CELL STRESS, METABOLIC REPROGRAMMING, AND CANCER

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CELL STRESS, METABOLIC REPROGRAMMING, AND CANCER

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Rod Brandsch "Vergaenglichkeit" ("transitoriness"), 100 x 100 cm, oil and mixed media on canvas, 2014.

The present eBook presents one review, five mini-reviews, and an opinion article on the achievements and perspectives of studies on important aspects of cancer cell metabolic reprogramming whose mechanisms and regulation are still largely elusive. It also sheds light on certain novel functional components, which rewires cell metabolism in tumor transformation.

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EDITORIAL
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Editorial: Cell Stress, Metabolic Reprogramming, and Cancer

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Editorial on the Research Topic

Cell Stress, Metabolic Reprogramming, and Cancer

The hallmarks of cancer comprise six biological capabilities acquired during the multistep development of human tumors: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (1). Mitochondria, beyond being the site of aerobic respiration, are at the crossroads of a variety of metabolic and signaling pathways resulting key regulatory organelles in cell life and death decision. Thus, it is no surprise that genomic, functional, and structural mitochondrial alterations have been associated with cancer and that mitochondria have become a pharmacological target in cancer therapy (2). Proliferating tumor cells show increased glycolysis and convert the majority of glucose to L-lactate, even in normoxic conditions. This is known as the Warburg effect. Actually, in many tumors, mitochondria are not defective in oxidative phosphorylation, and in the last decade, the molecular basis of Warburg effect has been reconsidered in the context of a set of concerted changes in energy metabolism and mitochondrial function that support tumorigenesis. This process, referred to as reprogramming of energy metabolism, is an emerging hallmark of cancer development (3, 4). This Research Topic presents one review, five mini-reviews, and an opinion article on the achievements and perspectives of studies on important aspects of cancer cell metabolic reprogramming whose mechanisms and regulation are still largely elusive. It also sheds light on certain novel functional components, which rewires cell metabolism in tumor transformation.

Metabolic reprogramming is driven by oncogenic changes of specific cell-signaling pathways and tumor microenvironment (5). The Mini-Reviews by Iommarini et al. (6) and Dahl and Aird (7) highlight what is currently known about the non-canonical function and regulation of hypoxia-inducible factor 1 alpha (HIF-1α) and ataxia-telangiectasia mutated (ATM) protein kinase, respectively. Iommarini et al. (6) review and discuss the non-canonical regulation of HIF-1α expression and stabilization in cancer cells, focusing on factors, which cause pseudohypoxia (HIF-1α stabilization in normoxic conditions) or fail to stabilize HIF-1α in low oxygen atmosphere (pseudonormoxia). The ATM protein kinase has been extensively studied for its role in the DNA damage response and its association with the disease ataxia telangiectasia. Dahl and Aird's review (7) highlights our current knowledge about ATM's regulation of carbon metabolism, the implication of these pathways in cancer, and the development of ATM inhibitors as therapeutic strategies for cancer.

It is well established that glucose is uniquely capable of supporting Warburg metabolism (or aerobic glycolysis), in which pyruvate is converted to lactate through a process that is coupled to ATP production in the cytoplasm. Such metabolic reprogramming and nutrient sensing is an elaborate way by which cancer cells respond to high bioenergetic and anabolic demands during tumorigenesis.

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Ždralević et al. (8) in their Mini-Review discuss the benefits and limitations of disrupting fermentative glycolysis at different levels of the pathway in order to find the most effective mode to overcome cancer cell metabolic plasticity that seriously limits the use of glycolysis inhibition for impeding tumor growth. With this respect, in view of the existence of a mitochondrial L-lactate dehydrogenase (m-L-LDH), Passarella and Shurr (9) propose in their Opinion a revision of the Cori cycle in all types of cells where mitochondrial metabolism of L-lactate is active.

Beyond the shift of glucose metabolism to aerobic glycolysis, some cancer cells are considered "glutamine addicted" because their growth and proliferation rates depend on the availability of this amino acid. This, together with the role of amino acid metabolism in tumorigenesis, is one of the key aspects of cancer cell metabolism, which is still matter of intense investigations. The Review by Vučetić et al. (10) provides the first unified review on the amino acid dependency of cancer antioxidant defense, a topic that has received more attention recently. Furthermore, the Mini-Review by Scalise et al. (11) provides a deep insight into glutamine transport and mitochondrial metabolism in cancer cell growth, highlighting glutamine transporters of plasma membrane, the key enzyme glutaminase, and other proteins involved glutamine metabolism as novel targets for anti-cancer drug development.

Beyond the metabolic shift toward glycolysis, typical of cancer cells, several evidences have shown that mitochondrial dysfunction provides survival advantage to cancer cells, suggesting that mitochondria have a tumor suppressor function (5). Mitochondrial dysfunction has been implicated in cancer chemoresistance (12). The association between mitochondrial dysfunction and progression to a metastatic phenotype is gradually emerging. Epithelial-to-mesenchymal transition (EMT) allows epithelial cancer cells to assume mesenchymal features, endowing them with enhanced motility and invasiveness, thus enabling cancer dissemination and metastatic spread. The Mini-Review by Guerra et al. (13) in this Research Topic gives an overview on the mechanistic link between EMT and mitochondrial dysfunction fostering the identification of the molecular determinants of the mitochondria-nucleus communication network linking mitochondrial dysfunction with EMT activation, which may provide useful therapeutic targets for treatment and prevention of metastatic cancer.

The contributions to this Research Topic deal with investigations at the leading edge of cancer research and provide an overview on key cellular processes and components, which are the basis of metabolic reprogramming of cancer cells. Inflammation has also been recognized as a hallmark of cancer and is known to play an essential role in the development and progression of most cancers, even those without obvious signs of inflammation and infection (14). Warburg metabolism is a hallmark of immune cells that have the potential to cause inflammation. Recently, Kornberg et al. gave proof of concept that aerobic glycolysis is a therapeutic target for regulating inflammation (15), further confirming the possibility that targeting key enzymes within metabolic pathways will provide new therapeutic options for cancer.

This Research Topic brings witness that research on metabolic reprogramming of cancer cells is coming of age and will still bring with it exciting results to lay the bases for the development of new therapies and the implementation of nutritional regimen for a healthy life as well as the improvement of anti-cancer therapies.

AUTHOR CONTRIBUTIONS

SG wrote the first draft of the manuscript; SG, MGM and CM contributed to manuscript revision.

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MINI REVIEW
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Non-Canonical Mechanisms Regulating Hypoxia-Inducible Factor 1 Alpha in Cancer

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Hypoxia-inducible factor 1 alpha (HIF-1α) orchestrates cellular adaptation to low oxygen and nutrient-deprived environment and drives progression to malignancy in human solid cancers. Its canonical regulation involves prolyl hydroxylases (PHDs), which in normoxia induce degradation, whereas in hypoxia allow stabilization of HIF-1α. However, in certain circumstances, HIF-1α regulation goes beyond the actual external oxygen levels and involves PHD-independent mechanisms. Here, we gather and discuss the evidence on the non-canonical HIF-1α regulation, focusing in particular on the consequences of mitochondrial respiratory complexes damage on stabilization of this pleiotropic transcription factor.

Keywords: hypoxia-inducible factor 1 alpha, cancer, mitochondria, oxidative phosphorylation, electron transport chain, prolyl hydroxylases, pseudohypoxia, pseudonormoxia

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Hypoxia-inducible factor 1 (HIF-1) is the major orchestrator of cellular adaptation to low oxygen environment (1). In normoxia, prolyl hydroxylases (PHDs) hydroxylate HIF-1α on two proline residues within the oxygen-dependent degradation domain, triggering von Hippel–Lindau (pVHL)-mediated ubiquitination and proteasomal degradation (**Figure 1**) (2). In parallel, the Factor Inhibiting HIF (FIH), an asparaginyl hydroxylase regulated similarly to PHDs, in an oxygen-dependent manner, suppresses HIF-1 transcriptional activity in normoxia by preventing co-activator recruitment (3, 4). Conversely, hypoxia inhibits PHDs and stabilizes HIF-1α, which then translocates into the nucleus and dimerizes with constitutively expressed HIF-1β, creating active HIF-1 complex and triggering the transcription of genes promoting glycolytic metabolism, angiogenesis, and survival (**Figure 1**) (5). Activation of HIF-1α is physiological during embryogenesis and in wound-healing processes, whereas in cancer, HIF-1α is associated with malignancy and poor prognosis (6, 7). Abnormal stabilization of HIF-1α and upregulation of its downstream targets have been described in a broad spectrum of solid tumors as they progress to malignancy (8).

Since the discovery of HIF-1α and the ingenious oxygen-dependent PHD-mediated regulation, a great number of additional modalities of HIF-1α control has been identified, independently from external oxygen concentrations and acting at the level of its transcription, translation, oxygen-independent stabilization/degradation, translocation from cytoplasm to the nucleus, and even affecting HIF-1 transcriptional activity. Here, we review and discuss the non-canonical regulation of HIF-1α expression and stabilization in cancer cells, focusing on factors which cause pseudohypoxia (HIF-1α stabilization in normoxic conditions) or fail to stabilize HIF-1α in low oxygen atmosphere (pseudonormoxia). Particular attention is given to the discussion of data showing that oxidative phosphorylation (OXPHOS) damage may block HIF-1α stabilization, since this controversial issue has seldom been reviewed elsewhere.

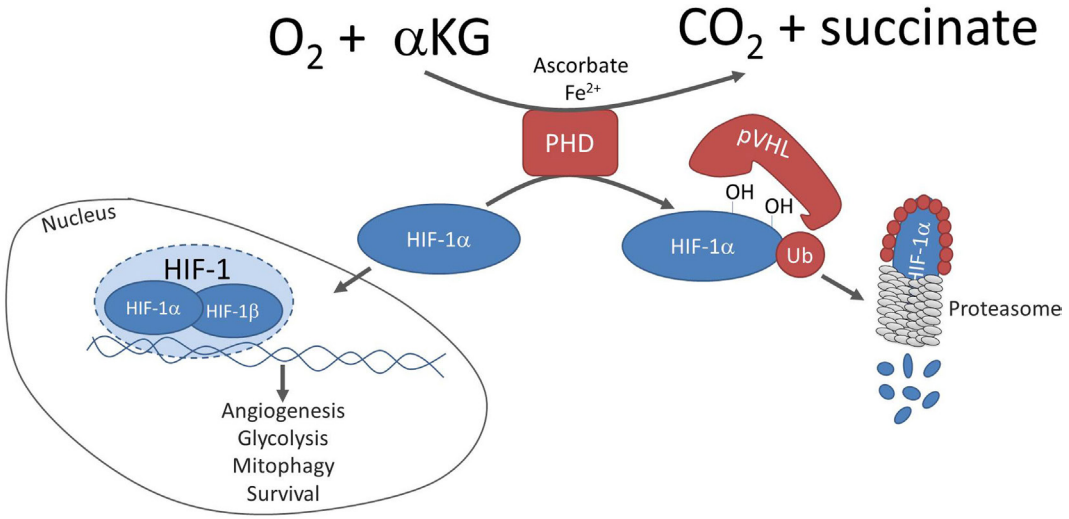

FIGURE 1 | Canonical regulation of HIF-1α stability. In normoxia, prolyl hydroxylases (PHDs) hydroxylate hypoxia-inducible factor 1 alpha (HIF-1α) on two proline residues, triggering pVHL-mediated ubiquitination and proteasomal degradation of hydroxylated HIF-1α. The hydroxylation reaction is coupled to conversion of αKG to succinate and requires co-factors ascorbate and ferrous iron. In hypoxia, hydroxylation is inhibited and HIF-1α dimerizes with constitutively expressed HIF-1β, creating an active HIF-1 complex, which transcribes genes promoting angiogenesis, glycolytic metabolism, mitophagy, and survival.

OXYGEN-INDEPENDENT HIF-1α STABILIZATION BY ONCOMETABOLITE-MEDIATED REGULATION OF PHDs ACTIVITY

The first evidence of an oxygen-independent regulation of HIF-1α stability *in vivo* was found in tumors harboring succinate dehydrogenase (SDH) and fumarate hydratase mutations (9). Soon after, it was demonstrated that SDH inhibition stabilizes HIF-1α in normoxia due to increased concentrations of succinate, a by-product and allosteric inhibitor of the PHD reaction (10). This finding gave birth to the concept of "oncometabolites," which initially regarded the accumulation of certain Krebs cycle intermediates, such as succinate and fumarate (11, 12), but may now be extended to any metabolite capable of triggering oncogenic or tumor suppressor signals. In the context of HIF-1α regulation, pyruvate and lactate were suggested to promote pseudohypoxia (13–15), whereas the PHD substrate alpha-ketoglutarate (αKG), as well as PHD co-factors ascorbate and Fe^{2+}, were all shown to confer a dose-dependent HIF-1α destabilization in hypoxia (16) (**Figure 2A**). For example, αKG increases the PHD affinity for oxygen and thus promotes HIF-1α hydroxylation and degradation even at low oxygen concentrations (17, 18). Accordingly, pseudonormoxia is observed in cells suffering nicotinamide nucleotide transhydrogenase deficiency or severe complex I damage, both conditions leading to NADH accumulation and consequent increase in αKG, due to the slowdown of the Krebs cycle rate (19–22). Conversely, the mitochondrial isocitrate dehydrogenase 3 alpha overexpression decreases αKG concentrations and promotes HIF-1α stability (23). Although mechanisms balancing oncometabolite concentrations represent intriguing therapeutic targets, their successful manipulation to fight cancer is still to be optimized, most likely due to the complexity of oncometabolite-mediated HIF-1α regulation. For instance, hypoxia-induced miR-210 expression was shown to contribute to the succinate accumulation by causing respiratory complex II defects (24, 25). Moreover, whereas (L)-2 hydroxyglutarate promotes HIF-1α stabilization (26), genetic lesions leading to the accumulation of the (R)-2 hydroxyglutarate enantiomer instead activate PHDs (27).

NON-CANONICAL OXYGEN-DEPENDENT REGULATION OF PHDs BY REDISTRIBUTION OF INTRACELLULAR OXYGEN FOLLOWING OXPHOS DAMAGE

As a solid cancer progresses, transformed cells usually activate HIF-1-mediated adaptations to hypoxic stress, which include downregulation of mitochondrial respiration to decrease the cells' requirement for oxygen (24, 28, 29). However, several xenograft studies, and a few examples from human tumors, demonstrate that severe OXPHOS damage induces a series of metabolic and molecular anti-tumorigenic events which, among other, include destabilization of HIF-1α (20, 21, 30–34). The anti-tumorigenic consequences of OXPHOS damage leading to HIF-1α destabilization come as a paradox to the known role of HIF-1 in promoting mitophagy and downregulation of OXPHOS genes (24, 28, 29) and are, therefore, discussed here in more detail. Hagen and colleagues pioneered in demonstrating that decreased oxygen consumption, due to OXPHOS inhibition in cancer cell lines, may result in redistribution of intracellular oxygen from respiratory enzymes to the PHDs, so that the latter become unable to sense external hypoxia (35, 36). As a result, HIF-1α is destabilized

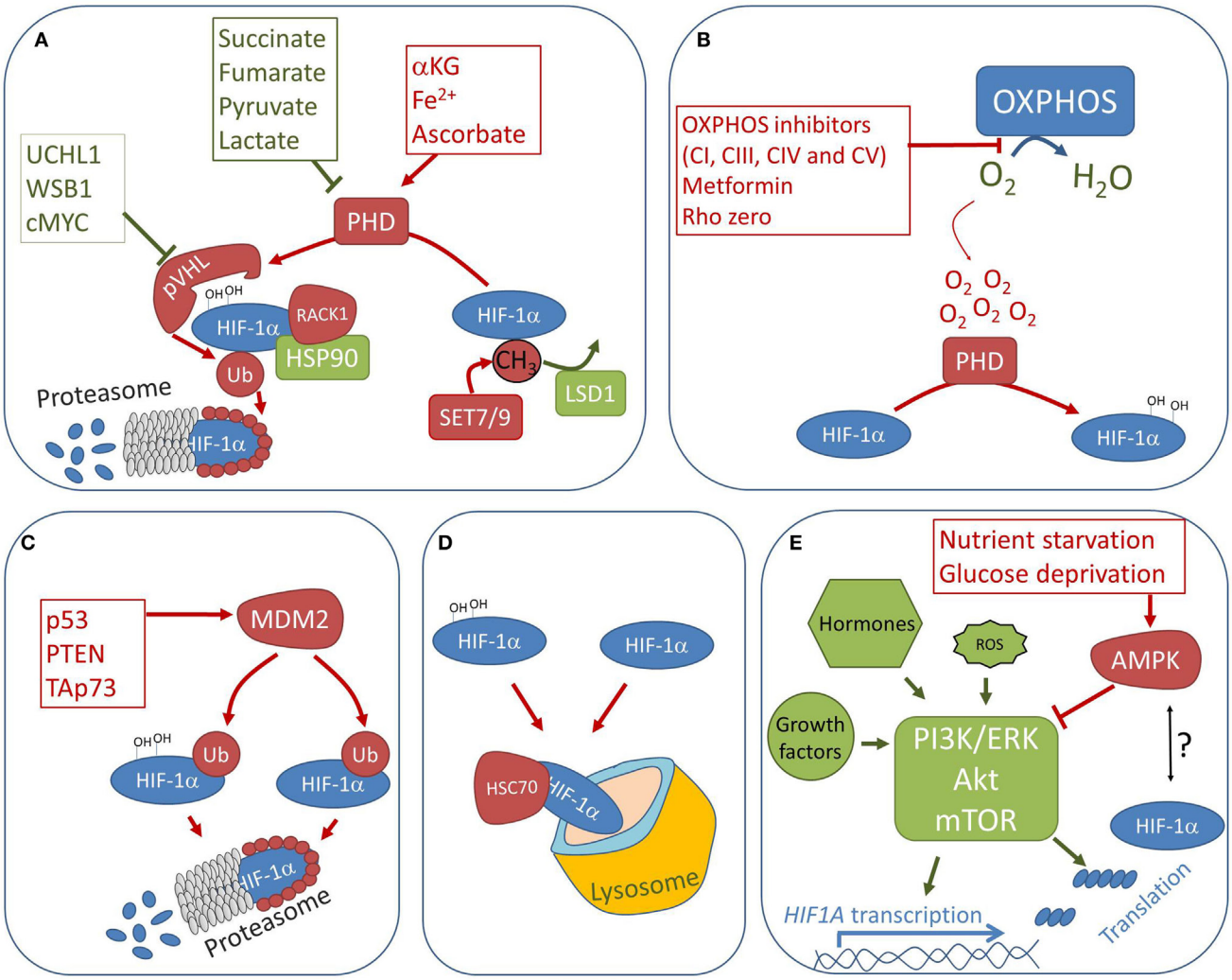

FIGURE 2 | Non-canonical regulation of HIF-1α stability. Factors promoting pseudonormoxia and pseudohypoxia are indicated in red and green, respectively. **(A)** Prolyl hydroxylase (PHD) activity may be blocked by accumulation of Krebs cycle metabolites succinate and fumarate, whereas αKG, and co-factors ascorbate and iron, boost PHDs activity regardless of oxygen levels. Activation of any factor promoting pVHL downregulation in normoxia will also lead to pseudohypoxic stabilization of HIF-1α. Finally, posttranslational modifications, such as methylation by SET7/9, or interactions with proteins, such as receptor of activated protein C kinase (RACK1) and HSP90, may regulate PHD accessibility to HIF-1α and promote or block hydroxylation regardless of oxygen concentrations. **(B)** Severe damage or inhibition of oxidative phosphorylation (OXPHOS) complexes I, III, IV, or V, reduces oxygen consumption, which in turn may increase intracellular oxygen concentrations and cause pseudonormoxia. **(C)** MDM2 is an ubiquitine ligase, which promotes HIF-1α degradation in hypoxic environment when associated with tumor suppressor proteins. **(D)** Proteasome-independent HIF-1α degradation *via* chaperone-mediated autophagy is mediated by HSC70. **(E)** PI3K/Akt/mTOR axis is the major pathway involved in promoting *HIF1A* transcription and translation, regardless of oxygen concentrations and upon numerous protumorigenic stimuli. For example, elevated reactive oxygen species concentrations were shown to promote *HIF1A* transcription and translation *via* Akt signaling. On the other hand, conditions counteracting mTOR pathway, such as nutrient starvation, and possibly adenosine monophosphate kinase (AMPK) activation, may lead to HIF-1α downregulation.

in cells with severe mitochondrial respiration damage, despite the outer hypoxic environment (**Figure 2B**). The association between mitochondrial respiration damage and HIF-1α inactivation despite hypoxia has also been observed in Rho zero cells and diverse cancer cell types, in which OXPHOS complexes I, III, IV, or V were pharmacologically inhibited (37–39). In accordance, by using a phosphorescent probe quenched by oxygen, a recent study showed that increasing concentrations of complex I inhibitor rotenone decrease intracellular hypoxia in a dose-dependent manner in a prostate cancer cell line (40). The conditions applied in these studies usually consisted of 3–6 h culture in the presence of 1–3% oxygen. On the other hand, studies applying 0.1–1% oxygen concentrations, reported that HIF-1α stabilizes in Rho zero cancer cells or upon rotenone treatment (41, 42), and Gong and Agani demonstrated that, in near-anoxic conditions, HIF-1α is stabilized despite OXPHOS damage (43). Therefore, OXPHOS damage does not seem to irreversibly prevent, but may rather attenuate HIF-1α stabilization, suggesting that the increased intracellular oxygen concentrations, caused by the lower oxygen consumption, may rapidly equilibrate with the extracellular

tensions. Such equilibration probably depends on the cellular membrane permeability to molecular oxygen, which among other is influenced by cholesterol levels and, therefore, lipid metabolism, which is conditioned by the OXPHOS status (44).

Notably, because of the short HIF-1α half-life (<5 min) in well oxygenated atmosphere, changes in ambient oxygen concentrations and variations of oxygen diffusion in the culture medium have a strong impact on HIF-1α stabilization when working *in vitro*. Therefore, precautions must be applied during cellular extraction and during cell washing, to avoid making biased conclusions regarding HIF-1α regulation. Moreover, for the time being, experimental limits prevent precise dissection of oxygen distribution in a growing tumor. Indeed, it must be noted that, to the best of our knowledge, the formal demonstration of the mechanism linking OXPHOS deficiency and HIF-1α destabilization *in vivo*, where selective pressures and microenvironment are radically different from *in vitro* conditions, has yet to be reported. Based on our data from complex I-deficient models, we hypothesize that more than one factor is involved in HIF-1α destabilization in OXPHOS-deficient tumors, since, if compared to counterpart controls, they display not only increased intracellular oxygen concentrations (unpublished data) but also higher αKG levels (20–22) and iron accumulation (unpublished data), all factors known to promote PHD-mediated HIF-1α hydroxylation.

To add complexity, OXPHOS damage is a known source of reactive oxygen species (ROS), which were suggested to promote HIF-1α stability in hypoxia and normoxia, although their role in HIF-1α regulation is still controversial (45, 46). Brunell and colleagues suggested that oxygen sensing in OXPHOS does not depend on oxygen consumption in human fibroblasts, but rather on ROS production deriving from decreased activity of complexes III and IV (47). On the other hand, by working on cancer cells, Chua and colleagues report that HIF-1α stabilization in hypoxia is not dependent on ROS and that re-establishing oxygen consumption in complex III-repressed cells is sufficient to induce HIF-1α stabilization, most likely due to a decrease of intracellular oxygen (48). The role of ROS in oxygen sensing has extensively been reviewed elsewhere (46, 49–51), and we discuss the role of ROS in promoting *HIF1A* transcription in the next paragraph. Still, it is interesting to note that OXPHOS damage leading to elevated ROS was suggested to promote HIF-1α stabilization (45), whereas severe respiratory deficiency associated to a decreased consumption of NADH results in pseudonormoxia. These apparently opposite effects may be explained by the fact that particularly severe damage, at least in the context of certain complex I mutations (20, 21), could destroy ROS-generating sites of respiratory multi-enzymes, resulting in unchanged or even decreased ROS concentrations. In this context, it is not surprising that mitochondrial DNA (mtDNA) mutations, not infrequent modifiers of tumorigenesis, may have opposing consequences on cancer progression, depending on the type of damage they induce (20). For example, mtDNA mutations increasing ROS production have been suggested to promote tumorigenesis and metastases, whereas those causing severe damage, such as complex I disassembly, compromise tumor progression (20, 21).

Taken together, the effects of OXPHOS deficiency on HIF-1α will depend on the type of damage inflicted, probably through different mechanisms depending on the mitochondrial respiratory complex involved. Nevertheless, while the down-regulation of mitochondrial respiration by HIF-1 is certainly a valid mechanism for adaptation of cancer cells to low oxygen tension, the block of OXPHOS may not be severe, since this would lead to HIF-1α destabilization. The latter is supported by studies such as the recent Hamanaka's work in epidermal keratinocytes, where the knock-out of mtDNA replication and transcription factor TFAM caused reduction of HIF-1α protein levels (52), indicating that HIF-1α destabilization in cells suffering mitochondrial respiratory damage seems to be a rather general phenomenon.

Interestingly, since severe OXPHOS damage seems to prevent cancer cells from experiencing hypoxia, they should be exempted from the need to adapt to low oxygen environment. Nevertheless, the growth of OXPHOS-deficient tumors is still challenged, as seen in complex I-deficient xenograft models (20, 21, 30, 31, 34) and in oncocytoma patients, who develop slowly proliferating masses, which rarely progress to malignancy (33). On one hand, this may be explained by the metabolic insufficiency, such as the recently described deficit in nucleotide biosynthesis, caused by aspartate shortage upon complex I inhibition (53). However, the consequences of the lack of HIF-1α in such tumors is not to be neglected, especially in the light of studies demonstrating that inhibition of HIF-1α is sufficient to block tumor growth (54, 55). In this context, it is intriguing to hypothesize that, in certain cancers, hypoxia may be advantageous, rather than a drawback for growing tumors, since the survival signals promoted by HIF-1 may actually be a requirement for malignant progression.

PHD-INDEPENDENT PATHWAYS REGULATING HIF-1α STABILIZATION

While PHDs control the oxygen-dependent HIF-1α stability, many other proteins are emerging as additional mediators of HIF-1α regulation, which act in an oxygen-independent manner and, therefore, regardless of the HIF-1α hydroxylation status. For example, several factors modulate pVHL activity (**Figure 2A**), such as WD repeat and SOCS box-containing protein 1 (WSB1), which was found to promote HIF-1α stabilization and metastases *via* ubiquitination and degradation of pVHL in renal carcinoma, breast cancer, and melanoma models (56). Similarly, ubiquitin C-terminal hydrolase-L1 was described to abrogate the pVHL-mediated ubiquitination of HIF-1α in mouse models of pulmonary metastasis (57), and c-Myc has been shown to weaken HIF-1α binding to pVHL complex, eventually leading to normoxic HIF-1α stabilization in breast cancer cells (58). Besides pVHL, E3 ubiquitin-protein ligase MDM2 was also found to ubiquitinate HIF-1α, but in a hydroxylation-independent manner, promoting its destabilization despite hypoxic atmosphere (**Figure 2C**). MDM2-mediated oxygen-independent HIF-1α degradation seems to occur upon binding with tumor suppressor proteins, such as TAp73 (59) or p53 (60). On a similar note, it has recently been shown that

PTEN and PI3K inhibitors promote HIF-1α destabilization by preventing MDM2 phosphorylation and subsequent translocation in the nucleus, suggesting that cytoplasmic MDM2 is then able to ubiquitinate HIF-1α and promote its degradation in hypoxia (61). Therefore, in cancers carrying mutations in tumor suppressor proteins such as *TP53*, MDM2-mediated HIF-1α degradation would be suspended, leading to synergic promotion of cancer progression, through blockage of the p53 pro-apoptotic stimuli and activation of the survival pathways upregulated by HIF-1α. Conversely, p53-independent binding of MDM2 to HIF-1α was associated with the increase in HIF-1α protein content (62), warning that the role of MDM2 in HIF-1α regulation might be more ambiguous than initially described. Further examples of oxygen-independent HIF-1α regulation involve factors, which may act either as promoters of HIF-1α degradation (**Figure 2A**), such as receptor of activated protein C kinase (RACK1), or as protectors from pVHL-mediated ubiquitination, such as heat shock protein (Hsp90) or Sentrin/SUMO-specific protease 1 (SENP1) (63–65). Inhibition of Hsp90 promotes the proteasome-mediated degradation of HIF-1α even in hypoxia or when functional pVHL is lacking (66). Moreover, it has been reported that gamma rays stimulate the mTOR-dependent synthesis of Hsp90 leading to HIF-1α stabilization and radiotherapy resistance of lung cancer cells (64). The mechanism of RACK1/Hsp90 competition in enhancing/decreasing HIF-1α-pVHL binding has already been reviewed (67), but it is interesting to note that, among other, calcium may influence RACK1 activity. For instance, calcium-activated phosphatase calcineurin prevented RACK1 dimerization and subsequent HIF-1α degradation in Hek293 and renal carcinoma RCC4 cells (68). Other studies also report a role for calcium in HIF-1α regulation (69, 70), suggesting that HIF-1α is not only an oxygen and nutrient sensor but may also promote adaptive responses to changes in cellular calcium homeostasis. It is probably due to its pleiotropic function that we find such intricate and multilayered control of HIF-1α, as testified by its numerous posttranslational modifications (1, 71, 72). Recently, SET7/9-mediated methylation of the HIF-1α lysine 32 residue was identified to destabilize HIF-1α, and promote its proteasomal degradation even in hypoxia (73). This reaction is contrasted by LSD1-mediated demethylation, which stabilizes HIF-1α, protecting it from ubiquitination (73). Furthermore, deacetylation of HIF-1α at lysine residue 709 by SIRT2 enhances PHD recognition of hydroxylating residues, promoting pseudonormoxia (74). It is interesting that, apart from proteasomal degradation, the mechanism of lysosomal digestion of HIF-1α has been described (**Figure 2D**). In particular, HIF-1α was first found to interact and co-localize with lysosome-associated membrane protein type 2A in HK2 human kidney and RCC4 renal cancer cells (75). The authors showed that the lysosomal digestion of HIF-1α is slower and less pronounced than its proteasomal degradation, but suggested it may become more important in circumstances where pVHL pathway is not working. Later, it was demonstrated that lysosomal degradation of HIF-1α is mediated by heat shock cognate 70-kDa protein (HSC70) *via* chaperone-mediated autophagy, which specifically targets individual proteins (76).

REGULATION OF HIF-1α ON TRANSCRIPTIONAL AND TRANSLATIONAL LEVEL

Besides the regulation of its protein stability and half-life, HIF-1α may also be regulated in a more conventional manner, *via* mRNA transcription and protein synthesis, in response not only to hypoxia itself but also to the stimulation by growth factors, cytokines and hormones, heat shock, irradiation, and nutrient availability. In this context, three major pro-survival pathways, namely ERK/MAPK, JAK/STAT, and PI3K/Akt/mTOR, concur to increase transcription and translation of *HIF1A*, especially in cancer (77). MAPK signaling *via* ERK1/2 was mainly associated with regulation of HIF-1 transactivation through phosphorylation of p300/CPB cofactors. On the other hand, JAK/STAT pathway triggers Akt-mediated *HIF1A* transcription *via* STAT3 (78, 79). The PI3K/Akt/mTOR signaling cascade directly increases *HIF1A* transcription and translation (80–82). Therefore, any aberrant stimulation of this pathway, which in cancer often occurs through growth factors, hormones, or oncogenes/tumor suppressor mutations, leads to the activation of HIF-1α, even in normoxic conditions (83–85). Concordantly, elevated ROS production caused by OXPHOS deficiency (86), and several other conditions leading to elevated ROS and reactive nitrogen species, including mtDNA mutations (87), chemical toxicants (88), intermittent hypoxia (89), and treatment with pro-inflammatory factors (90), have been associated with PI3K/Akt/mTOR-mediated increase of *HIF1A* transcription and translation (**Figure 2E**). Moreover, Akt pathway boosts HIF-1α-mediated response by stabilization and transactivation regardless of oxygen levels (91). For example, the ERK-PI3K/Akt mediate HIF-1α levels by stimulating protein synthesis of the molecular chaperone Hsp90, which in turn is able to stabilize HIF-1α in an oxygen-independent fashion (66, 92).

The PI3K/Akt-mediated activation of mTOR is antagonized by the 5′-adenosine monophosphate kinase (AMPK), the major sensor of cellular energy charge (93). In the context of a progressing cancer cell, PI3K/Akt/mTOR promotes survival and proliferation when conditions are fertile for cell proliferation, whereas AMPK serves as a sensor of nutrient starvation and ensures optimization of energetic sources when a cancer cell requires saving energy. Thus, it is intuitive to hypothesize that AMPK would counteract the effects of Akt-mediated increase of HIF-1α signaling. Indeed, an anticorrelation between active AMPK and HIF-1α has been confirmed by a recent system biology analysis (94) and, concordantly, by *in vitro* studies showing HIF-1α destabilization in hypoxia under glucose deprivation, suggesting that starvation dampens HIF-1α translation (95–97). However, the relationship between AMPK and HIF-1α is still unclear. On one hand, the lack of AMPK in MEFs stimulates HIF-1α expression in normoxia (98, 99), and mTORC1 activation and increased ROS production have been appointed for the normoxic stabilization of HIF-1α in AMPK-defective MEFs (99, 100). On the other hand, it has been reported that oxidative stress may induce AMPK activation leading to a reduction in HIF-1α degradation (101) and active AMPK was shown to

stimulate ROS-mediated increase of HIF-1α (102). It seems that the AMPK control of HIF-1α may be dependent on the contexts and phases of tumor progression, concordantly to the recently reviewed double-edged role of this energy sensor (103).

CONCLUDING REMARKS

Taken together, studies we discuss here show that, even though PHD-mediated hydroxylation of HIF-1α seems an impeccable mechanism to control its stability, many novel regulators of HIF-1α are emerging, especially in the context of cancer, where the selective pressures to activate this protumorigenic protein are particularly strong. Unraveling the complexity of HIF-1α regulation might lead to development of more precise anticancer treatments. In particular, considering the heterogeneous OXPHOS activity in different cancers, a better understanding of the mechanisms by which HIF-1α and mitochondrial respiratory chain complexes control oxygen sensing, may identify means for optimization of targeting HIF-1α, possibly based on the OXPHOS status of tumors. For example, therapies targeting HIF-1α could be avoided in tumors suffering OXPHOS deficiency, whereas targeting complex I could be adopted as a strategy to block HIF-1α in tumors which rely on the activity of this pleiotropic transcription factor.

AUTHOR CONTRIBUTIONS

IK designed the work. LI and IK wrote the manuscript. GG and AMP critically revised the manuscript.

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MINI REVIEW
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Ataxia-Telangiectasia Mutated Modulation of Carbon Metabolism in Cancer

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The ataxia-telangiectasia mutated (ATM) protein kinase has been extensively studied for its role in the DNA damage response and its association with the disease ataxia telangiectasia. There is increasing evidence that ATM also plays an important role in other cellular processes, including carbon metabolism. Carbon metabolism is highly dysregulated in cancer due to the increased need for cellular biomass. A number of recent studies report a non-canonical role for ATM in the regulation of carbon metabolism. This review highlights what is currently known about ATM's regulation of carbon metabolism, the implication of these pathways in cancer, and the development of ATM inhibitors as therapeutic strategies for cancer.

Keywords: ataxia-telangiectasia mutated, cellular metabolism, cancer, reactive oxygen species, senescence, p53, AKT, c-myc

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INTRODUCTION

Ataxia-Telangiectasia Mutated (ATM)

Ataxia-telangiectasia mutated is a serine/threonine kinase that is recruited to sites of DNA double-strand breaks and signals to various downstream targets to initiate cell cycle arrest and DNA repair (1). Although mainly nuclear, ATM is also found in the cytoplasm and mitochondria (2, 3). In the phosphatidylinositol kinase-related family, ATM consists of many conserved domains and is a tumor suppressor (4). Its kinase domain is flanked by a FAT (FRAT, ATM, and TRRAP) and FATC (C-terminus) domain (5, 6). The function of the FAT domain has yet to be elucidated; however, the FATC domain is essential for kinase activity (7, 8). In addition, ATM has a leucine zipper domain, which is important for its kinase function but not required for dimerization (9). Finally, the N-terminus of ATM encompasses HEAT (*h*untingtin, *e*longation factor 3, *A* subunit of protein phosphatase 2A, and *T*OR1) repeats, which form helices that interact with various macromolecules and play a role in ATM's kinase function (10, 11).

The activity of ATM in response to DNA damage has been extensively studied as ATM is known as the central regulator of the DNA damage response (DDR). During induction of DNA double-strand breaks, the MRN complex, containing Mre11, Rad50, and Nbs1, binds to the damage site (1). ATM is then activated and autophosphorylates its inactive dimer at serine 1981 (12). Monomeric, active ATM is then recruited to the damage site, where it phosphorylates downstream targets including SMC1, Nbs1, Chk2, BRCA1, and histone H2AX (13, 14). In addition, ATM phosphorylates p53 at serine 15 (15, 16). Activation and repression of ATM's downstream targets ultimately leads to senescence, genome repair, or apoptosis (17).

ATM is the primary gene mutation in ataxia telangiectasia (A-T) (18, 19). A-T is primarily documented as an immunodeficiency and neuronal degeneration disorder affecting 1:40,000–1:100,000 people worldwide (18, 20). Inherited in an autosomal recessive manner, patients typically produce

symptoms of delayed development due to neurodegeneration, deficient immune response, and predisposition to cancer. Approximately 10–15% of *ATM* null A-T patients develop childhood leukemia and lymphoma, specifically T-cell prolymphocytic leukemia (21, 22). In addition, patients are predisposed to breast cancer, pancreatic cancer, and melanoma (23). Renwick et al. conducted an unbiased screen in familial breast cancer patients and identified a number of premature truncations and missense variants in *ATM* that predispose patients to cancer (24). Furthermore, immunohistochemical staining of ATM and p53 in pancreatic tumor samples reveal that tumoral loss of ATM with wild-type p53 correlates with a decrease in patient survival, especially in families with a history of pancreatic cancer (25). Finally, somatic *ATM* mutations are implicated in increased melanoma risk (26). Moreover, ATM repairs mitochondrial genome defects, and loss of ATM leads to mitochondrial dysregulation (27). A-T patients have alterations in metabolism, including fluctuations in glucose metabolism (28). In addition, low NAD+ and SIRT1 levels are observed in rat models of A-T (29). These observations lead to the investigation of the role of ATM in metabolism.

Carbon Metabolism in Cancer

Carbon metabolism is defined as the breakdown of carbon sources, such as glucose and amino acids, to be utilized for cellular energy. Alteration in carbon metabolism is a hallmark of cancer (30). Highly proliferative cancer cells predominantly proceed through aerobic glycolysis rather than the TCA cycle, termed the Warburg effect, requiring high intake of glucose and glutamine (31). This allows cancer cells to compete in a nutrient depleted environment to reduce reactive oxygen species (ROS), generate ATP, and produce dNTPs for proliferation (32, 33). This emphasizes the importance in studying carbon metabolism in cancer and using this knowledge to discover novel, metabolic-based therapeutics.

METABOLIC ROLES OF ATM

ATM and ROS

Apart from its role in the DDR, ATM has more recently been implicated in sensing ROS. The role of ATM in ROS sensing has been extensively reviewed (34, 35). Here, we will focus on the coupling of ATM-mediated ROS sensing in cellular metabolism.

In 2011, Cosentino et al. published a pivotal paper linking ROS and the pentose phosphate pathway (PPP) (36). The PPP acts as the *de novo* pathway for deoxyribonucleotide (dNTP) synthesis, important for proliferation and DDR of cancer cells. ATM activates glucose-6-phosphate dehydrogenase (G6PD) through phosphorylation of heat shock protein 27 (Hsp27), which promotes shunting of glycolytic intermediates into the PPP to increase nucleotide synthesis. Furthermore, stimulation of the PPP increases NADPH production, which acts as a cofactor for antioxidants. Together, these data suggest the important role of ATM in the production of dNTPs and NADPH in the proliferation of cancer cells and protection against ROS.

Loss of ATM increases mitochondrial dysregulation, mitochondrial number, and ROS (3). A fraction of ATM localizes to the mitochondria, suggesting that A-T should be further classified as a mitochondrial disorder. Interestingly, this study suggested that the tumor predisposition of A-T patients may be in part due to the mitochondrial dysfunction observed.

Overall, ATM plays a key role in ROS prevention and sensing. The ability of cancer cells to sense ROS through ATM and reprogram metabolism by increasing PPP activity allows for cancer cell survival and resistance to therapy. Cells lacking wild-type ATM are prone to ROS accumulation and oxidative stress. However, the full mechanistic pathway for ATM activation after ROS accumulation is currently unclear.

ATM and Insulin Signaling

Although beyond the scope of this review, it is important to recognize the evident role of ATM in insulin signaling. The purpose of insulin is to reduce the amount of glucose circulating in the blood and promote cellular uptake of glucose (37). Insulin binds to its respective receptor and recruits GLUT4, a central regulator in glucose homeostasis, to the membrane. GLUT4 transports glucose into the cell where it is used for various processes including glycolysis. A-T patients have an increased risk of developing insulin resistance and type 2 diabetes. Early studies found that A-T patient monocytes have a decreased binding affinity for insulin when compared to unaffected controls (38). Furthermore, ATM signaling through p53 is vital to glucose homeostasis and insulin resistance. Together, these data suggest that ATM regulates glucose homeostasis in part through insulin signaling. Additional information on ATM and insulin signaling can be obtained in several excellent reviews (39–42).

ATM and Glycolysis

Glycolysis is the main carbon metabolism pathway occurring in the cytosol in which glucose is catabolized into pyruvate through a series of biochemical reactions. Importantly, glycolysis does not require oxygen to proceed and produces a net gain of two ATP molecules and two NADH molecules. Subsequently, in the presence of oxygen, pyruvate enters the mitochondria in the form of acetyl CoA and proceeds through the TCA cycle and oxidative phosphorylation. Conversely, pyruvate is converted to lactic acid in the absence of oxygen or in highly proliferative cancer cells as described above as the Warburg effect (31). ATM phosphorylates and activates the tumor suppressor p53 to regulate cell cycle arrest, apoptosis, senescence, and metabolism (43). p53 suppresses glycolysis through a number of pathways. Interestingly, p53 transcriptionally regulates metabolic genes, including glucose transporters *SLC2A* and *SLC2A4* (encoding for GLUT1 and GLUT4, respectively) (44). p53 also inhibits kinase IKK and targets NFκB, effectively suppressing glycolysis (45). In addition, p53 targets TIGAR, which reduces glycolysis by acting as a fructose-2,6-bisphosphotase (46). It is tempting to speculate that ATM activates p53 to modulate glycolysis through these pathways. Indeed, various DDR proteins are connected to mitochondrial signaling, as discussed in a recent excellent review (47).

ATM and the PPP

Metabolism is altered in cancer mainly due to the need for nutrients and essential macromolecules in a competing and

proliferative environment (32). The PPP is a key pathway in the breakdown of glucose and diverges from glycolysis at glucose-6-phosphate (G6P) (48). Indeed, the increase in proliferation of cancer cells requires the biosynthesis of dNTPs in order to faithfully replicate the genome and repair DNA damage (49, 50). The PPP is essential for *de novo* dNTP synthesis. The PPP produces ribose-5-phosphate, the sugar backbone precursor for purine and pyrimidine synthesis (51). The PPP is divided into the oxidative and non-oxidative pathways. The first irreversible step of the PPP converts NAD+ to NADPH during the conversion of G6P to 6-phosphate-gluconolactone (6PG). The production of NADPH acts as an antioxidant cofactor, protecting the cell from ROS and oxidative stress (52). Together these data suggest an important role of the PPP in the proliferation and reduction of ROS for cancer cell survival.

In response to DNA double-strand breaks, ATM activates Hsp27 and G6PD (36). This interaction increases the flux of G6P to enter the PPP, which increases dNTPs and NADPH to aid DNA repair and reduce ROS, respectively. Conversely, other groups found that ATM negatively regulates the PPP through p53 (52, 53). It is interesting to speculate that there is a balance between positive and negative regulation of the PPP downstream of ATM. It is possible that the amount of DNA damage differentially modulates PPP activity. Under low amounts of DNA damage, Hsp27 is activated to increase dNTP synthesis for DNA repair; however, significant DNA damage accumulation may hyperactivate p53 to inhibit the PPP to fully shut down biosynthetic pathways. Nevertheless, these data support the notion that ATM regulates the PPP to affect dNTP synthesis and NADPH production in cancer cells.

ATM AND CANCER

Tumor Suppressive Role of ATM in Senescence

Cellular senescence is defined as a stable cell cycle arrest (54) and is, therefore, a potent inhibitor of transformation (55). Senescence also plays a role in aging and is increased in age-related pathologies (56, 57). Senescence occurs due to multiple cellular insults, including telomere shortening, oncogene activation, termed oncogene-induced senescence (OIS), oxidative stress, and DNA damage (54). Senescence is characterized in part by alterations in metabolism (58). Senescence is now considered a reversible process (49, 53, 59–62). Therefore, dissecting how cells escape senescence is critical for understanding the earliest events in tumorigenesis.

One of the underlying mechanisms of OIS is increased replication stress, leading to DNA damage accumulation and cell cycle arrest (63, 64). Replication stress is due to a decrease in dNTP production *via* suppression of ribonucleotide reductase subunit 2 (RRM2), the rate-limiting enzyme in *de novo* dNTP synthesis (49). Replication stress due to decreased dNTPs activates ATM, correlating with senescence induction (53). Loss of ATM rescues senescence through restoration of dNTP levels. This is mediated by a p53-dependent modulation of PPP activity and increased c-myc stability to increase glucose and glutamine consumption. Consistently, a recent study found that pharmacological inhibition of ATM suppresses senescence (65). In this study, pharmacological ATM inhibition also modulated glucose consumption. Together, these data suggest that ATM functions in metabolic regulation and reprogramming in senescent cells.

Oxidative stress induced by ROS can also cause premature senescence in part through DNA damage accumulation. As discussed above, ATM senses and is activated by DNA damage (66). ATM signals through the AKT/p53/p21 pathway to induce senescence in human umbilical vein endothelial cells after oxidative stress (67). In addition, ATM activation is necessary for senescence due to nitric oxide (68). Finally, recent evidence suggests that loss of ATM in A-T mice increases NADPH oxidase 4 (NOX4) expression, leading to increased ROS and senescence (69). Together, these data demonstrate the importance of ATM signaling to induce senescence and suggest that ATM's role in modulating senescence status offers the possibility of a future therapeutic target in the fields of both aging and cancer.

ATM Suppresses c-myc

Many cancers upregulate oncogenes that modulate metabolism, including the well-known transcription factor c-myc (70, 71). Specifically, c-myc transcriptionally regulates various enzymes related to metabolic pathways (70, 71). In relation to cancer, c-myc increases the Warburg Effect through upregulation of lactate dehydrogenase, glucose transporters, and pyruvate dehydrogenase kinase. The regulation of c-myc by ATM has just begun to be elucidated. Loss of ATM increases c-myc protein stability, which in turn increases glucose and glutamine consumption (53). Consistently, ATM partially suppresses c-myc-induced lymphomagenesis in mouse models (72, 73). It is interesting to speculate whether this is due to suppression of pro-tumorigenic metabolism. Loss of ATM and c-myc amplification/overexpression are often mutually exclusive in multiple cancer types, suggesting a redundancy in the pathway. Altogether, this suggests an interplay between ATM and c-myc in cancer metabolism.

ATM Activates AKT

AKT is a well-known serine/threonine kinase that is activated by phosphatidylinositol-3-kinase (PI3K) and regulates many cellular processes related to cancer, including survival, cellular metabolism, and DNA repair (74, 75). ATM activates AKT in response to DNA damage (76–78). Activated AKT then promotes DNA repair (79) and inhibition of AKT decreases DNA repair (80, 81). Consistently, pharmacological inhibition of ATM inhibits AKT phosphorylation and survival in multiple cancer types (82–84). These findings suggest a vital role for AKT in the maintenance of genome integrity, and inhibition of this DNA repair function may result in accumulation of DNA damage and cell death.

AKT also modulates cancer metabolism (85–89). Active AKT increases glucose uptake by recruiting GLUT4 to the plasma membrane (90). In addition, pharmacological inhibition of AKT in primary effusion lymphoma decreases the rate of aerobic glycolysis (91). This suggests that ATM-mediated regulation of AKT activity in cancer reprograms metabolism by increasing glucose uptake and potentially shifting metabolism from aerobic glycolysis to oxidative phosphorylation. It is particularly interesting

that ATM-mediated AKT activation may be a double-edged sword, both increasing DNA repair to promote genomic integrity while at the same time increasing pro-tumorigenic metabolism. These data suggest that ATM inhibitors may both alleviate the metabolic changes induced by activated AKT and lead to DNA damage-induced death of cancer cells.

ATM Regulates p53

p53 is defined as the "guardian of the genome" as it serves to regulate genome stability as a tumor suppressor (92). *TP53* is one of the most mutated genes among all cancers. p53 is a transcription factor that can be activated by ATM (10). Activation of p53 by ATM was originally shown to be important for the regulation of genes essential in apoptosis and DNA repair (93). Further investigation into the interplay between ATM and p53 has revealed its importance in cancer metabolism. p53 regulates many pathways in cellular metabolism, including GLUT recruitment, glycolysis, and oxidative phosphorylation (94). Mutations in p53 lead to metabolic reprogramming in a cancer cells, allowing increased glucose intake through GLUT recruitment to the cell membrane, increased aerobic glycolysis, and decreased oxidative phosphorylation (94, 95). In addition, ATM directly impacts p53-mediated PPP metabolism as discussed above (53). Moreover, ATM loss and p53 mutation are often mutually exclusive in cancer, suggesting that these proteins act in the same pathway to promote cancer cell survival.

ATM Inhibitors for Cancer Therapy

A variety of ATM inhibitors are currently in pre-clinical and clinical trials for multiple cancer types. ATM inhibitors sensitize various cancer cell lines and tumors *in vitro* and *in vivo* to radiation treatment (83, 96–98). In addition, a phase I clinical trial is currently ongoing with an ATM inhibitor in combination with a PARP inhibitor in advanced cancer patients who are resistant to the standard-of-care (99). Together, these studies have found that cancer cells may be sensitized to DNA damage through inhibition of ATM.

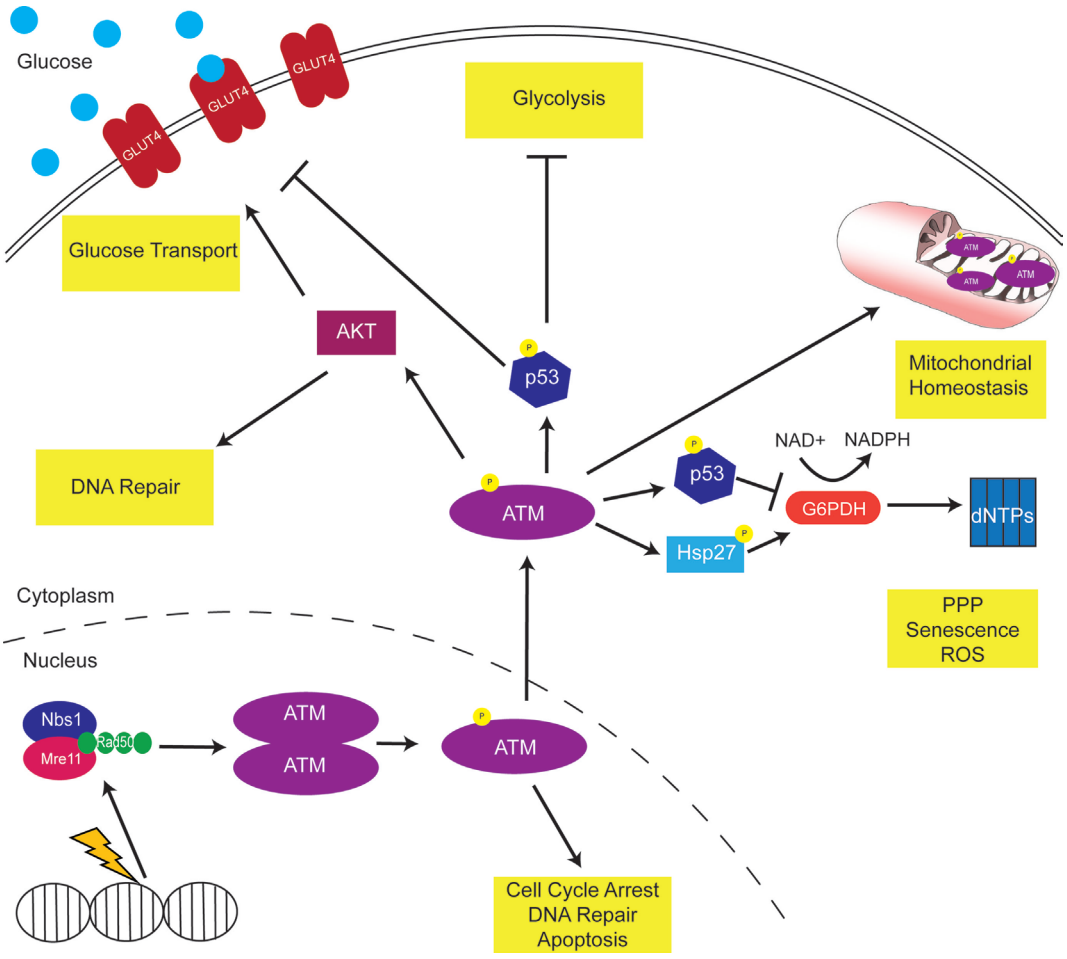

FIGURE 1 | Ataxia-telangiectasia mutated (ATM) modulates cellular metabolism. DNA damage activates ATM to phosphorylate multiple downstream proteins regulate cell cycle arrest, DNA repair, and apoptosis pathways. A non-canonical function of ATM is the regulation of cellular metabolism. Mitochondrial ATM acts to regulate mitochondrial homeostasis by repairing mitochondrial genome defects. ATM activates the tumor suppressor p53, which inhibits GLUT recruitment, glycolysis, and dNTP production. Consistently, p53 targets the oncogene c-myc, inhibiting the TCA cycle and increasing the Warburg effect. In addition, ATM activates AKT to increase GLUT recruitment to the membrane.

As discussed throughout this review, ATM modulates metabolism through various pathways, proteins, and enzymes (**Figure 1**). Thus, ATM inhibitors may offer a promising way to reprogram the metabolism of cancer cells to make them more vulnerable to anti-metabolic strategies. It will be important to dissect the role of metabolism in pre-clinical and clinical trials using ATM inhibitors.

CONCLUSION

Proliferation of cancer cells requires a metabolic shift allowing for an increase in cellular biomass in a highly competitive and nutrient-deprived environment. Although extensively studied for its role in the DDR, non-canonical roles of ATM in metabolic reprogramming have recently been elucidated. ATM modulates carbon metabolism through many pathways that are essential for cancer development, survival, and therapeutic response. Due to their radio- and chemo-sensitizing effects, ATM inhibitors are in pre-clinical and clinical trials as anti-cancer therapeutics. We suggest that ATM inhibitors may also be used to identify metabolic vulnerabilities that could be therapeutically exploited.

AUTHOR CONTRIBUTIONS

ED and KA jointly came up with the topic for this mini-review. Both ED and KA wrote and edited the text.

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MINI REVIEW
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Metabolic Plasiticy in Cancers—Distinct Role of Glycolytic Enzymes GPI, LDHs or Membrane Transporters MCTs

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Research on cancer metabolism has recently re-surfaced as a major focal point in cancer field with a reprogrammed metabolism no longer being considered as a mere consequence of oncogenic transformation, but as a hallmark of cancer. Reprogramming metabolic pathways and nutrient sensing is an elaborate way by which cancer cells respond to high bioenergetic and anabolic demands during tumorigenesis. Thus, inhibiting specific metabolic pathways at defined steps should provide potent ways of arresting tumor growth. However, both animal models and clinical observations have revealed that this approach is seriously limited by an extraordinary cellular metabolic plasticity. The classical example of cancer metabolic reprogramming is the preference for aerobic glycolysis, or Warburg effect, where cancers increase their glycolytic flux and produce lactate regardless of the presence of the oxygen. This allows cancer cells to meet the metabolic requirements for high rates of proliferation. Here, we discuss the benefits and limitations of disrupting fermentative glycolysis for impeding tumor growth at three levels of the pathway: (i) an upstream block at the level of the glucose-6-phosphate isomerase (GPI), (ii) a downstream block at the level of lactate dehydrogenases (LDH, isoforms A and B), and (iii) the endpoint block preventing lactic acid export (MCT1/4). Using these examples of genetic disruption targeting glycolysis studied in our lab, we will discuss the responses of different cancer cell lines in terms of metabolic rewiring, growth arrest, and tumor escape and compare it with the broader literature.

Keywords: cancer, CRISPR-Cas9, glycolysis, immune response, lactic acid, metabolism, oxidative phosphorylation, pentose phosphate pathway

INTRODUCTION

As opposed to normal, differentiated cells, which under aerobic conditions metabolize glucose mainly *via* oxidative phosphorylation (OXPHOS), cancer cells largely favor glycolytic pathway and subsequent lactate[1] formation for their energy production, regardless of oxygen availability. Warburg first observed this metabolic peculiarity of cancer cells (1) and postulated not only that cancer cells have damaged respiration and excessive glycolysis but also that the shift of energy

[1] The authors refer to L-lactate metabolism in this mini-review.

metabolism from aerobic to anaerobic is actually the cause of cancer (1). According to Warburg, the tumor is initiated by irreversible damage to respiration and persists because of increased anaerobic metabolism, which compensates energetically for the failure of respiration (1). However, today we know that many cancer cells have healthy mitochondria (2) and rely partly on oxidative metabolism (3), whereas fermentative glycolysis remains the "preferred" pathway by most hypoxic and rapidly growing tumors (4–6).

Following these pioneering studies, the field of cancer metabolism has been in a shadow of cancer genetics, which prevailed for decades, after the discovery of the role of oncogenes and tumor-suppressor genes in cancer. However, in the late 1990s, it was shown that lactate dehydrogenase A (*LDHA*) is a direct c-Myc-responsive gene (7), followed later on by the discovery that c-Myc and HIF-1 complementary induce all glycolytic enzymes with a concomitant inhibition of the pyruvate oxidation (8), reviving interest in connecting oncogenes and altered metabolism (4). At this time, altered metabolism was seen only as a consequence of oncogenic activation, since serum growth factors known to rapidly activate metabolism in the early 1970s (9) were shown to induce c-Myc. Interestingly, it was shown only later that loss-of-function mutations of the TCA cycle enzymes succinate dehydrogenase (10) and fumarate hydratase (11) were implicated in pathogenesis of several hereditary forms of cancer. These mutations in tumor-suppressor genes encoding for important metabolic enzymes raised the possibility that under certain conditions, altered metabolism could be the cause, not the effect, of cancer transformation (12).

Even if seemingly counterintuitive, given the much lower ATP yield from glycolysis with respect to the OXPHOS, this reprogramming of energy metabolism is thought to support large-scale macromolecule biosynthesis, necessary for rapid proliferation and growth (5, 6, 13) (**Figure 1**). Metabolic rearrangements are a feature of almost all cancer cells, which enables them to adapt to constantly changing conditions in nutrient microenvironment thereby promoting their aberrant proliferation. Aerobic glycolysis (Warburg effect) is just one component of the metabolic transformation, together with the reverse Warburg effect (14), metabolic symbiosis (15) and addiction to glutamine metabolism (16).

In this mini-review, we report the tumor growth consequences of re-routing fermentative glycolysis by genetic disruption at three key levels studied in our lab: glucose-6-phosphate isomerase (GPI), lactate dehydrogenase (LDHA and B isoforms), and at the level of export of lactic acid [monocarboxylate transporter (MCT) isoforms]. We discuss their responses in terms of metabolic rewiring, growth arrest, or tumor escape and compare it with a broader literature.

AEROBIC GLYCOLYSIS AND THE CONTROL OF THE METABOLIC SWITCH

Despite the remarkable genetic and phenotypic tumor heterogeneity, a specific set of signaling pathways appear to support the altered metabolic processing of glucose. Indeed, there is a dual set of universal mitogenic pathways: Ras-Raf-ERK and PI3K-AKT activated by growth factors/hormone receptor tyrosine kinases and G protein-coupled receptors. ERKs and AKTs protein kinases synergize in controlling growth and metabolism through activation of the master protein kinase (mTORC1). In cancer, oncogenes and tumors suppressors constitutively activate these mitogenic pathways to modify metabolism, nutrient, and oxygen sensing through c-Myc and HIF-1 (17–19). Regulation of cancer cells' metabolic rearrangements by oncogenes and tumor suppressors is complex and beyond the scope of this short review, but the fact that numerous pathways converge on glucose and glutamine reflects their central importance for energy metabolism.

The avidity of cancer cells for glucose is reflected by the upregulation of glucose transporters and clinical exploitation of the accumulation of radioactive ^{18}F-deoxyglucose is identified by positron emission tomography. Once inside the cell, glucose is metabolized by glycolysis, a pathway embedded in a complex metabolic network, directly providing precursors for nonessential amino acids (20) and through branching to the oxidative arm of pentose phosphate pathway (PPP), nucleotides (20) (**Figure 1**). Furthermore, NADPH is regenerated in the PPP and by the serine, glycine/C1-carbon synthesis glycolytic bypass thus contributing to reductive biosynthesis and redox homeostasis (21). As such, branching of the glycolytic pathway is strictly regulated at several different steps (22).

Recognition that the oncogenic activation leads to increased glycolysis (23), together with clinical evidence that correlated cell metabolism with cancer outcome, prompted many studies toward strategies to inhibit glucose metabolism in cancer (24, 25). In fact, some of the first metabolic anticancer therapies developed remain effective agents in clinic today, such as antifolate drugs and L-asparaginase (25). 2-deoxy-glucose (2-DG) has been recognized as a glycolysis inhibitor since the 1950s (26, 27), primarily by competitively inhibiting GPI (26, 28). However, 2-DG also inhibits glucose transport (29), hexokinase (HK) activity (30, 31) and the multiple points of action and its high toxicity have prevented its use in the clinic (32, 33).

GLUCOSE-6-PHOSPHATE ISOMERASE (GPI)

Glucose-6-phosphate isomerase (D-glucose-6-phosphate aldose-ketose-isomerase; EC 5.3.1.9) is a housekeeping cytosolic enzyme that plays a key role in glycolytic and gluconeogenic pathways, catalyzing the interconversion between G6P and fructose-6-phosphate (**Figure 1**). Its expression is induced by c-Myc (34) and HIF-1 (35, 36) and is increased in many cancers (37). GPI has also been described as a secreted multifunctional complex protein that could act as a cytokine under the name autocrine motility factor (38). However, this notion requires further confirmation.

In our lab a complete genetic ablation of *GPI* expression was accomplished by using CRISPR/Cas9 in two aggressive cancer cell lines, human colon adenocarcinoma (LS174T) and mouse melanoma (B16-F10) (39). Both *GPI*-mutant cell lines had no

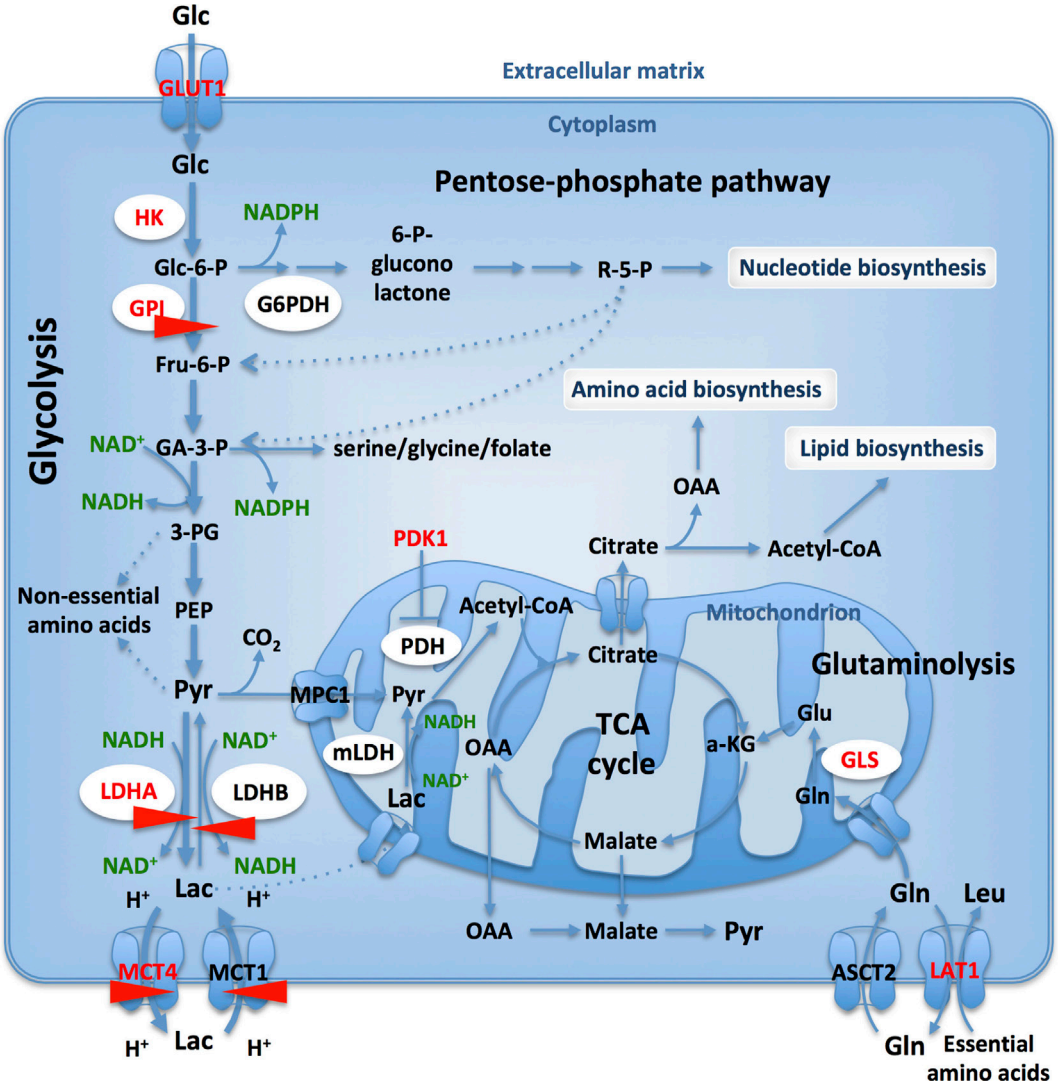

FIGURE 1 | Glucose and glutamine catabolism provide tumor cells with biosynthetic precursors. Glucose transport and glycolytic flux are accelerated in cancer cells, when compared to normal cells, due to increased expression of appropriate transporters and enzyme isoforms. Glucose-6-phosphate dehydrogenase (G6PDH) shunts G6P from the glycolysis into the oxidative branch of pentose phosphate pathway (PPP). Intermediates from glycolysis and TCA cycle replenish biosynthetic pathways to produce macromolecules (nucleic acids, lipids, and proteins) necessary for cell proliferation. Only those transporters and enzymes relevant to the text are shown: GLUT1, glucose-6-phosphate isomerase, lactate dehydrogenase A (LDHA)/-B, MCT1/4. HIF- targets are in red and CRISPR-Cas9 targets studied in our lab are identified with red arrows.

detectable GPI enzymatic activity, suppressed completely lactic acid secretion and grew by reprogramming their bioenergetic metabolism to OXPHOS (39). Surprisingly, in contrast to previous pharmacological inhibition studies (29, 37), *GPI*-KO cells growth was only reduced by twofold in normoxia with ATP produced by OXPHOS being sufficient to maintain their growth and viability. However, the growth rate of *GPI*-KO cells was severely reduced in hypoxia (1% O_2) while cells remained viable. Interruption of the glycolytic flow by *GPI*-KO increases the intracellular G6P pool, which in turn was proposed to elicit a short-term inhibition of HK and a long-term inhibition of glucose transport (40, 41). Indeed, we found that both *GPI*-KO cell lines had decreased GLUT1 expression, as well as induction of thioredoxin-interacting protein expression, a strong negative regulator of glucose uptake (42). We showed that increased OXPHOS dependence of *GPI*-KO cells made them extremely sensitive to inhibitors of the respiratory chain complexes, such as phenformin and oligomycin (39), in line with the findings of Pusapati et al. (37). Therefore, we speculate that pharmacological inhibition of tumor growth at the level of GPI was effective mainly because of the multiple targets of 2-DG.

In conclusion, we showed that complete suppression of glycolysis in two aggressive cancer cell lines slowed, but did not prevent *in vivo* tumor growth, in line with the findings of Pouysségur et al. (40) and Pusapati et al. (37). Particularly striking is the LS174T cell line that is highly glycolytic and almost

does not respire under normal conditions and is capable to achieve strong re-activation of OXPHOS when challenged by *GPI* ablation (**Figure 2**). Consequently, as shown with inducible shRNAs against *GPI*, the growth was significantly reduced only in combination with mTORC1 or OXPHOS inhibition (37). This remarkable metabolic plasticity of cancer cells revealed as well on several other cell lines (37) poses a big challenge for anticancer therapies targeting metabolism.

LACTATE DEHYDROGENASE (LDH) ISOFORMS

Lactate dehydrogenase [(*S*)-lactate:NAD^+ oxidoreductase; EC 1.1.1.27] is a family of NAD^+-dependent enzymes that catalyze the interconversion between pyruvate and lactate, with concomitant oxidation/reduction of the cofactor (NADH/NAD^+). LDH is a homo- or hetero-tetramer assembled from two different subunits: M and H, encoded by two separate genes, *LDHA* (M) and *LDHB* (H), respectively. A third subunit, LDHC, encoded by a separate *LDHC* gene, is expressed only in testes and sperm and is probably a duplication of the *LDHA* gene (43). LDH tetramers form at least six isoenzymes that differ in electrophoretic mobility, *K*m for pyruvate and lactate, immunological characteristics, thermal stability and inhibition by coenzyme analogs or excess pyruvate (44). The existence of mitochondrial LDH was shown in prostate cancer cells (45), and human hepatocellular carcinoma cells (46). Mitochondrial metabolism of lactate results in export of oxaloacetate, malate, and citrate outside mitochondria, therefore having an anaplerotic role (**Figure 1**) (46). In this mini-review, we will focus on the cytosolic LDH and refer readers to excellent reviews on this topic (47, 48).

LDHA

Lactate dehydrogenase A (LDH-5, or LDHA4) is composed of four LDHA subunits and has the lowest *K*m for pyruvate of the LDH isoforms and catalyzes pyruvate reduction to lactate, the

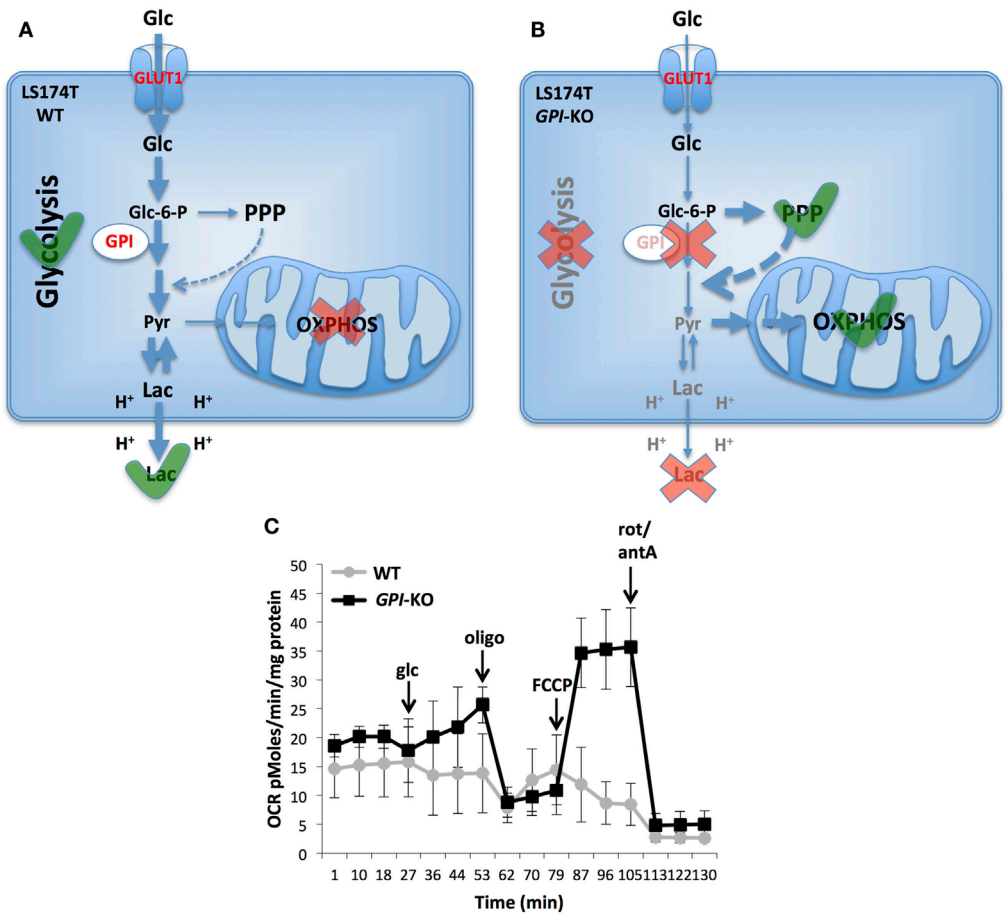

FIGURE 2 | Metabolic reprogramming in glucose-6-phosphate isomerase (*GPI*)-KO cells. A switch from glycolytic metabolism to oxidative phosphorylation (OXPHOS) caused by the complete *GPI* disruption is shown. LS174T WT cells are highly glycolytic and do not use mitochondria for ATP production **(A)**. Contrarily, cells survive *GPI* disruption by re-activating pentose phosphate pathway (PPP) and OXPHOS **(B)**. Oxygen consumption rate (OCR) of LS174T WT and *GPI*-KO cells was evaluated with Seahorse XF24 bioanalyzer **(C)**. The mean ± SEM is representative of four independent experiments performed in quadruplicate. The figure is adapted from Ref. (39). Glc, glucose, oligo, oligomycin, FCCP, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone, rot, rotenone, antA, antimycin A.

final step of the glycolysis, with concomitant regeneration of NAD^+ molecules, required for glycolysis to proceed. LDHA is located mainly in the cytoplasm, but it has also been found to bind single-stranded DNA in the nucleus (49). LDHA has been recognized as a valuable predictive/prognostic marker; its overexpression is associated with cancer invasiveness, and elevated serum lactate levels correlate with poor prognosis and resistance to chemo- and radiotherapy (50). LDHA expression is regulated by c-Myc (7), HIF-1 (51, 52), and micro-RNA miR-34a (53). The key role of LDHA in maintaining the Warburg phenotype in cancer cells was confirmed by several reports of LDHA inhibition or knock-down severely diminishing tumorigenicity in breast, lung, liver, lymphoma, and pancreas cancers (54–58). Decreased LDHA activity resulted in stimulation of OXPHOS and mitochondrial oxygen consumption and decrease of mitochondrial membrane potential (54) and increased apoptosis *via* ROS production (56–58). These data, together with the fact that LDHA deficiency has no serious consequences under normal conditions made LDHA a very attractive target for the anticancer therapy. Many LDHA inhibitors shown to suppress tumor growth *in vitro* and *in vivo* were developed by major pharmaceutical groups, but with moderate selectivity, particularly of those targeting the dinucleotide binding site common to many enzymes (50). These inhibitors were more powerful in combination with other therapies, but none have reached the stage of clinical trials (50). Recently, Genentech group described a novel LDHA inhibitor, GNE-140, capable of inhibiting both isoforms with nanomolar potency (59). Their work showed that predominantly glycolytic cell lines were more sensitive to LDHA inhibition, while cell lines relying more on OXPHOS were inherently resistant (59), and in these cells the combination of LDHA inhibition with OXPHOS inhibitors was synthetically lethal (59). However, GNE-140 was unable to inhibit tumor growth *in vivo*, alone or in combination with phenformin, due to its rapid clearance.

Conversely, our work with *LDHA*-KO cells in LS174T and B16 cell lines shows that LDHA is dispensable for *in vitro* tumor growth, both in normoxia and in hypoxia. These cells were still able to catalyze pyruvate conversion to lactate. Although reduced, this activity was sufficient to drive glycolysis and lactate production, which was only moderately decreased with respect to WT cells (60, 61). *LDHA*-KO cells moderately stimulated OXPHOS and, therefore, were more sensitive to respiratory chain inhibitors. However, residual LDH activity present in these cells, which we argue is due to the activity of the LDHB isoform, was sufficient to sustain cell growth and viability. Thus, we argue that most of the alterations due to LDHA inhibitors shown so far were due to off-target effects and not a specific decrease in LDHA activity. Similar results were observed in a study of *LDHA* silencing in breast cancer cell line, where stable *LDHA* knock down did not affect cell viability, lactic acid production, glucose consumption, or ATP (62). These cells contained twice as much LDHB isoform, again supporting the possibility of the LDHB isoform catalyzing the reverse reaction.

LDHB

LDHB is composed of four B subunits and catalyzes lactate oxidation to pyruvate, coupled with NADH formation. An increasing number of studies investigated the role of LDHB in several subtypes of cancer, but its role remains elusive and poorly characterized. LDHB was found to be positively regulated by the RTK–PI3K–AKT–mTOR pathway both in immortalized mouse cell lines and human cancer cells (63). Its expression was stimulated by signal transducer and activator of transcription STAT3, a key tumorigenic driver in many cancers (63). Furthermore, LDHB was found to be upregulated in triple-negative breast cancer, KRAS-dependent lung adenocarcinoma, maxillary sinus squamous cell cancer as well as in osteosarcoma and correlated with poor patient outcome (64–67). *LDHB* knock down inhibited cell growth, proliferation, and invasion and the loss of LDHB was shown to arrest tumor growth *in vitro* an *in vivo* (64, 66, 67). This is in line with the "reverse Warburg effect," proposing that stromal or cancer cells undergo aerobic glycolysis and produce lactate, which is then taken up by MCT1 to fuel oxidative cells *via* LDHB-catalyzed conversion to pyruvate (14, 68, 69). Indeed, MCT1 expression was found to correlate with high LDHB expression in TNBC (64).

Conversely, other studies found LDHB overexpression to be correlated with better prognosis (70), and accordingly, loss of *LDHB* expression was associated with metastatic progression (71). The underlying mechanism seems to involve *LDHB* promoter hypermethylation and consequent gene silencing at the transcriptional level (71), but exactly how loss of *LDHB* contributes to tumor progression is not clearly understood.

In our lab, *LDHB* gene knockout by CRISPR/Cas9 in LS174T and B16 cells did not significantly alter their growth and viability in normoxia or hypoxia (61). As expected, *LDHA/B*-DKO cells retained the ability to convert lactate into pyruvate by LDHA isoenzyme. Because our *LDHA*-KO cells were still capable to produce and secrete measurable levels of lactic acid we genetically disrupted the two *LDH* isoforms (*LDHA/B*-DKO) in LS174T and B16 cell lines. LDH enzymatic activity in both directions was completely abolished in these cells. As a consequence, they showed a distinctive phenotype—growth reduction, absence of glycolysis, and no lactic acid secretion, neither in normoxia nor in hypoxia (1% O_2). Furthermore, in order to overcome the imposed glycolytic blockade, these double *LDHA/B*-DKO cells re-directed their metabolism toward OXPHOS and relied on it for viability and growth. In contrast to wild-type or single *LDH*-KO cells, the double *LDHA/B*-DKO cells died rapidly in response to mitochondrial respiratory chain inhibitors, such as phenformin and oligomycin (*in submission*).

These findings, based on a genetic approach, demonstrate that both LDHA and B contribute to fermentative glycolysis (Warburg effect) and because of the bioenergetics metabolism re-routing these two enzymes are dispensable for tumor growth. In contrast, these results point that most of the LDHA inhibitors used so far, with the exception of GNE-140 from Genentech, inhibited tumor growth due to off-target effects.

MCT1 AND MCT4

Lactic acid, the end product of fermentative glycolysis abundantly released by cancer cells, has a strong impact in tumor microenvironment (72, 73). It can function as an oxidizable fuel,

gluconeogenetic precursor and a source of TCA cycle intermediates (46, 74, 75). In addition, it is an antioxidant promoting angiogenesis, migration (76), and its contribution to tumor acidosis was reported to blunt tumor-immune response by T and NK cells (60). Lactic acid is exported/imported in cells by a family of four reversible MCTs [for review, see Ref. (77)]. MCTs as H^+/Lactate$^-$ symporters facilitate net lactic acid exchange across the plasma membrane, whose direction depends on the concentration gradients of protons and monocarboxylate (77). Increasing experimental evidences support the cell–cell and intracellular lactate shuttles hypothesis proposed by Brooks (48), thus lactate is continuously formed and consumed in different cells under fully aerobic physiological conditions (48). MCT1 facilitates lactate and pyruvate transport, it is induced by c-Myc and expressed virtually in all cells. In contrast, MCT4 is an efficient lactate exporter induced by hypoxia and expressed in glycolytic tissues and cancer cells (77). Both MCT1 and 4 need assistance from the chaperone CD147 or basigine (BSG) to express active transporters at the plasma membrane.

Several reports from Baltazar's group (78–80) have shown that increased expression of MCT1 and MCT4 are associated with a poor prognosis in several types of human cancer, such as neuroblastoma, colorectal carcinoma, gastrointestinal stromal tumors, and prostate cancer. In parallel, our group, exploring pHi-regulating systems as putative anticancer targets in hypoxic tumors (81, 82), developed an interest in blocking lactic acid export. Pharmacological blockage with the specific AstraZeneca MCT1/2 inhibitor (AZD3965) was very efficient in arresting growth of tumors expressing only MCT1, like in transformed fibroblasts (83) or neoplastic B cells (84). However, it became clear that most aggressive cancers express both isoforms, like in colon adenocarcinoma, glioblastoma or non-small cell lung cancer. In these cancer types, genetic disruption of the chaperone (BSG), with zinc finger nucleases, reduced lactic acid export by 70–80%, an action sufficient to re-activate OXPHOS and maintain tumor growth (85). These tumor cells behaved like *GPI*-KO or *LDHA/B*-DKO with growth arrest and loss of cell viability induced by inhibitors of mitochondrial respiration (85, 86). However, pharmacological inhibition of MCT1 combined with a *MCT4*-KO was able to slow considerably *in vitro* growth and *in vivo* tumor xenografts (85, 86). We also confirmed that dual pharmacological inhibition of MCT1 and MCT4 considerably reduced cell growth. Removal of the inhibitors after a week allowed cells to form colonies, indicating a cytostatic, not cytotoxic effect induced by lactic acid sequestration in response to MCTs blockade.

CONCLUSION

Comparing the three independent approaches of interrupting the glycolytic flux, we reach a common consensus and a strong divergence. Genetic disruption of *GPI*, *LDHA/B*, or *MCT1/4* leads to re-activation of OXPHOS with tumor growth maintenance but increased sensitivity to mitochondrial inhibitors. The case of MCT1/MCT4 is interesting because the phenotype depends on the value of MCT suppression. Partial MCT suppression reached in *BSG*-KO cells, growth is maintained; total block with dual inhibition by AZD compounds, growth is compromised due to intracellular acidification.

Finally, targeting tumor metabolism *via* anti-glycolytic therapies remains an attractive therapeutic approach (82, 87), especially in combination with the inhibition of mitochondrial pathways, but it will have to be precisely administered in order to spare normal cells and limit toxicity (82).

AUTHOR CONTRIBUTIONS

MŽ isolated and characterized LDHA and B mutant cells, IM isolated and characterized MCT and BSG mutant cells and MP isolated and characterized GPI-mutant cells. SP helped with manuscript editing. MŽ and JP designed the project and wrote the manuscript.

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OPINION
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L-Lactate Transport and Metabolism in Mitochondria of Hep G2 Cells—The Cori Cycle Revisited

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In addition to being a glucose precursor in liver and kidney, L-lactate is now also being recognized as an energy substrate in most cells *via* its oxidation to pyruvate. This oxidation, assumed to occur in the cytosol, is catalyzed by L-lactate dehydrogenase with pyruvate subsequently catabolized in the mitochondria. However, recently mitochondria were recognized to play a role in L-lactate metabolism: the existence of a mitochondrial L-lactate dehydrogenase (m-L-LDH) was suggested by Dianzani (1), and later demonstrated by Baba and Sharma (2) to be located in the mitochondrial matrix (3). Indeed, L-lactate transport and metabolism was shown in various mitochondria, including skeletal muscle (4) rat heart (5), liver (6), brain (7–9), cerebellar granule cells (10), rabbit gastrocnemius (11), sperm cells (12), pig liver (13), and even plant (14). Thus, the existence of m-L-LDH, as reviewed by Passarella et al. (3), Brooks (15), and Schurr (16), was recognized with its inclusion in the MitoCarta (http://www.broadinstitute.org/pubs/MitoCarta/index.htrnl). As expected, in light of the presence of the L-LDH in the matrix, the occurrence of carriers for L-lactate has been shown in functional studies with purified, coupled mitochondria. These include the L-lactate/H^+ symporter and the L-lactate/pyruvate and L-lactate/oxaloacetate antiporters (3). Surprisingly, the overwhelming evidence for an m-L-LDH located inside mitochondria is not universally accepted, with some scientists still being skeptic about the existence of m-L-LDH, while others localizing m-L-LDH in the intermembrane space (17). It is our opinion that the skepticism could originate due to difficulties in isolating coupled mitochondria, not an easy task, in particular with skeletal muscle samples, or not being careful enough in selecting reaction media and in using inhibitors at the correct concentration (11). That m-L-LDH is localized inside mitochondria will be shown below.

IS L-LACTATE BEING TRANSPORTED AND METABOLIZED IN CANCER CELL MITOCHONDRIA?

Yes, it is. Although in the 1920s, Warburg found that cancer cells prefer to produce ATP by glycolysis with L-lactate production, to the best of our knowledge, the mitochondrial metabolism of L-lactate had not been investigated in cancer cells until 2010, when the first evidence for L-lactate mitochondrial metabolism in these cells (already reported in 2008 by Gabriella Chieppa in her PhD thesis at the University of Molise) was published (18). In this case, to study L-lactate transport and metabolism in mitochondria isolated from both normal and cancer prostate cells, spectroscopic and polarographic techniques were used, in which either m-L-LDH reaction or oxygen consumption by mitochondria, supplied with externally added L-lactate were monitored, respectively (19), rather than employing more involved procedures, available in molecular biology, genetics, and chemistry laboratories. The former two techniques were chosen since they afford the continuous monitoring of the kinetics of the investigated processes in experiments that last for several minutes where mitochondria remain coupled. By contrast, measurements using the latter methods are usually made once the processes have already been completed. Accordingly, an increase in the redox state of the

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intramitochondrial pyridine nucleotides, as shown by fluorimetric measurements, upon the addition of L-lactate to mitochondria indicates that L-lactate metabolism occurs inside the organelles *via* an NAD^+-dependent m-L-LDH; unfortunately, the occurrence of the mitochondrial L-lactate metabolism in cancer cells was not quoted in Ferguson et al. (17) possibly because the authors of the review consider the spectroscopic and polarographic techniques to be "problematic," despite its widespread use by numerous scientists. That theirs is a minority opinion might be exemplified by quoting from a review by Mayevsky and Rogatsky (20), which states that "The large numbers of publications by different groups testify to the valuable information gathered in various experimental conditions. The monitoring of NADH levels in the tissue provides the most important information on the metabolic state of the mitochondria." The existence of m-L-LDH can be also immunologically confirmed in mitochondria that are proven to be free of cytosolic contamination.

Notice that in the case where m-L-LDH is proposed to be localized in the intermembrane space, the increase in the intramitochondrial pyridine nucleotide fluorescence is explained as follows: L-lactate enters the mitochondrial intermembrane space where it is oxidized to pyruvate, which in turn crosses the mitochondrial inner membrane to be oxidized inside the mitochondria *via* the pyruvate dehydrogenase complex [for review, see Ferguson et al. (17)]. Such a mechanism is not supported by various experimental findings. For instance, in de Bari et al. (18), it was shown that NAD^+ reduction proceeds despite the presence of arsenite, an inhibitor of pyruvate dehydrogenase, but is inhibited by oxamate, an inhibitor of L-LDH. Additional evidence against the presence of m-L-LDH in the intermembrane space emerges from experimental results showing that L-lactate enters mitochondria under conditions where pyruvate is a non-penetrant compound (21) or where the pyruvate/H^+ symporter is blocked by an inhibitor (6). These experimental approaches can be also applied to measurements of oxygen consumption (in the presence or absence of ADP), proton efflux and membrane potential generation in the future. By applying the control strength criterion with various non-penetrant inhibitors (19) it can be established whether or not the rate of the above processes mirrors that of L-lactate transport across the mitochondrial membrane. Thus, L-lactate transport can be investigated quantitatively, including the occurrence of hyperbolic kinetics, pH profile, etc. Moreover, comparison made between the inhibition profiles of pyruvate and L-lactate-dependent mitochondrial processes through the use of compounds that are unable to enter mitochondria allows for a distinction between L-lactate and pyruvate carriers.

Briefly, it has also been shown that externally added L-lactate can enter both normal and cancer prostate cells and in particular, in a carrier-mediated manner, enters their mitochondria, where an L-LDH exists and is located in the inner compartment. The m-L-LDHs have been demonstrated to differ from the cytosolic enzymes that themselves differ from one another. Normal and cancer cells show differences with respect to m-L-LDH protein level and activity, where both the enzyme expression and activity are higher in cancer cells.

In 2011, the existence of monocarboxylate transporter (MCT) and LDH proteins in mitochondrial reticula of breast cancer cell lines was demonstrated (22). In that case, the expression of both MCTs and L-LDH was measured, and their mitochondrial localization was determined *via* immunofluorescence, a technique that does not allow for the identification of the submitochondrial localization.

A broader investigation of L-lactate transport and metabolism in cancer cell mitochondria was carried out in human hepatocellular carcinoma (Hep G2) cells (21) in which gluconeogenesis takes place (23). Hep G2 cell mitochondria (Hep G2-M) possess an m-L-LDH restricted to the inner mitochondrial compartment. Cytosolic and mitochondrial L-LDHs were also found to differ from one another in their saturation kinetics. The occurrence of a carrier-mediated L-lactate transport in these mitochondria has also been shown. Importantly, the efflux of various metabolites, including pyruvate, oxaloacetate, malate, and citrate, resulting from L-lactate addition to mitochondria was first shown, this giving a first insight into the role of mitochondrial metabolism of L-lactate; accordingly, the occurrence of an L-lactate/pyruvate shuttle devoted to the oxidation of the cytosolic NADH was also shown. Ultimately, the removal of the oxidation product by carrier-mediated transport and mitochondrial metabolism overcomes any theoretical thermodynamic difficulty which was considered to rule out any L-lactate oxidation in the mitochondria.

These findings strongly suggest that a revision of the dogmatic view of glucose metabolism is needed with a special focus on the role of L-lactate and m-L-LDH in gluconeogenesis. Hence, the Cori cycle (formulated in 1929 as an energy-requiring metabolic pathway in animals, where carbon atoms of glucose pass along the circular route: muscle glycogen → blood lactate → liver (where gluconeogenesis occurs) → blood glucose → muscle glucose → muscle glycogen) demands revision, too. In this regard, cellular L-lactate oxidation, which is necessary for the production of glucose in the Cori cycle, has been traditionally postulated to take place in the cytosol, but is it? The cytosolic-L-LDH (c-L-LDH) is a reducing enzyme, the final step of the glycolytic pathway, which converts pyruvate to L-lactate, and thus provides the regeneration of NAD^+. This reaction should proceed unabated, independently of the presence or absence of oxygen, as the standard free-energy $\left(\Delta G^{0'}\right)$ change of pyruvate conversion to L-lactate is about −6 kcal/mol. In addition, the high affinity of pyruvate to c-L-LDH would explain the fact that the normal [L-lactate]/[pyruvate] ratio in blood and other tissues is >10, a value that cannot correspond with the proposal of pyruvate as the end product of glycolysis under normal conditions. Therefore, the dogmatic portrayal of this reaction as bidirectional is misleading and has been accepted to date due to the absence of a possible alternative. We contend that L-lactate oxidation back to pyruvate does not take place in the cytosol, but rather, it occurs in the mitochondria. Indeed, there are only two options to prevent L-lactate accumulation in the cytosol, either L-lactate is transported out of the cell (under anaerobic conditions) and/or is oxidized *via* m-L-LDH upon its transport into the mitochondrion (under aerobic conditions). Therefore, even if we agree with Lu et al. (24) that "the majority of glycolysis-derived pyruvate is diverted to lactate fermentation," we cannot accept that L-lactate is "kept away from mitochondrial oxidative metabolism."

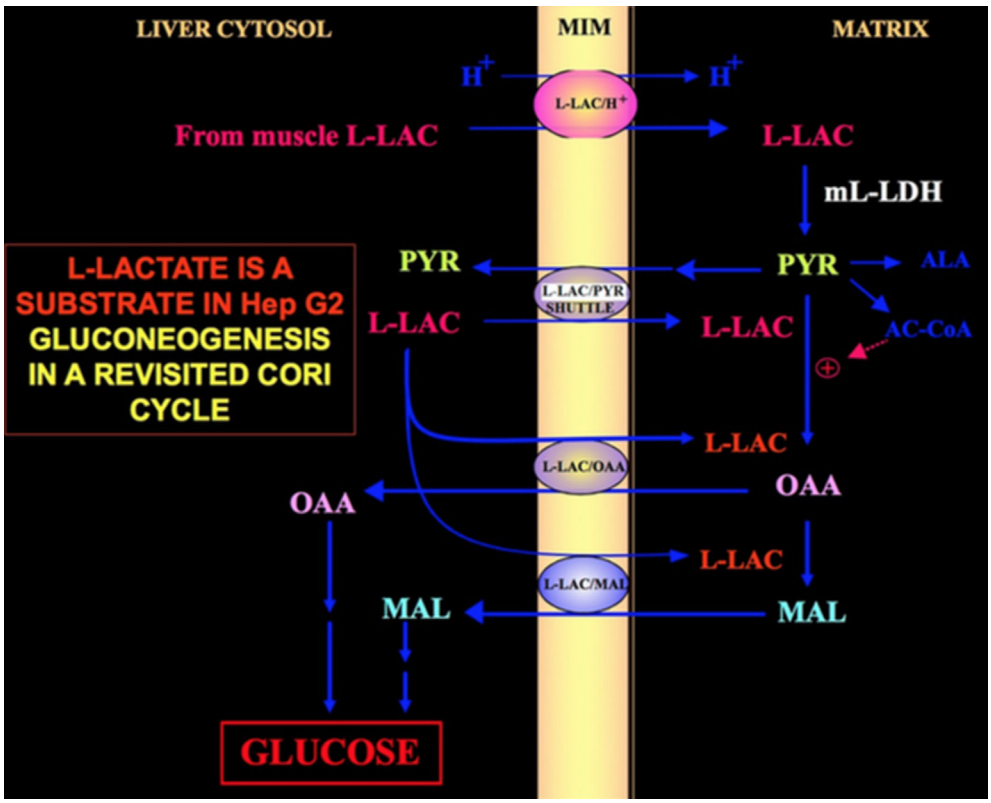

FIGURE 1 | Cori cycle revisited in Hep G2 cells. Given that pyruvate cannot enter Hep G2-M, as shown in Pizzuto et al. (21), L-lactate produced in the muscles reaches the liver *via* the blood stream and from the cytosol enters mitochondria; in the matrix L-lactate metabolism gives rise to pyruvate (PYR) *via* m-L-LDH and then to oxaloacetate (OAA) and malate (MAL) that are exported from the mitochondria to the cytosol via three putative carriers to be used for the l-lactate pyruvate shuttle and for gluconeogenesis to occur via a mechanism similar to that already shown by de Bari et al. (6).

Of special interest is the fact that pyruvate cannot enter Hep G2-M. In fact, contrary to malate + glutamate and L-lactate, externally added pyruvate fails to cause either oxygen consumption or membrane potential generation [see Pizzuto et al. (21) for details]. Notice that an impairment of pyruvate transport in cancer cells has been reported by Paradies et al. (25). Therefore, independently of the theoretical unfeasibility of L-lactate oxidation in the cytosol, as was explained above, the classic Cori cycle cannot occur in Hep G2cells. Therefore, we offer a revised Cori cycle (**Figure 1**), which involves both the mitochondrial carriers that mediate the L-lactate-dependent traffic and the m-L-LDH, which provides pyruvate inside mitochondria. Accordingly, the appearance outside mitochondria of oxaloacetate and malate derived from L-lactate uptake and metabolism *via* m-L-LDH, pyruvate dehydrogenase, pyruvate carboxylase, and malate dehydrogenase and by exchanges, likely due to the L-lactate/oxaloacetate and L-lactate/malate antiporters, confirms an anaplerotic role for L-lactate in gluconeogenesis in which mitochondria play a unique role. Importantly, the addition of L-lactate to Hep G2-M results in the appearance outside mitochondria of citrate, the fatty acid precursor. Accordingly, by using high-resolution mass spectrometry, L-lactate uptake into mitochondria of HeLa and H460 cells was found and proved to result in lipid synthesis; additionally, transmission electron microscopy confirmed that LDH is localized to the mitochondria (26). Surprisingly, the anaplerotic role of L-lactate mitochondrial metabolism has not been considered when cancer metabolism was "reexamined" (27).

We believe that the proposed revision of the Cori cycle, necessary for Hep G2 cells, should also be considered in all other types of cells where mitochondrial metabolism of L-lactate is active. For instance, partial reconstruction of *in vitro* gluconeogenesis arising from mitochondrial L-lactate uptake/metabolism was shown in the absence of LDH outside mitochondria (6).

The role of the mitochondrial L-lactate metabolism merits further focus: given that hydrogen peroxide production in the tumor microenvironment fuels the anabolic growth of cancer cells (28), a possible role of the putative mitochondrial L-lactate oxidase (LOX) which generates hydrogen peroxide in rat liver mitochondria (29) should be investigated; the LOX existence in Hep G2-M appears to be consistent with the evidence that rotenone, which blocks oxygen consumption induced by the addition of malate + glutamate fails to inhibit oxygen consumption induced by the addition of L-lactate.

AUTHOR CONTRIBUTIONS

SP conceived this opinion, shared it and wrote the paper with AS.

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REVIEW
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The Central Role of Amino Acids in Cancer Redox Homeostasis: Vulnerability Points of the Cancer Redox Code

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A fine balance in reactive oxygen species (ROS) production and removal is of utmost importance for homeostasis of all cells and especially in highly proliferating cells that encounter increased ROS production due to enhanced metabolism. Consequently, increased production of these highly reactive molecules requires coupling with increased antioxidant defense production within cells. This coupling is observed in cancer cells that allocate significant energy reserves to maintain their intracellular redox balance. Glutathione (GSH), as a first line of defense, represents the most important, non-enzymatic antioxidant component together with the NADPH/$NADP^+$ couple, which ensures the maintenance of the pool of reduced GSH. In this review, the central role of amino acids (AAs) in the maintenance of redox homeostasis in cancer, through GSH synthesis (cysteine, glutamate, and glycine), and nicotinamide adenine dinucleotide (phosphate) production (serine, and glutamine/glutamate) are illustrated. Special emphasis is placed on the importance of AA transporters known to be upregulated in cancers (such as system x_c-light chain and alanine-serine-cysteine transporter 2) in the maintenance of AA homeostasis, and thus indirectly, the redox homeostasis of cancer cells. The role of the ROS varies (often described as a "two-edged sword") during the processes of carcinogenesis, metastasis, and cancer treatment. Therefore, the context-dependent role of specific AAs in the initiation, progression, and dissemination of cancer, as well as in the redox-dependent sensitivity/resistance of the neoplastic cells to chemotherapy are highlighted.

Keywords: cancer, amino acids, redox homeostasis, glutathione, NADPH/$NADP^+$

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Abbreviations: AA(s), amino acid(s); ALDH1L2, 10-formyl-THF dehydrogenase; AOD, antioxidant defense; ARE, antioxidant response element; ASCT2, alanine-serine-cysteine transporter 2; ATM, ataxia telangiectasia mutated gene; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; ETC, electron transport chain; FOXO, forkhead box O; GCL, glutamate–cysteine ligase; GCN2, general control non-derepressable 2; GLS1/2, cytoplasmic/mitochondrial glutaminase; GLUD1, glutamate dehydrogenase; GOT1/2, aspartate transaminase 1/2; GR, glutathione reductase; GS, glutathione synthetase; GSH, glutathione; GSH-Px, glutathione peroxidase; GSTs, glutathione *S*-transferases; GSSG, glutathione oxidized; [H_2O_2], hydrogen peroxide; [$HO^{\cdot}$], peroxyl radical; [HO^-], hydroxyl anion; KEAP1, Kelch-like ECH-associated protein 1; LAT1, L-type amino acid transporter 1; ME1/2, malic enzyme 1/2; MTHFD, methylene tetrahydrofolate dehydrogenase; mTORC1, mechanistic target of rapamycin complex 1; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); NNT, energy-linked transhydrogenase; [$NO^{\cdot}$], nitric oxide; NOX, NADPH oxidase; NRF2, nuclear factor (erythroid-derived-2)-like 2; [$^1O_2$], singlet oxygen; [$O_2^{\cdot-}$], superoxide anion radical; PDAC, pancreatic ductal adenocarcinoma; PERK, protein kinase RNA-like endoplasmic reticulum kinase; PHGDH, phosphoglycerate dehydrogenase; PPP, pentose phosphate pathway; PRXs, peroxiredoxins; Rb, retinoblastoma; [$ROO^{\cdot}$], peroxyl radicals; [ROOH], organic hydroperoxides; ROS, reactive oxygen species; SHMT2, serine hydroxymethyl transferase 2; SNAT1-2, system A amino acid transporter 1–2; SOD, superoxide dismutase; SSP, serine synthesis pathway; TCA, tricarboxylic acid; THF, tetrahydrofolate; TRXs, thioredoxins; TSC, tuberous sclerosis complex; TSP, transsulfuration pathways; CySSCy, cystine (oxidized cysteine); xCT, system x_c-light chain.

INTRODUCTION

The potential of targeting redox homeostasis for both cancer prevention and development of novel anticancer treatments has been recognized during past decades. However, despite intensive efforts, development of an effective redox-based therapy remains challenging. A main reason for this is cancer cell plasticity but also our inability to adequately perceive the complexity of redox homeostasis. Namely, antioxidant prophylaxis led to the "antioxidant paradox" (1, 2), while use of chemotherapeutics that compromise the oxidative status of cancer cells encountered resistance (3) and the ability of some cancer cells to upregulate antioxidant protective mechanisms (4). Currently, most attention on targeting redox homeostasis focuses on the attack and downregulation of endogenous antioxidant tumor cell defense mechanisms (5). In this review, we approach cancer redox balance from a different perspective with the main players involving amino acids (AAs).

Although the idea of AA dependency of cancer antioxidant defense (AOD) has received more attention recently, a unified review on this subject is lacking. In 2015, Jones and Sies (6) labeled the nicotinamide adenine dinucleotide (NAD, NADP) and thiol/dysulfide [glutathione (GSH)/glutathione oxidized (GSSG) in the first place] systems together with thiol redox proteome as carriers of the cellular "Redox Code." According to this principle, spatiotemporal organization of these systems is fundamental for physiology, while its disruption inevitably leads to pathology. Interestingly, accumulating literature indicates that AA availability and metabolism are upstream and superior to these systems, especially in cancer cells. Our review will address this particular aspect of redox regulation in tumors. However, before considering the involvement of AA homeostasis in cancer redox balance, it is necessary to point out some important findings, as well as delusions, that exist in the complex cancer redox field.

PARTIALLY REDUCED OXYGEN—"ACTIVATED" OXYGEN

The first steps in understanding oxygen toxicity occurred in the mid-twentieth century when Gerschman et al. (7–9) proposed that the damaging effects of oxygen could be attributed to the formation of oxygen radicals. At approximately the same time, research with [$^{18}O_2$] and mass spectrometry showed that oxygen atoms from molecular oxygen [O_2] could be introduced into biomolecules (10, 11). The susceptibility of biomolecules to oxidation gave a biological frame to oxygen toxicity, and together with the discovery of superoxide dismutase [SOD; (12)] fueled research in the field of oxidative damage in biological systems. The term "oxidative stress" was introduced into scientific literature for the first time in 1985 (13).

Now it is clear that the oxidative capacity of molecular oxygen *in vivo* is minimal, but that is not the case for its partially reduced counterparts known as "reactive oxygen species—ROS." ROS is a term widely used to describe a number of reactive molecules and free radicals derived from molecular oxygen. However, we feel obliged to emphasize the generic nature of this term. ROS includes both radical (superoxide anion radical, [$O_2^{\cdot-}$]; hydroxyl radical, [$HO^{\cdot}$]; peroxyl radicals, [$ROO^{\cdot}$]; nitric oxide, [$NO^{\cdot}$]) and non-radical (hydrogen peroxide, [H_2O_2]; hydroxyl anion, [HO^-]; singlet oxygen, [$^1O_2$]; organic hydroperoxides, [ROOH]) species, which differ significantly in terms of half-life, water/lipid solubility and reactivity. For example, the cellular half-life of lipophobic [$HO^{\cdot}$] is only ~10^{-9} s because of its reactivity, compared to ~1 ms for [H_2O_2], which also can diffuse through lipid cellular compartments (14). However, use of the common term ROS is sometimes unavoidable (15) due to the complex nature of biological systems, an inability to exactly measure the species generated in a spatiotemporal manner in addition to the so-called theory of "kindling radicals" by which a few primary ROS "inflame" a cascade of ROS amplification by stimulating the sources of secondary ROS (16).

ROS IN CANCER

The terms "ROS" and "cancer" cover a wide range of molecules and diseases, which makes broad generalizations almost impossible. Is it possible, however, to conceptualize some common denominators of the cancer redox state? Widespread opinion is that virtually all malignant cells are in a pro-oxidative state, mostly due to oncogene-driven altered and/or intensified cell metabolism [reviewed in Ref. (17–21)]. However, Halliwell (20) raised important questions regarding ROS measurement in malignant (and other) cells in classical culture conditions that include 21% oxygen and media that is usually deficient in antioxidants/antioxidant precursors and contains free iron ions. These conditions, *per se*, favor ROS generation, and thus special attention should be paid in extrapolating results obtained *in vitro* to the *in vivo* state. Considering this point in combination with current advances in the cancer redox field, a major conclusion that can be drawn is that cancer cells indeed experience mild oxidative pressure in comparison to normal cells (**Figure 1**) that can help them to exhibit characteristic cancer hallmarks [for detailed review refer to Hornsveld and Dansen (22)].

According to the previous paragraph, it seems that a pro-oxidative state could facilitate initiation and progression of tumorigenesis. However, when reactive and very short living species such as ROS are considered, the situation is not so clear. Accordingly, studies on the effects of antioxidant supplements for cancer prevention and treatment showed opposed and mainly unpromising results, giving rise to confusion and the "antioxidant paradox" (1). Another redox consideration for cancer treatment includes increased ROS levels in cancer cells that already "walk on the edge of oxidative abyss" (23–25). This stand point arises from the very well know concept of hormesis that has been recognized since the XVI century by Paracelsus's—"*Die Dosis macht das Gift*" or "the dose makes the poison" (26). The concept of hormesis, which revolutionized modern toxicology, claims that the dose–response curve is U-shaped, generally meaning that a drug/stimulus can have opposite effects in small and large doses [for more details refer to Calabrese and Baldwin (27) and papers stemming from it]. Indeed, it has been shown that a mild oxidative state promotes all hallmarks of cancer cells; however, if the threshold is exceeded (reaching the top of the arm of the U-shaped curve), influence of the oxidative environment can easily become anti-carcinogenous, promoting cell-cycle arrest, senescence, programmed cell death, or necrosis (**Figure 1**).

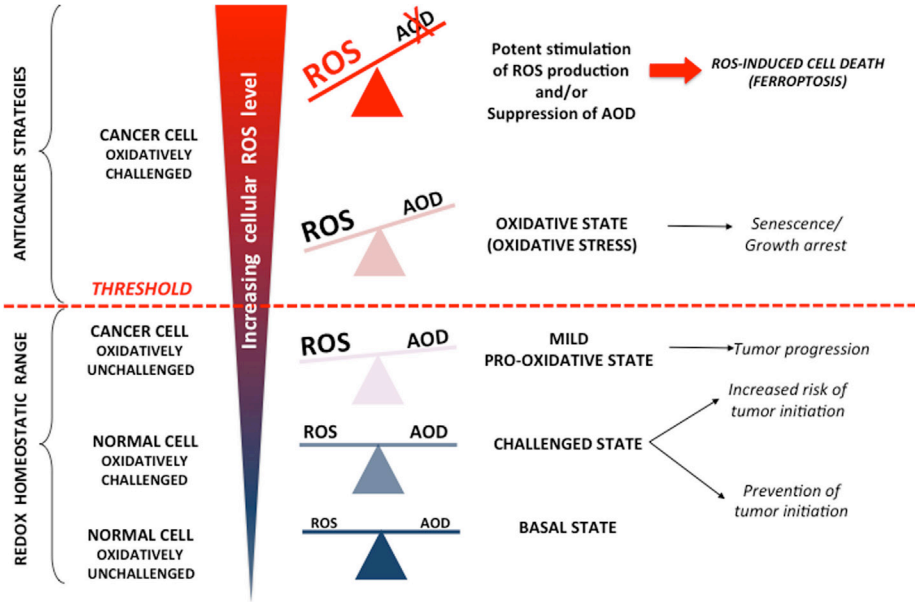

FIGURE 1 | Reactive oxygen species (ROS) can (i) promote cancer, (ii) cause growth arrest, and (iii) be cytotoxic. In normal cells, increased (endogenous or exogenous) oxidative pressure leads to adequate upregulation of cellular antioxidant defense (AOD), which prevent mutagenic events and initiation of cancer formation. However, AOD is not 100% efficient, and thus, these "challenging states" also represent well-known risk factors for cancer development. Once formed, cancer progression seems to be further stimulated by a mild pro-oxidative state due to intensified metabolism, ROS-producing foci, etc. Importantly, this state is still maintained within "redox homeostatic range" thanks to strongly upregulated AOD of cancer cells. However, due to maximized AOD, cancer cells do not support further increase in ROS levels and thus cross the threshold into the state of "oxidative stress." If ROS level increase further (e.g., due to chemotherapy), the only way for cancer cells to prevent further damage is by decreasing ROS production *via* cell-cycle arrest to repair damage and prevent cell death (cytostatic effects of ROS). However, if ROS burst induces irreversible damage and/or there is not enough components required for repair systems (e.g., glutathione), cancer cells experience programmed cell death or necrosis (cytotoxic effects of ROS).

Thus, it has been shown that increased oxidative pressure in the blood, if not adequately balanced by internal AOD, may limit the efficiency of melanoma cells to form distant tumors (28). These results are a textbook example of the antioxidant paradox suggesting how dietary supplementation of antioxidants may promote the metastatic potential of the cancer cells.

The anticancer effects of many conventional therapies, including irradiation and DNA-damaging chemotherapeutics (cisplatin, doxorubicin, gemcitabine, and 5-fluorouracil), rely mostly or partially on increased ROS production, due to mitochondria damage and dysfunction, as well as activation of NADPH oxidase (NOX) enzymes (29–33). However, these treatments encounter resistance with initial response being followed by the development of protective mechanisms against these oxidative/genotoxic insults. The mechanisms of resistance are complex involving drug modification, inhibition, degradation, and/or efflux [for further readings refer to Housman et al. (3)]. In spite of this complexity, the central role that AOD plays in these processes provided the rational for developing anticancer therapies targeting this aspect of cancer redox balance.

AOD IN CANCER

As mentioned previously, oncogenic mutations lead to a pro-oxidative state of cancer cells. However, these cells are still required to maintain ROS levels below the threshold that would become detrimental (**Figure 1**). Indeed, antioxidant pathways known to respond to increased oxidative pressure in normal cells are constitutively activated in some cancers. The best example is the nuclear factor (erythroid-derived-2)-like 2 (NRF2)-signaling pathway [reviewed elsewhere in great detail (34, 35)]. NRF2 is the main transcription factor regulating expression of AOD enzymes. Under normal conditions, NRF2 is constantly ubiquitinated by Kelch-like ECH-associated protein 1 (KEAP1) and degraded by the proteasome. Oxidants/electrophiles inactivate Keap1 and stabilize NRF2, which then translocates into the nucleus, binds to the antioxidant response element, and activates the transcription of many cytoprotective genes that encode detoxifying enzymes and antioxidant proteins. Constitutive activation of NRF2, due to gain-of-function mutations in NRF2 (36), or loss-of-function mutation in its negative regulator KEAP1, was observed in different types of cancers (37–41). In addition, several tumor-suppressor genes act to repress tumor cell proliferation or cause cells to enter permanent cell-cycle arrest in response to ROS overproduction. These include retinoblastoma, $p16^{INK4A}$, JNK, p38, p53, and forkhead box O. Most of these tumor-suppressor proteins sense changes in the cellular oxidative status and respond accordingly by inhibiting the cell cycle, and thus allowing cells time to recover after oxidative stress, and/or to induce expression of AOD enzymes (22).

Antioxidant defense is divided into enzymatic and non-enzymatic parts. Enzymatic AOD includes enzymes such as SODs,

catalases, gluthatione peroxidases (GSH-Px), and glutathione *S*-transferases, as well as redox proteins such as thioredoxins (TRXs), peroxiredoxins, and glutaredoxins. Non-enzymatic AOD components are low-molecular weight compounds such as the key AOD tripeptide glutathione (GSH), vitamins (vitamins C and E), β-carotene, and uric acid. Complementary to these AOD components is the reducing equivalent NADPH that maintains catalases in active forms, serves as a cofactor for TRX and glutathione reductase [which converts oxidized glutathione (GSSG) into its reduced state (GSH)], and acts as a reducing agent for regeneration of glutaredoxins.

The concept of the Redox Code proposed recently by Jones and Sies (6) secludes GSH and NADH/NADPH as main determinants of the dynamic nature of redox signaling and control in multidimensional biological systems. This is even more pronounced in cancer cells due to increased and imbalanced metabolism, mutation accumulation during tumor progression and activated ROS-producing foci (such as defected mitochondria or NOX enzymes). The main reason why GSH and nicotinamide adenine dinucleotide (phosphate) are in the spotlight is the fact that these are the ultimate reducing factors of the cell.

Glutathione

Glutathione, a tripeptide γ-glutamyl-cysteinyl-serine, appears in two forms: the predominant reduced form (GSH), which reaches millimolar concentrations in the cell, and the minor oxidized form (GSSG), which is estimated to be less than 1% of the total GSH (42). The bulk of GSH is found in the cytosol (~90%), while the rest is localized mainly in mitochondria and the endoplasmic reticulum (ER) (43). GSH functions to detoxify electrophilic compounds including xenobiotics, which makes it central to cellular anticancer drug resistance (44). Owing to the sulfhydryl (−SH) group of cysteine, GSH can serve as an electron donor for reduction of peroxides (reactions catalyzed by GSH-Px) or disulfides. GSH can also directly react with various oxidants in a non-enzymatic manner, although these reaction kinetics are generally very slow (45). In addition, GSH is important in its cysteine-storage function (γ-glutamyl cycle).

Similar to ROS, GSH effects can be pro- or antitumorigenic (46). Although it is important in carcinogen detoxification, increased GSH levels and GSH-dependent biotransformation in many tumors may increase resistance to chemotherapy and radiotherapy (47–50). In addition, high GSH levels are associated with cancer hallmarks such as genomic instability, suppression of apoptosis, invasion, and metastatic activity [for further reading refer to Balendiran et al. (46)].

NADPH/NADP$^+$ Couple

Antioxidant defense is completely ineffective without the NADPH/NADP$^+$ cofactor, which serves as a main electron donor for both antioxidant enzymes and catabolic reactions. NADPH supplies reducing equivalents to maintain vital AOD components including the maintenance of active catalase and the regeneration of glutathione, TRX, and glutaredoxin. The NADH/NAD$^+$ system is also involved in reversible 2-electron transfer catalysis and is connected with the NADPH/NADP$^+$ system by activity of mitochondrial energy-linked transhydrogenase (NNT) (51). However, these two nicotinamide nucleotide systems have somewhat different roles in metabolism. Namely, while NADH/NAD$^+$ is involved in catabolism and energy supply, NADPH/NADP$^+$ is central for anabolism, defense, and redox homeostasis [reviewed in Ref. (6)]. The redox potential of these two systems also differs significantly in cells. Namely, the cytosolic redox potential of NADH/NAD$^+$ is more oxidized (−241 mV) (52, 53) while in mitochondria, it operates at a more negative redox potential (−318 mV) (54), providing reductive force for ATP synthesis. Meanwhile, NADPH/NADP$^+$ operates at more negative redox potential than the NAD system both in cytosol (−393 mV) and mitochondria (−415 mV) (53).

The energy-linked mitochondrial enzyme NNT that transfers electrons from NADH to NADPH thus connecting the two systems is of utmost importance in cancers containing mutations in the tricarboxylic acid (TCA) cycle (fumarate hydratase or succinate dehydrogenase) or the electron transport chain (ETC, complex I or III), which have been shown to promote utilization of glutamine by reductive carboxylation (55, 56). Namely, adequate citrate production in these conditions requires high NADPH/NADP$^+$ ratios (57), which are achieved by the activity of the NNT (58).

NADPH production occurs *via* the pentose phosphate pathway (PPP), folate metabolism, and malic enzymes (MEs). The importance of AAs for NADPH-producing pathways, especially in cancer cells, is discussed below.

AAs SENSING FROM A REDOX PERSPECTIVE

Glucose, AAs, and fatty acids are the crucial building blocks of cellular biomolecules. Tight regulatory mechanisms have evolved to maintain the level of each within homeostatic range. The two main protein kinases involved in sensing and regulation of AA homeostasis are the mechanistic target of rapamycin complex 1 (mTORC1) and general control non-derepressable 2 (GCN2) [for an extensive reviews refer to Bar-Peled and Sabatini (59), Efeyan et al. (60), and Broer and Broer (61)]. Briefly, mTORC1 is a major sensor of specific AAs (Leu, Arg, and Lys), which also receives integrated, growth factors, hormonal, environmental and stress signals regulating growth, and proliferation. Although mechanisms of mTORC1 activation have progressed considerably in the past 20 years, the precise effects of individual AAs on mTORC1 activation have remained elusive. Sabatini's group has illuminated AA sensing by demonstrating that mTORC1 translocation to lysosomes, is critical for its activation (59). Interestingly, recent studies revealed that this lysosomal localization allows mTORC1 sensing of AA levels (Arg and Gln), not only in cytoplasm but also in lysosomal compartement *via* the lysosomal membrane-resident transport protein SLC38A9 that constitutes a physical and functional part of the AA-sensing machinery (62, 63). Conversely, GCN2-kinase senses AA-uncharged tRNA, resulting in a general suppression of protein translation, paralleled by induction of the mechanisms to increase the cellular AA pool. Data regarding redox dependency of these pathways are still scarce and mechanically unclear.

Earlier studies showed that UV radiation activates mTORC1 signaling through MAP kinase activation by promoting phosphorylation of its downstream target $p70^{S6k}$ in an $[H_2O_2]$ concentration and time-dependent manner (64, 65). mTORC1 activation was also observed when cells were treated with oxidizing agents, and surprisingly, even in AA-depleted conditions (66, 67). By contrast, subcellular localization of the mTORC1-interacting protein complex tuberous sclerosis complex at the peroxisome is responsible for mTORC1 repression and autophagy induction in response to ROS (68). Also, the tumor-suppressor ataxia telangiectasia mutated gene, appears to regulate autophagy through repression of mTORC1 in response to oxidative stress (69, 70). Thus, it seems that net effects of ROS on mTORC1 activity are context, time, and dose dependent. However, it should be emphasized that although the AAs leucine, arginine, and lysine are identified as key stimuli for mTORC1 activation, recent work on hepatoma HepG2 cells revealed significant sensitivity of both mTORC1 and GCN2 kinases to cysteine depletion (71). Prompt (within 60 min) inhibition of mTORC1 upon cysteine removal was observed. Considering that the Cys proteome coevolved with advanced $[O_2]$ sensing and $[H_2O_2]$ signaling systems (72–74), this effect of cysteine on mTORC1 from a redox perspective may be of higher importance than the effects of ROS, *per se*.

The main downstream target of activated GCN2 is the eukaryotic initiation factor 2α (eIF2α), whose phosphorylation results in a general reduction of translation initiation, while specific mRNAs containing upstream open-reading frames (e.g., ATF4) are actively translated. However, it has been recognized that GCN2 can be activated by a number of different stresses [osmotic, UV, oxidative (such as $[H_2O_2]$), and ER] independently of AA depletion/imbalance (75–77). Interestingly, although the mechanisms are not yet known, it is recognized that the response of GCN2 to stressors such as $[H_2O_2]$ or UV radiation are very fast in comparison to the gradual accumulation of uncharged tRNAs.

In turn, the AA-sensing pathways also influence cellular redox balance. Namely, ATF4, an effector molecule of the GCN2-pathway, also serves as a dimerization partner of the cap "n" collar transcription factor NRF2 (78, 79) promoting resistance to oxidative stress (79, 80). Consistently, it has been shown that mouse fibroblasts lacking *Atf4* depend on supplemental reducing substances, such as glutathione, *N*-acetyl cysteine, or β-mercaptoethanol in their growth media (81). Recent work on HT1080 and A549 tumor cells showed the phosphorylation of eIF2 by protein kinase RNA-like endoplasmic reticulum kinase increases the ability of these cells to cope with increased oxidative pressure in an ATF4-independent manner by activating Akt (82). The importance of the GCN2 kinase in maintaining redox balance was also proved *in vivo*. Mice lacking GCN2 exhibited an increase in protein carbonylation in response to a leucine-imbalanced diet (83).

As for the effect of mTOR on redox homeostasis, a recent study showed that mTORC1 controls ATF4 activity by regulating the translation and stability of its mRNA (84). These results indicate that mTORC1, besides promoting anabolism and consequently increased ROS production, may also contribute to maintenance of the cellular redox equilibrium through "antioxidant properties" of ATF4.

The results listed earlier favor the hypothesis that redox and AA balance are tightly intertwined. How AAs specifically influence the cellular "Redox Code" (GSH and NADPH levels) will be discussed below with special attention placed on the pathways that might represent "vulnerability points" for design of novel anticancer therapeutics.

CYSTEINE LEVELS DETERMINES GSH LEVELS

Two cytosolic ATP-dependent enzymes are involved in GSH synthesis: glutamate–cysteine ligase (GCL), which catalyzes formation of a particular gamma-peptidic bond between Glu and Cys, and glutathione synthetase. The rate-limiting step in GSH synthesis is the reaction catalyzed by GCL (85). Genetic deletion of the GCL catalytic subunit was lethal in the mouse embryo, while knockout mice for the modifier subunit of the enzyme, although viable and fertile, show a significant decrease of tissue GSH levels (9–16% of wt) (86). The K_m of mouse GCL for cysteine is estimated at ~0.2 mM (87), which is near the upper limit of typical cellular cysteine concentrations, while the K_m for glutamate is at or below the cellular glutamate concentration for *Drosophila*, mouse, or human GCLholo enzymes (88–90). Hence, it is not surprising that cysteine is the main regulator of GCL activity, and thus GSH synthesis (**Figure 2**).

In physiological conditions, cysteine is not an essential AA as it can be synthetized through trans-sulfuration pathways (TSP) from methionine, mainly in the liver. Approximately 50% of the cysteine in hepatic GSH is derived from methionine *via* TSP (91). However, high demand for cysteine in cancer cells, make TSP insufficient (**Figure 2**). Furthermore, some tumors have shown significantly lower expression of TSP enzymes mostly due to transcriptional silencing (92, 93). Consequently, Cramer and coworkers (94) showed that depletion of cyst(e)ine with pharmacologically optimized cyst(e)inase enzymes induced cell-cycle arrest and cancer cell death due to GSH depletion and ROS accumulation, both *in vitro* and *in vivo*.

x_c-Transport System

Multiple tissue-specific transporters are responsible for the import of cystine (CySSCy), the oxidized and predominant form of the AA in circulation (40–50 μM), and/or cysteine, which is present at substantially lower concentrations (8–10 μM) (95–97). However, increasing data in the literature points toward the x_c-system as being crucial for CySSCy import in cancer cells (**Figure 2**). The system x_c- acts as a Na^+-independent and Cl^--dependent antiporter of the anionic forms of cystine and glutamate and is composed of the transporter light-chain (xCT, encoded by *SLC7A11* gene) and a chaperone heavy-chain (CD98hc aka 4F2hc, encoded by *SLC3A2* gene) subunit [for a comprehensive review, see Lewerenz et al. (98)]. Interestingly, although the system x_c- seems to be a ubiquitous marker of almost all cells cultured *in vitro*, its *in vivo* distribution in humans appears restricted mainly to the CNS, pancreas, fibroblasts, and immune cells (99–105). According to Bannai et al.

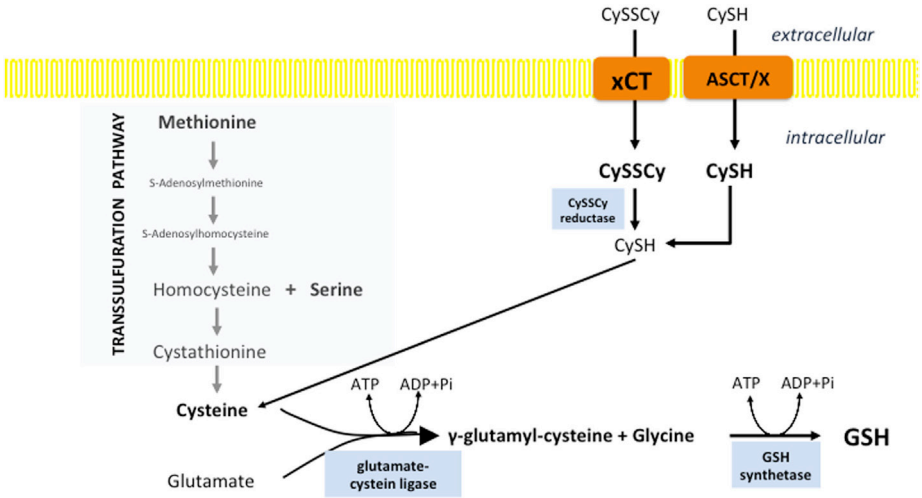

FIGURE 2 | Cystine import is the rate-limiting step in glutathione biosynthesis. Cysteine can be synthesized within the cell through the trans-sulfuration pathway. However, this pathway is often insufficient in cancer cells and therefore cysteine must be imported. Different transporters are involved in the import of the reduced, cysteine (CySH), and oxidized, cystine (CySSCy) form of this semi-essential AA. The heavy-chain transporter subunit of system x_c-light chain (xCT) seems to play a pivotal role in the import of CySSCy, the predominant form of cysteine in circulation. After import, CySSCy is reduced by cystine reductase and used for different purposes including GSH biosynthesis. Import of cysteine can occur *via* ASCT (alanine/serine/cysteine transporter) and other transporters (x).

(106), this induction of the system x_c- in culture conditions is caused by the high partial pressure of oxygen. Consistent with this hypothesis, prolonged cultivation of fibroblasts in reduced oxygen partial pressure caused a significant decrease in the system x_c-activity (106).

Considering that AA transporters are necessary for tumor cell proliferation, it is not surprising that xCT is upregulated in many patient samples and tumor cell lines including hepatoma, lymphoma, glioma, colon, breast, prostate, and pancreatic (95, 101, 107–113). Expression of the xCT subunit seems to be under direct control of oncogenes including NRF2 and Ets-1 (114–116). In addition, the promoter region of the *SLC7A11* gene contains an AA response element, which allows the transcription factor ATF4 to enhance expression of xCT in response to AA depletion and/or oxidative stress (115, 117).

System x_c-light chain mediates import of cystine into cells thus regulating GSH levels (118, 119). Since GSH is the most abundant non-enzymatic antioxidant within the cell, upregulation of xCT satisfies the highly proliferative phenotype of cancer cells. This is supported by complete growth inhibition of lymphoma cells and certain glioma, breast, prostate, lung, and pancreatic cancer cells upon pharmacological inhibition of xCT by sulfasalazine or by the cyclic glutamate analog (109, 111). Besides its role in tumor growth, knockdown or pharmacological inhibition of xCT increased adhesion and inhibited tumor cell invasion *in vitro* and decreased metastases *in vivo* (120). In addition, xCT was shown to associate with CD44v, a major adhesion molecule for the extracellular matrix, which is involved in tumor invasion and metastasis in lethal gastrointestinal tumors (121) along with the metabolic interplay between tumors and host tissue (122). Furthermore, xCT plays a pivotal role in the chemoresistance of tumor cells (123–125), particularly to anticancer drugs that produce high amounts of ROS, such as geldanamycin and celastrol (126, 127).

The importance of the cystine/glutamate antiporter in redox regulation was further implicated in the newly described type of cell death—ferroptosis (128, 129). Ferroptosis is described as an iron-dependent, programmed form of cell death driven by loss of activity of the lipid repair enzyme glutathione peroxidase 4 and subsequent accumulation of membrane lipid peroxides (130). The first described inducer of ferroptosis in Ras-mutated human foreskin fibroblasts was the xCT inhibitor erastin (131). Depletion of intracellular GSH levels due to inhibition of xCT and subsequent increase of ROS levels seems to be sufficient to trigger erastin-dependent cell death. The same results were observed with sulfasalazine, which is another inhibitor of xCT (109, 132). Interestingly, it has been shown that a loss of cysteinyl-tRNA synthetase might prevent erastin-induced cell death by inducing the TSP (133), suggesting that trans-sulfuration can contribute to resistance to inhibition of xCT and ferroptosis induction.

SERINE/FOLATE PATHWAY AND NADPH PRODUCTION

Textbooks have stated for years that the main cellular NADPH-producing system is the PPP. Surprisingly, a recent comprehensive study (134) showed that serine-driven one-carbon metabolism (folate cycle) gives almost the same contribution in the NADPH production as the PPP and MEs in proliferating cells. It is also interesting to note that enzymes of both PPP and the serine synthesis pathway (SSP, from which the folate cycle streams out) are induced by NRF2 (135, 136). The function of the folate cycle is ascribed to the collection of one-carbon units from AAs, and subsequent incorporation of these moieties into biomolecules in biosynthetic or methylation reactions. One of the major

branching points of the folate cycle is 10-formyl-tetrahydrofolate (10-formyl-THF), which in mitochondria may be used for ATP regeneration [methylene tetrahydrofolate dehydrogenase (MTHFD) reaction], formylation of the mitochondrial initiator *N*-formylmethionine-tRNA or metabolized to [CO_2], generating NADPH (10-formyl-THF dehydrogenase reaction). On the other side, in cytosol, 10-formyl-THF can be used for purine or NADPH synthesis, while its counterpart 5,10-methylene-THF is used for thymidylate synthesis and homocysteine remethylation in the methionine cycle. In cancer, mitochondrial 10-formyl-THF is mainly used for NADPH production due to overexpression of corresponding enzyme, while in citosol, this reaction is prevented so one-carbon unit, required for purine synthesis, would not be wasted (137, 138). Default mitochondria-to-cytosol directionality of the folate cycle is achived by different expression of enzymes in these compartments, as well as more reductive, i.e., oxidative environment in cytosol and mitochondria respectively (139).

Two mitochondrial reactions of the folate cycle contribute to NADPH production; one is catalyzed by MTHFD, and the other is catalyzed by 10-formyl-THF dehydrogenase (ALDH1L2) (**Figure 3**). Fan et al. showed that depletion of either of these enzymes decreased NADPH/$NADP^+$ and consequently GSH/GSSG ratios and impaired cellular resistance to imposed oxidative stress (134). Similarly, Piskounova et al. showed that redox balancing effects of these enzymes is fundamental for metastatic potential of melanoma cells *in vivo* (28). Namely, this study showed that knockdown of either MTHFD or ALDH1L2 prevents distant metastasis of melanoma cells that encounter high-oxidative pressure in the blood and visceral organs. Besides, it was reported that the first mitochondrial enzyme of the folate cycle,

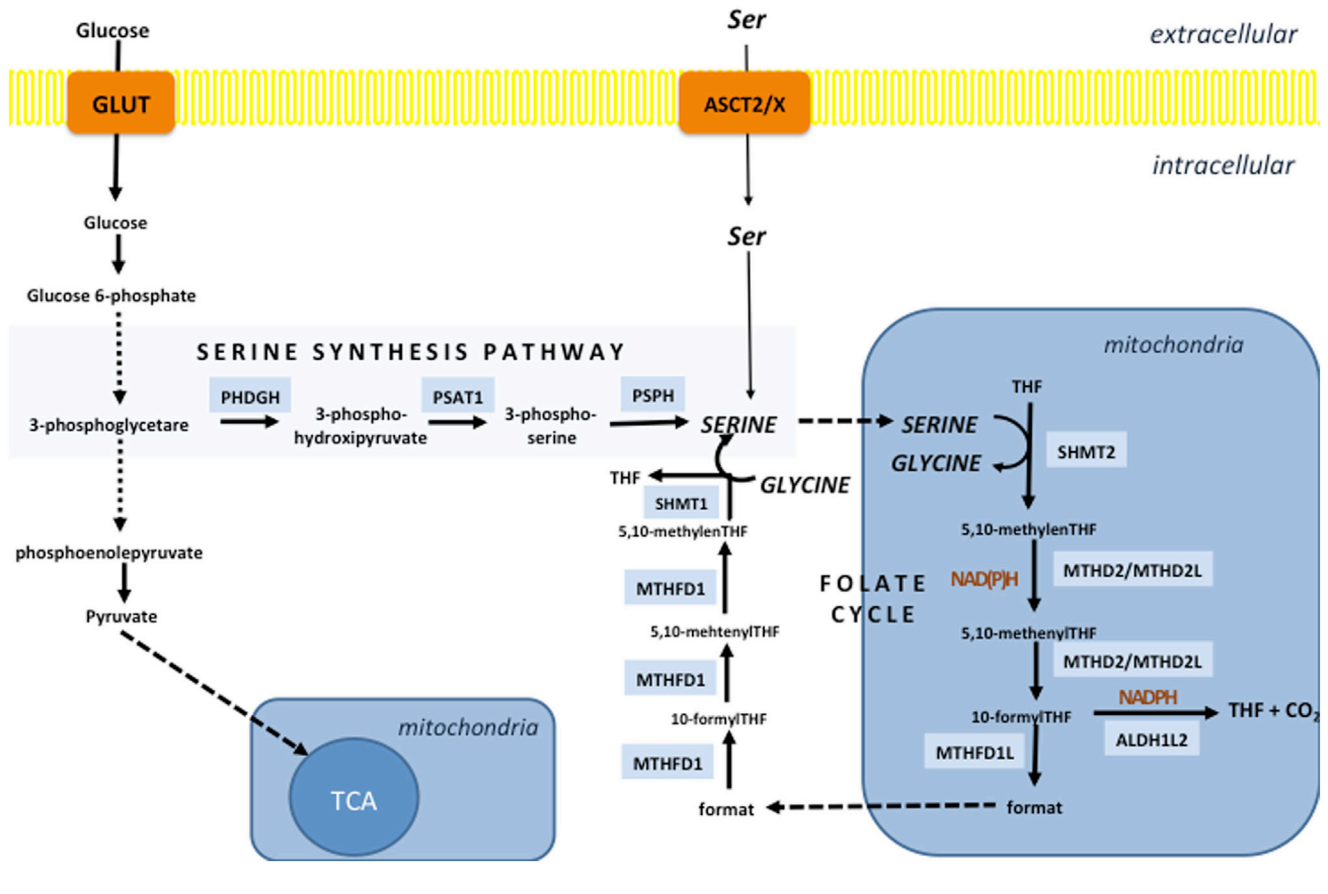

FIGURE 3 | The folate cycle is fueled by the serine synthesis pathway (SSP) and extracellular serine. SSP diverges from glycolysis at the level of 3-phosphoglycerate, which is converted into 3-phospho-hydroxipyruvate by the action of the enzyme phosphoglycerate dehydrogenase (PHDGH) and ultimately to serine following further enzymatic steps. This pathway is of great importance in cancers with mutated or overexpressed PHDGH, while serine import plays a pivotal role in maintenance of the serine cellular balance in cells with unaltered PHDGH activity. The folate cycle in the vast majority of the cells starts in mitochondria by the action of serine hydroxymethyl transferase 2 (SHMT2) which generates glycine and 5,10-methylene-tetrahydrofolate (5,10-methylene-THF). The next reaction can produce NADH or NADPH depending if methenyltetrahydrofolate dehydrogenase 2 (MTHD2) or MTHD2-like (MTHD2L) is used to convert 5,10-methylene-THF into 5,10-methenyl-THF. The same enzyme than generate one-carbon unit—10-formyl-THF, which can be used for ATP production by the enzyme (MTHD1L) or NADPH generation in the reaction catalyzed by 10-formyTHF dehydrogenase (ALDH1L2). If ATP is generated, 10-formylTHF is converted into a format that is transported into the cytosol and used by trifunctional MTHFD1 enzyme to regenerate 10-formylTHF for purine synthesis, 5,10-methylene-THF for thymidylate synthesis and homocysteine remethylation in the methionine cycle. The unidirectionality of the folate cycle seems to be provided by more oxidative mitochondrial redox state that favors use of $NAD(P)^+$ by mitochondrial MTHD2(L).

termed serine hydroxymethyl transferase 2 (SHMT2) is essential for maintaining mitochondrial NADPH and GSH level during hypoxia in neuroblastoma cell lines. This study detected a correlation between high expression of SHMT2 and poor prognosis in neuroblastoma patients (140). Expression of SHMT2 in neuroblastoma cells seems to be controlled by the collaborative action of c-Myc and HIF1α. However, numerous oncogenes are reported to affect enzymes of the folate cycle. For example, it is shown that common KRAS mutation associates with increased expression of MTHFD2 in non-small cell lung cancer cell lines (141), while mTORC1-dependent induction of MTHFD2 is reported in both normal and cancer cells (142).

Besides production of NADPH, the folate cycle contributes to production of GSH by intersecting with the methionine cycle (**Figure 4**). Considering the role of methionine and homocysteine in the TSP (cysteine synthesis), as well as that glycine is product of serine metabolism (folate cycle), it is not surprising that serine depletion results in reduced level of glutathione (143), while activation of serine synthesis is now well identified as a bypass of glycolysis flux contributing to GSH synthesis (136, 144).

Serine, just like cysteine, can be transported into the cell by different transporters [such as the sodium-dependent transport system ASC that will be mentioned later in the text, and transporter system A, as well as sodium-independent system asc (145, 146)], or synthesized *de novo* from glycolytic intermediate 3-phosphoglycerate through the SSP. Highly proliferating cancer cells both in culture conditions and *in vivo* consume significant amount of exogenous serine (143, 147). Consequently, serine depletion both *in vitro* and *in vivo* decreases proliferation and induces metabolic remodeling, commencing with SSP induction, to replenish cellular serine pool (143).

Serine Synthesis Pathway

The importance of serine for cancer physiology came from earlier studies that showed increased flux through the SSP in cancer cells (148). However, this was somewhere neglected until the recent discovery that the first enzyme of SSP, phosphoglycerate dehydrogenase (PHGDH), is genetically amplified in breast cancer and melanoma (149, 150), and overexpression of the SSP components are correlated with poorer prognosis in breast cancer patients (151). Consistently, suppression of PHGDH in cell lines characterized with elevated expression of this enzyme decreases cell proliferation and serine synthesis. What is even more interesting is that in non-tumorigenic breast cancer cells, overexpression of PHGDH alone lead to disruption of the acinar cellular morphology and predisposed them to neoplastic transformation (149, 152), making the PHGDH a *bona fide* oncogene (153).

Amplification of PHGDH de-sensitizes tumors to exogenous serine levels but also represents a vulnerability point for potential cancer treatment. Namely, PHGDH knockdown strongly decreased proliferation and some of the SSP outputs [such as α-ketoglutarate (α-KG)] only in cells with amplified PHGDH expression (150). Interestingly, PHGDH also prevents conversion of glycine to serine suggesting that the folate cycle relies exclusively on serine synthesis in PHGDH overexpressing

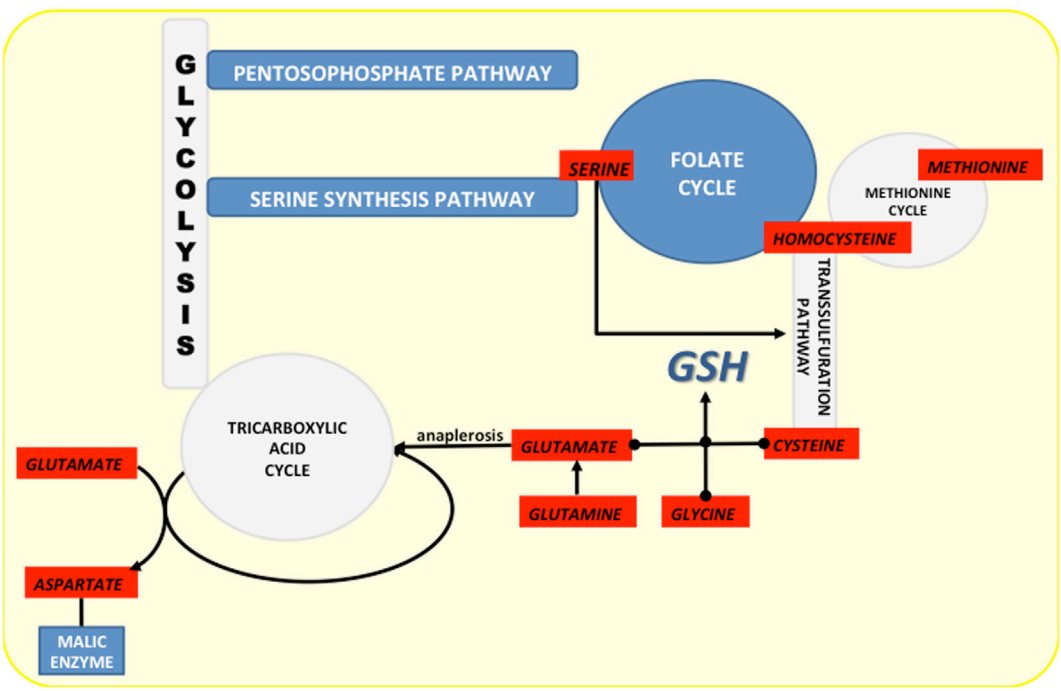

FIGURE 4 | Crossroads of NADPH-producing pathways (marked dark blue) and the pathways from which they diverge or to which they converge (marked light blue). Amino acids involved in these pathways are marked in red.

tumors (154). This was demonstrated by PHGDH knockdown decreasing cell proliferation even when exogenous serine was present (154).

Several other oncogenes also induce expression of the SSP enzymes, such as c-Myc and HER2 (155, 156). Also, in line with its involvement in maintaining redox balance, the SSP enzyme expression is induced by NRF2 in an ATF4-dependent manner in NSCLC cells (136). Interestingly, Maddocks and coworkers (143) showed that serine can be a vulnerable point of cancer metabolism even in tumors that do not have multiplication of the *PHGDH* gene, but lack p53. Namely, they showed that the p53–p21 axis is fundamental for metabolic adaptation upon serine deprivation, while loss of p53 in the conditions of serine depletion leads to impaired glycolysis and elevated ROS levels.

Interestingly, pharmacological inhibition of the SSP could also influence flux through the PPP. Namely, inhibition of the SSP would increase intracellular levels of 3-phosphoglycerate, which has been shown to inhibit 6-phosphogluconate dehydrogenase that catalyzes the second step in the oxidative PPP (157).

GLUTAMATE AND NADPH PRODUCTION

In addition to the PPP and folate cycle, MEs are known to regulate NADPH/NADP$^+$ balance, which is seemingly dependent of glutamine metabolism in cancer. One of the main metabolic characteristics of many cancers, besides the Warburg effect (158, 159), is increased consumption of glutamine to the extent where exogenous level of this AA limit tumor cell survival. This "glutamine addiction" has been recognized for more than 50 years (160, 161); however, diverse contributions of glutamine to intermediary metabolism, cell signaling, and gene expression are still not fully understood (162).

The vast majority of glutamine in the cell is converted into glutamate either by cytoplasmic glutaminase (GLS1) or by the mitochondrial isoform of this enzyme (GLS2). Glutamate is then converted to α-KG by the enzyme glutamate dehydrogenase. α-KG can then have one of two fates (**Figure 5**). (1) Canonically, produced α-KG enters the TCA and replenishes it, or (2) it is carboxylated to isocitrate, pushing the TCA in the opposite direction (163).

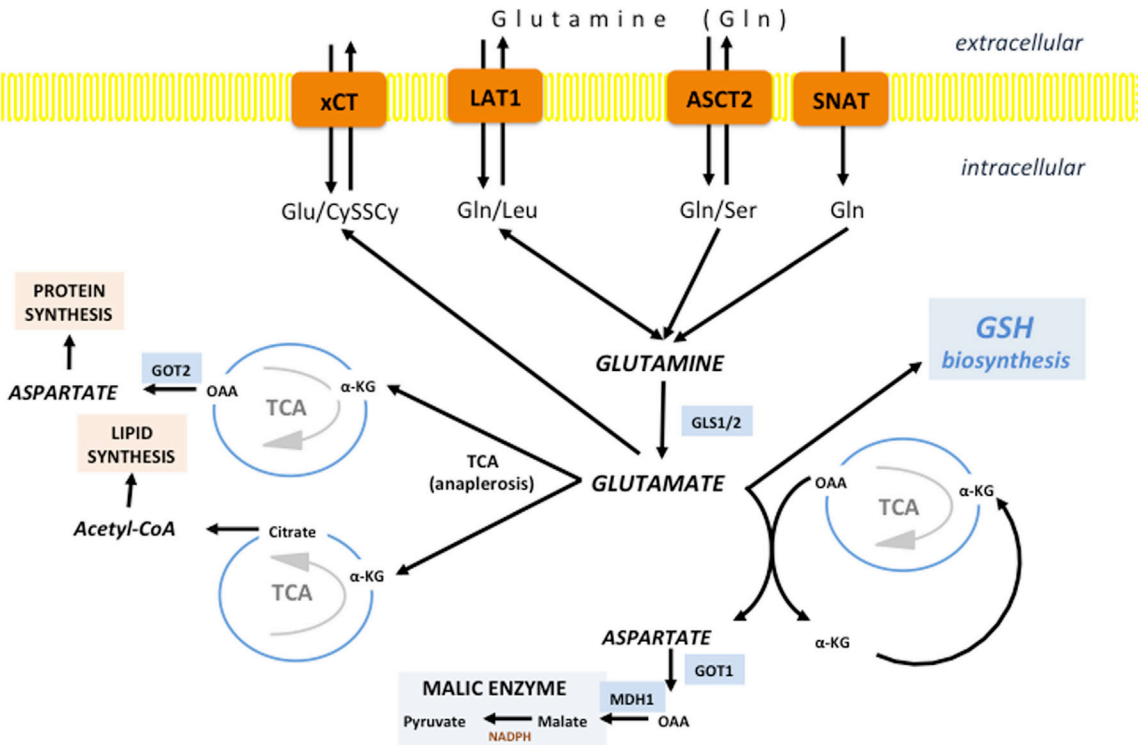

FIGURE 5 | Glutamine/glutamate fates in cancer cells. Different transporters are proposed to fuel the "Glutamine addiction" of cancer cells including alanine-serine-cysteine transporter 2 (ASCT2), SNAT1/2, and L-type amino acid transporter 1 (LAT1). Once inside the cell, Gln can be use for uptake of essential AAs by LAT1. However, the vast majority of Gln is promtly deaminated to glutamate by the action of cytoplasmic or mitochondrial glutaminase (GLS1 and GLS2, respectively). If deaminated in cytosol, Glu is transferred into mitochondria, and there it is further converted into α-ketoglutarate (α-KG) to replenish the tricarboxylic acid (TCA). However, the fate of α-KG can be dual. It can follow normal TCA flow until oxaloacetate (OAA), which is then converted into asparate by aspartate dehydrogenase (GOT2) and translocated into cytoplasm or used for synthesis of asparagine and arginine (protein synthesis). However, if the α-KG is carboxylated to isocitrate and then converted into citrate, citrate is exported into the cytosol where it is used for lipid synthesis in the form of acetyl-CoA. Glutamate-derived aspartate can also be converted into OAA by cytoplasmic GOT1, commonly induced in KRAS-mutated tumors. OAA is then converted first into malate by malate dehydrogenase 1 (MDH1) and then into pyruvate by malic enzyme (ME), generating reducing power in the form of NADPH. Besides involvement in anaplerosis and NADPH production, Glu has an important role as a component of GSH, as well as a substrate for system x_c-light chain (xCT) in allowing entrance of cystine into the cell.

When glutamine-derived α-KG follows the canonical pathway, the TCA works normally (clockwise) until oxaloacetate (OAA), which is usually converted into aspartate by aspartate transaminase (GOT2) and exported into the cytosol, or alternatively, it can be converted into asparagine and arginine and fuel protein synthesis. Interestingly, a recent study on KRAS-mutated pancreatic ductal adenocarcinoma (PDAC) showed that GOT2 regulates glutamine flux by producing α-KG and aspartate from glutamate and OAA (164). Aspartate is then shuttled into the cytosol where it is converted back into OAA by cytoplasmic GOT1. The OAA produced is converted first to malate and then to pyruvate and NADPH by the action of cytoplasmic malic enzyme 1 (ME1). Considering that KRAS-mutated PDACs have decreased flux through the PPP (165), glutamine-fueled ME1 in these cells may be seen as a major contributor to the NADPH homeostasis. Indeed, ME1 suppression increased ROS accumulation and decreased tumor cell growth both *in vitro* and *in vivo*, while suppressing glutamine utilization and sensitizing cells to oxidative damage (164). Conversely, it remains to be determined if inhibitors of glutamine import or its conversion to glutamate would have the same effects on oxidative status and cell growth.

Oppositely to KRAS, p53 has a negative impact on this NADPH-producing pathway. This was demonstrated by a strong upregulation of MEs (ME1/2) in the absence of functional p53 (166), which were crucial for maintenance of adequate NADPH levels. Here is important to recall the importance of the p53–p21 axis to serine starvation (143) and to anticipate potential resistance mechanisms for serine starvation, in the absence of p53, *via* upregulation of the ME1/2.

Alanine-Serine-Cysteine Transporter 2 (ASCT2)

Alanine-serine-cysteine transporter 2 (SLC1A5) is a Na^+-dependent transporter carrying small neutral AAs such as alanine, serine, cysteine, glutamine, and asparagine (K_m ~20 μM) in addition to long-chain AAs such as threonine, valine, and methionine with lower affinity (K_m ~300–500 μM). ASCT2 is proposed to play a central role in sustaining cancer cell glutamine homeostasis based on work from Myc-driven cancers, which are particularly addicted to glutamine, and fuel their "glutamine addiction" by promoting high ASCT2 expression (167–169). Also, ASCT2 together with xCT and L-type amino acid transporter 1 (LAT1), comprise the "minimal set" of transporters required for cancer AA homeostasis and the group known to be highly upregulated in cancer (170, 171). Consequently the glutamine import activity of ASCT2 has been proposed to be fundamental for the activity of other AA transporters upregulated in cancer, such as xCT and LAT1 (leucine-for-glutamine exchanger) (171–173). However, recent findings demonstrated that ASCT2 inhibition can be overcame in certain cancer cell types partly by expressing the Na^+-dependent glutamine transporters system A amino acid transporter 1–2, questioning the functional redundancy for certain AA transporters in tumor growth (174). Regardless, glutamine import (*via* ASCT2 or other transporters) is indeed of great importance for normal functioning of LAT1 and xCT. Recent studies showed that cancer cell glutamine addiction might be a direct consequence of xCT activity, which consumes large amounts of glutamate derived from extracellular glutamine thereby restricting nutrient flexibility of the cell (175, 176).

The importance of glutamine in cancer cells often dominates ASCT2 experimental interpretations. However, it is important to remember ASCT2's ability to transport other AAs such as serine. As mentioned, some cancer cells remain highly dependent on the uptake of exogenous serine (143). Since ASCT2 display a strong affinity for serine, it would be interesting to investigate the role of this transporter in serine metabolism and redox homeostasis in general. Furthermore, the name of ASCT2: alanine–serine–cysteine transporter may be misleading. Namely, ASCT2 is structurally related to the glutamate transporter and neutral AA transporter ASCT1 and when expressed in *Xenopus laevis* oocyte ASCT2 indeed exhibits Na^+-dependent uptake of AA similar to ASCT1 (177). However, the same study of Utsunomiya-Tate and collaborators revealed that ASCT2 exhibits different tissue distribution, as well as substrate selectivity and functional properties when compared to ASCT1. Thus, for example, glutamate uptake by ASCT1 is electrogenic, while in the case of ASCT2 lowering pH enhances uptake, which suggests electroneutral uptake. Also, it seems that cysteine is not a substrate for ASCT2, but an allosteric inhibitor of its activity. In accordance to this are recent findings that mark cysteine as a potent competitive inhibitor of ASCT2 that binds to the site different from the one for substrate and induces efflux of glutamine both in the case of proteoliposomes and in intact cells (178).

Considering that the "minimal set" of transporters required for cancer AA homeostasis comprises ASCT2, while its activity/specificity is still rather debatable, it is of utmost importance to continue research on the biology of this very intriguing AA transporter.

CONCLUDING REMARKS

For a long time, the mild pro-oxidative redox state of cancer cells has been recognized as a vulnerable point of these highly metabolically active cells. However, in the context of chemotherapy, we are still struggling to find the adequate approach to the vast majority of ROS-producing therapeutics that encounter cellular resistance and frequent disease relapse. During the past decade, an approach involving suppression of the internal AOD of cancer has attracted more attention. Within highly complex and intertwined AOD system, GSH and NADPH play the most universal and important role in determining the characteristic redox cellular profile. Considering that AA import and metabolism seems to be upstream of these AOD systems, we have emphasized here the specific molecules and pathways that show great, but still insufficiently examined, potential for anticancer therapy from a redox standpoint. In conclusion, the transport and internal synthesis pathways for cysteine, serine, glutamine, and to some extent glycine appear to be the most interesting targets for the development of novel redox-based therapeutics. Targeting AA transport systems (xCT, ASCT2, and SNAT) is promising considering that import of these semi-essential

AAs are not required in normal cells, while they are absolutely required for cancer cell survival.

AUTHOR CONTRIBUTIONS

MV and JP made substantial contributions to conception and design, revised manuscript critically, and gave final approval of the version to be submitted. YC and SP revised manuscript critically and gave final approval of the version to be submitted.

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Glutamine Transport and Mitochondrial Metabolism in Cancer Cell Growth

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The concept that cancer is a metabolic disease is now well acknowledged: many cancer cell types rely mostly on glucose and some amino acids, especially glutamine for energy supply. These findings were corroborated by overexpression of plasma membrane nutrient transporters, such as the glucose transporters (GLUTs) and some amino acid transporters such as ASCT2, LAT1, and $ATB^{0,+}$, which became promising targets for pharmacological intervention. On the basis of their sodium-dependent transport modes, ASCT2 and $ATB0^{+}$ have the capacity to sustain glutamine need of cancer cells; while LAT1, which is sodium independent will have the role of providing cancer cells with some amino acids with plausible signaling roles. According to the metabolic reprogramming of many types of cancer cells, glucose is mainly catabolized by aerobic glycolysis in tumors, while the fate of Glutamine is completed at mitochondrial level where the enzyme Glutaminase converts Glutamine to Glutamate. Glutamine rewiring in cancer cells is heterogeneous. For example, Glutamate is converted to α-Ketoglutarate giving rise to a truncated form of Krebs cycle. This reprogrammed pathway leads to the production of ATP mainly at substrate level and regeneration of reducing equivalents needed for cells growth, redox balance, and metabolic energy. Few studies on hypothetical mitochondrial transporter for Glutamine are reported and indirect evidences suggested its presence. Pharmacological compounds able to inhibit Glutamine metabolism may represent novel drugs for cancer treatments. Interestingly, well acknowledged targets for drugs are the Glutamine transporters of plasma membrane and the key enzyme Glutaminase.

Keywords: tumors, mitochondria, metabolism, proteoliposome, plasma membrane, drug design

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INTRODUCTION

A conspicuous number of scientific reports clearly show that cancer is a metabolic disease (1–3). Metabolic reprogramming is driven by changes in expression of specific genes that allow cancer cells escaping control mechanisms active in healthy cells. The knowledge of these variations is relevant for designing novel and more specific pharmacological strategies. Therefore, many unknown or controversial aspects of cancer cell metabolism are object of active investigation.

In this respect, mitochondria are crucial for cell survival and their features in cancer vary profoundly in terms of DNA content, electron chain functionality, and ATP production (4, 5). In this complex scenario, Glutamine is a key player since it is a versatile amino acid whose carbon skeleton is employed in different cell compartments for several purposes. Noteworthy, in physiological conditions as well, Glutamine is the most abundant amino acid in plasma, reaching a concentration of 0.8 mM and it can rise up to 40% of the total amino acids intracellular content (6). Glutamine is endogenously synthesized from α-Ketoglutarate, *via* Glutamate dehydrogenase and Glutamine synthetase. However, when cells are highly proliferative, the request of Glutamine increases and it has to be absorbed from external sources (7), making Glutamine a "conditionally essential" nutrient. Hence, some cancer cells are considered "glutamine addicted" because their growth and proliferation rates depended on availability of this amino acid (8, 9). Glutamine is engaged in different pathways, both cytosolic and mitochondrial, responsible for synthesis of many molecules (**Figure 1A**). Glutamine is also involved in other cell processes such as, Glutamine/Glutamate cycle in nervous tissue (**Figure 1A**) (10, 11). Glutamine ends its fate in mitochondria to be oxidized, producing ATP. Some aspects of the Glutamine transport and mitochondrial metabolism, which characterize cancer cells, will be dealt with. Noteworthy, Glutamine has been proposed to activate cell growth also independently from energy metabolism, by acting on signaling processes (11, 12).

GLUTAMINE SUPPLY TO CANCER CELLS

The higher demand of glutamine by some cancer cells requires the action of membrane transporters with two essential features: (i) specificity for Glutamine and (ii) high transport capacity. Membrane transporters for amino acids are characterized by a broad specificity. In other words, the same transporter is able to recognize different amino acids with a redundancy that is typical of this class of proteins (13). In particular, Glutamine is recognized as substrate by some of the members of four different SLC families, which are clustered on the basis of phylogenetic analyses: SLC1, SLC6, SLC7, and SLC38 (14). Each transporter can be indicated by either the SLC or the old nomenclature (**Figure 1**). Even though the genetic and biochemical characterization of Glutamine transporters began several years ago, many unclear aspects are still existing especially in the frame of concerted action and regulation of the transporters and to their importance in Glutamine homeostasis under physiological (**Figure 1A**) and pathological conditions (13, 14). A remark is, however, very clear: some of the transporters sharing specificity for Glutamine are overexpressed in many tumors, i.e., ASCT2, ATB^{0+}, and LAT1 (**Table 1**) (15–17); notwithstanding, not all of them are suitable for providing cells with high amount of this amino acid since they do not fulfill both the features above mentioned. A concise summary of the major players of Glutamine homeostasis is reported below together with an update on the most likely transport mechanisms underlying their role in cancer.

SLC1A5 is referred to as ASCT2, acronym standing for Alanine, Serine, Cysteine Transporter according to preliminary observations on substrate specificity (13). Recently, we showed that the actual preferred substrate is Glutamine and that Cysteine is not a substrate but, probably, a modulator of transport activity, in agreement with the previous reports describing a very low transport of Cysteine, if any (49, 50). The specificity of ASCT2 toward Glutamine correlates well with its overexpression in several human cancers (16, 51); to better explain its role in Glutamine addiction, many authors depicted ASCT2 as a Na^+-dependent symporter of Glutamine, thus apparently fulfilling the two constraints above listed, i.e., specificity and high transport capacity (52–55). However, the proposed mechanistic model does not correlate with the actual transport mode of ASCT2 that is a Na^+-dependent antiporter, according to both initial and more recent studies, including ours, which well clarify this aspect (16, 49, 56, 57) (**Figure 1A**). Therefore, at variance with the common view, the uptake of Glutamine, required by cancer cells, must be coupled to an opposite and quantitatively equal efflux of another neutral amino acid. Under a metabolic point of view, it is reasonable that the most probable exchanged amino acids are Asparagine, Threonine, or Serine; these, indeed, are high affinity substrates of ASCT2 (56) and the antiport with Glutamine will allow the net entry of 1–2 carbon atoms into the cell, which can be oxidized in the TCA to produce ATP (**Figure 1B**). This reaction is energetically favored by extracellular sodium gradient and membrane potential; the transporter is electrogenic due to net positive charge accumulation, as we recently highlighted (56). This "amino acid exchange" mechanism correlates well with the increased plasma concentration of Serine and Threonine, widely described in different cancers (58). Over the years, overexpression of ASCT2 has been associated also to another transporter of neutral amino acids, SLC7A5 referred to as LAT1 (59), as originally proposed by Fuchs and Bode (16). This protein is a Na^+-independent obligatory antiporter and it has an heterodimeric structure, being associated to an ancillary protein named CD98 (SLC3A2) which, however, does not play any role in the intrinsic transport function (**Figure 1A**) (60). LAT1/CD98 heterodimer is broadly expressed and provides cells with essential amino acids, such as Leucine, in those body districts where these are required for cell growth. Indeed, strong genetic alterations of LAT1 in embryo are not compatible with life and very few are found in families characterized by some cases of Autism Spectrum Disorders, in which the metabolic damage is ascribed to altered supply/excessive loss of essential amino acids, in particular Histidine, to/from brain (61). LAT1 is greatly overexpressed in tumors where it has a role in signaling function (**Table 1**) (16, 51). Leucine, indeed, modulates the activity of one of the master cell growth regulators: mTOR (62). This protein kinase senses amino acid availability and it is particularly responsive to Leucine, Glutamine, and Arginine levels across lysosomes (62). In this respect, it is worth to note that LAT1, besides in plasma membranes, has also been found in lysosomes together with the "transceptor" SLC38A9 (63–65). Moreover, Leucine is a positive allosteric regulator of Glutamate dehydrogenase, which is responsible of Glutamine fate in mitochondria (17). For all the stated reasons, both LAT1 and ASCT2 can be considered eminent targets for drugs (51). However, the commonly proposed model in which Glutamine is taken up *via*

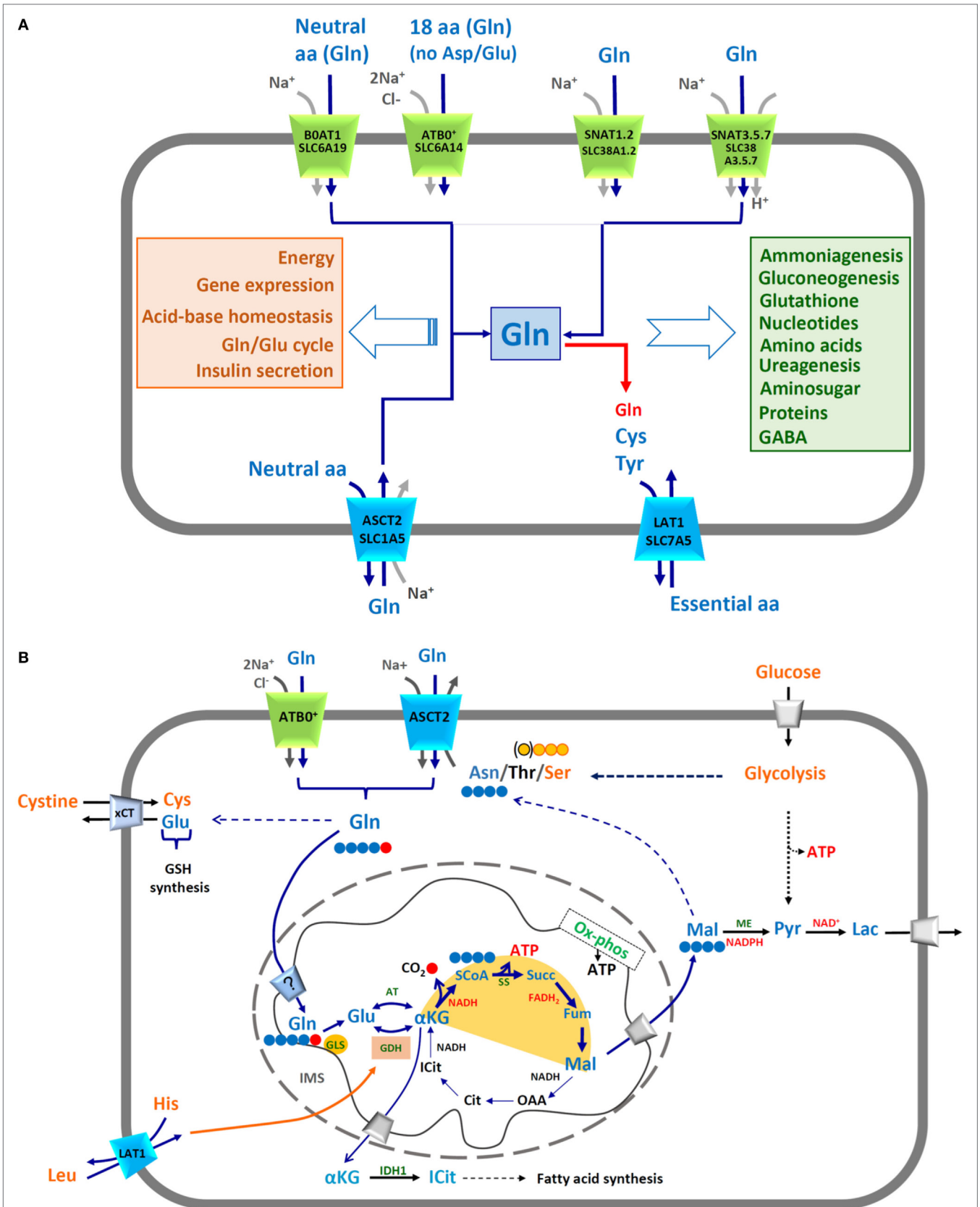

FIGURE 1 | Continued

FIGURE 1 | Continued
(A) Membrane transporters of glutamine and mechanisms of transport. The shape of the transporters reflects their asymmetry in membrane. Transporters are indicated by both conventional and SLC names. Different colors highlight different transport modes: in green symporters, in blue antiporters. Arrows represent direction of transported amino acids (blue) and ion (grey) fluxes; red arrow indicates possible Glutamine exit *via* LAT1 (SLC7A5). In the orange box, the list of cell pathways in which Glutamine is involved; in the light green box, the list of molecules synthesized from Glutamine. **(B)** Mitochondrial and cytosolic pathways responsible for energy production from Glutamine. In the scheme, Glutamine (Gln, blue) uptake occurs *via* membrane transporters $ATB^{0,+}$ and ASCT2 through a sodium coupled process. The pathways are indicated as solid or dotted (in the case of multistep pathways) arrows (in blue those related to Glutamine, in black those involved in other pathways). Carbon atoms of Gln are depicted in blue–red filled circles; Gln enters mitochondria *via* an inner membrane transporter whose existence is still questionable (?): it could be a Glutamine or a Glutamate transporter depending on the actual sub-localization of Glutaminase enzyme (GLS). Carbon atom derived from Gln and released as CO_2 is indicated in red, carbon skeleton of Malate and Asparagine (Asn) in blue, carbon skeletons of Serine (Ser) in orange circled in red and of Threonine (Thr) in orange circled in black. The truncated form of TCA is highlighted by a yellow hemicycle. ATP and reducing equivalent molecules produced by Glutamine metabolism are indicated in red. Leucine enters through LAT1 and allosterically regulates GDH in the orange box. Some metabolic pathways are indicated by names: GSH synthesis, fatty acid synthesis, Glycolysis, OX-phos. Membrane transporters of lactate and glucose in grey, xCT in light blue. Enzymes highlighted: GLS, Glutaminase; GDH, Glutamate dehydrogenase; AT, aminotransferases; SS, succinylCoA synthetase; ME, malic enzyme; IDH1, isocitrate dehydrogenase. Amino acids and other molecules involved in glutamine pathways (azure): Glu, Glutamate; α-KG, α-ketoglutarate; ICit, isocitrate; SCoA, succinyl coenzyme A; Succ, succinate; Fum, fumarate; Mal, malate; OAA, oxaloacetate; Cit, citrate; Pyr, pyruvate; Lac, lactate.

TABLE 1 | $ATB^{0,+}$, ASCT2, and LAT1-associated cancers.

SLC6A14 ($ATB^{0,+}$)	SLC1A5 (ASCT2)	SLC7A5 (LAT1)	Reference
Prostate cancer	Prostate cancer	Prostate cancer	(14, 18–22)
Colorectal cancer	Colorectal cancer	Colorectal cancer	(14, 23)
	Hepato cell carcinoma	Hepato cell carcinoma	(14)
	Lung cancer	Lung cancer	(14, 24)
Breast cancer	Breast cancer	Breast cancer	(14, 18, 25–28)
	Neuroblastoma and glioma	Neuroblastoma and glioma	(14, 29)
	Endometrioid carcinoma	Endometrioid carcinoma	(14, 30, 31)
	Ovarian cancer	Ovarian cancer	(14, 32)
	Renal cell carcinoma	Renal cell carcinoma	(14, 33, 34)
Pancreatic and biliary tract cancer		Pancreatic and biliary tract cancer	(14, 35, 36)
	Gastric cancer	Gastric cancer	(14, 37–40)
		Pleural mesothelioma	(14)
Cervical cancer	Cervical cancer		(41, 42)
	Oral squamous cell carcinoma	Oral squamous cell carcinoma	(43–45)
		Thymic cancer	(46)
		Melanoma	(47)
		Leukemia	(48)

List of cancer tissues in which $ATB^{0,+}$, ASCT2, and/or LAT1 have been found overexpressed with related references.

ASCT2 to boost the transport cycle of LAT1, for massive entry of Leucine, is questionable. Indeed, as above described, ASCT2 is not a symporter, but an antiporter, and Glutamine is a poor substrate of LAT1 (60) (**Figure 1**). Thus, it is necessary to reconsider an integrated view of metabolism, which takes into account other membrane transporters. In particular, two members of SLC6 family are characterized by both specificity for Glutamine and high transport capacity and are involved in supplying it to cells in physiological and pathological conditions (**Figure 1**): SLC6A14 and SLC6A19 known as $ATB^{0,+}$ and B^0AT1, respectively (66). In the case of $ATB^{0,+}$, Glutamine uptake has been proposed to be coupled with $2Na^+$ and $1Cl^-$ while, in the case of B^0AT1, it is coupled to Na^+ (**Figure 1A**). The transport cycle of the two proteins is electrogenic making $ATB^{0,+}$ and B^0AT1 high capacity transporters. Despite this, no involvement in cancer is reported for B^0AT1, so far. Altered expression of this protein is described only in an inherited disease referred to as Hartnup disorder (67). On the contrary, a number of studies shows overexpression of $ATB^{0,+}$ in human cancers (25, 51) (**Table 1**). Therefore, this protein can be considered one of the players in accomplishing metabolic needs of cancer cells and, hence, a druggable target (**Figure 1B**). However, at this stage, a plausible unified model, including ASCT2, LAT1, and $ATB^{0,+}$ cannot be predicted because the study on biology of the last one is still in embryonic form. The only available information concerns its broad specificity and localization (66). Another family characterized by a sizable number of Glutamine transporters is the SLC38, which accounts for 11 members, the best known of which are described as Glutamine transporters coupled to Na^+ or Na^+/H^+ fluxes (68) (**Figure 1A**). Wide proteomic/genomic data indicate that some of the SLC38 members are overexpressed in human cancers (69). Further studies are required to establish a direct role of these transporters in Glutamine supply and, hence, their possible consideration as drug targets. Noteworthy, an important advancement has been recently provided in the field of cell signaling linked to amino acid sensing with the discovery that SLC38A9 is a lysosomal transporter responsible for Glutamine and Arginine flux across lysosome with consequent activation of mTOR cascade (64, 65).

GLUTAMINE METABOLISM IN MITOCHONDRIA AND THE STILL UNSOLVED TRANSPORT ISSUE

The relevance of Glutamine for energy production underlies a truncated form of TCA characterizing the mitochondrial metabolism of several type of cancers. In this pathway, the cycle is not completed and the carbon skeleton of Glutamine, entering the TCA as α-Ketoglutarate, escapes as Malate with production of ATP at substrate level in the reaction catalyzed by the Succinyl-CoA Synthetase. According to this pathway, one out of the five carbon atoms of Glutamine, is released as CO_2 (**Figure 1B**). The four remaining carbon atoms of Glutamine are exported in cytosol as Malate that can give rise to different metabolic pathways. It can be converted into Pyruvate leading to NADPH production that can be used by fatty acid synthesis or other biosynthetic pathways (70). Pyruvate can, in turn, be transformed to Lactate, restoring NAD^+ needed for anaerobic glycolysis and production of ATP (**Figure 1B**). This typical anaerobic pathway occurs even in the presence of adequate oxygen supply, according to the well-acknowledged Warburg hypothesis (16, 71, 72). Alternatively, Malate can enter four carbon atom molecules among which Asparagine, i.e., one of the substrates necessary for ASCT2 transport cycle (**Figure 1B**). In this case, Malate is converted into oxaloacetate *via* malate dehydrogenase and then, to aspartate *via* aspartate aminotransferase (resumed by the dotted arrow of **Figure 1B**). The alternative efflux substrate of ASCT2, Serine can derive from glucose *via* a three enzymes pathway, i.e., phosphoglycerate dehydrogenase, phosphoserine aminotransferase, and phosphoserine phosphatase (resumed by the dotted arrow of **Figure 1B**). Noteworthy, the reaction catalyzed by the second enzyme (aminotransferase) requires Glutamate, which in turn derives from Glutamine. On the other hand, Threonine, which could be an efflux substrate of ASCT2 as well, is an essential amino acid; thus, it should derive from import through other transporters or, hypothetically, from protein degradation. Moreover, Glutamine skeleton can also fuel fatty acid synthesis in cytosol by reductive carboxylation of α-Ketoglutarate, exported from mitochondria, to isocitrate through the action of a cytosolic isoform of IDH (**Figure 1B**). This is a non-conventional reaction for producing citrate, occurring in cells that undergo metabolic switch (70, 73, 74). Glutamine is involved also in ROS metabolism, which is another crucial point for cancer development and progression (75). Cancer cells, indeed, need to keep the production of ROS under strict control *via* mechanisms involving both enhanced glutathione (Glutamate-Glycine-Cysteine—GSH) synthesis and decreased respiratory chain activity. Glutamate needed for GSH synthesis derives, under these conditions, from Glutamine (**Figure 1B**) (76). Cysteine is taken up by cells *via* the Glutamate/Cystine transporter xCT (SLC7A11), which has been found overexpressed in several cancers and is responsible for a novel way of cell death called ferroptosis (77). Thus, Glutamine withdrawal can have dramatic effects on cancer cell metabolism (75, 78). Despite the described importance of Glutamine in mitochondrial metabolism, the network of proteins involved in its flux to mitochondrial matrix is still underneath. Several efforts have been made to shed light on two mitochondrial molecular entities, which are still mysterious: the enzyme Glutaminase and the mitochondrial transporter for Glutamine (**Figure 1B**). Glutaminase is produced by two different genes: GLS1 and GLS2. The first one is known as kidney-type Glutaminase and is ubiquitously expressed. The GLS2 gene is known as liver-type glutaminase (LGA) and is mainly expressed in liver. The GLS1 type is subjected to alternative splicing producing a full isoform and a truncated one, which differs for its C-ter region and is known as Glutaminase C (79). These two isoforms have been found overexpressed in different cancers, in line with the increased metabolic demand of mitochondrial Glutamine (80). The importance of this enzyme in the fate of Glutamine is testified by a number of different pathways involved in its regulation among which, c-Myc, whose action is exerted through inhibition of a microRNA, miRNA-23a that results in increased GLS1 expression and, then, activity (81). Under a pharmacological point of view, Glutaminase represents an important target for anticancer therapy (82). However, the sub-localization of mitochondrial Glutaminase is not yet defined and, as a consequence, the need of a mitochondrial Glutamine transporter. In fact, if Glutaminase faces the intermembrane space, here, releases Glutamate then, a Glutamate transporter, not a Glutamine one, is required to allow entry of Glutamate in the TCA. On the contrary, if Glutaminase faces the intra-mitochondrial matrix, then a Glutamine transporter is necessary to allow Glutamine reaching the substrate active site of Glutaminase (**Figure 1B**). Biochemical data, even though indirect, agree with the second hypothesis and, hence, with the existence of a Glutamine transporter (**Figure 1B**) whose molecular identity is not yet revealed (82–86). We have conducted *in silico* analyses aligning a putative Glutamine binding motif with members of the mitochondrial transporter SLC25 family: the best score was obtained for three orphan SLC25 members resulting as possible mitochondrial Glutamine transporters (11).

GLUTAMINE METABOLISM AS TARGET FOR DRUGS

The complex network of enzymes/transporters involved in Glutamine metabolism explains the plethora of drug interventions to specifically target cancer cells. A big challenge is the metabolic adaptation of cancer cells that can survive also under stress conditions, such as Glutamine withdrawal (87, 88). Last, but not less important, is the great diversity of cancers; thus, it is not surprising that therapeutic interventions needs to be specifically designed. Being Glutamine a key player in multiple pathways, the most important makers of its fate represent potential crossroad for cancer therapy. In particular, inhibitors of the key enzyme Glutaminase have been designed over the years (7, 82) and their studies are at a more advanced stage, being Glutaminase a soluble protein, i.e., easier to handle also *in vitro*. Interestingly, murine Glutaminase 3D structure has been obtained (pdb 4JKT) and, very recently, the human one has been deposited in the database (pdb 5UQE), as well. Some inhibitors showed very good results in *in vitro* models of human cancers and few of them were promising in preclinical studies. In particular, one synthetic compound, i.e., CD-839 reached clinical trials due to its ability to block tumor growth *in vitro*, *in vivo*, and in mouse models

(89). The main challenges with respect to Glutaminase inhibitors are the presence of more than one isoform of GLS and the still unsolved issue of subcellular localization that can hamper the drug availability. The scenario around membrane transporters is even more complex. In fact, their relevance in pharmacology is obvious and relies on two main aspects: membrane proteins can be (i) target of designed drugs and/or (ii) responsible for drug traffic across membranes and, thus, for drug disposition. This second aspect is still not fully considered by the scientific community that did not include any transporter for amino acids in the list of the International Transporter Consortium for drug–transporter interactions (90). The frontiers of drug design are based on *in silico* models that, on the one hand, reduce the number of experimental analysis to be conducted; on the other hand, if the 3D model of the protein is obtained by homology, predictions may be uncertain. This circumstance, in the case of membrane transporter, occurs quite often because few 3D structures are available so far. The well-documented overexpression of some membrane transporters, above described (see Glutamine Supply to Cancer Cells; **Table 1**), boosted the research of potent and specific inhibitors; in particular, several reports dealt with the identification of inhibitors for ASCT2 (91) and LAT1 (92) *via* bioinformatics. The initial approach, attempted over the years, has been that of designing substrate analogs-based drugs to block either ASCT2 or LAT1 transport activities (93, 94). However, all the discovered molecules exhibited relatively low affinities and, hence, low effects on reducing cancer cell viability. The pitfalls of this strategy are explained by the frame schematically depicted in **Figure 1A**; in fact, membrane transporters of amino acids are poly-specific meaning that natural substrates can displace a hypothetical substrate-based drug. These compounds, in fact, interact only transiently with the target protein leading to scarce effects. In the recent years, we have exploited a combined approach of bioinformatics, *in silico* screening and biochemical assays using the *in vitro* experimental model of proteoliposomes in order to identify covalent inhibitors for both ASCT2 and LAT1. Being irreversible, covalent inhibitors should be in principle, more efficient in chemically knocking-out the transporters. This strategy has the advantage of facilitating the compound screening studying the effects on the sole target protein, without interferences deriving from other systems present in the whole cells (95). Then, we identified potent covalent inhibitors of the rat ASCT2 (96). Soon after, we obtained also a set of covalent inhibitors of human LAT1 with the highest affinity so far described (97). LAT1, as mentioned above, even if is probably not directly linked to Glutamine uptake in cancer cells, is responsible for providing essential amino acids, among which Leucine (see Glutamine Supply to Cancer Cells). Test in intact cells showed that the compounds were also able to impair viability of cancer cells.

AUTHOR CONTRIBUTIONS

MS and CI wrote the manuscript and designed the figures. MG, LP, and LC contributed to revision of the manuscript, figures, and bibliography.

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MINI REVIEW
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Mitochondrial Dysfunction: A Novel Potential Driver of Epithelial-to-Mesenchymal Transition in Cancer

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Epithelial-to-mesenchymal transition (EMT) allows epithelial cancer cells to assume mesenchymal features, endowing them with enhanced motility and invasiveness, thus enabling cancer dissemination and metastatic spread. The induction of EMT is orchestrated by EMT-inducing transcription factors that switch on the expression of "mesenchymal" genes and switch off the expression of "epithelial" genes. Mitochondrial dysfunction is a hallmark of cancer and has been associated with progression to a metastatic and drug-resistant phenotype. The mechanistic link between metastasis and mitochondrial dysfunction is gradually emerging. The discovery that mitochondrial dysfunction owing to deregulated mitophagy, depletion of the mitochondrial genome (mitochondrial DNA) or mutations in Krebs' cycle enzymes, such as succinate dehydrogenase, fumarate hydratase, and isocitrate dehydrogenase, activate the EMT gene signature has provided evidence that mitochondrial dysfunction and EMT are interconnected. In this review, we provide an overview of the current knowledge on the role of different types of mitochondrial dysfunction in inducing EMT in cancer cells. We place emphasis on recent advances in the identification of signaling components in the mito-nuclear communication network initiated by dysfunctional mitochondria that promote cellular remodeling and EMT activation in cancer cells.

Keywords: epithelial-to-mesenchymal transition, mitochondrial dysfunction, mitochondrial DNA, mitochondrial retrograde signaling, metastasis

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INTRODUCTION

Mitochondria are the cell powerhouse, on which amino acid, nucleic acid, lipid, and iron–sulfur cluster metabolic pathways converge. During the last decade, mitochondria have been recognized as key players in several aspects of cancer biology, including cancer development, metastasis, and drug resistance (1, 2), due to their central role as receivers, integrators, and transmitters of intracellular signals regulating various processes (3). Mitochondria are highly dynamic organelles whose biogenesis and functions, depending on cellular needs, is under tight nuclear control, through the so-called anterograde regulation, which allows mitochondria adaptation to the ever-changing cellular milieu (4). Only 1% of mitochondrial proteins are encoded by mitochondrial DNA (mtDNA), with all the others encoded by the nuclear genome, including proteins involved in mtDNA replication and transcription, such as mitochondrial single-stranded DNA-binding protein (mtSSB or SSBP1),

transcription factor A of mitochondria (TFAM), and mitochondrial DNA polymerase γ (POLG) (5). When cells require enhanced mitochondrial function, anterograde transcriptional regulation of mitochondrial biogenesis is mediated by a set of transcription factors whose activity is regulated by the PPARγ co-activator 1 family members (4).

Epithelial-to-mesenchymal transition (EMT) is a complex transdifferentiation process that allows epithelial cancer cells to transiently acquire a predominantly mesenchymal phenotype (6, 7). EMT is characterized by loss of epithelial cell polarity and cell–cell/cell–extracellular matrix contacts, supported by concomitant changes in stromal cells, that enable some tumor cells to migrate out of the primary tumor, cross the basement membrane barriers, and intravasate into the blood stream (8, 9) (**Figure 1A**). These circulating tumor cells (CTCs) become sources of metastasis at distant sites as the "seeds" in Paget's "seed and soil" theory (10). EMT requires a complex cellular reprogramming that may render the cells resistant to therapies designed against the primary tumor (11, 12) and has been connected with cancer cell stemness properties (6, 13, 14).

The mutual interplay between EMT and mitochondrial metabolism in cancer has been recently highlighted (15–17). In this relationship, mitochondrial metabolic alterations can drive EMT or, else, EMT activation can fine-tune cancer cell metabolism by affecting the expression of metabolic genes. Mitochondrial dysfunction has been widely implicated in cancer development and progression [for a recent review, see Ref. (2)]. The precise mechanisms underlying mitochondrial dysfunction are multiple and may involve deregulated autophagic processes, unbalance in reactive oxygen species (ROS) homeostasis, mutations in oxidative phosphorylation (OXPHOS) complexes, electron transport chain (ETC), or Krebs' cycle (TCA) enzymes. Despite the heterogeneity of the mechanisms, EMT induction has been described as one of the endpoint phenotypes in many epithelial tumor cells affected by mitochondrial dysfunction. In this review, we describe how dysregulation of the mitochondrial metabolism and genetics may promote EMT in cancer cells.

EMT IN CANCER

Epithelial-to-mesenchymal transition has been initially described as a physiological process occurring at different stages of the embryonic development (type I EMT) (18). Type II EMT occurs in wound healing and fibrosis (18). Type III EMT is associated with cancer progression (18) and is the focus of this review.

Epithelial-to-mesenchymal transition is a multistep process that involves several molecular changes, including down-regulation of the epithelial markers E-cadherin, claudins, desmosomes, and occludins (key components of intercellular

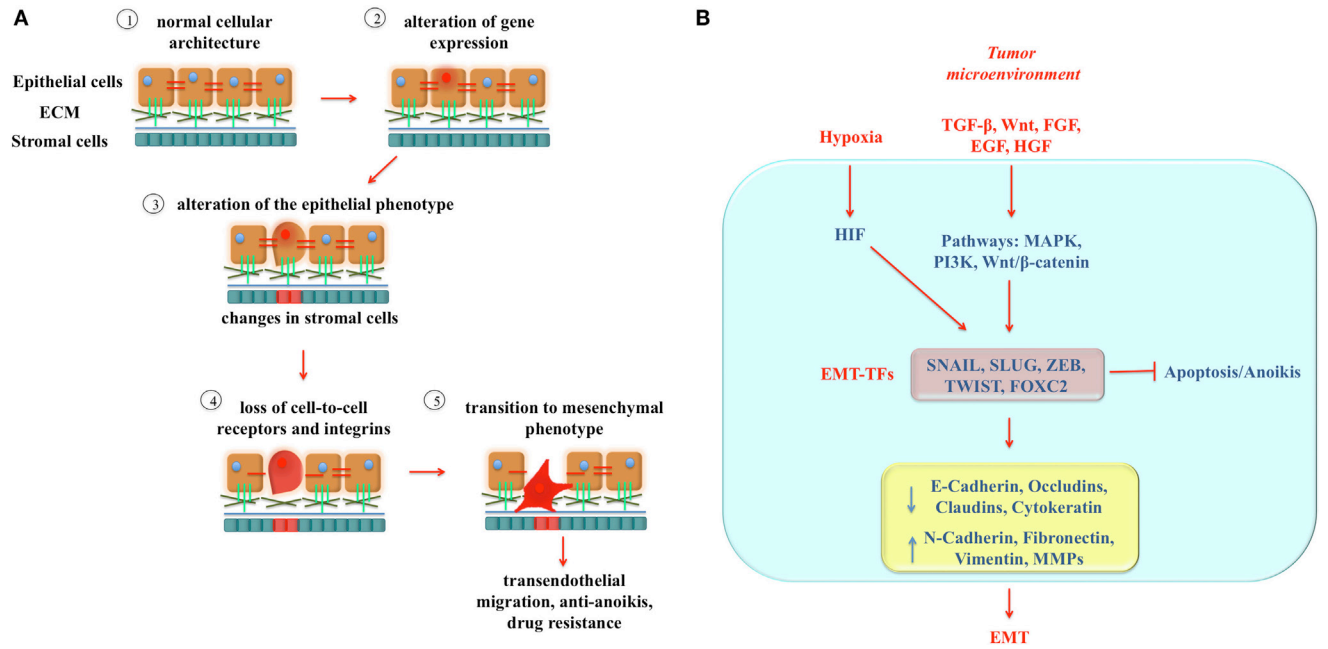

FIGURE 1 | The mechanism of epithelial-to-mesenchymal transition (EMT). **(A)** Cellular changes associated with EMT. Epithelial tumor cells are shown in light brown, and stromal cells are shown in cyan. EMT begins with alterations in gene expression of epithelial cancer cells (step 2) that determine loss of the epithelial phenotype accompanied by alterations in nearby stromal cells (shown as a shift of stromal cell color from blue to red) (step 3). Loss of cell-to-cell attachment receptors and integrins occurs and continues to step 4 and beyond. EMT allows the cells to increase their invasiveness determining degradation of extracellular matrix (ECM) proteins, cytoskeleton reconstruction, extravasation, angiogenesis, as well as anoikis and drug resistance (step 5). **(B)** The regulatory network of EMT. Some important extracellular molecules in the tumor microenvironment, such as TGF-β, HGF, FGF, EGF, and Wnt bind to their respective receptors to induce activation of intracellular pathway, such as MAPK, PI3K, and Wnt/β-catenin. In turn, they regulate induction of EMT-inducing transcription factors (EMT-TFs), including SNAIL, SLUG, ZEB, TWIST, and FOXC2, which are responsible for molecular and physical changes occurring during EMT. Also hypoxia contributes to trigger EMT and participates in the EMT regulatory network through activation of HIFs.

junctions) as well as upregulation of the mesenchymal markers N-cadherin, vimentin, and fibronectin, thus fostering motility and invasion (19) (**Figure 1B**). These changes are orchestrated by transcription factors known as EMT-inducing transcription factors (EMT-TFs), which include TWIST1 and TWIST2, SNAIL 1, SNAIL 2 (SLUG), ZEB1, and ZEB2 as well as non-canonical EMT-TFs such as KLF8, FOXC2, and GSC. EMT-TFs regulate directly or indirectly the expression of adhesive factors and can also induce the expression of matrix metalloproteinases (MMPs), which degrade the basement membrane facilitating invasion and intravasation. Some extracellular factors, such as Wnt, TGF-β, EGF, FGF, and HGF can drive EMT by activating different signaling pathways (MAPK, Wnt/β-catenin, and PI3K) thus promoting the expression of EMT-TFs (20). In addition, tumor hypoxia is considered one of the possible triggers of EMT by inducing hypoxia-inducible transcription factors, e.g., HIF-1α and HIF-2α, which regulate the hypoxic response by modulating the expression of EMT-TFs (21, 22) (**Figure 1B**).

The pro-metastatic role of EMT-TFs has been extensively demonstrated [for a review, see Ref. (23)]. For example, using genetic mouse models of breast cancer, Tran et al. (24) demonstrated that transient expression of SNAIL 1 in breast tumors was sufficient to increase metastasis. Ectopic expression of TWIST1 in Twist1-negative breast cancer cells also induces EMT and cancer stem cell-like features, including expression of the stem-cell marker CD44 (13, 25–27), suggesting that EMT and acquisition of stemness capacity may be part of the same pathway. Besides promoting migration, invasion and cancer stem-cell properties, EMT would also facilitate survival of CTCs in the peripheral system by inhibiting anoikis as well as apoptosis triggered by chemotherapy or radiotherapy (28, 29). Of note, EMT induction is also regulated by changes in the expression of splicing factors (30): suppression of epithelial-specific splicing proteins (ESPR) is an indicator of the EMT process (31). In addition, identification of epigenetic changes and microRNAs as potent EMT regulators adds further complexity to the regulatory network governing EMT (32, 33).

MITOCHONDRIAL DYSFUNCTION AND EMT

Mitochondrial dysfunction has been associated with increased invasiveness, metastatic potential, and drug resistance of cancer cells (2, 34–37). The mechanisms contributing to mitochondrial dysfunction may be multiple and may occur at the level of mtDNA- or nuclear-encoded mitochondrial proteins. In the next paragraphs, we will summarize current knowledge on factors promoting mitochondrial dysfunction that has been implicated in EMT induction in cancer cells.

Mutations/Changes in Expression of Nuclear-Encoded Mitochondrial Metabolic Enzymes

Mutations in the TCA cycle enzymes fumarate hydratase (FH), isocitrate dehydrogenase (IDH), and succinate dehydrogenase (SDH) have long been recognized as oncogenic but only recently, they have been associated with EMT activation.

Fumarate hydratase mutations suppress conversion of fumarate to malate and cause hereditary leiomyomatosis and highly aggressive renal cell cancer able to metastasize at an early stage even when the primary tumor is still very small (38). Accumulation of fumarate in FH-deficient cells would promote EMT through an epigenetic mechanism: fumarate suppresses the antimetastatic miRNA cluster mir-200ba429 by inhibiting demethylation of a regulatory region, thus resulting in expression of EMT-TFs (39). This novel mechanism provides a rationale to explain the aggressive nature of FH-mutated tumors.

Isocitrate dehydrogenase promotes oxidative decarboxylation of isocitrate to α-ketoglutarate. Mutations in IDH1/2 isoforms are common in oligodendrogliomas and astrocytomas and have been also found in leukemia, melanomas, prostate, colon, and lung cancers (40). Mutant IDHs are neomorphic and catalyze the transformation of α-ketoglutarate to 2-hydroxyglutarate, an oncometabolite that has been shown to induce EMT and to be associated with the presence of distant metastasis in colorectal cancer (41). The oncometabolite 2-hydroxyglutarate, an inhibitor of Jumonji-family histone demethylase, would induce EMT by increasing the trimethylation of H3K4 in the promoter of the *ZEB1* gene, thus increasing the expression of ZEB1, a master regulator of EMT (41).

Succinate dehydrogenase is another TCA cycle enzyme involved in EMT. It catalyzes the conversion of succinate to fumarate and loss-of-function SDH mutations predispose to hereditary pheochromocytoma, paraganglioma, gastrointestinal stromal tumor, and renal cell carcinoma (42). In metastatic pheochromocytomas and paragangliomas, mutations in the SDHB subunit are associated with activation of SNAIL and SLUG as a result of epigenetic remodeling due to hypermethylation of promoter CpG islands (43, 44). Focal deletions of SDHB have been also identified in serous ovarian (45) and colorectal (46) cancer and have been shown to promote EMT through an epigenetic mechanism.

Finally, a combined RNAseq and metabolomics profiling of different solid cancers has shown that downregulation of mitochondrial proteins, particularly those involved in OXPHOS, correlates with poor clinical prognosis across different cancer types and is associated with an EMT gene signature (47). Consistently, loss of OXPHOS genes was observed in metastatic cancer cell lines and in metastatic melanoma and renal cancer specimens. OXPHOS was downregulated in about 60% of low-survival patients, with subunits of Complex I and IV of the ETC being the most affected. In cancers exhibiting OXPHOS downregulation, EMT was the most upregulated cellular program, suggesting a causal role of mitochondrial dysfunction in EMT induction, and, consequently, in cancer aggressiveness and poor outcome.

mtDNA Modifications

Mutations in mtDNA-encoded proteins also contribute to mitochondrial dysfunction by directly affecting the ETC/OXPHOS system. Until a few years ago, mtDNA was believed to be very susceptible to damage because of absence of DNA

repair systems. Nowadays, it is widely accepted that both yeast and mammalian mitochondria are equipped with almost all known nuclear DNA repair pathways, including base excision repair, mismatch repair, single-strand break repair, and possibly non-homologous end joining and homologous recombination [for details, see Ref. (48, 49)]. Despite the presence of DNA repair systems, the mtDNA mutation rate is considerably higher than nuclear DNA, due also to the close proximity of mtDNA to ROS-generating sites. Accumulation of mtDNA mutations has been detected in several cancer types and has been associated with metastatic progression and/or chemoresistance (2, 50–52). In 2008, Ishikawa et al. (53) demonstrated that the mtDNA mutation G13997A in the NADH dehydrogenase (ND) subunit 6 gene promotes metastasis through an ROS-dependent mechanism. Other mtDNA mutations, such as C12084T and A13966G affecting ND4 and ND5, respectively, confer a metastatic phenotype to breast cancer cells but in an ROS-independent manner (54). Another mtDNA mutation affecting ND3 (A10398G) has been detected selectively in bone metastasis of 7/10 prostate cancer patients, suggesting that the A10398G mtDNA mutation may confer a selective advantage to prostate cancer cells to colonize the bone metastatic sites (55). Frequent mtDNA mutations in Complex I genes have been detected in both benign and malignant oncocytic thyroid tumors (56, 57). Intriguingly, oncocytic thyroid carcinomas, also known as Hurthle cell carcinomas, are more aggressive than non-oncocytic thyroid cancers (58, 59), suggesting a potential role of mtDNA mutations in acquisition of the aggressive phenotype. However, despite several evidences showing a link between certain mtDNA point mutations and metastasis, it remains to be investigated whether the mechanism involves EMT activation.

Besides single mtDNA mutations, reduction in mtDNA copy number has been reported in several cancer types and has been associated with metabolic reprogramming, increased metastatic potential, chemoresistance, and EMT activation. Different mechanisms have been proposed to explain reduction of mtDNA in cancer cells. Guo et al. (60) reported frequent truncating mutations in the mitochondrial transcription factor TFAM in colorectal cancer cells, which induced mtDNA depletion and apoptosis resistance. A recent study has shown that methylation of the mitochondrial polymerase POLG may also regulate the mtDNA copy number in cancer cells (61). Besides methylation, POLG mutations have been associated with mtDNA depletion in breast cancer tissues (62). Expression changes in other nuclear genes have been reported to affect mtDNA content and induce EMT: for instance, reduced β-catenin levels in basal ErbB2-positive breast cancer cells promote an EMT program through reduction of the mtDNA content, correlated with downregulation of mitochondrial biogenesis transcription factors TFAM and PGC-1α (63). A recent study performed on 207 primary breast tumor specimens shows a direct correlation between low mtDNA content and presence of distant metastasis: patients with ≤350 mtDNA molecules per cell showed a poorer 10-year distant metastasis-free survival compared with patients with> 350 mtDNA molecules per cell (64), suggesting that low mtDNA content might be a prognostic marker for distant metastasis in breast cancer. Reduced mtDNA content has been associated with aggressive features also in other cancer types, including prostate (35, 65, 66) and colorectal (60) cancers, and it has been directly correlated with induction of EMT through activation of mitochondria-to-nucleus signaling (retrograde signaling; **Figure 2**).

Mitophagy

Autophagy is the master mechanism of cell homeostasis through which destruction of unnecessary or dysfunctional molecules and organelles occur (67, 68). Withdrawal of nutrients and various stress conditions, such as alterations in glucose metabolism (69, 70), mitochondrial dysfunction, and oxidative stress (71, 72), induce autophagy with the aim of removing damaged macromolecules and organelles and/or to digest cell components to help the cell's own maintenance (73–76). Being a homeostatic process, autophagy may have a double and opposite role in cancer, behaving as both tumor-promoter and tumor-suppressor depending on cancer cell type and tumorigenic context (77, 78). Cancer cells may indeed activate autophagy to overcome microenvironmental (nutrient deprivation, cell detachment, and hypoxia) or therapeutic (radiotherapy and chemotherapy) stress, thus promoting cancer progression (79, 80).

Mitophagy is a selective form of autophagy that specifically removes dysfunctional mitochondria from the cells. Besides traditional autophagy-related (ATG) proteins, such as LC3 (ATG8) and Beclin1 (ATG6), mitophagy relies upon specific proteins, including the E3 ubiquitin ligase Parkin (PARK2) and mitochondrially targeted PTEN-induced kinase-1 (81, 82). In yeast cells, Atg32, an outer mitochondrial membrane protein, is essential for mitophagy (83–86). Recently, Bcl2-L-13 has been identified as the mammalian homolog of Atg32: it induces mitophagy in Parkin-deficient cells (87), but its role in cancer remains to be investigated. Impaired Parkin activity in mammals has been correlated with cancer progression, suggesting that mitophagy may represent a tumor suppression mechanism (82). On the other hand, Whelan et al. (88) have recently reported that mitophagy supports EMT-mediated conversion of low CD44- to high CD44-expressing keratinocytes through modulation of oxidative stress and Parkin-dependent mitochondrial clearance. In this model, mitophagy was associated with mtDNA depletion, an event known to induce EMT and high-CD44 cell generation in mammary epithelial cells (89). It remains to be established if mitophagy drives EMT-mediated high-CD44 cell generation or is a permissive factor during this process. An independent recent study confirmed a positive role of mitophagy during EMT: Marín-Hernández et al. (90) reported that simultaneous exposure of cancer cells to hypoxia and hypoglycemia results in EMT activation and increased invasiveness, accompanied by activation of mitophagy and impaired mitochondrial functionality.

Taken together, these studies indicate a possible dichotomous nature of the relationship between EMT and mitophagy, which may be ascribed to cell type- and context-dependent factors, but much remains to be investigated.

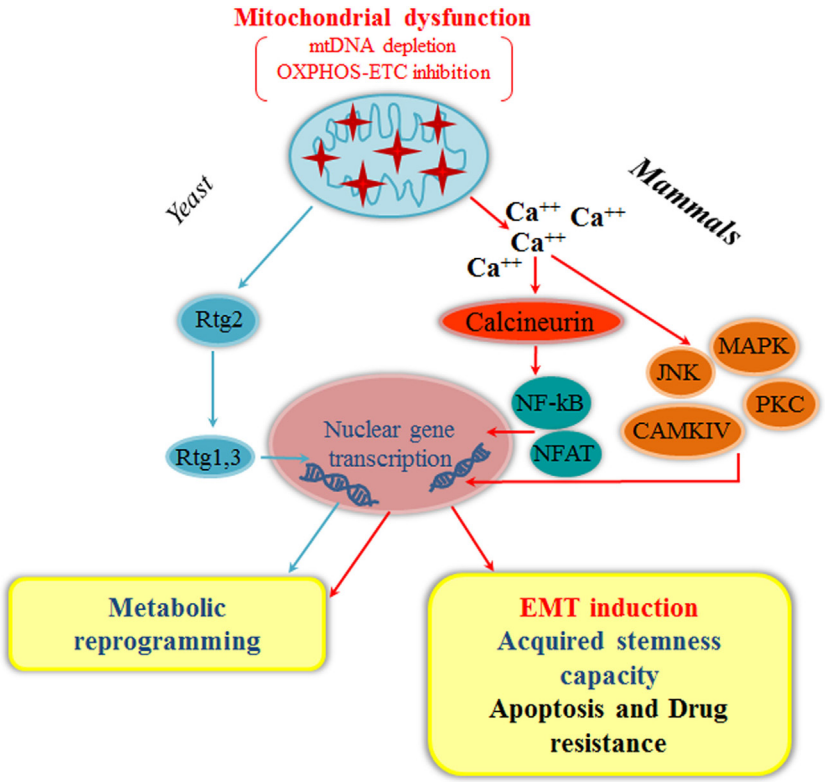

FIGURE 2 | Mitochondrial retrograde signaling and epithelial-to-mesenchymal transition (EMT). Mitochondrial dysfunction, such as mitochondrial DNA (mtDNA) depletion or oxidative phosphorylation (OXPHOS) inhibition, triggers mitochondrial retrograde signaling, which is evolutionary conserved from yeast to mammals. In yeast, Rtg2 regulates the Rtg1,3 translocation into the nucleus eliciting a metabolic reprogramming through the upregulation of specific genes involved in anaplerotic reactions (cyan arrows). In mammals, deregulation in calcium homeostasis due to mitochondrial stress [mtDNA depletion, OXPHOS/electron transport chain (ETC) inhibition] can activate a Ca^{++}-dependent retrograde signaling that converges on two possible branches: one mediated by calcineurin for the nuclear translocation of NF-κB or NFAT, and the other directly dependent on activation of Ca^{++}-dependent protein kinases, such as PKC, JNK, MAPK, and CAMKIV. These pathways culminate with the activation of different transcription factors that lead to metabolic reprogramming, EMT induction, acquired stemness capacity, apoptosis resistance, and drug resistance (red arrows). Alternative RTG signaling pathways in yeast, *Caenorhabditis elegans*, and mammals are discussed in the text.

MITOCHONDRIAL RETROGRADE SIGNALING AND EMT

Dysfunctional mitochondria can generate a wide range of retrograde responses, i.e., intracellular signals relayed from mitochondria to the nucleus, leading to changes in the expression of nuclear genes for metabolic adjustments and cytoprotection (91–93). The first mitochondrial retrograde signaling was discovered by Butow (94) in yeast *Saccharomyces cerevisiae*. The main positive regulators of mitochondria-to-nucleus in yeast are three retrograde response (RTG) genes: *RTG1* and *RTG3*, encoding for a heterodimeric transcription factor activating RTG target gene expression (95). *RTG2*, coding for a cytoplasmic protein with an N-terminal ATP-binding domain, acts as a sensor of the mitochondrial dysfunction and regulates Rtg1/3p localization (96). RTG genes dynamically interact with other regulators and signaling pathways to elicit a metabolic reprogramming through activation of anaplerotic reactions, supplying intermediates in response to respiratory defects initiated by mtDNA depletion/mutations or disruption of ETC/OXPHOS (97) (**Figure 2**). Interestingly, *AUP1* encoding for a conserved mitochondrial protein phosphatase required for mitophagy in yeast has been shown to induce the *RTG3*-dependent retrograde signaling pathway (98), suggesting a possible interplay between mitophagy and mitochondrial retrograde signaling.

Another mitochondrial retrograde pathway, induced by mitochondrial proteotoxic stress, was discovered in mammalian cells by the pioneering work of Hoogenraad (99), but its detailed regulation has recently been elucidated in *Caenorhabditis elegans* (100). Disturbance of mitochondrial protein homeostasis and/or an increase in unassembled components initiates an retrograde response named mitochondrial unfolded-protein response (UPR^{mt}). The current paradigm suggests that peptides resulting from proteolytic degradation of improperly folded mitochondrial proteins are released from mitochondria. However, mitochondrial import efficiency is reduced during mitochondrial dysfunction, causing ATFS-1, a pivotal transcription factor of the UPR^{mt}, to accumulate in the cytosol and subsequently be imported into the nucleus. ATFS-1 in the nucleus regulates a transcriptional response to recover mitochondrial function including induction

of mitochondrial proteases and chaperones, ROS detoxifying genes, and metabolic regulators leading to metabolic reprogramming (93, 100). The transcription factor ATF5 was recently identified as the mammalian ortholog of ATFS-1 (101). While a body of literature is already present on the function of ATF5 in cancer biology, notably in the regulation of survival and apoptosis (102, 103), it will be interesting to explore the role of ATF5 in the context of UPR^{mt} and cancer, particularly in EMT regulation and metastasis.

The mitochondrial retrograde signaling is conserved in mammals both in response to energy metabolism impairment and to proteotoxic stress (93, 104). Of the multiple retrograde signaling pathways activated in mammals by mitochondrial dysfunction (91, 105), Ca^{++}/calcineurin-mediated retrograde signaling has been involved in EMT activation (105) (**Figure 2**). Ca^{++} homeostasis strictly depends on mitochondria and its deregulation due to different mitochondrial stresses, such as mtDNA depletion or ETC/OXPHOS inhibition, can elicit an increase in cytosolic Ca^{++} that activates a Ca^{++}-dependent retrograde signaling. Depending on cell type and conditions, there are essentially two branches in this pathway: (i) a Ca^{++}-calcineurin-mediated retrograde signaling, through the nuclear translocations of transcription factors, NF-κB, NFAT, CREB, and HnRNPA2; (ii) a direct activation of Ca^{++}-dependent protein kinases, such as PKC, JNK, MAPK, and CAMKIV (94, 104). Activation of these signaling pathways in epithelial cells converge on the upregulation of genes affecting several cellular functions, including apoptosis resistance, multidrug resistance, invasion, and EMT (66, 89, 106). Mitochondrial dysfunction induced by mtDNA depletion promotes EMT in breast epithelial cells through a calcineurin A-mediated mitochondrial retrograde signaling that triggers transcriptional activation of SLUG, SNAIL, and TWIST, the MMP-9 metalloproteinase, and the mesenchymal markers fibronectin, vimentin, and N-cadherin, with a corresponding decrease in the epithelial marker E-cadherin. In addition, mtDNA-depleted breast cells exhibited loss of the ESPR such as ESPR1, indicative of their mesenchymal phenotype, and expressed stem-cell markers, suggesting generation of cancer stem cells (13) (**Figure 2**). Of note, mtDNA-depleted cells exhibit also unorganized trajectory and higher mitochondrial fission, characteristic of cells with high metastatic ability (105). The potential link between mitochondrial dysfunction and EMT was also reported in prostate and breast adenocarcinoma cell lines depleted of mtDNA, which acquired a mesenchymal phenotype and showed TGF-β overexpression (107). More recently, mtDNA depletion was shown to induce EMT in hepatocellular carcinoma cells through TGF-β/SMAD/SNAIL signaling (108). In addition, suppression of SSBP1 promoted triple-negative breast cancer cell metastasis through mtDNA depletion, which triggered calcineurin A-mediated mitochondrial retrograde signaling resulting in c-Rel/p50 translocation to the nucleus, increased levels of TGF-β and TGF-β-driven EMT (109).

CONCLUDING REMARKS

Epithelial-to-mesenchymal transition endows cancer cells with the ability to detach from the primary tumor bulk and survive during invasion, dissemination, and metastasis. The observation that mitochondrial dysfunction can drive EMT is important as it unfolds novel therapeutic scenarios: EMT could be potentially blocked by targeting mitochondrial stress-specific EMT marker genes, effectors of the mitochondrial retrograde signaling, specific metabolic enzymes, or metabolism-dependent epigenetic reprogramming, with the aim to limit or prevent cancer metastasis. Several questions, however, remain to be answered. For instance, how and why different types of mitochondrial dysfunction converge on EMT remains a puzzle. It is possible that transient transition to a mesenchymal phenotype may confer a survival advantage to epithelial cancer cells under nutrient or oxygen stress, or in the presence of genetic defects in metabolic enzymes. In this context, EMT would represent a strategy to equip cancer cells with the necessary "armor" (increased survival) and "skills" (increased motility, invasion) to strive while exploring more advantageous metabolic microenvironments. Further studies aimed at understanding the interplay between mitochondrial retrograde signaling pathways and changing microenvironments as well as identifying the molecular determinants of the mito-nuclear network linking mitochondrial dysfunction with EMT activation may provide useful therapeutic targets for treatment and prevention of metastatic cancer.

AUTHOR CONTRIBUTIONS

LM and SG designed and outlined structure and contents of the review. FG, NG, AA, CB, SG, and LM contributed to the literature analysis, interpretation, and writing of the review.

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