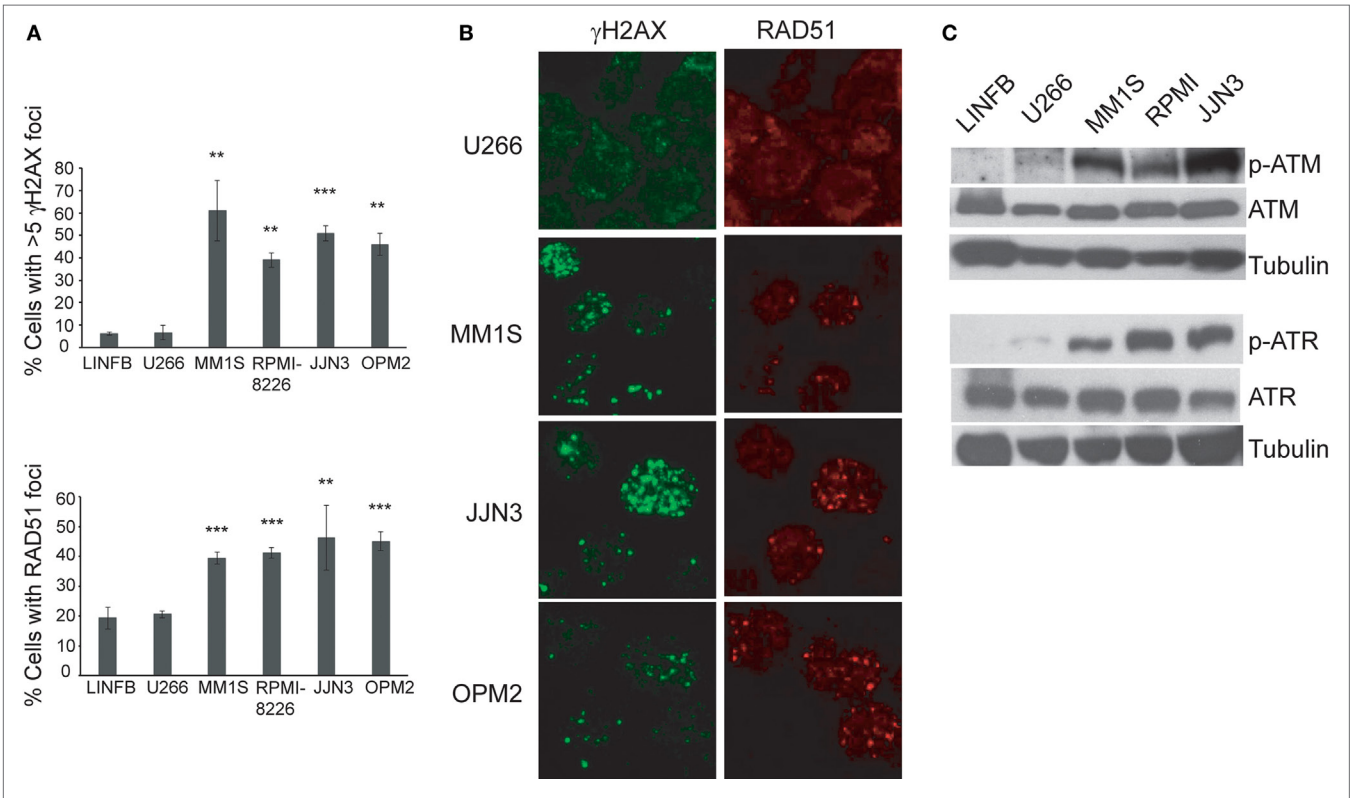


FIGURE 1 | **Several human myeloma cell lines exhibit a high level of endogenous DNA damage**. **(A)** Percentage of cells with γH2AX or RAD51 foci in the indicated cell lines. Data are the mean of three independent experiments. At least 75 cells per experiment were counted. Error bars correspond to the SD (***$p < 0.001$ and **$p < 0.01$ compared with LINFB cells, Student's *t*-test). **(B)** Representative immunofluorescence images of cells stained with anti-γH2AX or anti-RAD51 antibodies. **(C)** Western blot of kinase proteins involved in the DNA damage checkpoint.

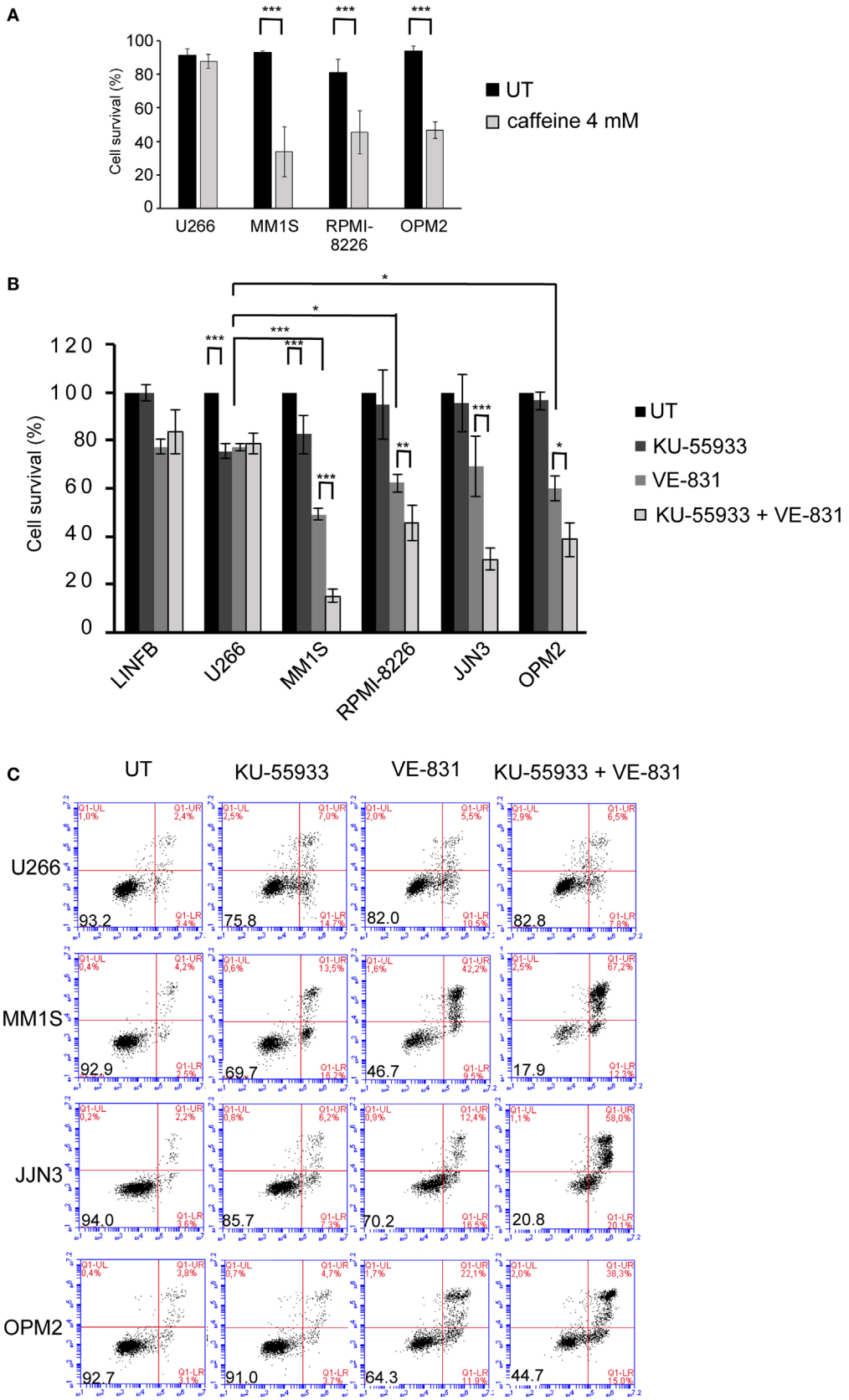


FIGURE 2 | Continued

FIGURE 2 | **Continued**
Multiple myeloma cells with ongoing DNA damage are hypersensitive to the simultaneous inhibition of ataxia telangiectasia-mutated protein and ataxia telangiectasia and Rad3-related protein kinases. **(A)** Cell survival after 48 h of treatment with 4 mM caffeine determined by Annexin V/propidium iodide (Annexin V/PI) staining. **(B)** Cell survival after 72 h in the absence (untreated = UT) or the presence of 10 μM KU-55933, 5 μM VE-821, or both. Cell viability in the absence of treatment (only DMSO) was taken as 100%, and values obtained for all other conditions were normalized with respect to the UT value. **(C)** Examples of flow cytometry dot plots of cells stained with Annexin V/PI after 72 h of treatment with the indicated chemical inhibitors. Data are the mean of at least three independent experiments (***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$).

to normalize measurements with respect to the transfection efficiency and were incubated in the presence or absence of various DDR inhibitors. Live cells were selected by FSC/SSC gating, and live GFP+ and DsRed+ cells were quantified by flow cytometry. HR efficiency was calculated as the ratio of GFP+ to DsRed+ cells.

RESULTS

HMCLs Exhibiting DNA Damage Are Hypersensitive to a Combination of ATM and ATR Inhibitors

Recent reports have shown ongoing constitutive DNA damage in several HMCLs and in plasma cells isolated from patients (6, 8). To corroborate these findings we quantified γ-H2AX and Rad51 foci, markers of DSBs, in HMCLs and in LINF903, a lymphoblastoid B cell line obtained from normal lymphocytes (7). HMCLs exhibited a higher percentage of cells with γ-H2AX and Rad51 foci than control lymphoblastoid cells, with the exception of U266 (**Figures 1A,B**), in agreement with recent published data (8). Activation of the DDR was detected in HMCLs with ongoing DNA damage by the presence of p-ATM and p-ATR (**Figure 1C**). The confirmation of high levels of DNA damage in most of the HMCLs prompted us to investigate the effect of various inhibitors of proteins involved in the DDR on MM cell viability. First, we analyzed the sensitivity of MM cell lines exhibiting low (U266) or high levels of DNA damage to caffeine, a well-known inhibitor of both ATM and ATR kinases. MM1S, RPMI-8226, and OPM2 were found to be more sensitive to the drug than U266, as revealed by Annexin V-FITC/PI staining (**Figure 2A**). Specific inhibitors were then used to determine whether cell death induced by caffeine in HMCLs was due to inhibition of ATM, ATR, or both kinases. Inhibition of ATM with 10 μM KU-55933 resulted in a small reduction in cell survival in U266 and MM1S cells relative to untreated cells, whereas no significant effect was detected in any other HMCLs (**Figure 2B**). On the other hand, pharmacological inhibition of ATR with 5 μM of the specific inhibitor VE-821 (23) triggered a stronger apoptotic response in MM1S, RPMI-8226, and OPM2 than in LINF903 and U266 cell lines, in agreement with a recent report (8). Interestingly, inhibition of both kinases using a combination of the two drugs significantly increased cell death caused by ATR inhibition only in cells with high endogenous DNA damage (**Figures 2B,C**). These results indicated that ATM compensates for the absence of ATR, since the level of cell death caused by both inhibitors was higher than that observed with the individual inhibitors.

ATM and ATR Participate in DSB Repair by HR in MM, and Inhibition of Both Kinases Produces a Stronger Inhibitory Effect

Ataxia telangiectasia-mutated protein and ATR kinases are mainly known for phosphorylating their substrates CHK2 and CHK1 leading to cell cycle arrest in G1 or G2 (12, 24–26). However, even though many HMCLs exhibited endogenous DNA damage we found that cell cycle profiles did not change significantly after treatment with the combination of ATM and ATR checkpoint inhibitors (**Figures 3A,B**). This result suggests that ATM and ATR may carry out other functions in cells with DNA damage that make them dependent on the presence of both kinases. It has previously been shown that unrepaired DSBs can lead to cell death (27) and also that ATM is important for DSB repair by HR, since its downregulation reduces HR efficiency (14). Based on these findings, we hypothesized that inhibition of both kinases might strongly affect DSB repair by HR, leading to the death of MM cells with accumulated DNA damage. To analyze whether inhibition of ATM, ATR, or both caused defects in recombination, we took advantage of an HR substrate that was integrated within the chromatin of the MM cell lines JJN3 and U266. DSBs were then generated by transfection of the resulting JJN3-HR and U266-HR cells with an I-*Sce*I endonuclease-expressing plasmid, and HR efficiency in the presence or absence of the kinase inhibitors was estimated. We found that ATM inhibition by treatment with KU-55933 (10 μM) strongly reduced HR efficiency in JJN3-HR and U266-HR (**Figures 3C,D**), although cells were still able to perform HR to some extent. ATR inhibition by VE-821 also resulted in a notable reduction in HR that was lower than that induced by ATM inhibition in JJN3-HR, but higher in U266-HR. A stronger suppressive effect on HR was observed when both kinases were simultaneously inactivated, indicating that ATM partially compensates for the lack of ATR and *vice versa*.

Total Inhibition of Recombination Severely Affects Survival of MM Cells with Ongoing DNA Damage

Based on the results reported above we speculated that sensitivity to ATR inhibition is caused by an additional role of this protein to that performed in DSB repair. Cell death induced by the chemical inhibition of both kinases could be caused by the individual effect of ATR inhibition along with the more marked decrease in HR capability. It has previously been shown that inhibition of ATR in conditions of RS leads to replication fork collapse and the accumulation of DSBs (28, 29), so we hypothesized that such accumulation, together with defects in DSB repair, might underlie hypersensitivity to ATR inhibition in MM. To

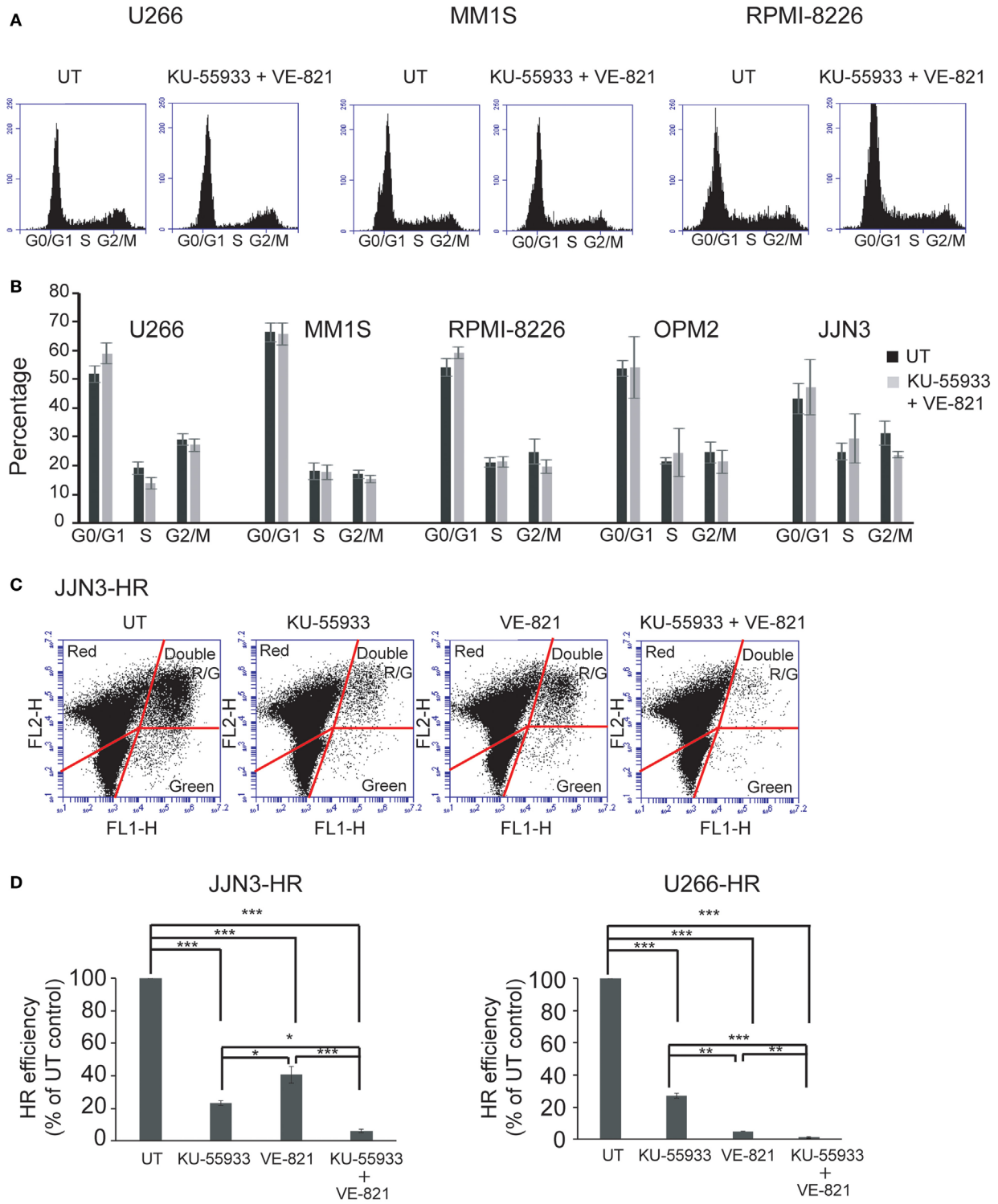


FIGURE 3 | Inhibition of ataxia telangiectasia-mutated protein and ataxia telangiectasia and Rad3-related protein does not affect cell cycle profiles of multiple myeloma cells but greatly reduces the efficiency of homologous recombination (HR) by gene conversion. **(A)** Cell cycle profiles of the indicated human myeloma cell lines untreated (UT) or treated with 10 μM KU-55933 and 5 μM VE-821. Cells were collected after 30 h of treatment and stained with propidium iodide. **(B)** Distribution of cells in G1, S, and G2/M phases of the cell cycle. **(C)** GFP+ and DsRed+ cells after 30 h of transfection with 5 μg of I-*Sce*I endonuclease-expressing plasmid and 0.5 μg pf pDSRed2-N1. **(D)** HR efficiency was calculated as the ratio of GFP+ to DsRed+ cells. The ratio in UT cells was taken to be 100% and in all other situations calculated as the percentage of UT controls. Data are the mean of three independent experiments. Error bars represent the SD (***$p < 0.001$, **$p < 0.01$, and * $p < 0.05$).

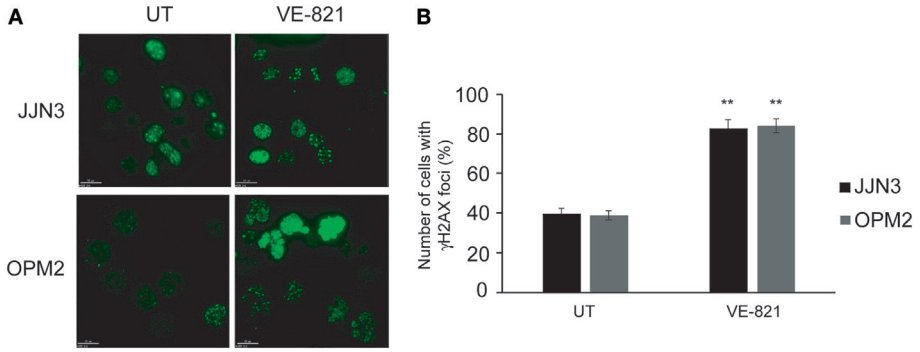


FIGURE 4 | **Inhibition of ataxia telangiectasia and Rad3-related protein results in the accumulation of DNA double-strand breaks**. **(A)** γH2AX foci in untreated (UT) cells and after 48 h of treatment with 5 μM VE-821. **(B)** Quantification of cells with foci. A total of 100 cells per cell line and condition were counted. Data are the mean of three independent experiments. Error bars represent the SD (**$p < 0.01$ compared with UT cells).

explore this possibility, cells were treated with VE-821, and the presence of DSBs was analyzed by monitoring γH2AX foci. As shown in **Figures 4A,B**, treatment of MM cells with VE-821 clearly increased the number of cells with γH2AX foci relative to untreated controls. Number of γH2AX foci per cell also increased and in several cells became uncountable.

To explore whether the strong inhibition of HR causes the death of MM cells with ongoing DNA damage we used mirin, an MRE11 inhibitor that abolishes HR by impeding DSB resection (30). We also explored the contribution of the other main pathway involved in DSB repair, NHEJ, to the survival of MM cells exhibiting DNA damage. For this purpose, we used NU7441, a NHEJ inhibitor that targets DNA-PK (31). We found that treatment for 72 h with mirin reduced survival of MM1S, RPMI-8226, and JJN3 in a dose-dependent manner, whereas survival of LIN903 and U266 cells was higher, as expected, and remained similar at the different doses of mirin employed (**Figures 4A,B**). On the other hand, inhibition of NHEJ with concentrations of NU7441 up to 40 μM did not affect survival of any of the cell lines analyzed, but clearly decreased survival of HMCLs with a high level of ongoing DNA damage when treated with 25 and 50 μM mirin. Based on these results we concluded that mirin at 25 or 50 μM might not completely inhibit HR, and, if so, cells might survive HR inhibition by the compensatory NHEJ pathway. To explore this hypothesis, JJN3-HR and U266-HR cells carrying the HR reported cassette were treated with different doses of mirin, and HR efficiency was analyzed after 30 h of culture (**Figure 5C**). We found that the recombination efficiency of live cells decreased with increasing doses of mirin, from a partial loss of efficiency at 25 μM to a nearly total loss at 100 μM. These results indicated that HMCLs with ongoing DNA damage are hypersensitive to HR inhibition and also that defects in DNA resection induced by mirin can be compensated by NHEJ, a mechanism able to rejoin broken molecules before total resection occurs (32). Importantly, the reduction in HR was very similar in JJN3-HR and U266-HR (**Figure 5C**), which demonstrated that U266 was more resistant to mirin because it suffered less endogenous DNA damage, and not because mirin was not active in these cells.

To confirm the dependency of MM cells on HR we used B02, a recently discovered inhibitor of Rad51 (33). HMCLs were treated with different doses of B02, and HR efficiency and cell viability were analyzed. As expected, B02 clearly inhibited HR in both U266-HR and JJN3-HR reporter cell lines (**Figure 6A**), although inhibition of HR resulted in more cell death in JJN3 and RPMI-8226 than in U266 (**Figures 6B,C**), further confirming that MM cells with ongoing DNA damage are hypersensitive to HR inhibition. We found that simultaneous inhibition of HR using B02 and NHEJ with NU7441 did not increase cell death induced by B02 alone (**Figure 6B**). This result clearly indicated that endogenous DNA damage in MM is mainly repaired by HR and that if this pathway is inhibited after DNA resection occurs the break can no longer be repaired by a compensatory NHEJ mechanism. An illustration summarizing all the results is shown in **Figure 7**.

DISCUSSION

High levels of DNA damage can make tumor cells dependent on a proper DDR and represent a vulnerability of cancer cells that could be targeted therapeutically (34). In this study, we show that inhibition of the DDR is particularly toxic for MM cells exhibiting high levels of DSBs. Specifically, MM cells were found to be hypersensitive to agents that inhibit DSB repair by HR, a pathway upregulated in MM and important for the resolution of breaks associated with RS.

Recently, Cottini et al. showed that several HMCLs suffer RS, which is induced, at least in part, by the overexpression of MYC. In fact, silencing this oncogene in HMCLs exhibiting DNA damage and MYC overexpression reduced the number of lesions. On the other hand, its overexpression in U266, an HMCL with low levels of DNA damage and no MYC overexpression, triggered an increase in DSBs (8). The authors also found that ATR, the main kinase involved in the RS response, promoted survival of MYC-overexpressing cells. Here, we confirm the presence of high levels of DNA damage in several HMCLs, with the exception of U266, whose level of endogenous DNA damage was similar to that found in normal lymphoblastoid B cells, which were used in this work as healthy controls. We also confirmed that HMCLs

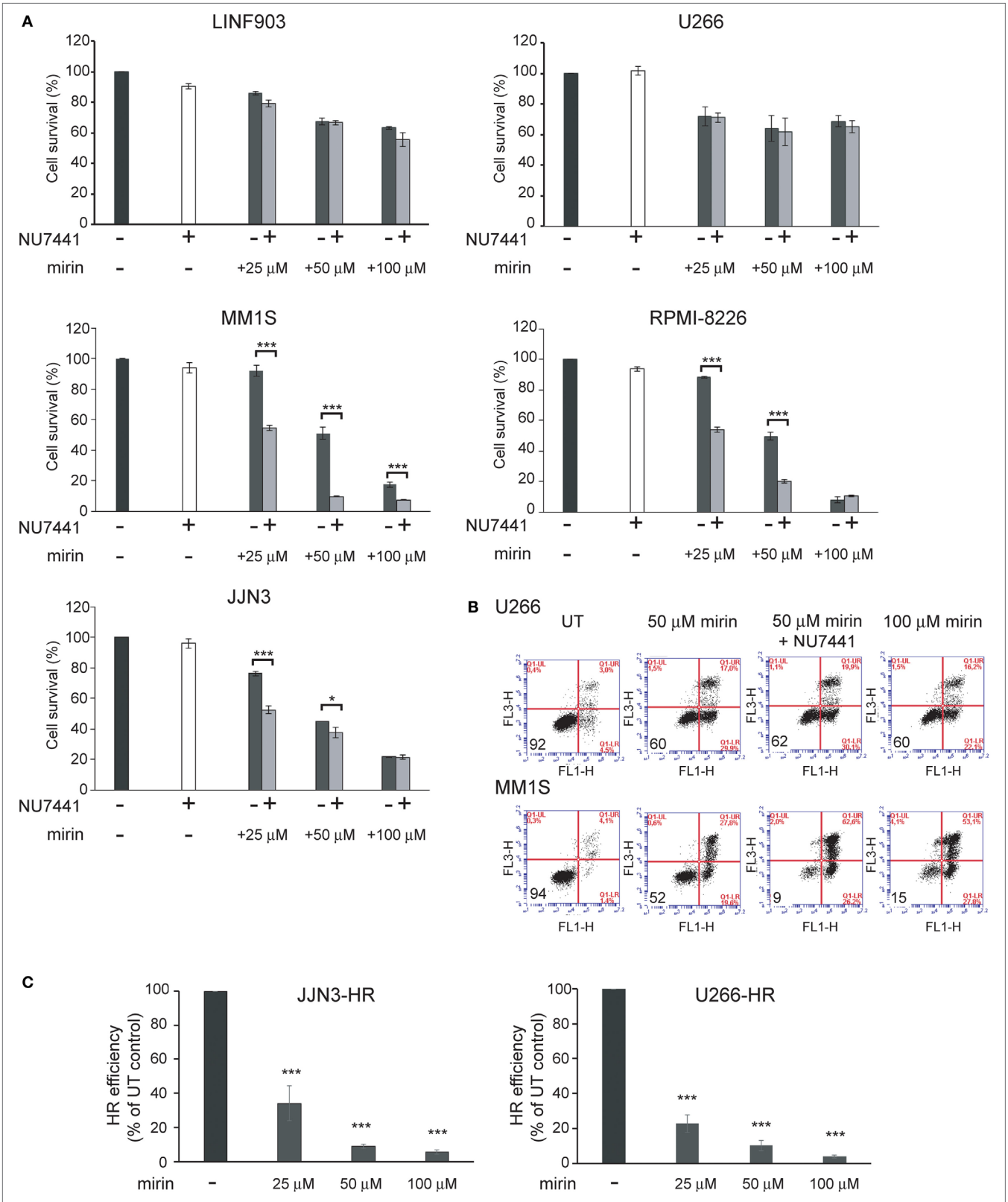


FIGURE 5 | Mirin induces cell death of multiple myeloma cells with endogenous DNA damage and reduces homologous recombination (HR) efficiency in a dose-dependent manner. **(A)** Cell survival determined by Annexin/propidium iodide (Annexin/PI) staining after 72 h of treatment with the indicated compounds. **(B)** Representative flow cytometry dot plots of cells stained with Annexin/PI. **(C)** HR efficiency in the indicated conditions. Data are the mean of three independent experiments. Error bars correspond to the SD (***$p < 0.001$ and *$p < 0.05$).

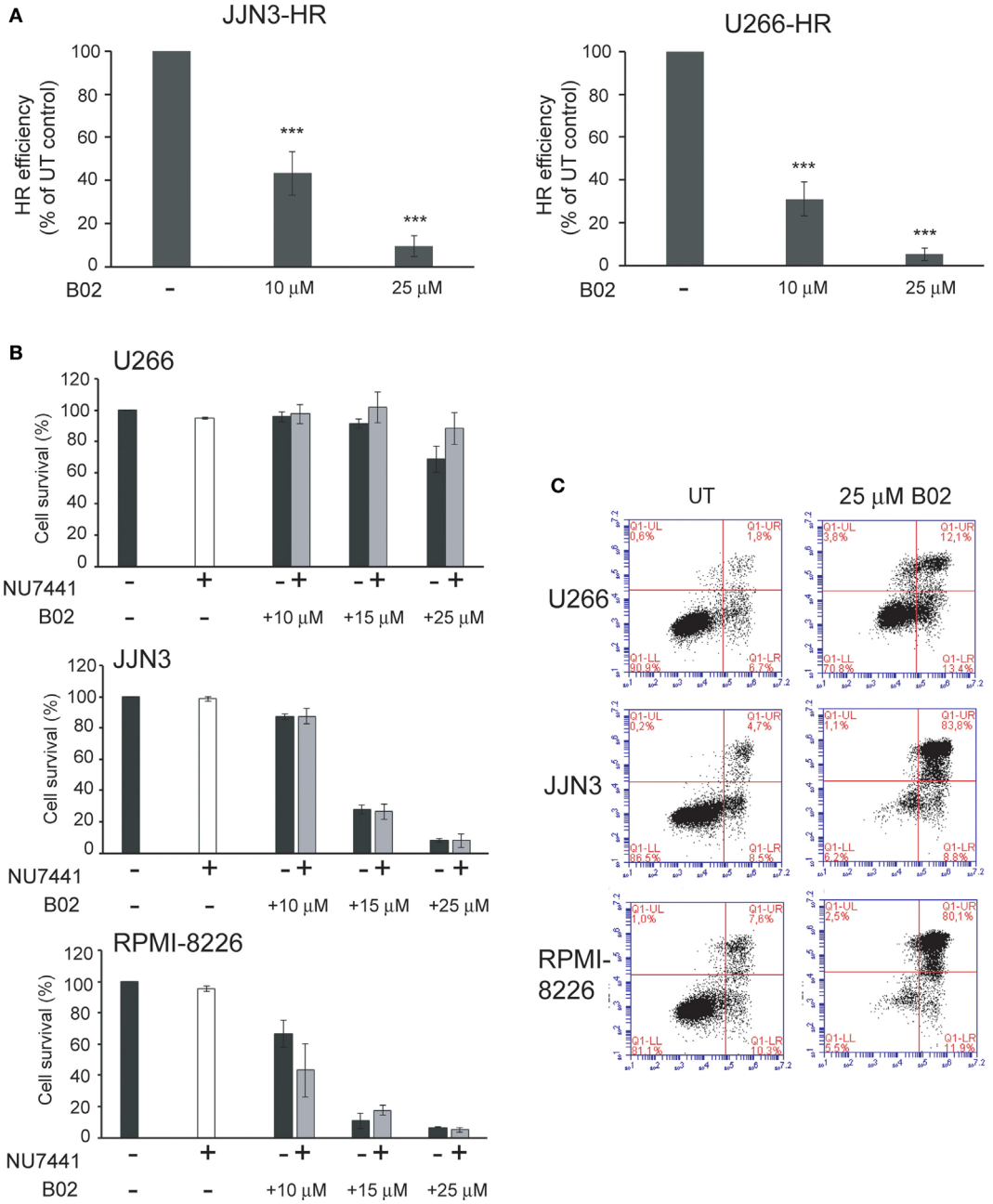


FIGURE 6 | **B02 reduces homologous recombination (HR) efficiency and induces multiple myeloma cell death**. **(A)** Efficiency of HR after treatment with B02. **(B)** Cell survival determined by Annexin–fluorescein isothiocyanate/propidium iodide staining of human myeloma cell lines (HMCLs) treated for 72 h with the indicated inhibitors. A total of 10,000 events are shown. **(C)** Representative dot plots of the indicated HMCLs. Data are the mean of three independent experiments. Error bars represent the SD (***$p < 0.001$).

exhibiting DNA damage rely on activated ATR for survival. Moreover, we observed that these cells were more sensitive to ATR than to ATM inhibition, probably because the first induces replication fork collapse (28, 29), leading to the accumulation of DSBs (**Figure 4**). However, we found that the absence of ATR was compensated by ATM, since cell death induced by the inhibition of both kinases was more extensive than that obtained using single kinase inhibitors. It is especially noteworthy that the lethal effects of ATM and ATR inhibitors were found only in cells with extensive endogenous DNA damage, which bolsters the therapeutic opportunities. ATR and ATM transduction pathways were initially considered as two parallel pathways: IR induced the ATM pathway, whereas RS activated the ATR pathway. However, the regulation of ATM and ATR was later

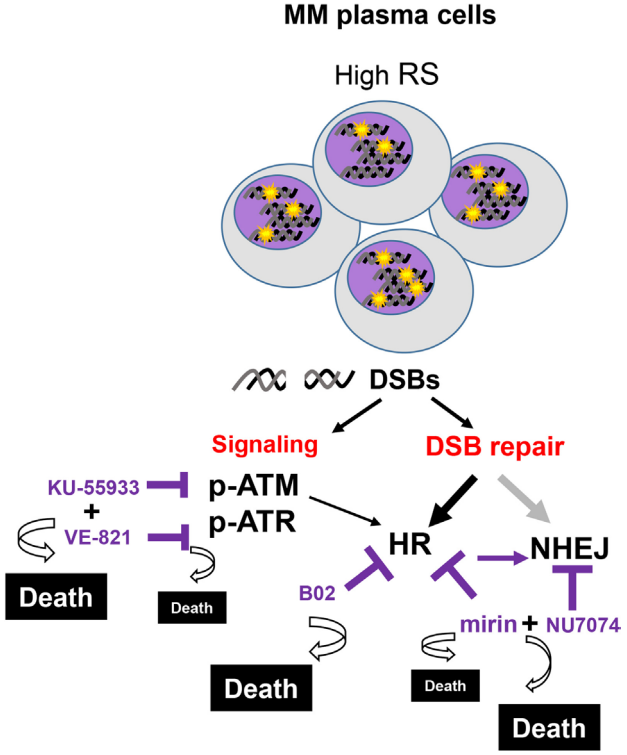


FIGURE 7 | Several multiple myeloma patients present PCs with high levels of replication stress (RS), leading to double-strand breaks (DSBs). These cells activate the DNA damage response that coordinates the signaling and the repair of the lesions. Replication-associated DSBs are mainly repaired by homologous recombination (HR). Targeting HR by dual inhibition of ataxia telangiectasia-mutated protein and ataxia telangiectasia and Rad3-related protein or by using the HR inhibitor B02 would be particularly toxic for these cells. High levels of cell death will also be achieved using mirin and NU7471, since the last inhibits the compensatory non-homologous end joining mechanisms. In contrast, normal cells with minimal levels of RS are expected to survive these treatments.

shown to be mutually dependent in response to various DNA-damaging conditions like UV light, IR, and replication stalling (35–39). Thus, ATM has been demonstrated to play a role in maintaining fragile site stability, which is revealed only in the absence of ATR (40). Our results showing an increase in MM cell death with the combination of the two kinase inhibitors indicate the importance of both proteins for coping with endogenous DNA damage in MM and suggest that a double therapy could be more efficient for killing malignant cells. In this regard, it has been reported that in *ATM*-defective chronic lymphocytic leukemia cells, inhibition of ATR signaling by AZD6738 leads to an accumulation of unrepaired DNA damage that results in cell death by mitotic catastrophe (41). Dual inhibition of ATR and ATM has also been shown to potentiate the activity of the DSB-inducing drugs trabectedin and lurbinectedin by perturbing the DDR and HR repair (42).

We show for the first time that ATM and ATR are involved in DSB repair by HR in MM and also that inhibition of both kinases completely abolishes HR efficiency. These results, obtained using an integrated-GFP-based DNA repair reporter substrate, support a previously described model in which ATM and ATR collaborate to maintain the activity of CtIP for efficient DNA end resection during HR (43). We found that ATM/ATR inhibition did not affect cell cycle distribution of HMCLs with high endogenous DNA damage (**Figures 3A,B**), which led us to hypothesize that the effect on HR, together with the accumulation of DSBs due to ATR inhibition, might underlie cell death induction. This hypothesis was supported by the finding that abolition of HR using the MRE11 inhibitor mirin, or the RAD51 inhibitor B02, severely decreased survival of HMCLs exhibiting high levels of endogenous DNA damage. Here, we show that inhibition of the other route involved in DSB repair, NHEJ, using the DNA-PK inhibitor NU7441 did not affect MM cell viability, which clearly demonstrated that endogenous DNA damage in MM is repaired by HR. We found that NHEJ inhibition did not increase cell death when HR was simultaneously inhibited with B02, but did clearly enhance cell death when HR was inhibited with mirin, which interferes with recombination before DNA resection occurs. These results are consistent with previous observations by Shibata et al., who showed that inhibition of the endonuclease activity of MRE11 promoted NHEJ (32). They are also consistent with the notion that 3′-ssDNA ends generated after DNA resection as substrates for HR can no longer be channeled toward NHEJ (18). Importantly, our results indicate that inhibition of HR after the initial step of end resection might be more appropriate for inducing MM cell death, since it prevents the occurrence of a compensatory NHEJ repair mechanism.

Many studies have shown that combinations of genotoxic agents with inhibitors of the DDR produce greater cell death of tumor cells compared with single agents (21, 44–46). In MM, the cytotoxic effects of bendamustine, melphalan, and doxorubicin on p53-deficient cells are enhanced by AZD7762, a CHK1/CHK2 inhibitor (47). In addition, inhibition of RAD51 has been found to sensitize MM cells to IR and to the DSB-inducing drug doxorubicin (48). However, the present study is the first to indicate that the single-agent activity of HR inhibitors induces apoptosis of MM cells with intrinsic DNA damage, providing a therapeutic window. It is important to note that partial inhibition of HR did not result in complete myeloma cell death at the times assayed, probably because residual traces of activity may be enough to ensure repair of endogenous DSBs. However, doses that completely abolished HR, such as the combination of ATM and ATR inhibitors, or concentrations of B02 greater than 15 μM drove most of the MM cells with intrinsic DNA damage to apoptosis. It is possible that the high doses of HR inhibitors needed to induce complete MM cell death could have a toxic effect, especially over prolonged periods, but maybe one that is less severe than that induced by combinations of DDR inhibitors with genotoxic agents, since the latter induce DNA damage and are also toxic to non-tumor cells. Nevertheless, specific (or temporary) inhibition of HR could be efficient not only for reducing MM cell survival but also for mitigating genomic instability and disease progression, given that we and others have demonstrated a high level of RAD51 expression and increased HR that lead to the genome instability characteristic of the disease (5, 7). Taken together, our findings suggest that HR inhibition could be a promising target for the treatment of MM. Therapeutic outcomes and toxicity

profiles of the different inhibitors used as single agents or in combinations need to be tested in clinical trials.

AUTHOR CONTRIBUTIONS

AH conceived and designed the work, performed the experiments, wrote the manuscript, and approved the final version of the manuscript. NG contributed to design the work, revised it critically for important intellectual content, ensured that any part of the work was appropriately investigated and resolved, corrected the manuscript, and approved the final version to be published.

ACKNOWLEDGMENTS

We thank Dr. V. Gorbunova for the HR reporter plasmid and Dr. Wiesmueller for pCMV-I-SceI plasmid. We are grateful to A. L. Prieto for his help with the Deltavision microscope.

FUNDING

This work was supported by the Spanish Association for Cancer Research (AECC, GCB120981SAN), the INNOCAMPUS program (CEI10-1-0010), and the Samuel Solórzano Barruso foundation (FS/25-2015).

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. *CA Cancer J Clin* (2017) 67:7–30. doi:10.3322/caac.21387
2. Chesi M, Bergsagel PL. Molecular pathogenesis of multiple myeloma: basic and clinical updates. *Int J Hematol* (2013) 97:313–23. doi:10.1007/s12185-013-1291-2
3. Mateos MV, Ocio EM, Paiva B, Rosinol L, Martinez-Lopez J, Blade J, et al. Treatment for patients with newly diagnosed multiple myeloma in 2015. *Blood Rev* (2015) 29:387–403. doi:10.1016/j.blre.2015.06.001
4. Bergsagel PL, Mateos MV, Gutierrez NC, Rajkumar SV, San Miguel JF. Improving overall survival and overcoming adverse prognosis in the treatment of cytogenetically high-risk multiple myeloma. *Blood* (2013) 121:884–92. doi:10.1182/blood-2012-05-432203
5. Shammas MA, Shmookler Reis RJ, Koley H, Batchu RB, Li C, Munshi NC. Dysfunctional homologous recombination mediates genomic instability and progression in myeloma. *Blood* (2009) 113:2290–7. doi:10.1182/blood-2007-05-089193
6. Walters DK, Wu X, Tschumper RC, Arendt BK, Huddleston PM, Henderson KJ, et al. Evidence for ongoing DNA damage in multiple myeloma cells as revealed by constitutive phosphorylation of H2AX. *Leukemia* (2011) 25:1344–53. doi:10.1038/leu.2011.94
7. Herrero AB, San Miguel J, Gutierrez NC. Deregulation of DNA double-strand break repair in multiple myeloma: implications for genome stability. *PLoS One* (2015) 10:e0121581. doi:10.1371/journal.pone.0121581
8. Cottini F, Hideshima T, Suzuki R, Tai YT, Bianchini G, Richardson PG, et al. Synthetic lethal approaches exploiting DNA damage in aggressive myeloma. *Cancer Discov* (2015) 5:972–87. doi:10.1158/2159-8290.CD-14-0943
9. Ciccia A, Elledge SJ. The DNA damage response: making it safe to play with knives. *Mol Cell* (2010) 40:179–204. doi:10.1016/j.molcel.2010.09.019
10. Mazouzi A, Velimezi G, Loizou JI. DNA replication stress: causes, resolution and disease. *Exp Cell Res* (2014) 329:85–93. doi:10.1016/j.yexcr.2014.09.030
11. Branzei D, Foiani M. Maintaining genome stability at the replication fork. *Nat Rev Mol Cell Biol* (2010) 11:208–19. doi:10.1038/nrm2852
12. Matsuoka S, Huang M, Elledge SJ. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. *Science* (1998) 282:1893–7. doi:10.1126/science.282.5395.1893
13. Shiloh Y, Ziv Y. The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. *Nat Rev Mol Cell Biol* (2013) 14:197–210. doi:10.1038/nrm3546
14. Bakr A, Oing C, Kocher S, Borgmann K, Dornreiter I, Petersen C, et al. Involvement of ATM in homologous recombination after end resection and RAD51 nucleofilament formation. *Nucleic Acids Res* (2015) 43:3154–66. doi:10.1093/nar/gkv160
15. Branzei D, Foiani M. Regulation of DNA repair throughout the cell cycle. *Nat Rev Mol Cell Biol* (2008) 9:297–308. doi:10.1038/nrm2351
16. Goodarzi AA, Jeggo P, Lobrich M. The influence of heterochromatin on DNA double strand break repair: getting the strong, silent type to relax. *DNA Repair (Amst)* (2010) 9:1273–82. doi:10.1016/j.dnarep.2010.09.013
17. Helleday T, Lo J, van Gent DC, Engelward BP. DNA double-strand break repair: from mechanistic understanding to cancer treatment. *DNA Repair (Amst)* (2007) 6:923–35. doi:10.1016/j.dnarep.2007.02.006
18. Brandsma I, Gent DC. Pathway choice in DNA double strand break repair: observations of a balancing act. *Genome Integr* (2012) 3:9. doi:10.1186/2041-9414-3-9
19. Symington LS, Gautier J. Double-strand break end resection and repair pathway choice. *Annu Rev Genet* (2011) 45:247–71. doi:10.1146/annurev-genet-110410-132435
20. Curtin NJ. DNA repair dysregulation from cancer driver to therapeutic target. *Nat Rev Cancer* (2012) 12:801–17. doi:10.1038/nrc3399
21. Gavande NS, VanderVere-Carozza PS, Hinshaw HD, Jalal SI, Sears CR, Pawelczak KS, et al. DNA repair targeted therapy: the past or future of cancer treatment? *Pharmacol Ther* (2016) 160:65–83. doi:10.1016/j.pharmthera.2016.02.003
22. Mao Z, Bozzella M, Seluanov A, Gorbunova V. Comparison of nonhomologous end joining and homologous recombination in human cells. *DNA Repair (Amst)* (2008) 7:1765–71. doi:10.1016/j.dnarep.2008.06.018
23. Reaper PM, Griffiths MR, Long JM, Charrier JD, Maccormick S, Charlton PA, et al. Selective killing of ATM- or p53-deficient cancer cells through inhibition of ATR. *Nat Chem Biol* (2011) 7:428–30. doi:10.1038/nchembio.573
24. Matsuoka S, Rotman G, Ogawa A, Shiloh Y, Tamai K, Elledge SJ. Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. *Proc Natl Acad Sci U S A* (2000) 97:10389–94. doi:10.1073/pnas.190030497
25. Kumagai A, Kim SM, Dunphy WG. Claspin and the activated form of ATR-ATRIP collaborate in the activation of Chk1. *J Biol Chem* (2004) 279:49599–608. doi:10.1074/jbc.M408353200
26. Bartek J, Lukas J. Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell* (2003) 3:421–9. doi:10.1016/S1535-6108(03)00110-7
27. Bennett CB, Westmoreland TJ, Snipe JR, Resnick MA. A double-strand break within a yeast artificial chromosome (YAC) containing human DNA can result in YAC loss, deletion or cell lethality. *Mol Cell Biol* (1996) 16:4414–25. doi:10.1128/MCB.16.8.4414
28. Couch FB, Bansbach CE, Driscoll R, Luzwick JW, Glick GG, Betous R, et al. ATR phosphorylates SMARCAL1 to prevent replication fork collapse. *Genes Dev* (2013) 27:1610–23. doi:10.1101/gad.214080.113
29. Fernandez-Capetillo O, Nussenzweig A. Naked replication forks break apRPArt. *Cell* (2013) 155:979–80. doi:10.1016/j.cell.2013.10.049
30. Dupre A, Boyer-Chatenet L, Sattler RM, Modi AP, Lee JH, Nicolette ML, et al. A forward chemical genetic screen reveals an inhibitor of the Mre11-Rad50-Nbs1 complex. *Nat Chem Biol* (2008) 4:119–25. doi:10.1038/nchembio.63
31. Leahy JJ, Golding BT, Griffin RJ, Hardcastle IR, Richardson C, Rigoreau L, et al. Identification of a highly potent and selective DNA-dependent protein kinase (DNA-PK) inhibitor (NU7441) by screening of chromenone libraries. *Bioorg Med Chem Lett* (2004) 14:6083–7. doi:10.1016/j.bmcl.2004.09.060
32. Shibata A, Moiani D, Arvai AS, Perry J, Harding SM, Genois MM, et al. DNA double-strand break repair pathway choice is directed by distinct MRE11 nuclease activities. *Mol Cell* (2014) 53:7–18. doi:10.1016/j.molcel.2013.11.003
33. Huang F, Motlekar NA, Burgwin CM, Napper AD, Diamond SL, Mazin AV. Identification of specific inhibitors of human RAD51 recombinase using high-throughput screening. *ACS Chem Biol* (2011) 6:628–35. doi:10.1021/cb100428c
34. Toledo LI, Murga M, Fernandez-Capetillo O. Targeting ATR and Chk1 kinases for cancer treatment: a new model for new (and old) drugs. *Mol Oncol* (2011) 5:368–73. doi:10.1016/j.molonc.2011.07.002

35. Jazayeri A, Falck J, Lukas C, Bartek J, Smith GC, Lukas J, et al. ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat Cell Biol* (2006) 8:37–45. doi:10.1038/ncb1337
36. Myers JS, Cortez D. Rapid activation of ATR by ionizing radiation requires ATM and Mre11. *J Biol Chem* (2006) 281:9346–50. doi:10.1074/jbc.M513265200
37. Stiff T, Walker SA, Cerosaletti K, Goodarzi AA, Petermann E, Concannon P, et al. ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling. *EMBO J* (2006) 25:5775–82. doi:10.1038/sj.emboj.7601446
38. Cuadrado M, Martinez-Pastor B, Murga M, Toledo LI, Gutierrez-Martinez P, Lopez E, et al. ATM regulates ATR chromatin loading in response to DNA double-strand breaks. *J Exp Med* (2006) 203:297–303. doi:10.1084/jem.20051923
39. Yoo HY, Kumagai A, Shevchenko A, Shevchenko A, Dunphy WG. Ataxia-telangiectasia mutated (ATM)-dependent activation of ATR occurs through phosphorylation of TopBP1 by ATM. *J Biol Chem* (2007) 282:17501–6. doi:10.1074/jbc.M701770200
40. Ozeri-Galai E, Schwartz M, Rahat A, Kerem B. Interplay between ATM and ATR in the regulation of common fragile site stability. *Oncogene* (2008) 27:2109–17. doi:10.1038/sj.onc.1210849
41. Kwok M, Davies N, Agathanggelou A, Smith E, Oldreive C, Petermann E, et al. ATR inhibition induces synthetic lethality and overcomes chemoresistance in TP53- or ATM-defective chronic lymphocytic leukemia cells. *Blood* (2016) 127:582–95. doi:10.1182/blood-2015-05-644872
42. Lima M, Bouzid H, Soares DG, Selle F, Morel C, Galmarini CM, et al. Dual inhibition of ATR and ATM potentiates the activity of trabectedin and lurbinectedin by perturbing the DNA damage response and homologous recombination repair. *Oncotarget* (2016) 7:25885–901. doi:10.18632/oncotarget.8292
43. Peterson SE, Li Y, Wu-Baer F, Chait BT, Baer R, Yan H, et al. Activation of DSB processing requires phosphorylation of CtIP by ATR. *Mol Cell* (2013) 49:657–67. doi:10.1016/j.molcel.2012.11.020
44. Garrett MD, Collins I. Anticancer therapy with checkpoint inhibitors: what, where and when? *Trends Pharmacol Sci* (2011) 32:308–16. doi:10.1016/j.tips.2011.02.014
45. Hosoya N, Miyagawa K. Targeting DNA damage response in cancer therapy. *Cancer Sci* (2014) 105:370–88. doi:10.1111/cas.12366
46. O'Connor MJ, Martin NM, Smith GC. Targeted cancer therapies based on the inhibition of DNA strand break repair. *Oncogene* (2007) 26:7816–24. doi:10.1038/sj.onc.1210879
47. Landau HJ, McNeely SC, Nair JS, Comenzo RL, Asai T, Friedman H, et al. The checkpoint kinase inhibitor AZD7762 potentiates chemotherapy-induced apoptosis of p53-mutated multiple myeloma cells. *Mol Cancer Ther* (2012) 11:1781–8. doi:10.1158/1535-7163.MCT-11-0949
48. Alagpulinsa DA, Ayyadevara S, Shmookler Reis RJ. A small-molecule inhibitor of RAD51 reduces homologous recombination and sensitizes multiple myeloma cells to doxorubicin. *Front Oncol* (2014) 4:289. doi:10.3389/fonc.2014.00289

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer, ES, and handling editor declared their shared affiliation, and the handling editor states that the process nevertheless met the standards of a fair and objective review.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership