METABOLIC ADAPTATION TO CELL GROWTH AND PROLIFERATION IN NORMAL AND PATHOLOGICAL CONDITIONS

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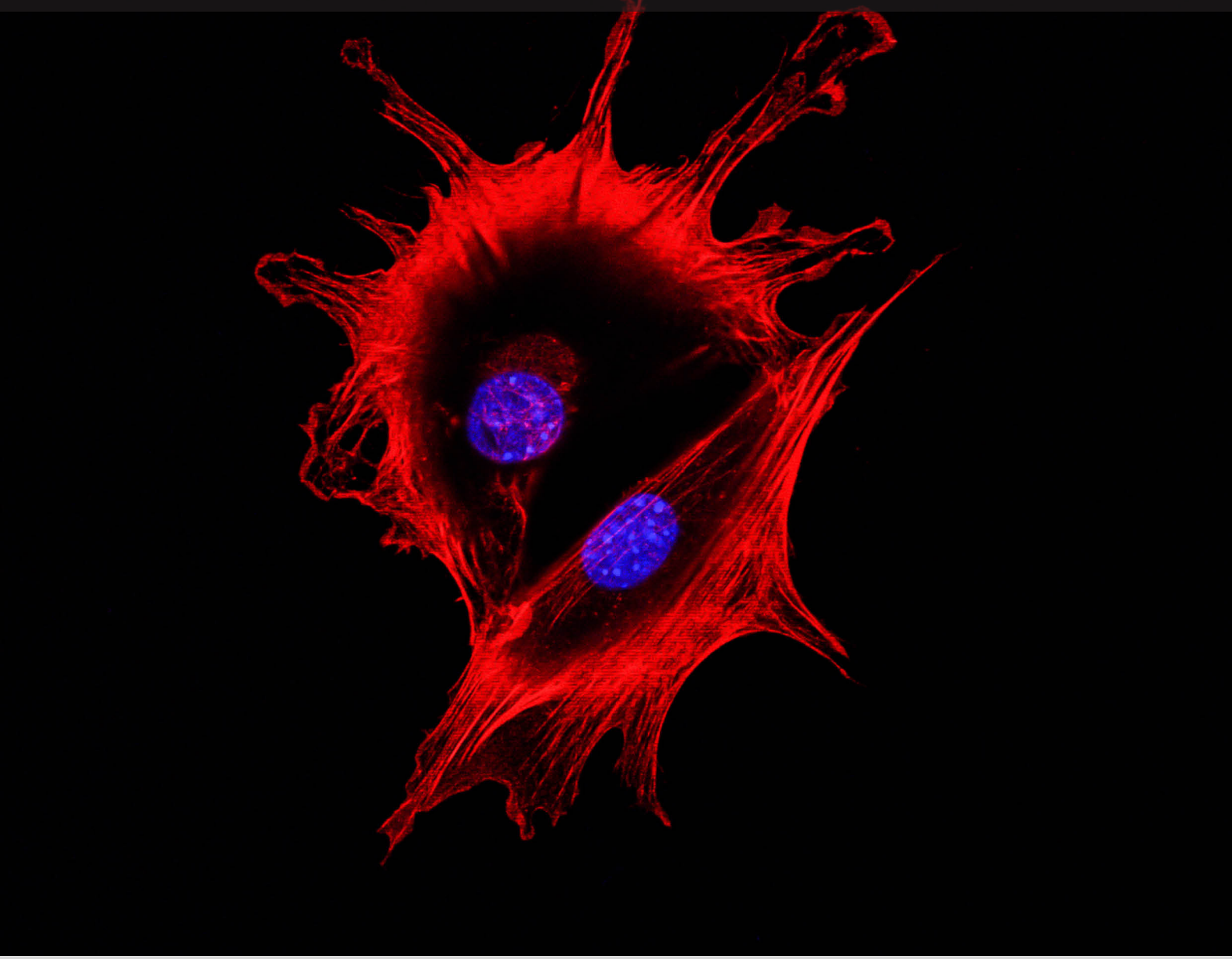

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METABOLIC ADAPTATION TO CELL GROWTH AND PROLIFERATION IN NORMAL AND PATHOLOGICAL CONDITIONS

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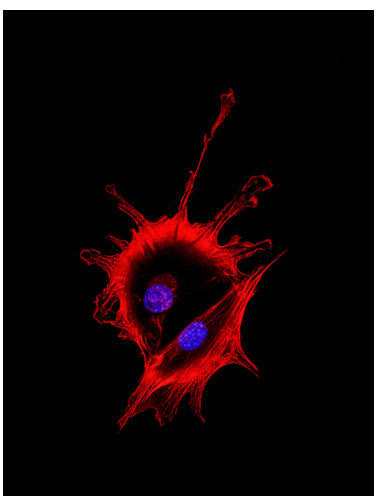

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Proliferating cells must adapt their metabolism to fulfill the increased requirements for energy demands and biosynthetic intermediates. This adaptation is particularly relevant in cancer, where sustained rapid proliferation combined with the harsh conditions of the tumor microenvironment represent a major metabolic challenge. Noteworthy, metabolic reprogramming is now considered one of the hallmarks of cancer. However, the one size fits all rarely applies to the metabolic rewiring occurring in cancer cells, which ultimately depends on the combination of several factors such as the tumor's origin, the specific genetic alterations and the surrounding microenvironment. In the present Research Topic, we compile a series of articles that discuss different metabolic adaptations that proliferating cells undergo to sustain growth and division, as well as the potential therapeutic window to treat certain pathologies, with a special focus on cancer.

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EDITORIAL
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Editorial: Metabolic Adaptation to Cell Growth and Proliferation in Normal and Pathological Conditions

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Keywords: Warburg effect, cancer metabolism, macropinocytosis, cell cycle proteins, epigenetic modifications, mitochondrial diseases, *N*-acetylaspartate, tumor microenvironment

Editorial on the Research Topic

Metabolic Adaptation to Cell Growth and Proliferation in Normal and Pathological Conditions

Proliferating cells must adapt their metabolism to fulfill the increased requirements for energy demands and biosynthetic intermediates. This adaptation is particularly relevant in cancer, where sustained rapid proliferation combined with the harsh conditions of the tumor microenvironment represent a major metabolic challenge. Noteworthy, metabolic reprogramming is now considered one of the hallmarks of cancer (1). However, the one size fits all rarely applies to the metabolic rewiring occurring in cancer cells, which ultimately depends on the combination of several factors such as the tumor's origin, the specific genetic alterations and the surrounding microenvironment (2). In the present Research Topic, we compile a series of articles that discuss different metabolic adaptations that proliferating cells undergo to sustain growth and division, as well as the potential therapeutic window to treat certain pathologies, with a special focus on cancer.

One of the most common and well-described metabolic adaptations occurring in cancer cells is the so-called Warburg effect, which consists on high rates of glycolysis and lactate export, even in the presence of oxygen (3). Abdel-Haleem et al. show that this metabolic phenotype, far from being an exclusive feature of tumors, is a common characteristic of the proliferative state and also a usual metabolic adaptation when robust transient responses are required. Interestingly, when Otto Warburg described this phenomenon almost a century ago, he proposed that the exacerbated aerobic glycolysis observed in cancer cells was due to defective mitochondria. However, as Herst and collaborators highlight in an extensive review about the role of mitochondria in health and disease, these organelles are not only usually functional in cancer cells but also all the more essential to generate metabolic intermediates for biosynthesis, to maintain redox balance and to trigger signaling pathways that promote cell growth and proliferation.

Cell cycle progression, cellular division, and metabolism are intricate processes that regulate each other. One of the mechanisms by which proliferating cells orchestrate these phenomena in a timely manner is by the use of the cell cycle machinery to control metabolism (4). On this subject, Denechaud et al. describe how the transcription factor E2F1 couples the progression of cell cycle with the expression of genes involved in several metabolic pathways and show that dysregulation of E2F1 activity contributes to the pathophysiology of metabolic disorders such as obesity and type 2 diabetes. Another emerging link between metabolism and proliferation is the epigenetic regulation of gene expression, which is treated here in two articles. On the one hand, Rabhi and collaborators and collaborators discuss how, in response to the nutritional status, variations in the intracellular levels of certain metabolites are sensed by epigenetic cofactors that in turn promote changes in gene expression. On the other hand, Bogner-Strauss

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comments on the very recent literature about the novel roles of the metabolite *N*-acetylaspartate in lipogenesis and cancer progression, which include, but are not limited to, epigenetic modulation.

In the recent years, the importance of the interactions between tumor cells and their surrounding microenvironment has become clear (5). Two reviews describe different strategies developed by tumors to acquire external nutrients to sustain biomass production. Recounvreux et al. highlight the relevance of macropinocytosis as a protein source for cancer cells under nutrient-deprived conditions, whereas Blücher et al. show how lipids and other molecules delivered by adipocytes fuel tumor growth in breast cancer, unveiling a possible link with obesity.

One of the most important aspects about the study of the metabolic adaptations occurring during proliferation is the possibility of developing novel therapies to treat cancer. Fendt discusses in an opinion article the opportunities, but also the challenges, for metabolism-based anticancer strategies.

Overall, in the present Research Topic, we cover some of the different metabolic adaptations that take place during proliferation and show that they ultimately depend on both internal and external cues (cell type, history, metabolic context, etc.). Importantly, understanding the specific metabolic profile of proliferating cells may contribute to the identification of metabolic vulnerabilities in the tumors that could be exploited to increase the efficacy of the current treatments.

AUTHOR CONTRIBUTIONS

AG wrote the Editorial and LF edited it.

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PERSPECTIVE
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The Emerging Facets of Non-Cancerous Warburg Effect

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The Warburg effect (WE), or aerobic glycolysis, is commonly recognized as a hallmark of cancer and has been extensively studied for potential anti-cancer therapeutics development. Beyond cancer, the WE plays an important role in many other cell types involved in immunity, angiogenesis, pluripotency, and infection by pathogens (e.g., malaria). Here, we review the WE in non-cancerous context as a "hallmark of rapid proliferation." We observe that the WE operates in rapidly dividing cells in normal and pathological states that are triggered by internal and external cues. Aerobic glycolysis is also the preferred metabolic program in the cases when robust transient responses are needed. We aim to draw attention to the potential of computational modeling approaches in systematic characterization of common metabolic features beyond the WE across physiological and pathological conditions. Identification of metabolic commonalities across various diseases may lead to successful repurposing of drugs and biomarkers.

Keywords: Warburg effect, cancer, immune cells, malaria, angiogenesis, pluripotency, rapid proliferation, constraint-based metabolic modeling

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INTRODUCTION

While all cells need a source of energy to maintain homeostasis, proliferating cells require a substantial amount of nutrients to produce biosynthetic building blocks and macromolecules for the newly produced daughter cells (1). Both glycolysis and respiration through oxidative phosphorylation (OxPhos) can generate free energy in the form of adenosine-5′-triphosphate (ATP) (1). Most cells metabolize glucose to pyruvate *via* glycolysis, and under normoxic conditions, the generated pyruvate is further oxidized to CO_2 in the mitochondria through OxPhos, generating up to 36 ATP molecules per glucose molecule. When oxygen becomes limiting, mitochondrial OxPhos is restricted and pyruvate is converted to lactate instead. However, it has been widely observed across different cell types that the latter can predominate when oxygen is plentiful (2). A common feature among cells exhibiting this phenomenon of aerobic glycolysis (3, 4) is "rapid proliferation." Although it seems counterintuitive, most rapidly proliferating cells seem to rely on aerobic glycolysis despite the fact that it yields significantly less ATP/glucose compared to OxPhos (5, 6). Although different proposals have been put forward to rationalize the cell's unique feature of using the Warburg effect (WE),

it is still unclear whether aerobic glycolysis is "causal" or if it is just a phenotype of rapidly proliferating cells due to metabolites overflow (7).

Although aerobic glycolysis is now an established hallmark of cancer (8), relatively fewer studies have investigated the WE in non-cancerous cells (**Figure 1**). Here, we discuss the role of aerobic glycolysis as a "hallmark of rapid proliferation" as part of cellular dysregulation (cancer, inflammation, and autoimmune diseases), physiologically regulated process (T-cell activation and angiogenesis), and pluripotency. Beyond mammalian cells, the WE has also been central to the developmental stages of rapidly proliferating parasites, such as Plasmodium and Toxoplasma. Furthermore, the use of aerobic glycolysis and the secretion of organic acids are common in most rapidly growing microbes (e.g., yeast and *E. coli*). We argue that cells adopt aerobic glycolysis in the cases where a rapid transient action is needed while respiration tends to support long-term constitutive (more stable) processes. Because the span of cells exhibiting the WE is wide, we propose that a systems biology approach based on constraint-based modeling (CBM) of metabolism (**Figure 2**) can be useful as a means of systematic characterization of common and distinct features of the WE across different diseases and cell types.

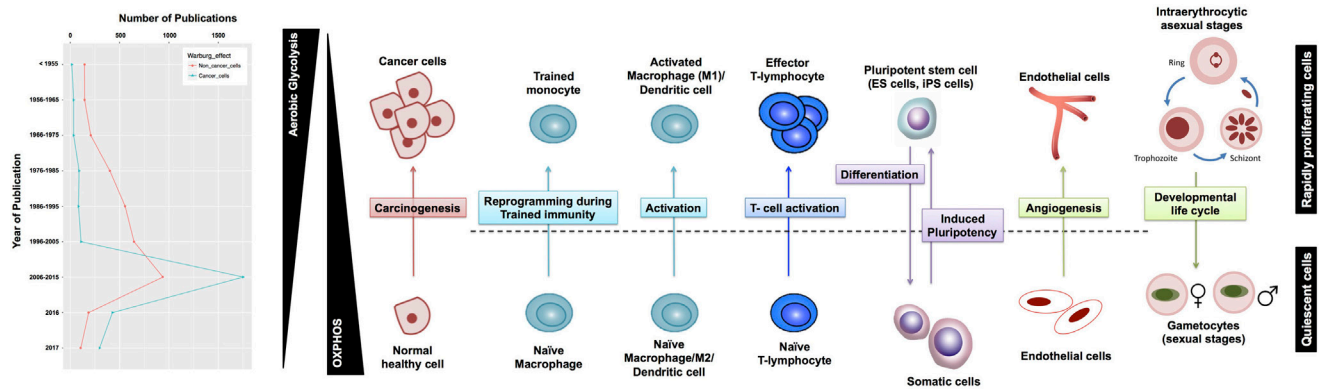

FIGURE 1 | Warburg effect (WE) in cancerous and non-cancerous cells. **(A)** The frequency of publications on the WE in cancer cells has not witnessed a parallel surge in studies investigating the same phenomenon in non-cancerous context. **(B)** WE has been observed across a plethora of rapidly proliferating cells in both physiological and pathological contexts. T-cells shape is adopted from Ref. (9), the malaria life cycle stages image is adopted from Ref. (10), and the blood vessel icon is adopted from Ref. (11). ES, embryonic stem cells; iPS, induced pluripotent stem cells.

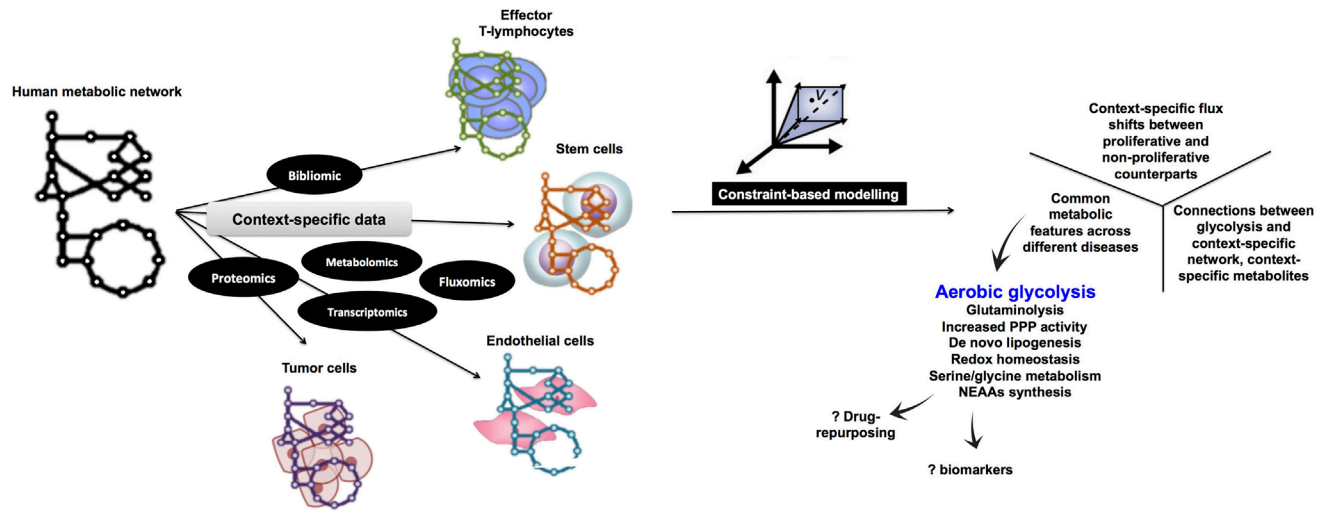

FIGURE 2 | Proposed computational metabolic modeling approach to systematically identify common metabolic features within pathological conditions as well as across normal and disease states. Starting with a curated human metabolic network, high-throughput data specific for each normal tissue (e.g., proliferating endothelial cells and effector T lymphocytes) or disease (e.g., cancer, autoimmune diseases, and inflammation) will be used to develop the corresponding context-specific metabolic network which is amenable to simulations under the constraint-based modeling framework being subject to different levels of constraints. By assigning an appropriate objective function (e.g., biomass production), it is possible to enumerate metabolic processes that are tightly coupled to growth and proliferation. Eventually, since all context-specific models are developed under a uniform integrative framework, it is legitimate to cross compare metabolic networks potentially identifying common metabolic features (e.g., aerobic glycolysis). NEAAs, non-essential amino acids metabolism.

WE PLAYS A ROLE IN ACTIVATION OF EFFECTOR T-HELPER LYMPHOCYTES (ADAPTIVE IMMUNITY)

In order to perform their function in protecting the body against pathogens and allergens, naïve T-cells need to be activated. This involves rapid proliferation (clonal expansion) and differentiation of naïve T-cells into antigen-specific T-effectors (Teff) or T-regulatory cells that function to mediate or suppress immune responses, respectively (12–14). T-cell activation requires energy and metabolic precursors for macromolecular biosynthesis (15). The role of WE in T-cell activation has recently become more apparent, and efforts are underway to understand its role.

Upon stimulation, effector T-cells exhibit high levels of glucose uptake and glycolysis (16). In both cancer cells and activated effector T-cells, elevated expression of glucose transporter, particularly GLUT1, has been reported (12, 16, 17). However, it is unclear whether upregulation of GLUT1 happens as a prerequisite or consequence of T-cell activation as Teff was selectively increased in transgenic mice when Glut1 was overexpressed (18). Enhanced glucose uptake is also linked to increased expression and activity of glycolytic enzymes. In humans, chronically activated T-cells in allergic asthma patients produce high levels of lactate and overexpress pyruvate dehydrogenase kinase that inhibits pyruvate dehydrogenase, thus restricting the entrance of pyruvate into the mitochondrial TCA (19, 20). In addition to transporters and glycolytic enzymes, carcinogenesis shares many metabolic regulators with T-cell activation, including phosphoinositide 3-kinase [PI(3)K]/Akt, mammalian target of rapamycin complex (15, 16, 21), and Myc (15, 22) as well as the hypoxia-inducible factor-1α (HIF-1α) (2, 20). Altogether, activation of T-cells parallels carcinogenesis with respect to adopting glycolysis rather than OxPhos in presence of oxygen.

It is critical to highlight that T-cell activation is not accompanied merely by a switch from oxidative metabolism to glycolysis, but that both pathways coordinate to support bioenergetic demands (15). In fact, mitochondrial activity is enhanced in stimulated lymphocytes compared to their resting counterparts. The same observation has been extensively reviewed in the field of cancer biology, which contradicts the initial "WE" theory that cancer cells opt for aerobic glycolysis due to a defective mitochondria. Hence, the WE does not require a defective mitochondria to be the predominant metabolic program in rapidly dividing cells (1, 21, 23).

An important difference between glycolysis in T-cell activation and carcinogenesis is that carcinogenesis is a form of cellular dysregulation (24). By contrast, T-cell activation happens in both pathological (e.g., autoimmune diseases) and physiological contexts (25, 26). This further underscores that aerobic glycolysis is a feature that is tied to rapid proliferation independent of the context being normal or pathological state.

MARCOPHAGES (INNATE IMMUNITY) UTILIZE AEROBIC GLYCOLYSIS

Inflammatory cells, such as activated macrophages, upregulate glycolysis (2) in response to tissue injury or infection to cope with increased production of host defense factors, enhanced phagocytosis, and antigen presentation (2). In both activated inflammatory cells and cancer cells, glucose transporter GLUT1 is upregulated, lactate production is increased, and oxygen consumption by the mitochondria is decreased in the downstream events to PI(3)K-Akt1 signaling (2). There is also a marked switch from expression of the liver isoform of 2,6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (encoded by PFKFB1) to the PFKFB3 isoform, the type of which is also commonly found in tumor cells. This leads to accumulation of fructose-2,6-bisphosphate ($F2,6P_2$) as an allosteric activator of 6-phosphofructo-1-kinase (PFK1), and therefore, glycolysis takes place (2, 27, 28). In addition, hexokinase, the first enzyme involved in glycolysis as well as in the pentose phosphate pathway (PPP), is also upregulated in activated macrophages (29).

In contrast to pro-inflammatory M1 macrophages, anti-inflammatory M2 macrophages have higher rates of OxPhos and lower rates of glycolysis (2). Further, M2 macrophages have no detectable PFKFB3 and expressing PFKFB1 instead (2). In addition, the transcription factor HIF-1α and AMP-activated protein kinase play critical roles in regulating the metabolic alterations between inflammatory and anti-inflammatory responses (2, 30, 31). It is noteworthy that both M1 and M2 macrophages are highly active and can proliferate; nevertheless, the pro-inflammatory M1 relies on glycolysis while the anti-inflammatory and tissue-repair-promoting M2 relies on OxPhos. We thus hypothesize that OxPhos is more suited to long-term reparative roles (e.g., anti-inflammation), while aerobic glycolysis being suited for rapid, robust, and transient responses (e.g., inflammation). However, fitting this premise in the context of the behavior of quiescent adult stem cells, which opt for glycolysis to avoid senescence due to increased ROS load generated by OxPhos (5, 32), warrants further investigation.

In the pathological states discussed here, we observe that the WE is a phenotype of rapidly dividing cells irrespective of whether the context is triggered by an external or internal cue. For instance, autoimmune diseases might arise from somatic mutations in antigen receptors according to the "Clonal Selection Theory" (26). Similarly, cancer cells might arise due direct or indirect oncogenic mutations. In both cancer and autoimmune diseases, the trigger is an internal cue. However, in the case of physiological T-cell activation, external signals (cytokines in this case) mediate the appropriate immune response (2, 33).

Taken together, the WE is a hallmark of rapidly proliferative cells across wide spectrum of pathological and physiological processes that are triggered by either internal or external cues.

ENDOTHELIAL CELLS (ECs) UTILIZE AEROBIC GLYCOLYSIS DURING ANGIOGENESIS

Blood vessels deliver oxygen and nutrients to all of the tissues and organs in the body. ECs and vascular smooth muscle cells are the two main cellular components of blood vessels. Consequently, these cells are involved in a variety of physiological processes as well as pathological dysfunctions, including atherosclerosis (34).

Angiogenesis relies on the proliferation and migration of ECs (35). Once the vessel is perfused, ECs become quiescent phalanx cells (27). Similar to other rapidly proliferating cells, ECs are exposed to sufficient oxygen for oxidative metabolism, yet they prefer aerobic glycolysis. Radioactive-tracer labeled substrates in EC monolayers showed that glycolytic flux in ECs was largely comparable to those in tumor cells but much higher than in various other healthy cells (27). Notably, glycolytic flux was more than 200-fold higher compared to glucose oxidation, fatty acid oxidation, and glutamine oxidation (27), while mitochondrial respiration was lower in ECs than in other oxidative cell types (27). In addition, glycolysis generated up to 85% of the total cellular ATP content (27) and regulated vessel sprouting (27). Overall, glycolysis is the predominant bioenergetic pathway for proliferating ECs.

Similar to other rapidly dividing cells discussed here, PFKFB3 is critical for EC proliferation where PFKFB3 silencing reduced vessel sprouting (27) while inhibition of respiration did not have a significant effect. Similar findings have been reported in cancer cells and M1 macrophages (36,37), highlighting a potentially conserved critical role for PFKFB3 in aerobic glycolysis.

AEROBIC GLYCOLYSIS IS A METABOLIC FEATURE IN PLURIPOTENT EMBRYONIC STEM CELLS

In contrast to somatic cells and in analogy to rapidly proliferating cells, embryonic stem (ES) cells rely on glycolytic ATP generation regardless of oxygen availability (38–40). The reliance on glycolysis was suggested to be due to a low copy number of mitochondrial DNA (mtDNA) as well as low numbers of nascent mitochondria (38, 41). Differentiation increases mtDNA abundance and promotes mitochondrial biogenesis to form networks of elongated and cristae-rich mitochondria in support of competent oxidative metabolism (38, 42, 43).

High-resolution metabolomics showed that induced pluripotent stem (iPS) cells upregulate glycolytic enzymes and downregulate electron transport chain subunits enabling a switch that converts somatic oxidative metabolism into a glycolytic flux-dependent and mitochondria-independent state that underlies pluripotency induction (32, 38). To maintain high glycolytic rates, human embryonic stem cells as well as cells of the inner cell mass (which becomes the embryo proper) upregulate GLUT1, GLUT3, HK, and PFK1 (6, 32, 44–46) leading to increased lactate synthesis (44, 47). In iPS cells, the upregulation of glycolysis precedes the reactivation of pluripotency markers (48, 49) implicating that the glycolytic phenotype is more tied to rapid proliferation rather than pluripotency. Further, despite the low levels of oxygen consumption in undifferentiated ES, ATP synthesis is decoupled from oxygen consumption and depends on glycolysis instead, possibly consuming oxygen through the mitochondrial ETC (44). As ES progress toward differentiation, their glycolytic fluxes decrease dramatically while mitochondrial OxPhos fueled by glucose and fatty acids increases (44).

Elevated levels of PFKFB3 have been also reported in human embryonic kidney 293 cells (28) and cancer stem (CS) cells. However, iPS cells express a very low level of PFKFB3 while expression of PFK1 was comparable to that in CS cells. This indicates that PFK1 activation could be PFKFB3-independent in iPS cells (50). Taken together, PFKFB3 is upregulated in a wide spectrum of rapidly proliferating cells adopting Warburg metabolism.

MALARIA ADOPTS A GLYCOLYTIC METABOLIC PROGRAM DURING ITS ASEXUAL INTRAERYTHROCYTIC LIFE CYCLE STAGES

Malaria forms that are injected into human blood following an infected-mosquito bite, migrate to the liver, and then are released into the blood stream were they rapidly proliferate inside the red blood cells (RBCs), eventually causing malaria symptoms and pathology due to RBCs lysis (51). A small fraction (<1%) of these rapidly proliferative stages commit to sexual development and is responsible for transmitting infection to another mosquito vector (52). Because the malaria parasite encounters different metabolic niches across its developmental stages, its growth matches its nutritional requirements by rewiring its metabolic network [(53) and our unpublished work]. During the intraerythrocytic developmental stages, the asexual stages of the malaria parasite increase their glucose uptake by more than 10-fold (51) with 93% of their glucose uptake being converted into lactate (53), consistent with a high metabolic demand that is imposed by parasite division. This percentage drops to 80% in the non-proliferative/quiescent gametocyte stages (53). Hemoglobin digestion generates ROS and increases the redox burden, so that favoring aerobic glycolysis could be a means to minimize redox burden (compared to using OxPhos). Nevertheless, the asexual forms still rely on electron transport activity for regeneration of ubiquinone that is required as the electron acceptor for dihydroorotate dehydrogenase, an essential enzyme for pyrimidine biosynthesis (54). Knocking out the mitochondrial ATP synthase β-subunit gene that disrupted the parasite transmission cycle while only marginally reducing growth of the asexual rapidly proliferating stages, reflecting a higher essentiality of mitochondrial function in the non-rapidly proliferative mosquito stages (54). In another study, a genetic investigation of TCA metabolism across the malaria life cycle (55) showed that knocking out of six of the eight TCA cycle enzymes does not affect asexual growth while affecting life cycle progression in later stages. Collectively, these studies (51, 54, 55) show that the overall flux of pyruvate into the TCA cycle is low in the rapidly dividing sexual stages while aerobic glycolysis is more prominent. In contrast, elevated levels of the TCA cycle activity sustained by increased catabolism of pyruvate dominates in Plasmodium gametocytes.

In this context, the asexual forms of the malaria parasite converge metabolically with the rapidly proliferating counterparts of other cancerous and non-cancerous cells, as discussed here. Malaria is an obligate intracellular parasite and has lost several of its genome content leading to a reduced metabolic capacity compared to its host (56). The fact that the asexual rapidly proliferating forms of the parasite opt for the WE despite their reduced metabolic capacity compared to other rapidly proliferating

eukaryotic cells implies that the synthesis of biosynthetic precursors does not necessarily come on top of the reasons for why cells preferentially undergo the WE.

IN SILICO METABOLIC MODELING CAN SYSTEMATICALLY ELUCIDATE COMMON METABOLIC FEATURES ACROSS DIFFERENT CELL TYPES AND DISEASES

Constraint-based modeling (57, 58) uses genome-scale metabolic models (GEMs) as platforms for integrating and interpreting different levels of high-throughput data (59–62) (**Figure 2**). Under the constraints of substrate availability, mass conservation limits reaction products and their stoichiometry, while thermodynamics constrain reaction directionality. This information can be obtained from genome sequences and annotation (e.g., human genome annotation); organism-specific database (e.g., http://plasmodb.org for malaria) along with bibliomic data that support the presence of each metabolic functionality before being added to the metabolic reconstruction. A metabolic reconstruction is then converted to a stochiometric matrix (57, 58), based on the stochiometric coefficients of each reaction, which is amenable to computation and simulations. Data-driven network boundaries (e.g., uptake and secretion products) are then applied. Taken together, these constraints would define the allowable "solution space" to achieve a certain cellular objective (e.g., growth that can be simulated by biomass precursors production) (58, 63). Additional context-specific constraints (e.g., enzyme gene expression levels or metabolites concentration) would shrink the solution space leading to context-dependent predictions about the utilization of alternate pathways across the metabolic network.

Many CBM methods for analyzing genome-scale metabolic networks (64, 65) have been developed (58). Since certain enzymes are only active in specific cell types, COBRA methods can be used for tailoring a generic metabolic network (**Figure 2**) by integrating high-throughput data to extract a cell type or disease-specific metabolic model from a GEM.

To comprehensively identify common metabolic features across the range of rapidly proliferating cells we discuss here, we suggest a CBM-based workflow (**Figure 2**) to enable integration of different levels of data to model the widely variable types of rapidly proliferating cells. Because of the ability of CBM to predict gene essentiality by simulating single-gene knockouts (58, 63), GEMs can provide a means to address the systematic interactions between the different biological components of the WE along with elucidating how they influence the entire metabolic network. Furthermore, model-predicted knockout phenotypes that selectively inhibit growth of rapidly proliferating cell models but not their quiescent counterparts can be integrated in drug development pipelines to predict druggable targets (63, 66–68) as well as new drug combinations. Using a metabolite essentiality analysis (69), instead of gene-knockout experiments, biomarkers for identification of cells undergoing the WE can be predicted. The advantage of using metabolites prompt searching for structural analogs of the essential metabolites to inhibit enzymes that relied on them as substrates (59).

Constraint-based modeling methods have also been used to model interactions between different cell types (70). Following a similar workflow, it is possible to use GEMs of the cancerous and non-cancerous cells in a tumor microenvironment to identify essential metabolites whose inhibition would disrupt the symbiotic relationship between cancerous cells and non-cancerous cells in their surroundings. For instance, recent data have indicated that glycolysis-targeting interventions such as the depletion of PFKFB3 may exert antineoplastic effects by limiting vessel sprouting (27), hence targeting both proliferative endothelial and cancer cells. Thus, outlining the common metabolic features between normal and pathological cells can be of potential clinical value.

Constraint-based modeling allows prediction of numerous metabolic phenotypes, including growth rates, nutrient uptake rates, and gene essentiality. They are, thus, well-suited to the search for common metabolic features across a span of pathological and physiological conditions as well as for integration in the early stages of target-based drug development pipelines.

CONCLUDING REMARKS

Although WE is one of the most extensively studied bioenergetic processes that are being shared between cells that undergo rapid proliferation, other bioenergetic and anabolic processes contain similar potential to being metabolic phenotypes of rapid proliferation. For instance, glutamine dependency and glutaminolysis increased PPP activity, serine and glycine metabolism as well as *de novo* lipogenesis (71, 72). Likewise, several intermediate metabolites bear the potential of being biomarkers of rapid proliferation (e.g., serine, sarcosine, and kyneurin). However, whether a therapeutic window for the clinical application of these processes exists remains to be determined. Noteworthy is that the response of the glycolytic pathway to drug perturbations is non-linear (71–73). Thus, careful considerations will be needed to develop a biomarker that can determine the context in which it would be efficacious to exploit any diagnostic or therapeutic potential for the WE. The clinical success of antimetabolites (71) lends support to the argument presented here that metabolic events can be therapeutically exploited while being shared between both normal and pathologic rapidly proliferating cells. Nevertheless, drug inhibitors developed against other metabolic events have not progressed beyond the pre-clinical stages yet (74, 75). Taken together, the arguments and discussion presented here suggest that grouping diseases and cell types according to common metabolic phenotypes can provide mechanistic understanding of the observed phenotypes in relation to the context-specific repertoire of metabolic interactions as well as expediting drug development pipelines.

AUTHOR CONTRIBUTIONS

AMA and TG conceived and synthesized the study. The manuscript was written by AMA and TG with input from NL, NJ, KM and XG.

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REVIEW
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Functional Mitochondria in Health and Disease

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The ability to rapidly adapt cellular bioenergetic capabilities to meet rapidly changing environmental conditions is mandatory for normal cellular function and for cancer progression. Any loss of this adaptive response has the potential to compromise cellular function and render the cell more susceptible to external stressors such as oxidative stress, radiation, chemotherapeutic drugs, and hypoxia. Mitochondria play a vital role in bioenergetic and biosynthetic pathways and can rapidly adjust to meet the metabolic needs of the cell. Increased demand is met by mitochondrial biogenesis and fusion of individual mitochondria into dynamic networks, whereas a decrease in demand results in the removal of superfluous mitochondria through fission and mitophagy. Effective communication between nucleus and mitochondria (mito-nuclear cross talk), involving the generation of different mitochondrial stress signals as well as the nuclear stress response pathways to deal with these stressors, maintains bioenergetic homeostasis under most conditions. However, when mitochondrial DNA (mtDNA) mutations accumulate and mito-nuclear cross talk falters, mitochondria fail to deliver critical functional outputs. Mutations in mtDNA have been implicated in neuromuscular and neurodegenerative mitochondriopathies and complex diseases such as diabetes, cardiovascular diseases, gastrointestinal disorders, skin disorders, aging, and cancer. In some cases, drastic measures such as acquisition of new mitochondria from donor cells occurs to ensure cell survival. This review starts with a brief discussion of the evolutionary origin of mitochondria and summarizes how mutations in mtDNA lead to mitochondriopathies and other degenerative diseases. Mito-nuclear cross talk, including various stress signals generated by mitochondria and corresponding stress response pathways activated by the nucleus are summarized. We also introduce and discuss a small family of recently discovered hormone-like mitopeptides that modulate body metabolism. Under conditions of severe mitochondrial stress, mitochondria have been shown to traffic between cells, replacing mitochondria in cells with damaged and malfunctional mtDNA. Understanding the processes involved in cellular bioenergetics and metabolic adaptation has the potential to generate new knowledge that will lead to improved treatment of many of the metabolic, degenerative, and age-related inflammatory diseases that characterize modern societies.

Keywords: mitochondrial DNA, mitochondrial DNA mutations, mitochondriopathies, mito-nuclear cross talk, mitochondrial transfer, mitochondrial stress signals, mitopeptides

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INTRODUCTION

Mitochondria are maternally inherited multifunctional organelles that form a comprehensive network in many cells maintained by an intricate balance between fission and fusion, mitochondrial biogenesis, and mitophagy (1, 2). Although mitochondria are best known for harvesting and storing energy released by the oxidation of organic substrates under aerobic conditions through respiration, their many anabolic functions are often overlooked (see **Figure 1**). Arguably, the biosynthetic functions of mitochondria are at least as important for tumorigenesis and tumor progression as ATP generation [recently reviewed by Ahn and Metallo (3)]. Tumor cells easily survive in hypoxic conditions by recycling NADH to NAD^+ *via* lactate dehydrogenase (LDH) and plasma membrane electron transport (PMET) to allow for continued glycolytic ATP production (4). Cells without mitochondrial (mt) DNA (ρ^0 cells) are incapable of mitochondrial electron transport (MET) coupled to oxidative phosphorylation (OXPHOS), but proliferate if supplemented with pyruvate and uridine (5, 6). Pyruvate addition appears to be necessary to maintain the pyruvate/lactate couple which generates NAD^+ for continued glycolysis, even though the majority of pyruvate produced through glycolysis will be reduced to lactate rather than entering the Krebs cycle, which limits biosynthetic intermediates required for several metabolic pathways (3, 5). For example, α-ketoglutarate is a precursor of glutamate, glutamine, proline, and arginine while oxaloacetate produces lysine, asparagine, methionine, threonine, and isoleucine. Amino acids in turn are precursors for other bioactive molecules, such as nucleotides, nitric oxide, glutathione, and porphyrins. Citrate can be transported out of mitochondria *via* the pyruvate-citrate shuttle and metabolized to cytosolic acetyl-CoA, which is the substrate for the biosynthesis of fatty acids and cholesterol as well as protein acetylation (3). Uridine is necessary for ρ^0 cells to bypass metabolic reliance on MET, allowing continued pyrimidine biosynthesis and thus DNA replication to continue. Dihydroorotate dehydrogenase (DHODH), a flavoprotein found on the outer surface of the inner mitochondrial membrane (IMM), oxidizes dihydroorotate to orotate. Electrons from this oxidation are used to reduce coenzyme Q just prior to complex III in MET (6). In the absence of MET, DHODH is unable to oxidize dihydroorotate, blocking pyrimidine biosynthesis.

Whereas many biosynthetic processes occur in the mitochondrial matrix, respiratory complexes that form the functional respirasome are positioned in the IMM, which is heavily folded into cristae in many cell types with high energy requirements. Electrons from NADH and $FADH^2$ are transported to oxygen as the terminal electron acceptor through respiratory complexes I, II, III, and IV of MET. The energy released in this process is stored in the form of a proton gradient, which produces an electric potential across the IMM. This membrane potential drives the generation of ATP through OXPHOS *via* the F_0F_1 ATP synthase (respiratory complex V) [summarized in Ref. (7)]. The mitochondrial membrane potential also regulates influx of Ca^{2+} ions into the mitochondria to buffer cytoplasmic calcium as well as facilitate the import of nuclear-encoded, mitochondrially targeted proteins (n-mitoproteins) (7–10). MET ensures low $NADH/NAD^+$ ratios to facilitate sustained glycolysis. An important byproduct of MET is the production of reactive oxygen species (ROS) which at low levels act in cell signaling pathways. These radicals are balanced by strong mitochondrial antioxidant defense systems to prevent oxidative damage to mitochondrial DNA (mtDNA), and to protein and lipids at higher concentrations (11, 12). Mitochondria are also involved in regulation of apoptosis through activation of the mitochondrial permeability transition pore whenever ROS and the AMP/ATP ratio increases and Ca^{2+} levels in the mitochondria increase (13, 14).

Mitochondria play a vital role in bioenergetic and biosynthetic pathways and can rapidly adapt to meet the metabolic needs of

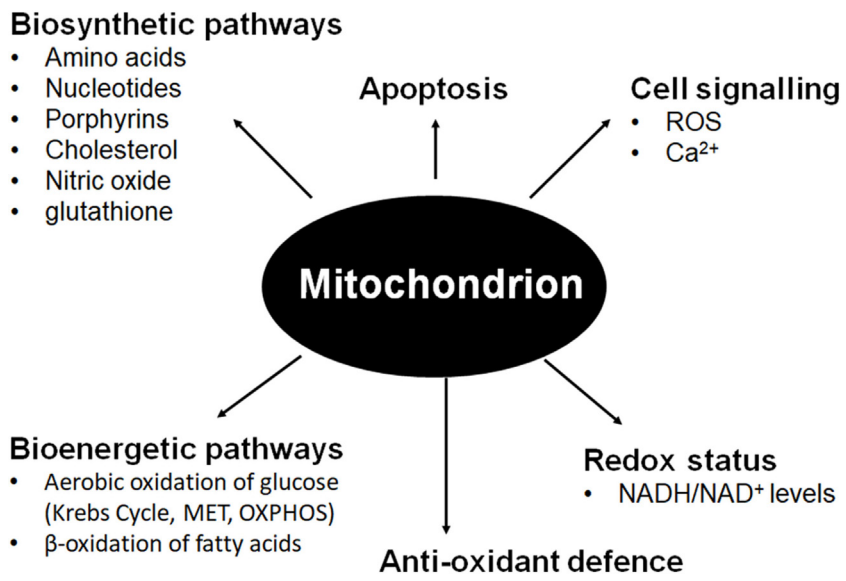

FIGURE 1 | Mitochondrial involvement in fundamental cellular pathways and processes.

the cell. Increased demand is met by mitochondrial biogenesis and fusion of individual mitochondria into dynamic networks, whereas a decrease in demand results in the removal of superfluous mitochondria though fission and mitophagy (1, 2, 15, 16). This level of adaptability to cellular needs is achieved by effective communication between the nucleus and the mitochondria. Factors that compromise mito-nuclear cross talk will affect the cell's ability to respond to stresses caused by changes in the microenvironment. Effective mito-nuclear cross talk is also of vital importance in tumorigenesis and tumor progression but remains largely unexplored (17). The role of mitochondria in different stages of tumor biology has been reviewed recently (10, 18–20). This review will discuss how cells respond when mtDNA mutations accumulate, mito-nuclear cross talk falters, and mitochondria do not deliver important functional outputs. In some cases, drastic measures such as acquisition of new mitochondria from donor cells occur to ensure cell survival.

EVOLUTIONARY ORIGIN OF MITOCHONDRIA

To better understand the need for and intricacies of ongoing mito-nuclear communication, we provide a brief summary of the events that led to mitochondria becoming an integral part of eukaryotic cells. The idea that mitochondria originated from free living bacteria that became incorporated inside an archaeal cell *via* endocytosis (21) is supported by phylogenetic analysis of conserved ribosomal RNA (rRNA) (22, 23). It is now widely accepted that all multicellular life originated from a common eukaryotic ancestor that evolved more than two billion years ago. Although the exact timing of the acquisition of the α-proteobacterium is still debated (24, 25), the metabolic advantages that this endosymbiotic relationship brought are indisputable. Protomitochondria conferred on early eukaryotes the ability to use previously toxic oxygen to fuel a much more efficient way of releasing energy from organic substrates, aerobic respiration. This allowed them to colonize new and diverse ecological niches and set the scene for the advent of complex multicellularity, eventually giving rise to fungal, plant, and animal cells. A comparison of non-ribosomal proteomes of α-proteobacteria and eukaryotes reveals that protomitochondrial metabolism was likely based on the aerobic catabolism of lipids, glycerol, and amino acids provided by the host. Over time, considerable endosymbiotic gene transfer from the protomitochondrion to the host nucleus occurred while many genes were lost through redundancy. As a result, the nuclear genome has become larger and more complex, while the mitochondrial genome has dwindled. A comparison of proteomes suggests that only 22% of human mitochondrial proteins are of protomitochondrial descent (26).

INTEGRITY OF mtDNA

Organization of mtDNA in Nucleoids

The human mitochondrial genome is a double-stranded, closed-circular molecule of 16,569 nucleotide pairs. It was first sequenced in 1981 (27) and revised in 1999 (28). mtDNA does not contain introns and encodes just 13 polypeptides, 22 transfer RNAs (tRNAs), and the 12S and 16S rRNA genes for mitochondrial protein synthesis (29). The 13 polypeptides encode subunits of the respiratory complexes (7 of 45 for RC-I, 1 of 11 for RC-III, 3 of 13 for RC-IV, and 2 of 16 for RC-V). The four subunits that make up RC-II are nuclear encoded along with the remaining 85% of the other RC subunits (29). Nuclear DNA encodes more than 22,000 proteins, about 1,500 of which contribute to the mitochondrial proteome. These n-mitoproteins include enzymes required for the TCA cycle, amino acid, nucleic acid and lipid biosynthesis, mtDNA and RNA polymerases, transcription factors, ribosomal proteins in addition to all components of DNA repair pathways. N-mitoproteins are expressed in the cytoplasm and folded upon entry through the mitochondrial outer membrane *via* the TOM/TIM complex. From there, they locate to their specific sites: the outer mitochondrial membrane (OMM), the IMM, the intermembrane space (IMS), or the mitochondrial matrix (30).

Mitochondrial DNA is not structurally associated with histones, as is nuclear DNA. Instead, it is closely associated with a number of proteins in discreet nucleoids, approximately 100 nm in diameter. Nucleoids are anchored to the IMM, facing the matrix (31). One cell can contain tens to thousands of nucleoids, each with a single mtDNA molecule as shown by super-resolution microscopy (31). Transcription factor A of mitochondria (TFAM), the mtDNA helicase Twinkle, and mitochondrial single-stranded DNA-binding protein (mtSSB) all co-localize with mtDNA within nucleoids (32). TFAM binds to mtDNA and forces U-turns in the circular molecule, which allows compacting and packaging of mtDNA into nucleoids (33). TFAM plays an important role in both the transcription and maintenance of mtDNA and has been shown to recognize and bind to cisplatin-damaged and oxidized mtDNA. TFAM is also expressed in the nucleus and regulates nuclear genes. mtDNA replication is enhanced by an increase in expression of the master regulator of the mitochondrial biogenesis, transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1alpha (PGC-1α) expression *via* co-activation of the nuclear respiratory factor 2 (NF2), and NF1 (34). Overexpression of mitochondrial TFAM after cisplatin exposure promotes treatment resistance and cancer growth (35). High TFAM expression in tumors has been found to be correlated with poor outcomes in patients with ovarian cancer, pancreatic adenocarcinoma, endometrial adenocarcinoma, and colorectal cancer, with a poor response to chemotherapy [reviewed by Kohno et al. (35)]. TFAM, Twinkle, and mtSSB are essential components of nucleoids. Other n-mitoproteins associated with mtDNA replication, transcription, translation, and repair are transiently associated with the nucleoid and are referred to as mitochondrial nucleoid associated proteins (30). Mito-ribosomes, although found close to nucleoids, are not attached and are, therefore, not part of the nucleoid. In contrast to prokaryotes, mitochondrial transcription and translation occur as separate processes, with the polycistronic RNA needing further processing before being translated. Any apparent association with the nucleoid is likely to be related to the small spaces between cristae in the mitochondrial matrix (30).

The mitochondrial and nuclear genomes differ in size by more than five orders of magnitude. However, each somatic cell contains

10 to several thousand mtDNA copies (31) and only two copies of nDNA. This disproportionate representation of protein-encoding mitochondrial to nuclear genes in most cells exceeds two orders of magnitude (36), requiring ongoing mito-nuclear communication to ensure appropriate stoichiometry of RC subunits. Both decreased and increased mtDNA copy numbers have been associated with increased cancer incidence, with contradictory findings between some studies for the same type of tumor. For example, both increased (37) and decreased copy numbers (38, 39) have been reported to increase the incidence of renal cancer.

Given the density of open reading frames in mtDNA, one could argue that loss of mtDNA integrity can have serious consequences for individual cells as well as the entire organism. It is generally accepted that mtDNA mutates more rapidly than nDNA because of its close proximity to mitochondrially generated ROS, lack of protective histone proteins, and comparatively less effective repair processes (40, 41). An earlier study showed that mtDNA damage is not only more severe but also persists longer than nuclear DNA damage after H_2O_2 exposure (42). However, other studies have shown that the DNA-binding proteins of mitochondrial nucleoids can be equally protective of mtDNA as histones are of nuclear DNA when exposed to H_2O_2 or X-rays (43). In addition, the effect of a single mtDNA mutation may have fewer consequences than a single nDNA mutation. Impaired MET due to mtDNA mutations results in depolarized mitochondria which are unable to re-fuse with the mitochondrial network after fission (44). Protection from damage by nucleoid proteins combined with the removal of mitochondria with damaged DNA through mitophagy may result in a more robust response to oxidative stressors such as H_2O_2 than previously thought.

Cells of most outbred populations contain more than one mitochondrial genotype. This heteroplasmy can be quite variable within tissues and cell types of one organism, complicating interpretation of mitochondrial genetics and influencing disease presentation in the case of pathological mtDNA mutations (36). Interestingly, most mtDNA mutations are recessive and easily complemented by wild type mtDNA copies. There seems to be a threshold ratio of mutated/wild type mtDNA of approximately 70% before disease symptoms become evident, depending on the mutation and the type of tissue (40, 45).

Both nDNA mutations that affect n-mitoproteins and mtDNA mutations in the 13 genes encoding subunits of the respiratory chain compromise OXPHOS (40, 41, 45). Germline mutations, resulting in a decrease in or loss of expression of succinate dehydrogenase (SDH), fumarate hydratase (FH), and isocitrate dehydrogenase have been reported in inherited paragangliomas, gastrointestinal stromal tumors, pheochromocytomas, myomas, SDH, papillary renal cell cancer (FH), and gliomas (46).

Mutations in mtDNA have been implicated in neuromuscular and neurodegenerative mitochondriopathies (47–49) and complex diseases like diabetes (50), cardiovascular diseases (51, 52), gastrointestinal disorders (53), skin disorders (54), aging (55, 56), and cancer (41).

A recent review by van Gisbergen et al. (41) describes several studies showing that mtDNA germline variations can play a role in tumor growth for hemopoietic cancers, prostate cancer, breast cancer, and renal cancer. The authors also report that somatic mtDNA mutations can be involved in breast, colorectal, bladder, esophageal, head and neck, ovarian, renal, lung and thyroid cancer, and leukemia and can influence cancer progression and metastasis. The effect of somatic mtDNA mutations on tumorigenesis depends on the functional and threshold effects of the mutation (57). Different human populations have different human mtDNA haplotypes, each with a unique fingerprint of mtDNA polymorphisms, passed on through the maternal germline. These haplotypes correlate to the geographic origin of the population. Certain human haplotypes carry a higher risk of developing a particular type of cancer or a neurodegenerative disease during their lifetime than others (8, 41, 58).

More than 50% of mtDNA mutations involved in carcinogenesis are located in the 22 mitochondrial tRNA genes (58). The most common mtDNA mutation is the single nucleotide polymorphism, 3243A > G, which alters leucine mt-tRNA and thus affecting translation of the 13 respiratory subunits, resulting in fewer mitochondrial subunits and impaired OXPHOS (59, 60). Individuals with 10–30% faulty copies of $tRNA^{Leu}$ may develop maternally inherited diabetes and deafness. People with 50–90% faulty copies are likely to develop mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) (50, 59–65). The $tRNA^{Leu}$ mutation results in variable forms of mitochondrial RC deficiency in different patients. By far, the most common finding in MELAS is complex I (RC-I) deficiency, whereas some patients have combined deficiencies of RC-I, RC-III, and RC-IV (59, 66). Other mt-tRNA mutations that play a role in human disease are: $tRNA^{Lys}$, which is associated with myoclonic epilepsy, $tRNA^{Ser}$ with deafness, and $tRNA^{Ile}$ with cardiomyopathies (51).

In addition to mutations affecting the respirasome and tRNAs, a recent review by Gopisetty and Thangarajan (67) summarizes possible roles for mutations in mitochondrial ribosomal small subunit genes (MRPS) in human disease. The authors describe the roles of 30 new MRPS as well as the effect of known MRPS mutations on different cancers and other diseases, including developmental and neurodegenerative diseases, mitochondriopathies, cardiovascular diseases, obesity, and inflammatory disorders. They further provide evidence of the role of MRPS18-2 in carcinogenesis as a potential oncogene. Differential expression of specific MRPS genes has been associated with breast cancer, cervical cancer, non-small cell lung cancer, thyroid tumors, invasive glioblastoma, Burkitt's lymphoma, pediatric hyperdiploid acute lymphoblastic leukemias, testicular germ cell tumors, endometrial carcinoma, and head and neck squamous cell carcinoma (67). Expression levels in cancers were heterogeneous both within the same tumor type and between different cancers. For example, MRPL42 overexpression has been described for breast, carcinoid, liver, endometrial, melanoma, and ovarian cancers. Downregulation was seen in pancreatic, renal, and urothelial cancer. Expression profiles change in response to cisplatin chemotherapy treatment and radiation, indicating a potential role for MRPS genes in cellular responses cytotoxic drugs or serve as biomarkers. Single nucleotide polymorphisms in MRPS genes have also been linked to cancer risk [reviewed in Ref. (67)].

Drivers and Timing of mtDNA Mutations

Until recently, the generally accepted view was that mtDNA mutations are generated by ROS-mediated oxidative damage (36, 41). Generation of ROS in the respiratory chain is inherently part of OXPHOS. ROS play an important part in several signaling processes and their levels are kept in check by antioxidant enzyme systems in the mitochondrial matrix and IMS. However, in situations where OXPHOS is compromised due to misshapen respiratory complexes resulting in increased leakage of electrons to oxygen, ROS levels may overwhelm the antioxidant defense system and damage nearby mtDNA (11, 12).

DeBalsi and colleagues propose that mistakes made by the mtDNA replication and repair machinery can also generate mtDNA mutations (68). Human cells contain 17 distinct human DNA polymerases, but only polymerase gamma (Pol-γ) functions in mtDNA replication and repair. Nuclear-encoded Pol-γ holoenzyme consists of a catalytic subunit and an accessory subunit [reviewed by DeBalsi et al. (68)]. Pol-γ replicates mtDNA with high fidelity due to nucleotide selectivity and proofreading ability with one mis-insertion in every 500,000 new base pairs (69). Over 300 Pol-γ mutations have been linked to human disease, some manifest in adulthood and these are associated with aging, such as various forms of progressive external ophthalmoplegia (PEO) and Parkinson's disease (PD) [reviewed in Ref. (68)]. The importance of Pol-γ in limiting mtDNA mutations was demonstrated by homozygous, but not heterozygous, mutator mice with a proofreading-deficient Pol-γ developing several age-related conditions and having a shortened lifespan. They accumulated mtDNA mutations that were not caused by oxidative damage, as their antioxidant capacities were the same and the extent of oxidative damage was similar to wild-type mice. The mutator mice acquired somatic point mutations, large deletions and multiple linear deleted mtDNA fragments. Another n-mitoprotein involved in mtDNA replication is the mtDNA-specific helicase Twinkle, which unwinds mtDNA for synthesis by Pol-γ [reviewed in Ref. (70)]. Overexpression of Twinkle in transgenic mice led to increased mtDNA copy number and OXPHOS and several twinkle mutations are associated with mitochondrial myopathy (68). Both oxidative damage and faulty replication are likely to contribute to the total mtDNA mutational load of a cell and the contribution of each mutational driver is likely to change over time.

Repair of Faulty mtDNA

For the most part, mtDNA repair pathways mirror those that occur in the nucleus with the same or similar proteins alternatively spliced and targeted to the mitochondria [reviewed by Kazak et al. (71)]. Mitochondria have a robust base excision repair (BER) system, which mainly fixes oxidative DNA damage of mtDNA physically associated with IMM. Single strand breaks are sensed by PARP-1 and repaired by the BER enzymes. There is also evidence of double strand break (DSB) repair, with alternatively spliced nuclear DSB repair proteins or mitochondrial homologs from the non-homologous end joining and homologous recombination pathways present in the mitochondrial matrix. Mismatch repair for replication errors is present in mitochondria but the proteins involved are distinct from those in nuclear mismatch repair (71). The various mtDNA repair pathways employ a myriad of proteins, all of which are nuclear encoded. Although there is a certain amount of redundancy, upregulation, downregulation, and/or point mutations in mtDNA repair proteins will affect mtDNA integrity. Cancer cells with a compromised mtDNA repair capability will accumulate more mtDNA mutations over time. In the event that mtDNA mutations are not removed through fission and mitophagy, increased mtDNA burden will compromise OXPHOS and force a switch to a purely glycolytic metabolism, as described in the next part of this review.

MITO-NUCLEAR CROSS TALK

Most mito-nuclear cross talk is focused on meeting the bioenergetics demands of cells. This will be related to the speed with which cellular demands change and the consequences if these demands are not met. Mitochondria continually update the nucleus of their bioenergetics status (retrograde signaling) by producing a number of energy metabolites (mitostress signals). The nucleus responds by activating stress response signaling pathways aimed at adjusting ATP production to suit the cell's energy requirements. The different mitostress signals and nuclear stress response pathways are summarized in **Figure 2**.

Mitostress Signaling Overview

Decreased MET results in a *decrease in mitochondrial membrane potential* which leads to several mitostress signals that trigger specific nuclear transcriptional responses described in more detail by Arnould et al. (72) and summarized in **Figure 2**. *Decreased mitochondrial ATP levels* cause energy deprivation (high AMP/ATP ratio). This induces AMP-activated protein kinase (AMPK) signaling, which activates peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α), promoting mitogenesis. PGC-1α also decreases mammalian target of rapamycin (mTOR) activity which downregulates energy-demanding anabolic processes, which is mirrored by a lack of Krebs cycle metabolites available for anabolic pathways. *Increased cytosolic Ca^{2+} levels* activate transcriptional regulators such as activating transcription factor, CREB1, NFkB, p53, MEF-2, and PGC-1α. *Increased mitochondrial NADH/NAD^+ ratios* affect the membrane and cytosolic redox potential, causing reductive stress. Changing the NADH/NAD^+ ratio also affects activity of the NAD^+-dependent poly[ADP-ribose] polymerase-1 (PARP-1), involved in DNA repair, and the Sirtuin family. *Increased mitochondrial ROS* result from leakiness of the respiratory chain caused by misshapen respiratory complexes. In most healthy mammalian cells, 95–98% of total oxygen consumption occurs at respiratory complex IV. Premature electron leakage to oxygen occurs at respiratory complexes I, II, and III generating superoxide, although other possible sites of superoxide production have been identified (73). Superoxide is converted to hydrogen peroxide by superoxide dismutases (Cu-ZnSOD in the IMS and MnSOD in the matrix). Hydrogen peroxide in mitochondria is detoxified to water and oxygen by glutathione peroxidase and peroxireductase (73). Under normal circumstances, these antioxidant enzymes neutralize most of the ROS, leaving enough hydrogen peroxide to

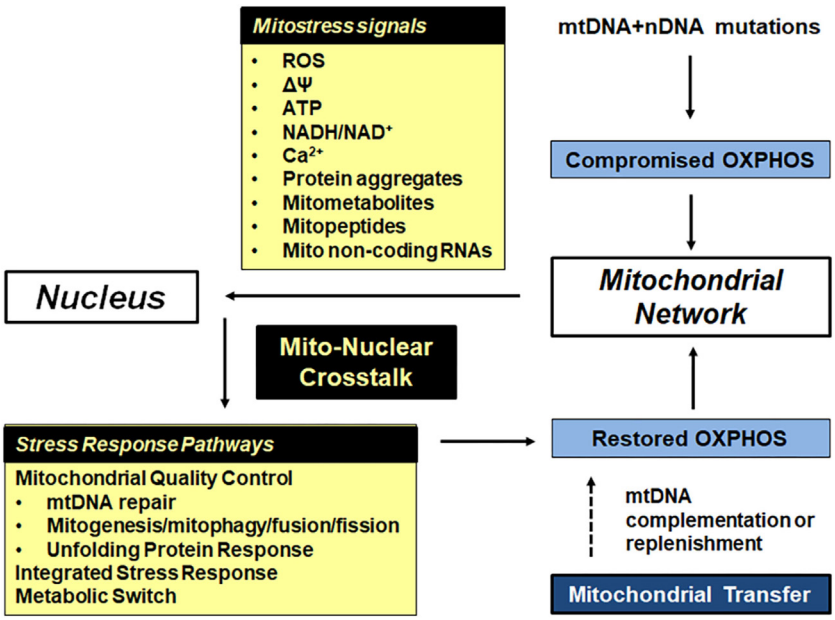

FIGURE 2 | Bioenergetic plasticity is central to the ability of cells to adapt to their ever-changing microenvironment. Mitochondrial and nuclear mutations in respiratory subunits, mitochondrial chaperones and proteases and other mitochondrial proteins can lead to compromised oxidative phosphorylation (OXPHOS). In response, damaged mitochondria send out mitostress signals such as increased reactive oxygen species (ROS) and exhibit decreased inner mitochondrial membrane potential. The latter causes a drop in mitochondrial ATP levels, increased mitochondrial and cytoplasmic NADH, and increased cytoplasmic calcium. In addition, mtDNA and nDNA mutations as well as increased ROS cause aggregation of damaged and misfolded proteins in the mitochondrial matrix, leading to prototoxic stress. Mitometabolites affect epigenetic modifications. The nucleus responds to these mitostress signals by activating one or more stress response pathways, depending on the type and duration of the stress signal. Repair of damaged DNA, dilution of faulty mtDNA copies by fusion and mitogenesis, removal of faulty mtDNA copies by fission followed by mitophagy, removal of protein aggregates by the mitochondrial unfolded protein response (mtUPR), removal of faulty mitochondria by the integrated stress response (ISR) and a shift from mitochondrial to glycolytic/lactate metabolism all ensure that the energy demands of cells are met. If OXPHOS cannot be restored, failing mitochondria are removed though mitophagy. In extreme cases, the cell undergoes apoptosis or replaces faulty mitochondria with functional mitochondria from other cells.

pass through membranes to promote redox signaling through modifications of cysteine residues on redox sensitive proteins, resulting in posttranslational modifications. Redox signaling has been implicated in anti-aging and longevity, promoting protective stress responses and enhanced immunity [reviewed in Ref. (74)]. Excess hydrogen peroxide can be transformed into the highly aggressive hydroxyl radical, responsible for oxidative damage to mitochondrial proteins, lipid and DNA. Both SIRT1 and SIRT3 are activated under increased ROS and help orchestrate increased antioxidant gene expression as well as mitophagy.

Prototoxic Stress

Prototoxic stress is caused by a mismatch in the number/shape of respiratory chain subunits and results in a buildup of redundant/misshapen/unfolded respiratory subunits in the mitochondrial matrix. Both proteotoxic stress and depolarization of mitochondria activate the mitochondrial unfolding protein response (mtUPR), resulting in accumulation of PINK 1 in the IMS, recruitment of PINK2 to the mitochondria, and the removal of defective mitochondria through mitophagy (75).

Mitometabolite Levels

Mitometabolite levels such as acetyl-CoA and S-adenosyl methionine (SAM) affect acetylation and methylation of the nuclear genome, respectively. Changes in the nuclear and mtDNA profiles can directly affect epigenetic regulation and thus cancer progression and metastasis (76). SAM is the primary methyl donor molecule utilized in cellular methylation of proteins, DNA, RNA, and lipids and is synthesized directly from methionine. Both existing DNA, as well as newly synthesized DNA can be dynamically methylated and demethylated (76). In mouse embryonic stem cells, lack of threonine in the growth medium decreased accumulation of SAM and decreased histone methylation, resulting in slowed growth and increased differentiation (77).

Mitopeptides

Recent mitochondrial transcriptome analysis have revealed the existence of several small open reading frames (sORFS) within the 16S and 12S rRNA gene sequences. These sORFS corresponded with small mitochondria-derived peptides (MDPs). The first MDP, humanin, was discovered in 2001 by Hashimoto et al. (78), followed by the discovery of MOTS-c by Lee et al. in 2015 (79) and small humanin-like peptides (SHLP 1–6) by Cobb et al. in 2016 (80). Emerging evidence suggests that MDPs play important roles in the regulation of cellular bioenergetics and system metabolism by modulating insulin sensitivity and glucose homeostasis [reviewed by Kim et al. (81)], but whether these hormone-like peptides are *bona fide* retrograde signaling

molecules that modulate nuclear gene expression or induce epigenetic changes intracellularly or in other cells remains to be determined. The 24 amino acid humanin (located within the 16S rRNA gene) has been shown to be strongly neuroprotective, antiapoptotic, and protects against ischemia/reperfusion injury possibly due to a decrease in ROS generation (78). Humanin directly affects mitochondrial bioenergetics by increasing basal OCR, respiration capacity, and ATP production and increases mtDNA copy number and the number of mitochondria. Humanin also plays a role in lipid metabolism by decreasing body weight gain, visceral fat, and hepatic triglyceride accumulation together with an increase in activity level in high-fat diet-fed mice (81). Injecting humanin improved pancreatic islet function and insulin sensitivity in non-obese diabetic mice and prevented diabetic progression in some animals (82). In humans, humanin is found in the brain, hypothalamus, heart, vascular wall, blood plasma, kidneys, and testes (83). Humanin could be considered a mitohormone with plasma levels adjusting to cellular oxidative stress levels. In support of this notion, low levels of oxidative stress, as seen in prediabetic patients with slightly increased blood glucose levels, had significantly lower plasma humanin levels than healthy control patients (83). This could be considered a positive adaptation to mild-moderate oxidative stress which may promote longevity in a similar manner to dietary caloric restriction (84). As oxidative stress increases, mitochondria would then significantly upregulate humanin levels as seen in patients with advanced mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS), and chronic progressive external ophthalmoplegia (85, 86).

The six SHLP peptide sequences (20–38 amino acids long) are also found within the 16S rRNA gene (80), with SHLPs1-5 being on the antisense light strand. SHLP2 and SHLP3 have similar protective effects as humanin and both improved mitochondrial metabolism by increasing oxygen consumption rate and ATP production, mitochondrial biogenesis and by reducing apoptosis and ROS levels. SHLP2 and SHLP3 enhance insulin sensitizing effects *in vitro* and *in vivo* (81). MOTS-c is a 16 amino acid peptide located within the 12S rRNA gene [reviewed in Ref. (87)]. MOTS-c increases glucose uptake and glycolysis through AMPK activation, whereas it suppresses mitochondrial respiration in cultured cells and skeletal muscle. This resembles a Crabtree effect-like phenomenon, namely, decreased mitochondrial OCR in response to high glucose uptake. MOTS-c is also closely associated with amino acid and lipid metabolism. MOTS-c enhances whole body insulin sensitivity, acting primarily through the muscle. MOTS-c further prevents HFD-induced obesity and insulin resistance in CD-1 mice and prevents HFD-induced obesity independent of caloric intake in C57BL/6J mice (87).

Mitochondrial Non-Coding RNAs

Large parts of the non-coding nuclear genome, which itself represents more than 98% of the total genome, are transcribed into various types of non-coding RNA, which include rRNAs, tRNAs, small nucleolar RNAs, small nuclear RNAs, and the more recently identified microRNAs (miRNAs), and long non-coding RNAs (lncRNAs). According to the last GENCODE release (v25), the human genome contains more than 4,000 miRNA and 15,000 lncRNA genes. A very recent review by Vendramin and colleagues describes in detail the roles of different types of non-coding RNAs as modulators of mitochondrial function (88). The 22 nucleotide long miRNAs are highly conserved non-coding RNAs that have been implicated in a large variety of patho-physiological processes including aging and cancer. They inhibit translation of mRNA targets in the cytoplasm, by binding to them and recruiting the RNA-induced silencing complex. Many non-coding RNAs have evolved to allow cells to cope with stress and several miRNAS have been shown to play a role in tumorigenesis, both as oncogenes and tumor suppressors (88).

MicroRNAs have been found inside mitochondria of mtDNA competent cells while being absent in their mtDNA deficient ρ^0 counterparts (89) These mito-miRNAs could have been nuclear or mitochondrially encoded and would have the potential to bind and prevent translation of mito-messenger RNAs. A decrease in the number of mitochondrially encoded respiratory subunits would affect respiratory subunit stoichiometry and thus mito-nuclear cross talk, resulting in cells switching to a more glycolytic metabolism, which is described in more detail below. Two mitochondrially encoded miRNAs were described very recently by Gao et al. after re-analyzing a public PacBio full-length transcriptome dataset, producing the full-length human mitochondrial transcriptome (90). The authors propose that these miRNAs, through sense–antisense interactions with mRNAs, regulate the transcription of the RC subunits, and thus control MET and OXPHOS activity. Interestingly, the transcription level of these miRNAs was significantly higher in normal tissues compared with hepatocellular carcinoma, indicating a loss of regulatory control (90).

Metabolic Shift

Glycolysis is the common energy-generating pathway used by all mammalian cells; it oxidizes glucose to pyruvate in the cytoplasm, generating 2ATP/glucose through substrate phosphorylation. In the presence of oxygen, cells with a functional respiratory chain will further oxidize pyruvate to carbon dioxide in the Krebs cycle, generating 2ATP/glucose. Reoxidation of NADH and $FADH_2$ during MET ideally generates an additional 30-32ATP/glucose through OXPHOS. Under hypoxic conditions, some normal cells (myocytes, hepatocytes, erythrocytes, and adipocytes) and most cancer cells reoxidize NADH produced during glycolysis *via* LDH that reduces pyruvate to lactate, and through a short evolutionarily conserved electron chain in the plasma membrane (PMET). PMET could be a potential evolutionary remnant of an ancient pathway responsible for preventing intracellular reductive stress due to buildup of NADH during glycolysis. A number of different PMET pathways have been described in yeasts, plants and mammalian cells [reviewed in Ref. (4)]. A PMET system, active in highly proliferative glycolytic cells (both non-transformed and cancer cells), uses oxygen as a terminal electron acceptor, reminiscent of MET (91, 92). Cell surface oxygen consumption has been reported for a number of cancer cell lines and can be 2–3× higher in cells devoid of mtDNA (ρ^0 cells) (91–93). Cell surface oxygen consumption

also contributes to the acidification seen in glycolytic cells, due to increased LDH activity resulting in increased lactate production (92). Cell surface oxygen consumption together with LDH activity are required for maintaining intracellular NADH/NAD^+ balance of highly glycolytic cancer cells, and thus their invasive and metastatic potential. In support of this, inhibition of PMET by the external redox cycler, phenoxodiol, was shown to promote apoptosis in a range of cancer cell lines (94–96) as well as leukemic blasts from patients with myeloid and lymphoid leukemias (97, 98). Cell-impermeable drugs targeting PMET may, therefore, represent useful additional tool in preventing growth, invasion, and metastasis of highly glycolytic cancers (4, 99–101).

The metabolic shift from OXPHOS to aerobic glycolysis in rapidly proliferating cells, including cancer cells is controlled by hypoxia-inducible factor 1α which is highly expressed in most solid cancers [reviewed in Ref. (102)]. However, even under aerobic conditions, many, but not all, cancer cells rely to a large extent on glycolysis to meet their energy demands. This allows them to use glycolytic intermediates for anabolic processes and escape the effects of high ROS levels at the expense of additional OXPHOS energy. Otto Warburg was the first person to describe the phenomenon of aerobic glycolysis (the Warburg effect) in the 1920s in Ehrlich ascites cells (103). However, other than for cells unable to use OXPHOS due to an assortment of mutations, this scenario has proven to be too simplistic. Many cancer cells still use OXPHOS to increase their bioenergetic potential and generate low levels of ROS for signaling purposes. It seems that the glycolysis to OXPHOS shift is more like a rheostat, facilitating a dynamic adjustment of the proportion of energy gained from glycolysis and OXPHOS depending on demand and the microenvironment (20). For example, ionizing radiation causes re-oxygenation in previously hypoxic tumors. In this scenario, cancer cells with the flexibility to adjust readily between glycolysis and OXPHOS would have a distinct survival advantage. In support of this, Lu and colleagues recently showed that exposing human MCF-7 breast cancer cells, HCT116 colon cancer, and U87 brain cancer cells to a single dose of 5 Gy caused a switch from aerobic glycolysis to OXPHOS, increasing their bioenergetic capacity and conferring radiation resistance (104). They reported that mTOR, a serine/threonine kinase of the PIK3 family and highly expressed in cancer cells, translocated to the OMM after radiation. There, mTOR bound to and inactivated hexokinase II, inhibiting glycolysis and reactivating OXPHOS (104).

Mitochondrial Quality Control

In general, a shortfall in ATP levels is caused by a lack of respiratory units, increased energy demands by the cell, or transient hypoxia. In these cases, mitochondrial quality control restores the bioenergetics capacity of the cell for differentiated function by increasing the mitochondrial network through mitogenesis. In contrast, increasing glycolysis and removing excess mitochondria through autophagy (mitophagy) favors rapid cell proliferation (see **Figure 3**).

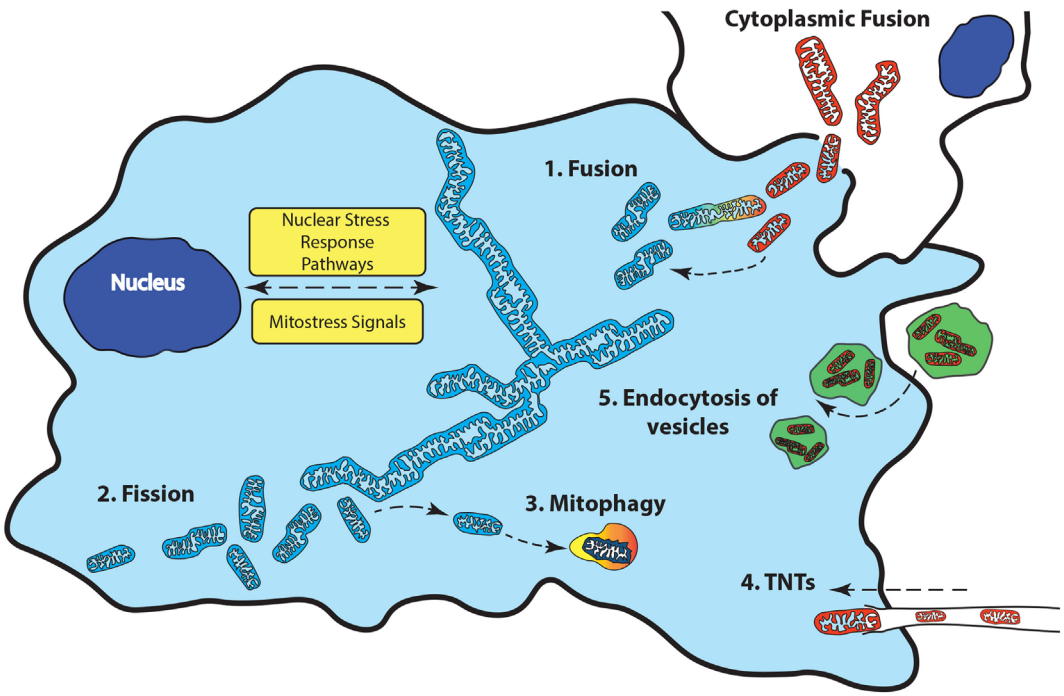

FIGURE 3 | Mitochondrial quality control involves changes to the mitochondrial network to maintain bioenergetics homeostasis. Fusion (1) of additional mitochondria to the existing network occurs when the bioenergetics demands of the cell are not met. When damaged mitochondria cannot be repaired, they can be removed from the network through fission (2) followed by removal from the cell though mitophagy (3). Transfer of functional mitochondria from external sources through tunneling nanotubules (TNTS) (4), vesicles (5), or direct cytoplasmic fusion (6) can replenish a damaged or deficient mitochondrial network.

Mitogenesis

Mitogenesis is regulated by mitochondrial and nuclear-encoded structural proteins as well as n-mitoproteins involved in mtDNA transcription, translation, and repair. Expression of the master regulator of mitochondrial biogenesis, PGC-1α is increased with increased energy demands after exercise in skeletal and cardiac muscle as well as upon fasting in the liver, or after cold exposure in brown fat cells. PGC-1α regulates the transcription levels of a number of genes involved in metabolic pathways such as gluconeogenesis, fatty acid synthesis, and oxidation, promoting mitochondrial biogenesis, angiogenesis, and aerobic respiration [reviewed in Ref. (105)].

Regulation of PGC-1α occurs at the level of expression and a variety of posttranslational changes that regulate its activity and stability (phosphorylation, acetylation, and ubiquitination). Activation of the p38 mitogen-activated protein kinase (p38 MAPK) increases PGC-1α expression and stability of the protein in brown fat, muscle and liver, which increases gluconeogenesis. The energy sensor AMPK also induces PGC-1α transcription and enhances its activation through Sirt1-mediated deacetylation when ATP/ADP levels are low (105). This in turn allows for the regulation of the downstream pathways controlled by PGC-1α. One of the targets of PGC-1α, is the newly discovered peptide hormone, irisin, secreted by muscle cells after exercise (106). Increased irisin expression caused browning of subcutaneous adipose tissue (thermogenesis), increased oxygen consumption, reduced obesity and insulin resistance in mice given a high fat diet (106).

The effects of PGC-1α in cancer cells mimic those in normal cells including mitochondrial biogenesis and increased OXPHOS, with the added effect of promoting invasion and metastasis (107). Circulating breast cancer cells have been found to exhibit enhanced mitochondrial biogenesis and respiration as a result of increased PGC-1α expression, leading to an increased rate of metastasis (107). PGC-1α also increased resistance to cisplatin of ascites-derived cancer cells from ovarian cancer patients with advanced disease (108). In addition, expression of PGC-1α and TFAM were increased in high grade serous ovarian cancers that were highly chemoresistant (109).

Mitophagy

Mitophagy is crucially important in removing superfluous or faulty mitochondria from the cell. Mitophagy is triggered by the PTEN-induced putative kinase 1 (PINK1)/Parkin pathway. This pathway is activated by membrane depolarization which is a signal of mitochondrial dysfunction caused by hypoxia, lack of NADH, and/or a limited number or ill-fitting dysfunctional respiratory complexes. Stabilization of PINK1 on the depolarized OMM directly phosphorylates Parkin, which ubiquinates a number of OMM proteins leading to their degradation through the 26S proteasome and recruitment of the autophagosome. Other Parkin-dependent and independent mitophagy pathways have been described and have been reviewed in detail by Gumeni and Trougakos (110).

Faulty mtDNA copies can be diluted out through continued cycles of fusion/fission events. Fusion of several individual mitochondria into the larger network allows for complementation of mtDNA variants to maintain mitochondrial function. Parts of the mitochondrial network with a high mutational load can be isolated through fission and eliminated through mitophagy (111, 112). *Fusion* is orchestrated by three GTPases; the mitofusins, Mfn1, and Mfn2, are involved in fusion of the OMM, whereas optic atrophy-1 (Opa1) is responsible for fusing the IMM and is also involved in cristae remodeling (111–113). *Fission* is driven by recruitment of dynamin-related protein 1 (Drp1) to receptors on the OMM where it causes constriction of both the OMM and IMM (1). Drp1 translocation and activity is regulated by multiple kinases that respond to distinct cell cycle and stress conditions (113).

Removal of Protein Aggregates by mtUPR

Accumulation of ROS-damaged/unfolded/misfolded proteins in the mitochondrial matrix is called prototoxic stress. The mitochondrial unfolded protein response (mtUPR) is responsible for degrading protein aggregates in the mitochondrial matrix and IMS. Two comprehensive reviews describe the process of unfolding, translocation, and refolding of precursor proteins, as well as degradation of damaged/misfolded/unfolded proteins by ATP-dependent and ATP-independent proteases and oligopeptidases (72, 110). Most of our knowledge of the mtUPR pathway and its integration with other stress pathways has been obtained from research with *C. elegans*. The mtUPR in mammals is still poorly defined with respect to signaling pathways and target genes. Two separate mtUPR pathways have been described, one deals with protein aggregates in the mitochondrial matrix and the other resolves protein aggregates in the mitochondrial IMS (72, 110). An interesting recent review by Nuebel and colleagues explores the roles of many newly identified proteins unique to the IMS in mitochondrial and cellular homeostasis (114). This mitochondrial compartment represents a uniquely oxidizing redox environment. The IMS proteome is involved in protein and lipid transport across the OMM and IMM, apoptosis, redox homeostasis, ROS signaling, and MET. Accumulation of unfolded or aggregated proteins is a hallmark of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. Prototoxic stress in the mitochondrial matrix is also a common occurrence in cancer cells and many, but by no means all cancer types, have an activated mtUPR response (110).

Integrated Stress Response (ISR)

The ISR is an evolutionary conserved adaptive stress pathway. In mammals, the ISR is activated under oxidative stress, ER stress, depletion of amino acids, glucose and haem, viral infection, or UV irradiation. Four different kinases phosphorylate the eukaryotic translation initiation factor 2α (eIF2α), a key event in ISR [described in detail in Ref. (72)]. Activation of eIF2α reduces ATP-consuming processes such as protein synthesis, helps stabilize Ca^{2+} storage in the ER and mitochondria, and maintains mitochondrial function. If mitochondrial function cannot be recovered, the ISR can initiate autophagy or apoptosis. Mitochondrial ISR is triggered by mitochondrial dysfunction caused by mtDNA damage, mtDNA depletion, and oxidative

stress, whereas mtUPR is specifically triggered by protein aggregates buildup in the mitochondrial matrix. However, it is likely that these two stress pathways overlap as phosphorylation of eIF2α is a common feature of both pathways.

MITOCHONDRIAL TRANSFER BETWEEN CELLS

So far, this review has covered the many different ways that cells address inadequate mitochondrial performance. However, what happens when mito-nuclear cross talk fails, mtDNA and nDNA mutations that affect mitochondrial function accumulate, ATP levels fall and biosynthetic pathways wind down? Until fairly recently, the answer to these questions would have been clear: a decrease in metabolic rate and ultimately, cell death would ensue. However, recent research has shown that cells may be able to obtain functional mitochondria from other cells in order to satisfy their bioenergetics and biosynthetic needs.

The traditional cell biology dogma that mitochondria and mtDNA remain within the constraints of their host cell has recently been questioned. Several studies have shown that mitochondria can move between cells *in vitro* (115–124). Furthermore, we recently showed that tumorigenesis of murine melanoma and breast cancer cell lines without mtDNA depended on their ability to obtain mtDNA from host mouse cells in the microenvironment (125, 126).

Transfer of functional mitochondria was also shown to confer a survival advantage in several mouse models. For example, bone marrow-derived stromal cells were able to rescue lipopolysaccharide-induced acute lung injury in alveolar epithelia of mice (127) while transfer of mitochondria from mesenchymal stem cells (MSCs) protected epithelia by decreasing mitochondrial ROS in a mouse model of airway injury and allergic airway inflammation (128). Transfer was enhanced when the donor cells overexpressed Miro1, a mitochondrial Rho-GTPase. In other recent publications, astrocytes were shown to increase ATP levels and viability of neurons in a mouse ischemia model by donating healthy mitochondria contained in vesicles (129), while stromal cells transferred mitochondria to immortalized acute myeloid leukemia (AML) cells in an immunocompromised mouse xenograft model in response to chemotherapy-induced apoptosis (130). This transfer occurred *via* endocytosis from stromal cells to the AML cells, and increased ATP production, viability, and survival of AML cells was reported. Mitochondrial transfer has also been shown to rescue aerobic respiration in carcinoma cells (116) and increase survival in an adrenal gland cell line (117). Together, these results suggest that intercellular mitochondrial transfer plays a role in cellular communications, intracellular metabolic homeostasis or exists as a mechanism to support cells under physiological stress. In support of this, recent research demonstrated that cells exposed to injury have improved survival when introduced to healthy cells as an extracellular source of mitochondria (115, 127, 128, 131). When exposed to intentional injury such as chemotherapy or radiation, fragmentation of mtDNA occurs alongside damage to the nuclear genome. The resulting mitochondrial dysfunction in the absence of nuclear DNA damage can be toxic (132) and/or contribute to the mechanism of action of several cancer therapies. Circumvention of mtDNA damage by uptake of mitochondria from other cells could lead to treatment resistance.

Although most published work refers to mitochondrial transfer as a way to replace dysfunctional mitochondria, cells could also transfer dysfunctional mitochondria (133, 134). This may be particularly relevant in both Alzheimer's and Parkinson's disease where an increase in mitochondrial dysfunction is correlated with progressive degenerative phenotypes (111–113). Mutations in mtDNA and disrupted mitochondrial homeostasis are common across many neurodegenerative diseases and lead to mitochondrial dysfunction (114). Affected cells could manage the increase in faulty mtDNA copies by intercellular transfer of dysfunctional mitochondria that escape mitophagy.

The types of cells that are able to donate mitochondria, as well as the communication between recipients and donor cells that drive this transfer remain largely unknown. The exact mechanism(s) of mitochondrial transfer has also not been fully elucidated. Tunneling nanotubes (TNTs), extracellular vesicles and direct cellular contact have all been suggested to facilitate mitochondrial transfer between cells (see **Figure 3**).

Tunneling Nanotubes

Emerging research into the role of TNTs has revealed that these open-ended, F-actin containing intercellular structures can act as a conduit for intercellular transfer of various biomaterials, inclusive of, but not limited to mitochondria (115, 131, 135–138). First described in 2004 (139), movement of organelles through TNTs has received much attention, particularly from investigators engaged in MSC research. Numerous recent studies conclude that intercellular mitochondrial transfer contributes to the protective or restorative properties of MSC seen both *in vitro* and in multiple animal injury models [reviewed in Ref. (140)]. The formation of TNT-like structures is closely associated with the physiological state of the cells, making TNTs likely candidates for facilitating intercellular mitochondrial transfer *in vivo* (133, 141–143). However, due to the challenges in identifying and characterizing open-ended TNTs among a multitude of other subcellular tube-like structures, definitive evidence in support of TNT-mediated mitochondrial transfer *in vivo* remains elusive (144). Identification of TNTs is currently limited to generalized morpho-temporal characteristics determined by time-lapse confocal fluorescence microscopy *in vitro* (129). Identification of specific markers for TNTs and other similar structures engaged in intercellular mitochondrial transfer is a prerequisite for further progress in the field.

Extracellular Vesicles

Microvesicles, exosomes, apoptotic bodies, and oncosomes are biological particles which fall under the broad categorization of "extracellular vesicles." Vesicle size, molecular content, and the origin of the particles determines their specific nature and biological role [reviewed in Ref. (145)]. Microvesicles make up the largest category of extracellular vesicles and consist of particles of up to approximately 1 μm in size. Like other extracellular vesicles, microvesicles bear proteomic signatures that allow cellular uptake *via* endocytosis or phagocytic mechanisms. Their molecular contents can exert a broad range of effects on cell physiology.

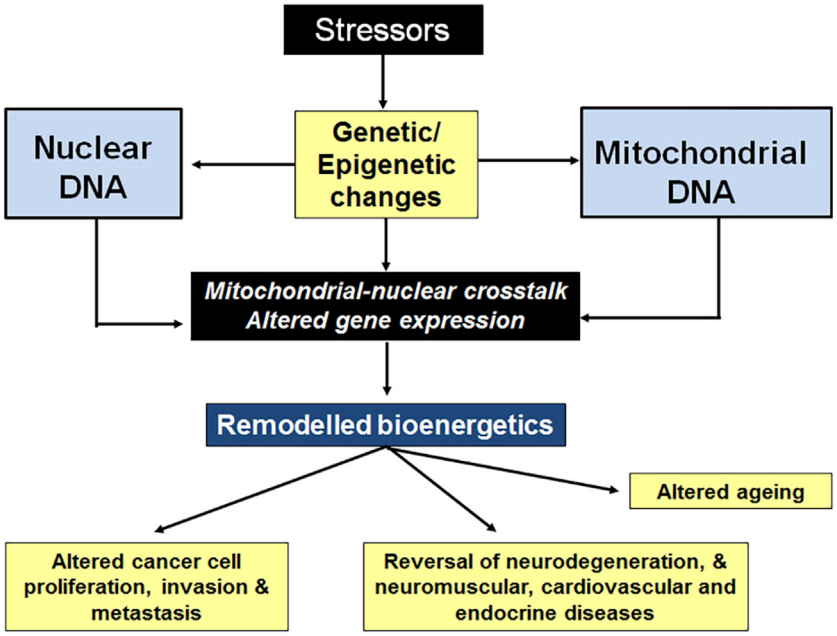

FIGURE 4 | Stressors affect nuclear cross talk through changes in gene expression. Stressors such as oxidative stress, radiation, chemotherapeutic drugs, hypoxia, etc., cause genetic and epigenetic changes to both nDNA and mitochondrial DNA (mtDNA). The resulting changes in gene expression result in altered cellular bioenergetics, often leading to decreased oxidative phosphorylation. Mitochondrial stressors (a decrease in mitochondrial membrane potential, ATP levels, NADH levels, and increased mitopeptide expression, etc.) elicit nuclear stress responses. Stress pathway activation (mtDNA damage repair, mitochondrial biogenesis and fusion, switching to glycolytic metabolism, etc.) results in a return to bioenergetics homeostasis, restoring cellular function.

Numerous studies of intercellular mitochondrial transfer report mtDNA as well as intact mitochondria can be partitioned into microvesicles from specific cell types, suggesting a vesicular mechanism for uptake of exogenous mitochondria by recipient cells. The first published study of intercellular mitochondrial transfer by Spees et al. (115) reported secretion of extracellular vesicles containing mitochondrially targeted fluorescent proteins by human mesenchymal stem cells into growth medium and uptake by recipient cells. These particles were also found to play a role in intercellular transfer of mitochondria by Islam et al. (127) who observed connexin43-mediated uptake of bone marrow-derived MSC mitochondria in microvesicles by lung epithelium. Evidence for mitochondrial and mtDNA transfer mediated by extracellular vesicles has seen steady development across many different cell types, and continues to expand as a new field of intercellular communications (129, 133, 141, 143, 144).

Partial or Complete Cell Fusion

Perhaps the least explored mechanism in the existing mitochondrial transfer literature, the acquisition of exogenous mitochondria *via* partial or complete cell fusion, is an interesting concept. Given the notion that the majority of cells exist in a state of individual compartmentalization, cells engaged in these types of intimate interactions may not be limited to traditional syncytial candidates such as osteoclasts or skeletal muscle cells. Cell fusion may be more widespread and important within normal biological function than traditionally thought—this is a somewhat challenging proposition. There is precedence for certain cell types, particularly those derived from the bone marrow, to spontaneously fuse with other cell types including cardiomyocytes, hepatocytes, and Purkinje neurons (146–150). Partial fusion events or alternatively, syncytial mixing through intercellular structures, provides opportunities for the acquisition of mitochondria from surrounding cells. An example of this would be the tumor networks interconnected by tumor microtubes (distinct from TNTs) in primary glioblastomas, that communicate *via* connexin43 gap junctions (151, 152). Spees and colleagues (115) demonstrated mitochondrial transfer without the uptake of nuclear associated polymorphisms, excluding complete cell fusion in their system. Regardless, restoration of bioenergetic status and cellular regeneration *via* fusion-like mechanisms (150, 153) remains a potential mechanism in future studies of intercellular mitochondrial transfer.

CONCLUDING REMARKS

The ability to adapt cellular bioenergetics capabilities to meet rapidly changing environmental conditions is mandatory for cellular function and for cancer progression. Any compromise in this adaptive response has the potential to compromise cellular function and render the cell more susceptible to external stressors such as oxidative stress, radiation, chemotherapeutic drugs, hypoxia, etc. Mito-nuclear cross talk, involving the generation of different mitochondrial stressors as well as the nuclear stress response pathways to deal with those stressors is capable of maintaining bioenergetics homeostasis under most conditions (see **Figure 4**). Although many mito-nuclear stress signaling pathways have been described (see Mito-Nuclear Cross Talk), a

detailed understanding of how these pathways work together to ensure that mitochondrial and nuclear transcription are closely coordinated to meet the dynamic bioenergetic and metabolic demands of the cell remain poorly understood. For example, the way in which the 13 mitochondrially encoded proteins of the mitochondrial RC that are made in mitochondria are combined with 80 nuclear-encoded proteins that are translated on distinct cytoplasmic protein synthetic machinery and imported into mitochondria where they are assembled into functional RCs is not fully understood. In addition, the role of the eight mitopeptides encoded by mitochondrial *rRNA* genes in bioenergetics and metabolic regulation is under intense scrutiny. Whether or not mitopeptides, or the lncRNA molecules transcribed from the light chain of mtDNA play a role in intracellular mito-nuclear cross talk is not known. The existence of many serious diseases caused by mitochondrial dysfunction, such as the neuromuscular and neurodegenerative mitochondriopathies (47–49), diabetes (50), cardiovascular diseases (51, 52), gastrointestinal disorders (53), skin disorders (54), aging (55, 56), and cancer (41), shows that mito-nuclear cross talk can fail. The ability to replace a dysfunctional mitochondrial network with fresh functional mitochondria from healthy cells is a recently discovered and currently poorly understood phenomenon. Mitochondrial transfer poses a series of intriguing questions: is mitochondrial transfer between cells a fundamental physiological process, silent until recently because of limited tools available for tracking mitochondrial movement between cells? If mito-nuclear cross talk is fundamental to cellular bioenergetic homeostasis, how does this process change when the original nucleus is faced with mitochondria from different cell types and/or with different genetic backgrounds following transplantation? Are the signals that promote mitochondrial transfer between cells related to those that are involved in mito-nuclear cross talk? Is this a process of trial error that takes time to be perfected?

On another front, comparative transcriptome analysis of both protein coding and non-coding mitochondrial and nuclear genomes, under various conditions could provide unbiased information about the immediate consequences of mito-nuclear cross talk that result in respiration and metabolic remodeling. Such analyses are likely to reveal unexpected regulatory roles for transcripts that could challenge current dogma about the stoichiometry and function of mitochondrial- and nuclear-encoded RC subunits, and the control of respiration. Large datasets often contain both mitochondrial and nuclear transcript information but these have rarely been mined for their comparative transcript information.

Finally, we are optimistic about the potential to look beyond current technological and conceptual horizons to better understand how bioenergetic and metabolic remodeling play out in health and disease. Cancer cells display enhanced plasticity with respect to metabolic remodeling at different stages of initiation, invasion, and metastasis, and in response to the many stressors they encounter in their rapidly changing microenvironment.

AUTHOR CONTRIBUTIONS

Concept of the review: MB; design of the review: MB and PH. PH wrote the review with contributions from MB, MR, and GC; PH made **Figures 1**,**3** and **4**; MR made **Figure 2**. All authors were involved with finalizing the manuscript and all approved the final version.

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REVIEW
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E2F1, a Novel Regulator of Metabolism

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In the past years, several lines of evidence have shown that cell cycle regulatory proteins also can modulate metabolic processes. The transcription factor E2F1 is a central player involved in cell cycle progression, DNA-damage response, and apoptosis. Its crucial role in the control of cell fate has been extensively studied and reviewed before; however, here, we focus on the participation of E2F1 in the regulation of metabolism. We summarize recent findings about the cell cycle-independent roles of E2F1 in various tissues that contribute to global metabolic homeostasis and highlight that E2F1 activity is increased during obesity. Finally, coming back to the pivotal role of E2F1 in cancer development, we discuss how E2F1 links cell cycle progression with different metabolic adaptations required for cell growth and survival.

Keywords: E2F1, cell cycle regulators, cancer metabolism, obesity, metabolic diseases

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INTRODUCTION: A CELL CYCLE PROTEIN WITH NEW SKILLS

The E2F transcription factors were first identified as proteins that were able to bind to the promoter of the adenoviral gene E2 (1). Eight E2F genes (*E2F1-8*) have been described to date, which can be classified based on their protein structures, their interaction partners, and their transcriptional properties (2). E2F1 was the first member of the E2F family to be identified because of its ability to bind the retinoblastoma protein (pRB), a tumor suppressor mutated in many types of cancer (3, 4). The activity of E2F1 is dependent on its binding partners, which include dimerization proteins (DP) and the retinoblastoma family proteins (also known as "pocket proteins"), composed by pRB (*RB1*), p107 (*RBL1*), and p130 (*RBL2*) (5). E2F1–pRB interaction blocks the transcriptional activation domain of the E2F1–DP complex and prevents the recruitment of transcriptional co-activators to the promoters of its target genes (6). During cell cycle progression, cyclin-dependent kinases (CDKs) phosphorylate pRB, releasing E2F1, which is then available to promote the expression of genes involved in S-phase entry, DNA synthesis, and mitosis (7–9).

Three decades after its discovery, it is now clear that the control of cell cycle represents only a subset of the E2F1 roles, which include the regulation of apoptosis (10), senescence (11), and DNA-damage response (12). Indeed, genome-wide location studies have revealed that E2F1 binds to hundreds of promoter regions of genes involved in a myriad of cellular pathways (13–16). What ultimately determines E2F1 distinct biological functions are its protein levels, the combination of several posttranscriptional modifications and its interaction with different partners (17). The intricate role of E2F1 as a master regulator of cell fate has been extensively examined before and is out of scope for this review (17, 18). Instead, here, we want to focus on the recent research evidencing that E2F1 is a master regulator of metabolism both in normal and pathological conditions.

E2F1 REGULATES METABOLISM IN NON-PROLIFERATIVE CONDITIONS AND CONTRIBUTES TO GLOBAL METABOLIC HOMEOSTASIS

Role of E2F1 in Normal Physiology

Despite the critical function of E2F1 in cell proliferation, *E2f1*$^{-/-}$ mice undergo normal development, likely due to the compensation by other E2F family members (19, 20). However, *E2f1*$^{-/-}$ mice present some metabolic perturbations that highlight its specific role in the regulation of metabolism independently from cell cycle control. E2F1 participates in the development and the differentiation of several tissues involved in global metabolic homeostasis, but it is also implicated in specific metabolic functions of fully differentiated organs like pancreas, adipose tissues, muscle and liver (**Figure 1**).

E2f1/E2f2 mutant mice show severe exocrine atrophy of pancreatic β cells, primarily resulting from E2F1 mutation, which leads to insulin-dependent diabetes (21). E2F1 promotes β cell proliferation and differentiation through the regulation of the endocrine markers PDX-1 and Neurogenin 3 (22, 23). In addition, in fully differentiated β cells, E2F1 directly controls the expression of the major subunit of the ATP-sensitive K^+ channel Kir6.2, hence promoting glucose-stimulated insulin secretion (24). These studies show that E2F1 participates in pancreas development, maintenance, and endocrine function, hence contributing to global glucose homeostasis.

In the adipose tissue, E2F1 promotes adipogenesis though the regulation of *PPARG* and *RIP140* gene expression, two master regulators of adipocyte fate and differentiation (25, 26). Moreover, in mature adipocytes E2F1 can form a repressor complex with TRIP-Br2—a transcriptional co-regulator—which inhibits lipolysis and mitochondrial β-oxidation (27). Interestingly, CDK4, the main E2F1 upstream activator during cell cycle progression, also promotes adipogenesis though PPARG activation and in mature adipocytes it sustains insulin signaling by phosphorylation of the Insulin Receptor Substrate 2(28, 29). Altogether, these findings show that the canonical CDK4-pRB-E2F1 axis is essential for adipogenesis and to maintain adipocyte function.

In contrast to white adipose tissue, E2F1 represses mouse myogenic differentiation by inhibiting the transcription factors MyoD and Myogenin (30, 31). MyoD in turn, promotes the expression of the Kelch Repeat and BTB Domain Containing Protein 5 (Kbtbd5), which represses E2F1 activity in a negative feedback loop by the ubiquitination and degradation of DP1 (32). Conversely, in *Drosophila*, depletion of the dE2F1 blunts the expression of late myogenic markers during muscle differentiation, which is critical for survival (33). The differences between the two organisms are puzzling and require further exploration, but they may rely on the fact that in *Drosophila* there are only two E2F isoforms, dE2F1 and dE2F2, which work as activators and repressors of transcription, respectively.

Chromatin immunoprecipitation (ChIP) analysis revealed that in basal conditions E2F1 and pRB form a repressor complex in the promoters of several genes involved in oxidative metabolism and mitochondrial biogenesis in muscle, but also in brown adipose tissue (34). In response to exercise or cold exposure, pRB is phosphorylated in muscle and brown adipose tissue, respectively, and mitochondrial and thermogenic genes are expressed

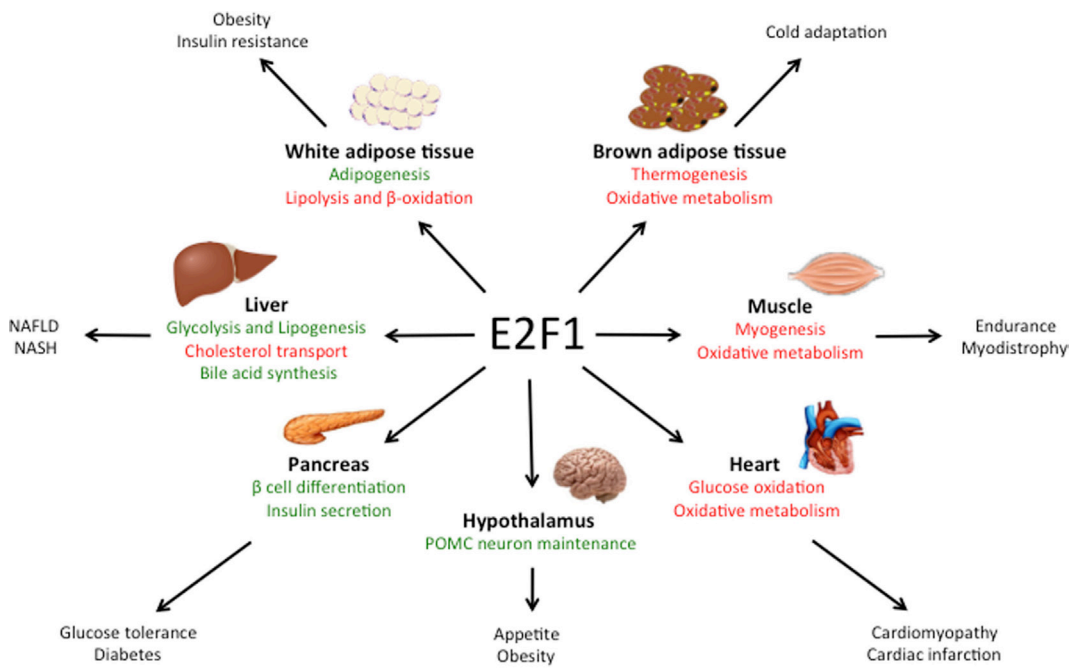

FIGURE 1 | Main roles of E2F1 in metabolic tissues. E2F1 participates in the differentiation of several tissues, but also in the regulation of specific metabolic functions in fully differentiated organs, thus contributing to global metabolic homeostasis. Moreover, during obesity, E2F1 activity is increased and it contributes to some of the comorbidities of this pathological condition. Pathways activated by E2F1 are represented in green while pathways repressed by E2F1 are in red.

(34, 35). As a consequence, deletion of E2F1 in mice results in better resistance to fatigue during exercise and higher body temperature upon cold stimulation due to increased oxidative metabolism (34). Strikingly, E2F1 depletion in a dystrophic mouse model significantly improved muscle performance by increasing muscle oxidative metabolism (36).

Some studies using pRB lack of function models support the role of the E2F1–pRB complex as a negative regulator of oxidative metabolism. For instance, adipose-specific RB1-deficient mice are resistant to high-fat diet (HFD)-induced obesity and display increased mitochondrial activity in white and brown adipose tissues (37). This was reproduced in RB1-haplosufficient mice (38). However, the HFD-resistant phenotype of RB1-deficient mice could also be attributed to the role of pRB in promoting white versus brown fat cell differentiation (35, 39), as evidenced by the increased expression of the thermogenic protein UCP1 in both white and brown adipose tissue depots (37, 38). Additionally, acute loss of pRB or depletion of p170 increased mitochondrial content and activity in muscle cells (40, 41). Conversely, other studies report that pRB may in fact promote mitochondrial biogenesis. Deletion of *RB1* led to impaired mitochondrial function in myocytes (42) and erythrocytes (43). More recently, it was shown that acute pRB loss in adult mice results in a decreased content of oxidative phosphorylation proteins in the lung and in the colon (44), while RB1 depletion blocked muscle differentiation due to an impairment in oxidative metabolism (45). The above confounding studies evidence the relevance of the E2F1-pRB complex in the control of oxidative metabolism in highly metabolic tissues, but they highlight that its specific function may be context dependent. It should also be taken into account that pRB loss of function also leads to multiple E2F1-independent effects (4). Moreover, the fact that E2F1 can activate or repress its target genes often complicates the understanding of the phenotype of E2f1 knockout models.

Role of E2F1 in Metabolic Diseases

Obesity is associated with increased risk of developing cardiovascular diseases, type 2 diabetes, and cancer (46). As we will discuss in this section, E2F1 expression and activity are increased during obesity in several tissues involved in metabolic homeostasis, suggesting that E2F1 could contribute to some of the comorbidities of this condition.

E2f1 mRNA and protein levels are increased in the visceral white adipose tissue of obese human subjects and positively correlated with insulin resistance and circulating free-fatty acids (47). E2F1 expression was also increased in the visceral adipose tissue of two widely used mouse models of obesity: mice fed a HFD and leptin-deficient (ob/ob) mice (48). This effect was reversed when HFD-fed mice were treated with resveratrol, which in parallel decreased body weight gain and the levels of pro-inflammatory cytokines levels in white adipose tissue (49). In addition, pRB levels and repressor activity decrease in white adipose tissue during obesity both in rats and in humans (50), which is consistent with increased E2F1 activity. These evidences are supported by ChIP analysis in human white adipose tissue that revealed increased E2F1 binding to the promoters of stress signaling genes during the progression of obesity (51). Interestingly, E2F1 has been shown to enhance NF-κB-mediated inflammatory response (52, 53). However, the contribution of E2F1 to the inflammation of white adipose tissue during insulin resistance remains to be explored.

Obesity is a well-known inducer of cardiac hypertrophy, which often contributes to heart failure (54). Pathological cardiac hypertrophy occurs in parallel with the development of metabolic inflexibility and a re-activation of the cell cycle machinery (55). Similar to the effects observed in the white adipose tissue, HFD increased E2F1 levels and increased RB phosphorylation in mouse heart. This correlated with elevated expression of the E2F1 transcriptional target pyruvate dehydrogenase kinase 4 (PDK4) (56, 57). PDKs inhibit pyruvate dehydrogenase, blocking pyruvate conversion into acetyl-CoA, which results in decreased glucose oxidation. Hence, upregulation of the E2F1–PDK4 axis during obesity may account for the impairment in glucose oxidation that characterizes cardiomyopathy. Moreover, through the regulation of PINK1 translation via miR-421 expression, E2F1 promotes mitochondrial fragmentation in cardiomyocytes, which can lead to myocardial infarction (58). Additionally, E2F1 has been shown to suppress cardiac neovascularization by downregulating VEGF and PIGF expression. Consequently, *E2f1*$^{-/-}$ mice present better cardiac function after myocardial infarction than their wild-type littermates (59). Altogether, these studies suggest that increased E2F1 activity occurring during obesity contributes to the development of cardiomyopathy through the re-entry in the cell cycle and the re-wiring of cardiac metabolism.

Some laboratories, including ours, have recently demonstrated the importance of E2F1 in the physiopathological context of non-alcoholic fatty liver disease (NAFLD), which is highly related to the epidemic of obesity. NAFLD is a progressive disease that starts with a benign accumulation of lipids in the liver (hepatic steatosis) that can develop to non-alcoholic steatohepatitis (NASH) which, in its worst prognosis, can lead to liver fibrosis, cirrhosis, and hepatocarcinoma (60). Hepatic E2F1 levels are increased in steatotic liver in mice but also in humans (16). Additionally, NAFLD correlated with the phosphorylation of pRB in the liver in different mouse models of obesity and diabetes (16, 61), altogether consistent with increased E2F1 activity in these conditions. One major contributor to NAFLD is an increase in hepatic *de novo* lipogenesis, a process in which E2F1 plays an important role. Indeed, E2F1 directly activates the expression of key glycolytic and lipogenic genes and E2F1 depletion protects against NAFLD (16). Remarkably, hepatic E2F1 expression is increased in patients with NASH and in different mouse models of liver fibrosis (62, 63). Perturbed bile acid metabolism and/or cholesterol homeostasis are major contributors to NASH. The importance of E2F1 in bile acid synthesis was found in a mouse model of NASH—bile duct ligation and 3, 5- diethoxycarbonyl-1, 4-dihydrocollidine (DCC) feeding—in which bile acid accumulation in the liver contributes to fibrosis. Indeed, knockout of E2F1 in mice reduced bile acid synthesis, which protected from the development of biliary fibrosis under DCC feeding (62). We also recently revealed that E2F1 participates in cholesterol homeostasis by enhancing the expression of PCSK9, a negative regulator of the LDL receptor and cholesterol uptake (63). Importantly, anti PCSK9 antibodies were recently approved for the treatment of cardiovascular diseases due to

their capacity to lower LDL cholesterol levels (64). *E2f1*$^{-/-}$ mice present decreased circulating levels of cholesterol as a consequence of increased cholesterol uptake by several tissues, including the liver. However, when subjected to a high cholesterol diet, *E2f1*$^{-/-}$ mice presented increased liver fibrosis, likely due to the combination of exacerbated cholesterol uptake and a defect in bile acid secretion (63). Taken together, these studies imply that the convenience of targeting E2F1 to treat liver fibrosis could be context dependent and that this approach requires further investigation. Nevertheless, in humans, the increase of E2F1 during NASH was more substantial than the induction of standard fibrosis markers such as α-SMA and α1-collagen, which suggest that E2F1 could be potentially used as a new diagnostic marker for increased risk of developing liver fibrosis and cirrhosis (62).

Long-term HFD also increased E2F1 protein levels and pRB phosphorylation in hypothalamic Arcuate nucleus neurons, which are involved in global energy balance (65). This in turn led to a de-repression of E2F1-target genes involved in cell cycle regulation and apoptosis. Lu et al. found that the E2F1–pRB repressor complex is necessary for POMC neuron maintenance, whereas specific RB1 depletion in these neurons led to hyperphagia, obesity and diabetic syndrome in an E2F1-dependent manner (65). These results indicated that dysregulation of E2F1 at the central level also contributes to the development of the metabolic syndrome during the progression of obesity.

Altogether, recent work has highlighted the importance of the pRB-E2F1 pathway in the pathophysiology of obesity.

E2F1 CONTRIBUTES TO THE METABOLIC REPROGRAMMING OF CANCER CELLS

Cancer cells adapt their metabolism in order to promote growth, proliferation, survival, and metastasis. The specific metabolic profile of a tumor ultimately depends on the tissue of origin, the oncogenic alterations, the tumor stage, and the tumor microenvironment. Metabolic reprogramming is now considered one of the hallmarks of cancer and selectively targeting tumor metabolism has been proposed in the recent years as a therapeutic strategy to treat cancer (66, 67). Remarkably, some oncogenes such as p53 and Myc regulate cancer metabolism (68, 69) and, as we will discuss in this section, so does E2F1 (**Table 1**).

TABLE 1 | E2F1 contributes to the metabolic reprograming of cancer cells.

	E2F1-target genes	Reference
Nucleotide synthesis	DHFR, TK	(85,86)
Lipid synthesis	FAS	(89)
Glycolysis	PFKB, Sirt6, PDK	(71,72,73,75)
Oxidative metabolism	TOP1MT, EVOVL2, NANOG	(76–78)
Autophagy	v-ATPase, ATG1, DRAM1, MAP1LC3	(91,92)

E2F1 regulates the expression of several genes that have an impact on cancer metabolism.

DHFR, dihydrofolate reductase; TK, thymidine kinase; FAS, fatty acid synthase; PFKB, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; PDK, pyruvate dehydrogenase kinase; Sirt6, Sirtuin 6; TOP1MT, mitochondrial topoisomerase I; EVOVL2, ELOVL fatty acid elongase 2; ATG1, autophagy-related gene-1; MAP1LC3, microtubule-associated protein-1 light chain-3; DRAM, damage-regulated autophagy modulator.

E2F1 Contributes to the Warburg Effect

One metabolic feature of many cancer cells is the so-called Warburg effect, which consists on increased aerobic glycolysis and decreased glucose oxidation, resulting in high rates of glucose utilization and lactate production (66, 70). It has been shown that, against the assumption of Otto Warburg, who first described this phenomenon almost a century ago, in most cancers this is not caused by defective mitochondria. Several hypotheses have been proposed on how the Warburg effect benefits cancer cells, including higher rates of ATP synthesis, the generation of glycolytic intermediates for biosynthetic reactions or the remodeling of the tumor microenvironment; however, this phenomenon is still not fully understood (70). It has been shown that E2F1 can promote this metabolic switch by both enhancing glycolysis and by repressing glucose oxidation in the mitochondria (**Figure 2**). During the development of HCC, increased E2F1 levels progressively recruit Pontin and Reptin (two putative DNA helicases) to promote the expression of genes involved in glycolysis and in lactate export, which contributes to the Warburg effect (15). During cell division, E2F1 also promotes the expression of the F-type isoform of the enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, which results in the synthesis of fructose-2,6-bisphosphate, a potent stimulator of glycolysis (71, 72). Moreover, E2F1 also enhances glycolysis in bladder and prostate cancer cell lines through the suppression of the expression of Sirtuin 6, a NAD(+)-dependent deacetylase that inhibits the transcription of several key glycolytic genes (73, 74). Besides enhancing glycolytic gene expression, as previously mentioned, E2F1 also blocks glucose oxidation in the mitochondria by promoting the expression of the PDK enzymes. While in the heart E2F1 regulates PDK4 (57), in pancreatic cancer cells E2F1 enhances the expression of PDK1 and PDK3 isoforms, which results in increased aerobic glycolysis and proliferation (75).

E2F1 and Oxidative Metabolism

In addition to regulating oxidative metabolism in non-proliferative conditions (34), E2F1 also repress mitochondrial biogenesis during proliferation. Like in the muscle, knocking down E2F1 in HeLa cells led to increased expression of several genes involved in mitochondrial biogenesis and oxidative phosphorylation, which resulted in increased ATP production (76). E2F1 depletion in Mesenchymal Stem Cells also increased mitochondrial biogenesis and oxygen consumption (77). Additionally, it has been shown that E2F1-mediated repression of oxidative metabolism results in a self-renewal of tumor-initiating stem-like cells that contributes to the progression of HCC (78). Some evidences show that mitochondrial function, in turn, also impacts E2F1 activity. For instance, inhibition of ATP synthase or of the electron transport chain leads to the downregulation of E2F1 activity and to cell cycle arrest (79, 80). On the other hand, mitochondrial ROS production can promote E2F1-mediated apoptosis (81, 82). For a more detailed perspective of the complex interplay between E2F transcription factors and the mitochondrial function, we address you to recent specific reviews about the topic (83, 84).

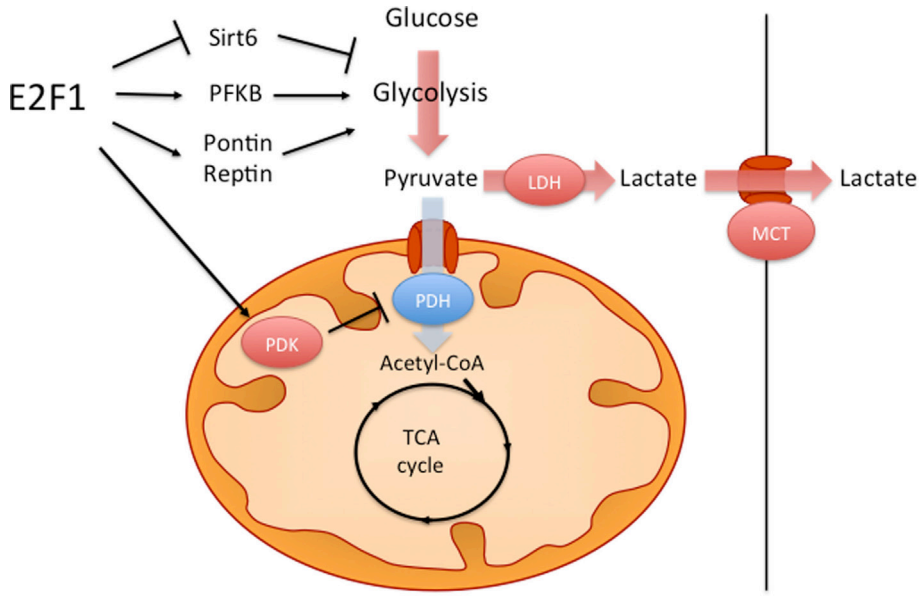

FIGURE 2 | E2F1 contributes to the Warburg effect. E2F1 participates in the characteristic aerobic glycolysis observed in many tumors by different mechanisms. E2F1 promotes glycolysis by repressing the expression of Sirtuin 6 (Sirt6), a negative regulator of glycolytic gene expression and by promoting the expression of the F-type isoform of 6-phosphofructo-2-kinase/fructose-2,6-bissphosphatase (PFKB). E2F1 also recruits a Pontin/Reptin complex to promote the expression of genes involved in glycolysis and lactate export. Additionally, E2F1 blocks glucose oxidation in the mitochondria by promoting the expression of pyruvate dehydrogenase kinase (PDK) enzymes, which inhibit the pyruvate dehydrogenase complex (PDH).

E2F1 and Anabolic Metabolism

Cancer cells undergo different anabolic processes to fulfill the high demand of macromolecules required for proliferation. E2F1 participates in DNA synthesis by regulating the expression of several genes involved in nucleotide metabolism such as Thymidine kinase and Dihydrofolate reductase (85, 86). Tumors also normally present high rates of lipid synthesis, which are used both for membrane production and as signaling molecules (87). Lipogenesis is not only important during proliferation; it also contributes to the metastatic capacity of cancer cells (88). Besides promoting lipogenesis in the liver (16), in medulloblastoma E2F1 enhances fatty acid synthase expression in response to Sonic hedgehog signaling (89).

mTORC1 is a master regulator of cell growth and survival, and it is involved in the progression of many cancers (90). It was recently shown that E2F1 promotes mTORC1 activity by enhancing the expression of lysosomal v-ATPase. This in turn, blocked autophagy, one of the main metabolic processes regulated by mTORC1 (91). Conversely, it was shown that E2F1 can also stimulate upregulation of genes involved in autophagy in response to DNA damage (92). Hence, the contribution of E2F1 to autophagy is still a matter of debate. Additionally, numerous studies have highlighted the crosstalk between E2F1 activity and other signaling pathways involved in cancer metabolism, such as the AKT or the HIF pathways (93–95). Whether E2F1 promotes anabolic reprogramming in cancer cells through the interaction with these signaling pathways remain to be explored.

Overall, these studies show that the transcription factor E2F1 plays a pivotal role integrating the cell cycle regulatory machinery with metabolic pathways essential for cell growth and survival. This, in turn, determines cell fate by affecting cell stemness, proliferation rate, or apoptosis. Therefore, inhibiting E2F1 activity could potentially impact tumor development at different levels simultaneously by blocking cell cycle progression and by impairing metabolic flexibility in cancer cells. In this regard, CDK4/6 inhibitors that block pRB phosphorylation and that are currently used for treating hormone-positive breast tumors have been reported to block proliferation, in part, by inducing a metabolic reprogramming in cancer cells (96, 97).

CONCLUSION AND PERSPECTIVES

Here, we have collected the current and emerging evidence showing that E2F1 regulates metabolism in non-proliferating conditions and, more importantly, that dysregulation of E2F1 activity leads to complications associated with obesity. Many studies have focused on the mitogenic signals that drive E2F1 activation in cancer cells, but how E2F1 is activated in other pathological conditions such as obesity is just beginning to be understood. The CDK4-pRB-E2F1 pathway can be stimulated both by glucose and by insulin in different tissues involved in global metabolic homeostasis (16, 24, 29, 95, 98). One possibility is that during obesity, hyperglycemia and/or hyperinsulinemia render pRB hyperphosphorylated (50, 61, 65). This in turn, would increase E2F1 activity and, in a positive feedback loop, E2F1 could promote its own expression (99). Other possible candidates for exacerbated E2F1 activation during obesity could be chronic inflammation or increased ROS production due to mitochondrial

stress, two factors that promote E2F1 activity in other contexts (52, 82). Despite the specific mechanisms that lead to E2F1 hyperactivation during obesity, targeting E2F1 could potentially be used to ameliorate some of the deleterious effects of this condition. Notably, *E2f1*$^{-/-}$ mice present increased insulin sensitivity and are resistant to HFD-induced obesity (25, 34). However, it should be considered that systemically inhibiting E2F1 activity would likely impair insulin secretion (100), which could be detrimental in the initial phases of insulin resistance, when insulin production is enhanced to maintain normoglycemia.

Given its dual role in proliferation and metabolism, it is tempting to speculate that E2F1 might be a central actor in the interplay between obesity and some types of cancer. One of those cases could be HCC, for which there is an increased risk in obese patients (101). We have recently shown that hepatic E2F1 expression is augmented during obesity (16), while numerous studies have demonstrated that increased E2F1 activity promotes the development of HCC (15, 78, 102, 103). Notably, it was also recently reported that E2F1 mediates the proliferative effects of insulin in hepatocytes (95). Indeed, obesity-associated hyperinsulinemia is one mechanism proposed to explain the epidemiological observations of increased HCC in obese patients (104). Therefore, under obesity conditions, enhanced hepatic E2F1 activity—maybe in response to hyperinsulinemia—may first lead to enhanced *de novo* lipogenesis, NAFLD development and fibrosis (16, 62). Subsequently, E2F1 may contribute to HCC progression by promoting the expression of genes involved in cell cycle machinery and cancer metabolism (15).

In conclusion, research over the past 15 years has given an increasingly complex picture of the multiple roles of E2F1. Beyond being a mere cell cycle regulator, this transcription factor has emerged as a novel player in the control of metabolism not only in normal physiology but also under pathological conditions such as obesity and cancer.

AUTHOR CONTRIBUTIONS

PDD, LF, and AG conceived and wrote the manuscript.

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REVIEW
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Cofactors As Metabolic Sensors Driving Cell Adaptation in Physiology and Disease

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Chromatin architectures and epigenetic fingerprint regulation are fundamental for genetically determined biological processes. Chemical modifications of the chromatin template sensitize the genome to intracellular metabolism changes to set up diverse functional adaptive states. Accumulated evidence suggests that the action of epigenetic modifiers is sensitive to changes in dietary components and cellular metabolism intermediates, linking nutrition and energy metabolism to gene expression plasticity. Histone posttranslational modifications create a code that acts as a metabolic sensor, translating changes in metabolism into stable gene expression patterns. These observations support the notion that epigenetic reprograming-linked energy input is connected to the etiology of metabolic diseases and cancer. In the present review, we introduce the role of epigenetic cofactors and their relation with nutrient intake and we question the links between epigenetic regulation and the development of metabolic diseases.

Keywords: metabolism, nutritional status, cofactors, epigenetics, metabolites

INTRODUCTION

During their lifetime, cells receive several external signals, including hormones, growth factors, cytokines and other extracellular factors. Cells translate those signals to make crucial adaptive decisions, such as quiescence, proliferation, or differentiation. Recent works highlighted a fundamental role of environmental cues and nutrient availability in cell metabolism and adaptation. This flow of metabolites, through complex but well characterized metabolic networks, constitutes a fuel for diverse epigenetic cofactors thus relaying nutrition and diet changes into cytoplasmic signaling and chromatin remodeling.

Through their ability to sense internal and external cues, several transcriptional cofactors allow a cell to rapidly adapt by introducing reversible protein posttranslational modifications (PTMs). Hundreds of PTMs have been identified (1–3). However, only few have been directly linked to metabolic fluxes. PTMs include histone and non-histone modifications and represent a key physiological signal for cell adaptation (4–12). For the purposes of this review, we will only focus on PTMs linking changes in metabolism to histone modification.

Histone modifications—all with DNA methylation, RNA interference, and non-coding RNA—encompassed by the term epigenetics represent diverted ways by which cells control the expression of genes without any alteration in the underlying genetic material. Since each cell has the same

genetic code, epigenetic modifications allow a fine regulation of the gene expression and determine cell identities. Thereby, various chromatin modification patterns, such as acetylation, methylation, phosphorylation, *O*-linked glycosylation, ubiquitination, and SUMOylation, result in a particular configuration that determines chromatin accessibility to the transcriptional machinery. For example, acetylation of lysine nine residues of histone H3 (H3K9), H3K14, and/or (mono-, di-, or tri) methylation of H3K4, H3K36 and H3K79 are often associated with transcriptionally active chromatin. By contrast, methylation of H3K9, H3K27, and H4K20 are markers of transcriptional silencing (13–15).

The generated metabolites remain the same for a given cell. Yet, the tissue function and nutriment availability will determine the metabolite requirements. Moreover, metabolic challenges, such as caloric or oxygen restriction or even a high-fat diet (16–19), will drive cell fate decisions. Consistent with this, dramatic epigenetic changes have been linked to metabolic disorders, such as obesity, insulin resistance, type-2 diabetes, and cancer (20–25). In this perspective, this review will focus on cofactor families linking nutritional input and metabolism to epigenetic pattern modifications.

ACETYL-CoA AND HISTONE/LYSINE ACETYLTRANSFERASE ENZYMES

Lysine/Histone acetyltransferases (KAT/HAT) catalyze the transfer of an acetyl group from acetyl-CoA to ε-amino group of a histone lysine residue (26), which allows a transcriptional access to DNA by either neutralizing the positive histone charge, or serving as a binding site for chromatin remolding complexes. HAT can be divided on the basis of their subcellular localization or of the structural and functional similarity of their catalytic domains (27).

Acetyl-CoA availability is a major input for histone acetylation. A rise in acetyl-CoA level is sufficient to drive a yeast growth program by promoting histone acetylation at specific growth genes through the General control of amino acid synthesis protein 5-like 2 (GCN5, KAT2A) (28). In mammalian cells, histone acetylation with acetyl-CoA generated from glucose metabolism controls the early differentiation of embryonic stem cells (ESCs) (29). The limiting ATP citrate lyase enzyme that controls the conversion of citrate into oxaloacetate and acetyl-CoA was shown to be important for histone acetylation in response to glucose and growth factor stimulation (30).

As demonstrated for yeast, the mammalian GCN5 activity is required for histone acetylation during cell differentiation (30, 31). Tracing experiments using 13C-carbon combined with acetyl-proteomics showed that up to 90% of histone acetylations on certain histone lysines are derived from fatty acid even in glucose excess. Acetyl-CoA generated from fatty acid β-oxidation seems to be important for the control of a gene expression program involved in lipid metabolism (32). Cytosolic acetate is another acetyl-CoA source that leads to an increase in H3K9, H3K27, and H3K56 histone acetylations of specific promoter regions, enhancing *de novo* lipid synthesis under hypoxic conditions (33). KAT2b is a KAT that acetylates H3K9 and H3K14. During embryogenesis, GCN5 mRNA is already expressed at high levels by day 8, whereas KAT2b mRNA is first detected on day 12.5, suggesting that KAT2b and GCN5 play distinct roles by controlling the expression of a distinct set of genes (34). We have demonstrated that KAT2b is required for pancreatic β-cell adaptation to metabolic stress by promoting histone acetylation and gene expression of several unfolded protein response markers (35). While a β-cell-specific deletion of Kat2b in mouse has no effect under normal diet, Kat2b deficiency leads to a dramatic effect on β-cell morphology and function upon high fat feeding. KAT2b is thereby a major sensor of acetyl-CoA under hyperglycemic condition (35). Altogether, those data suggest that distinct histone acetyltransferases can sense acetyl-CoA upon different conditions and translate the appropriate cell response by activating different sets of genes. Moreover, the origin of acetyl-CoA seems to be important for this selectivity. Sutendra et al. recently demonstrated that acetyl-CoA is generated in the nucleus through a dynamic translocation of the mitochondrial pyruvate dehydrogenase complex (PDC), raising new questions about intracellular acetyl-CoA compartmentalization and the way its origin can regulate a specific set of genes (36–38). A better understanding of KAT activation, of the origin of acetyl-CoA and of its fluctuations within subcellular compartments upon different nutritional challenges can be of interest for the development of new therapeutic strategies against metabolic disease and cancer.

NAD$^+$-DEPENDENT AND INDEPENDENT HISTONE/LYSINE DEACETYLASES

Lysine/Histone deacetylases (KDAC/HDAC) are the enzymes that catalyze the removal of the acetyl group from lysine residues of histones. On the basis of their mechanistic similarities, they can be divided into two groups: classical HDAC and NAD$^+$-dependent sirtuin deacetylase families (39, 40).

The mammalian NAD$^+$-dependent KDACs consist of seven sirtuin members (SIRT1 to SIRT7), with distinct subcellular localizations. Three sirtuins are located in the mitochondria (SIRT3–SIRT5), while SIRT1, SIRT6, and SIRT7 are predominantly located in the nucleus, and SIRT2 is found in the cytoplasm (41, 42). NAD$^+$ levels rise in energy deficiency situations, such as exercise, caloric restriction, and fasting, leading to sirtuin activation (43, 44). In contrast, when energy is in excess, NAD$^+$ is depleted, generating a higher NAD$^+$/NADH ratio, which inhibits sirtuin activity (6, 41, 42). This notion further argues toward a direct link between the nutritional status and epigenetic control.

SIRT1, one of the most studied KDAC, controls circadian rhythm and liver metabolism through the deacetylation of H3K9 and H3K14 at the promoter of clock genes (45, 46). Furthermore, through its interaction with Menin, SIRT1 enhances histone deacetylation and controls hepatic triglyceride accumulation (47, 48). SIRT1 can also deacetylate H4K16, functionally linking metabolic activity to genome stability and aging (49, 50). SIRT6, another nuclear sirtuin, is linked to aging by controlling a specific deacetylation of H3K9 at NF-κB target gene promoters (51). In cancer cells, SIRT7 is involved in the stabilization of their

transformed phenotype by inducing the deacetylation of H3K18 at specific oncogene promoter regions (52).

The second families of KDAC are classical HDAC, and, in spite of their independent activity on endogenous metabolite, they have been linked to cellular metabolism. Shimazu et al. showed that β-hydroxybutyrate produced under fasting, starvation or intense exercise condition is a natural endogenous HDAC inhibitor leading to increased H3K9 and H3K4 acetylation (18, 53). It also increases histone acetylation at the Foxo3a and Mt2 promoters through the inhibition of HDAC1 and HDAC2 (18).

Lactate production, as a result of an increased rate of glycolysis, has also been shown to upregulate the expression of genes associated with HDAC proteins (54). The authors have demonstrated that the primary effect of lactate on gene expression depends on HDAC inhibition (54). Therefore, lactate may be an important transcriptional regulator, linking the metabolic state of the cell to gene transcription. Further work is needed to corroborate whether the lactate produced *in vivo* has a tissue specific effect on HDAC cofactors. Moreover, lactate has been implicated in the modulation of the DNA damage and repair processes as well as in the resistance of carcinoma cells to anticancer therapy (55).

Altogether, those data provide evidence for a direct link between metabolism products and cellular adaptation through the modulation of KDAC activity.

HISTONE METHYLATION AND S-ADENOSYLMETHIONINE (SAM)

S-adenosylmethionine, generated by the methionine cycle, contains the active methyl donor group used by methyltransferases to methylate RNA, DNA, and proteins, including histones (56–61). While extensive studies focused on the changes of methylation status upon embryonic development, physiology, and diseases, the link between intracellular SAM fluctuation and their conversion into specific epigenetic modifications remains poorly understood. For instance, only histone methylation has been linked to methionine availability, an essential amino acid obtained from the diet (62).

Histone methylation can occur on arginine or lysine residues. While lysine can be mono-, di-, or trimethylated, arginine can only be mono-methylated. There are three classes of histone methyltransferase: SET domain lysine methyltransferases, non-SET domain lysine methyltransferases (disruptor of telomeric silencing 1-like, DOT1L), and arginine methyltransferases (PRMT) (63–65).

In mouse ESCs, mitochondrial threonine dehydrogenase (TDH), an enzyme that catabolizes threonine into glycine and acetyl-CoA, has been shown to be important in maintaining the intracellular SAM level (66). Threonine depletion in culture medium or TDH knockdown in mouse ESCs decreases SAM accumulation and H3K4me3 mark, whereas no effect was observed in other methylation marks (66). In cancer cells, an aberrant expression of Nicotinamide N-methyltransferase—a limiting enzyme that metabolizes SAM—exerts specific control over the cells methylation potential (67). Moreover, recent works provide evidence in both mouse and human that methionine status is sufficient for the control of numerous physiological processes including the activity of genes involved in cell fate through the modulation of histone methylation levels (62).

As observed for HDAC, PMRT activity can be controlled by intermediary metabolites. Three recent reports showed that an increased intracellular concentration of methylthioadenosine (MTA) in cancer cells harboring 5-methylthioadenosine phosphorylase (MTAP) deletions leads to PMRT5 inhibition (68–70). MTAP is the enzyme controlling MTA cleavage to generate precursor substrates for methionine and adenine salvage pathways. In cancer cells, MTAP deficiency leads to partial metabolite-based inhibition of PRMT5 by altering the ratio of MTA to SAM, which results in a decreased H4R3me2s mark (68–70). More studies are needed to understand whether the MTA-to-SAM ratio can also be controlled by physiological metabolic nutritional states.

FLAVIN ADENINE DINUCLEOTIDE (FAD) AND HISTONE DEMETHYLASES

Histone methylation was originally considered as a permanent chromatin alteration until the landmark discovery of histone lysine-specific demethylase 1 (LSD1) by Shi Yang's group, established both *in vitro* and *in vivo* methylation reversibility (71). LSD1 uses FAD formed from ATP and riboflavin (vitamin B2) in mitochondria as a cofactor to demethylate mono- and di-methylated H3K4 and H3K9 (72, 73). Although LSD1 demethylase activity appears to control the metabolism in favor of *de novo* fatty acid synthesis over gluconeogenesis in hepatocyte and brown adipose tissue thermogenic activity, a direct link between nutritional status and LSD1 activity still needs to be established (74–78). For instance, recent works demonstrate that livers from mouse fed with folate-deficient diet present an increased dimethyl-H3K4 and decreased LSD1 activity (79). More studies are needed to decipher the metabolic consequence of FAD fluctuation upon physiological and pathophysiological conditions.

α-KETOGLUTARATE (αKG) AND HISTONE DEMETHYLASES

α-ketoglutarate is produced from isocitrate through the activity of two key-enzymes of the Krebs cycle, isocitrate dehydrogenase 1 and 2 (IDH 1 and IDH2) (16, 80). αKG can also be produced anaplerotically from glutamate by oxidative deamination, using glutamate dehydrogenase (49). Under fasting or caloric restriction, the accumulation of αKG is used by the αKG-depending dioxygenase to influence the epigenetic status of the cells (81, 82). Several chromatin-modifying enzymes are regulated by αKG availability, including demethylase enzymes containing a Jumonji C domain (JmjC) and ten-eleven translocation (TET) protein families (83–86).

The JmjC subfamily comprises the largest identified family of lysine demethylases (KDMs) with more than 60 enzymes identified in humans (87). In addition to αKG, JmjC-dependent histone demethylation requires iron Fe(II) (88). Each JmjC

family member exhibits preference to reverse lysine or arginine trimethylated histone. Considering the key-determinant role of methylation on gene expression and demethylase specificity, KDM2 and KDM5 families have been shown to promote a repression chromatin status, while KDM3, KDM6 and KDM7 act as chromatin activators (63).

Ten-eleven translocation protein family can catalyze 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) in three consecutive Fe(II)- and 2-oxoglutarate (2-OG)-dependent oxidation reactions (89, 90). Gene expression depends on the location of the 5hmC marks. Indeed, the presence of 5hmC in the gene bodies was found to correlate positively with gene expression, whereas no correlation with gene expression was found when 5hmC peaks are located at transcription start sites (91, 92).

α-ketoglutarate can be derived from glucose and glutamine. However, few studies have demonstrated a direct link between αKG generation and histone demethylation. A direct manipulation of intracellular αKG/succinate ratio is sufficient to regulate chromatin state in ESCs. The accumulation of αKG promotes self-renewal of ESCs through JMJD3 and Tet1/Tet2 demethylation of H3K9me3, H3K27me3, and H4K20me histone marks (93). Gas chromatography coupled to mass spectrometry analysis revealed a rapid increase in hepatic α-KG levels following intraperitoneal glucose injection in mice. Strikingly, 5hmC and 5fC marks are reported to increase in various mouse tissues including the liver, kidney, and muscle without any change in TET protein expression or localization leading to a change in gene expression (94). Changes in demethylase activity may thereby contribute to cellular and tissue dysfunction under persistent hyperglycemic conditions.

In cancer cells, a loss of function mutation of TCA cycle enzymes, such as mitochondrial succinate dehydrogenase or fumarate hydratase, promotes succinate and fumarate abundance (95, 96). Both metabolites inhibit α-KG-depending demethylase leading to a decreased 5hmC mark and a specific increase in H3K9me3 levels (96, 97). Somatic mutations of IDH1 and IDH2 have been identified in glioblastomas, acute myelogenous leukemia, chondrosarcomas and lymphomas and other solid tumors (98–103). These gain-of-function mutations lead to a new enzymatic activity promoting the conversion of α-KG to produce D(R)-2-hydroxyglutarate (R2HG) (104, 105). This oncometabolite, which accumulates in tumors with IDH mutations, is a competitive inhibitor of TET and JmjC protein family activity (106–109).

Two recent reports describe another metabolite generated under hypoxic condition by the conversion of α-KG to produce L(S)-2-hydroxyglutarate (S2HG) (110, 111). Both reports demonstrate that S2HG is the product of malate dehydrogenase 1, malate dehydrogenase 2, and lactate dehydrogenase A. The accumulation of this metabolite leads to α-KG-depending demethylase activity inhibition toward TET1/2 and KDM4C (110, 111). Interestingly, this effect is not cancer-specific since a similar level of S2HG production was observed in endothelial cells (110). Moreover, manipulating S2HG is sufficient to increase the methylation of histone repressive marks, suggesting that this metabolite may be generated in other conditions than hypoxia. Further studies are needed to understand the role of S2HG in controlling proliferation versus fate in ES cell.

NUCLEAR LOCALIZATION OF METABOLITES

The cytosol and nucleus are dense and very viscous. This may restrict the diffusion of small molecules and slow down biochemical reactions. Moreover, several metabolite pathways are organized in multiprotein complexes to allow reaction channeling to facilitate signaling. A multiprotein complex (molecular assembly line) has been proposed to promote efficient substrate channeling from one enzyme to the next (112). Accumulated evidence suggests a close coupling of the histone-modifying enzymes with their critical cofactor synthesis enzyme in the nucleus. Their nuclear translocation aims to provide *in situ* metabolite synthesis in response to metabolic stress. For example, Katoh and colleagues demonstrate that the SAM-generating enzyme, methionine adenosyltransferase II (MATIIα), is localized in the nucleus and interacts with the Swi/Snf and NuRD complexes, supplying SAM for methyltransferases (113). MATIIα will maintain a local high SAM concentration, which is used by an H3K9-specific histone methyltransferase to repress the oncogene MafK transcriptional activity (113).

Similarly, a pyruvate conversion to acetyl-CoA is processed in the nucleus through the nuclear translocation of the mitochondrial PDC. Nuclear PDC levels, as well as the histone H3 and H4 global acetylation levels, increase in a cell-cycle depending manner upon epidermal growth factor, serum, or mitochondrial stress (36). Nuclear PDC inhibition leads to a specific decrease in the acetylation of the histone that is important for the gene expression of G1-S phase progression and S phase markers (36). Moreover, nuclear concentration of acetyl-coA has been shown to be important for osteoblast differentiation (114). In line with this, recent works showed that pyruvate is critical for the TCA cycle enzyme nuclear localization in mammalian zygotic genome activation (115). The authors demonstrated that nutrients, such as pyruvate, are essential for an early pre-implantation development in mouse and human. Mechanistically, Nagaraj and colleagues showed that pyruvate controls the nuclear localization of multiple TCA enzymes in addition to proteins related to TCA cycle entry, including pyruvate carboxylase, pyruvate dehydrogenase, pyruvate dehydrogenase phosphatase, citrate synthase, aconitase-2, and isocitrate dehydrogenase 3A (115). Moreover, acetate-dependent acetyl-coA synthase 2 (ACSS2) binds to chromatin nearby regions of genes that are upregulated during neuron differentiation. A decrease in ACSS2 lowers nuclear acetyl-coA levels, histone acetylation, and neuronal genes in hippocampus, leading to defective spatial memory (116). Those data support a critical role of ACSS2, linking acetate metabolism to localized acetyl-coA production, histone acetylation, and gene expression. In hepatocellular carcinoma cells, the nuclear localization of ACCS2 promotes cancer cell survival by increasing H3K9, H3K27, and H3K56 acetylation levels at the promoter regions of lipogenic genes such as acetyl-CoA carboxylase alpha and fatty acid synthase and enhances *de novo* lipid synthesis (33).

Finally, NAD^+, the critical cofactor of sirtuin deacetylase, can also be generated in the nucleus through the conversion of nicotinamide by nuclear NMNAT1. NMNAT1 enzymatic activity is required to provide NAD^+ for SIRT1 (117) and PARP1 (118) during transcriptional regulation and DNA repair.

The precise nuclear localization of critical cofactor-generating enzymes supports the presence of localized subdomains within the chromatin that may promote the clustering of relevant PTMs at specific genomic loci. This model raises a new question on how the nutritional state and metabolism products control the nuclear localization and activity of those microdomains. The second question is to know whether those processes are tissue and cell specific and if they are disturbed under pathophysiological conditions such as obesity or cancer. Then, the final question is: what is the functional and physiological significance of this process?

CONCLUSION

Scientific evidence clearly supports that nutrition and diet are the most influential lifestyle factors that contribute to health and the development and progression of chronic diseases, including metabolic disorders, neurodegenerative diseases, cancers, and cardiovascular diseases.

The recent exciting advances surveyed herein show that eating habits and nutritional input is deciphered by a metabolic sensor and translated into an adaptive epigenetic code that controls major biological processes such as cell survival, proliferation, DNA damage, and cellular energy production and/or storage (**Figure 1**). The next major challenge for epigenetic research will depend on the ability to translate the lessons learned from epigenomic profiling, structural studies, and regulatory mechanisms to treatment.

However, it will also be important to strengthen our understanding on how metabolite fluctuations can control a specific gene set in a given tissue. Importantly, it will be of interest to understand how all those pathways integrate into a specific physiological and/or pathophysiological state. The mechanisms controlling the concentration of metabolites in microdomains within the nucleus and the ability for this chromatin compartmentalization of critical cofactor synthesis enzyme to coordinate specific responses to metabolite changes are two other intriguing questions.

Finally, the most important question might be to determine whether cofactors can be successful targets for metabolic diseases. Although this review highlights how far we have come in less than two decades, those findings shed light on a wide

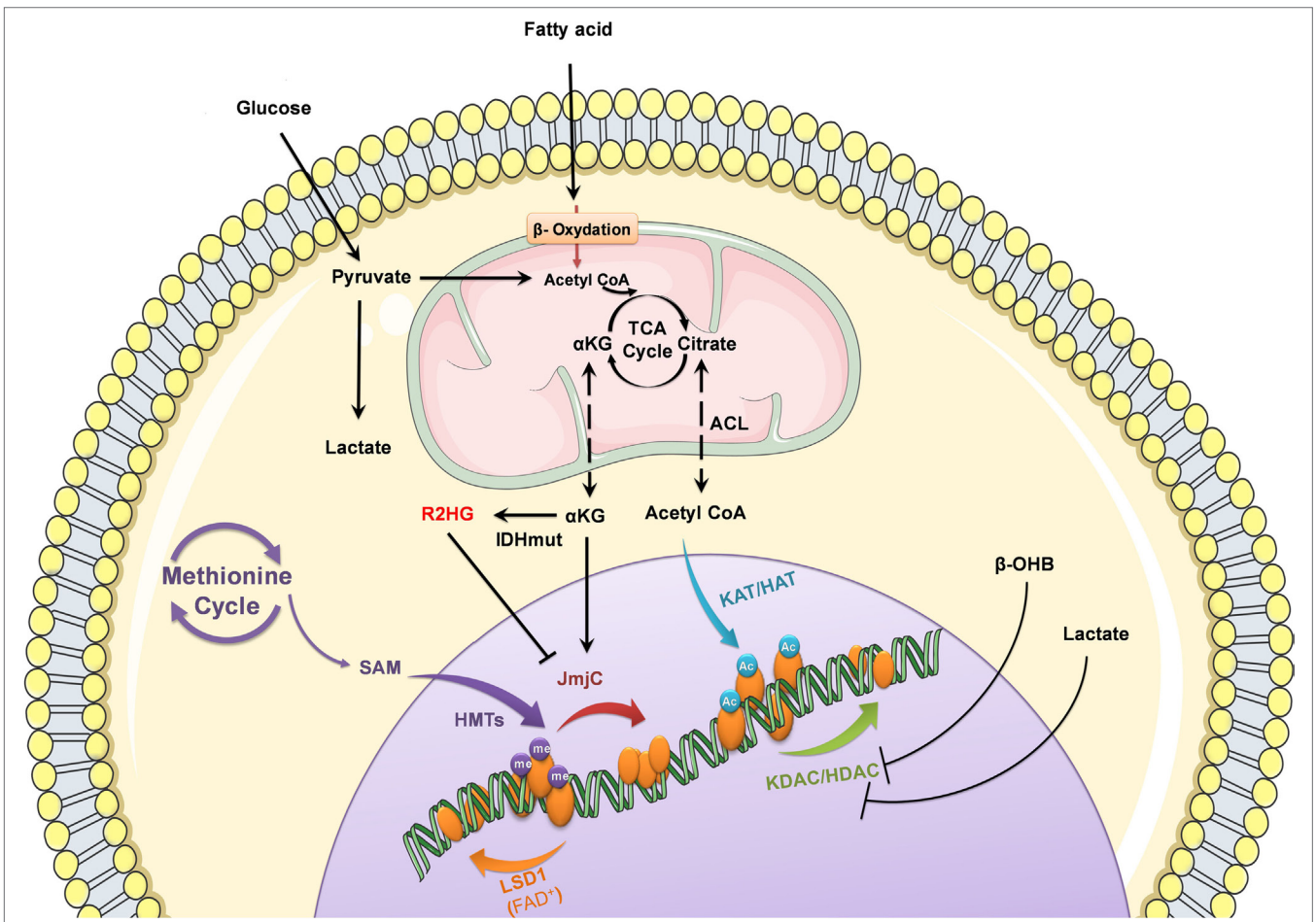

FIGURE 1 | Nutrient-derived metabolites control epigenetic processes. Metabolic status and circulating nutrients, such as glucose and free fatty acids, provide intermediate metabolites required for histone modifications to control gene expression.

range of more open questions to understand the role of cofactors in nutritional sensing and the epigenetic control of gene expression.

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NR, SAH, J-SA, and PF discussed and wrote the manuscript.

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N-Acetylaspartate Metabolism Outside the Brain: Lipogenesis, Histone Acetylation, and Cancer

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N-acetylaspartate (NAA) is a highly abundant brain metabolite. Aberrant NAA concentrations have been detected in many pathological conditions and although the function of NAA has been extensively investigated in the brain it is still controversial. Only recently, a role of NAA has been reported outside the brain. In brown adipocytes, which show high expression of the NAA-producing and the NAA-cleaving enzyme, the metabolism of NAA has been implicated in lipid synthesis and histone acetylation. Increased expression of *N*-acetyltransferase 8-like (*Nat8l*, the gene encoding the NAA synthesizing enzyme) induces *de novo* lipogenesis and the brown adipocyte phenotype. Accordingly silencing of aspartoacylase, the NAA-cleaving enzyme, reduced brown adipocyte differentiation mechanistically by decreasing histone acetylation and gene transcription. Notably, the expression of Nat8l and the amount of NAA were also shown to be increased in several tumors and inversely correlate with patients' survival. Additionally, Nat8l silencing reduced cell proliferation in tumor and non-tumor cells, while NAA supplementation could rescue it. However, the mechanism behind has not yet been clarified. It remains to be addressed whether NAA *per se* and/or its catabolism to acetate and aspartate, metabolites that have both been implicated in tumor growth, are valuable targets for future therapies.

Keywords: *N*-acetylaspartate, acetate, acetyl-CoA, lipid metabolism, brown adipocytes, ATP-citrate lyase, NAA catabolism

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INTRODUCTION

N-acetylaspartate (NAA) is the second most abundant brain metabolite with concentrations around 10 mM (1). NAA is synthesized from aspartate and acetyl-CoA by aspartate *N*-acetyltransferase (Asp-NAT, encoded by the gene *Nat8l*) and cleaved by aspartoacylase (Aspa) yielding aspartate and acetate. Acetyl-CoA synthetase (AceCS) can then use acetate to generate acetyl-CoA which is a general energy metabolite and second messenger (2) and essential for lipid synthesis. In this respect, NAA has been suggested as acetyl-CoA source for myelin lipid synthesis in oligodendrocytes during brain development and loss-of-function mutations of Aspa lead to hypomyelination as well as NAA accumulation in the central nervous system (CNS) (3, 4). However, other studies proposed roles for NAA as a precursor for *N*-acetylaspartylglutamate synthesis (the most concentrated neuropeptide in the human brain), in osmoregulation, and in axon-glial signaling (5). Although the role of NAA in the CNS has been studied over decades and several mouse models with either deletion of Nat8l (6), Aspa (7), or both (8), have been investigated with regard to its physiological function, the role of NAA remains still controversial. Even though disruption of NAA metabolism leads to clear effects in human and mice, to this day, the question whether NAA itself or its breakdown to acetate and aspartate is essential for CNS awaits to be answered. An overview of a plethora of studies which

tried to answer this question was given in excellent reviews in the past (5, 9). The present review focuses on the role of NAA in physiological and pathological conditions outside the CNS which has appeared in the focus of research only most recently. **Table 1** shows tissues/conditions in which NAA concentrations and/or the NAA yielding/catabolizing enzymes have been detected outside the CNS. Details about a potential role of NAA in the differentiation of adipocytes and the proliferation of cancer cells are given in separate sections subsequently.

NAA METABOLISM AND LIPID SYNTHESIS IN BROWN ADIPOCYTES

Over the past decades, NAA synthesis has only been described in the CNS. However, its uptake and consumption was also observed in other tissues. Kidney metabolizes NAA to CO_2, while other tissues like mammary gland convert NAA into lipids (16). We recently discovered that *Nat8l* mRNA is highly expressed in brown adipose tissue (BAT) (10). Although many other metabolic tissues were screened for *Nat8l* expression, robust expression of *Nat8l* was only observed in BAT while its expression in white adipose tissue is much weaker and is negligible in skeletal muscle, heart and liver. Interestingly, the expression of *Nat8l* is massively increased during adipocyte differentiation of both murine and human cells, suggesting that NAA could be involved in lipid metabolism (10). Aspa expression is also upregulated in differentiating brown adipocytes suggesting that NAA catabolism is required for its function in adipocytes (11). However, NAA is not a primary source for acetyl-CoA and its downstream usage for lipogenesis as it requires acetyl-CoA for its synthesis. Thus, as suggested by us for brown adipocytes (11) and others for the CNS (17), NAA might be a storage and transport form of acetate that can be subsequently used for synthesis of acetyl-CoA by acetyl-CoA synthase-1 (AceCS1) when required. In agreement, silencing of Aspa in brown adipocytes led to a massive accumulation of NAA and reduced cytosolic acetyl-CoA concentrations (11) while overexpression of Nat8l (and concomitant Aspa upregulation) strongly increased *de novo* lipogenesis (10), arguing that NAA catabolism and acetate availability is important for adipocytes. Wang et al. (18) showed that NAA supplies around one third of the acetyl-CoA for myelin lipid synthesis during brain development while citrate provides the other two thirds, suggesting that the NAA pathway might be an alternative pathway for lipogenesis in adipocytes as well. Citrate is produced in mitochondria and exported to the cytosol where it is cleaved by ATP-citrate lyase (Acly) to yield acetyl-CoA and oxaloacetate. We hypothesized that NAA might complement citrate to deliver acetyl-CoA to the cytosol. In alignment, Nat8l localizes to mitochondria in brown adipocytes (10), while Aspa is found in the cytosol (11). Notably, the expression of Acly was strongly enhanced in brown adipocytes silenced for Nat8l and in BAT from Nat8l-knockout mice suggesting a compensatory upregulation of the Acly pathway if NAA is not available (10).

NAA CATABOLISM AND HISTONE ACETYLATION IN BROWN ADIPOCYTES

Wellen et al. showed that Acly silencing leads to reduced histone acetylation. They also proposed that AceCS1 could provide an alternative acetyl-CoA source for protein acetylation in the presence of acetate (19). A role in protein acetylation has also been discussed for NAA-derived acetate in the brain as Aspa and AceCS1 have even been found to colocalize (9). Hence it seemed logical that, if the NAA pathway is an alternative way for cytosolic acetate delivery, NAA catabolism could play a role in posttranslational protein modification as well. In brown adipocytes, silencing of Aspa diminished cytosolic acetyl-CoA levels and reduced acetylation of histone H3 and the locus-specific lysine residues H3K9 and H3K27 (11). The latter histone modifications have been shown to regulate transcription. Accordingly, the transcription of many genes, amongst others adipogenic marker genes, was downregulated thereby leading to reduced

TABLE 1 | Body regions/conditions in which NAA concentrations and/or Nat8l/Aspa expression have been determined in physiological and pathological conditions outside the CNS.

Tissue/condition	NAA concentration (method used for detection)	Nat8l	Aspa	Literature
Brown adipose tissue		mRNA, protein	mRNA, protein	Pessentheiner et al. (10), Prokesch et al. (11)
Brown adipocytes	Up to 20 nmol/mg protein (HPLC/HRMS; LC-MS/MS)	mRNA, protein	mRNA, protein, activity	Pessentheiner et al. (10), Prokesch et al. (11)
White adipose tissue, human white adipocytes		mRNA	mRNA	Pessentheiner et al. (10), Prokesch et al. (11)
Non-small cell lung cancer (NSCLC)	Blood (up to 200 nM) Tumor (5–15 μM) Cells (relative) (HPLC, GC-MS)	Tumor (mRNA) Cells (protein)		Lou et al. (12)
High-grade serious ovarian cancer (HGSOC)	Ovarian cancer (~60 μM) (NMR)	mRNA, Protein	mRNA	Zand et al. (13)
Inflammatory breast cancer (IBC)	Cells (relative) (LC-MS)	mRNA		Wynn et al. (14)
Duodenum of obese/diabetic mouse model			Protein, activity	Surendran et al. (15)
Adipose tissues of obese/diabetic mouse model		mRNA	mRNA	Pessentheiner et al. (10)

differentiation potential in Aspa-silenced adipocytes. To date, a system boosting NAA catabolism by overexpressing Aspa has not yet been investigated in brown adipocytes. However, it can be speculated that increased NAA cleavage would lead to increased availability of cytosolic acetyl-CoA and higher histone acetylation. Interestingly, the addition of NAA to brown adipocytes led to a similar decrease in gene transcription as observed upon Aspa-silencing but without affecting cytosolic acetyl-CoA levels (11). Thus, it is conceivable that NAA *per se* impacts the activity of protein deacetylases or is even "toxic" as it can easily be taken up by cells as also observed for brown adipocytes (11). A couple of studies (20–23) showed that NAA is bioavailable and can be taken up by several tissues in rats (but cannot pass the blood brain barrier) when administered either by oral gavage or when incorporated into diets. At doses under 2,000 mg/kg, these investigators did not observe NAA-related adverse effects with regard to motor activity, hematology, coagulation, organ weight, or gross pathology evaluations. Thus, they concluded that NAA does not evoke systemic or reproductive toxicity at given doses. It is worth mentioning that acute toxicity leading to death within 2 days in female rats has been observed with a single gavage of 5,000 mg/kg NAA. NAA is present in a number of foods (24) and although very low in concentration, its biological effect in humans should probably not be underestimated. In this regard, long-term studies that investigate the effects of NAA in diets except for reproduction and development might be required to exclude a toxic effect of NAA at the molecular level.

THE ROLE OF NAA IN CANCER

Nowadays, metabolic reprogramming is a well-accepted hallmark of cancer. Distinctive metabolic dependence of cancer cells on alternative sources for energy and biomass production can provide new possibilities for early diagnosis and targeted therapies. During the past decade, alternative metabolites as acetate have been suggested for the use of lipid generation which supports cell proliferation (25–27). Although previous work found NAA to be more abundant in tumors when compared to non-cancerous tissues (28–32), only very recently, the biological and clinical role of NAA/Nat8l in cancer was addressed in more detail in some nearly simultaneously published studies. Lou et al. detected NAA in non-small cell lung cancer (NSCLC) while it was undetectable in normal lung epithelium (12). Concomitantly, they found increased expression of Nat8l in approximately 40% of investigated adenocarcinoma and squamous cell carcinoma cases while the expression of Nat8l was minimal in non-malignant lung tissues. Expectedly, reducing Nat8l expression in NSCLC through siRNA also reduced NAA content of these cells. These investigators suggested that the biosynthesis of NAA depends on glutamine availability in NSCLC cells. Glutamine dependency was also confirmed in an *in vitro* model for inflammatory breast cancer (IBC) that also shows NAA enrichment (14). Lou et al. also investigated whether NAA, as it has the potential to be secreted, could serve as a circulating biomarker and found blood NAA concentrations increased in 46% of the NSCLC patients at the age of 55 years or younger when compared to age-matched, healthy controls (12). However, this data should be interpreted with caution as NAA concentrations were also found to be influenced by age, obesity and diabetes (12, 33, 34). Another group used metabolic flux analysis that also revealed biosynthesis of NAA in lung cancer cells (35). Additionally, they found that Nat8l silencing inhibits the proliferation of several human cancerous and non-cancerous cell lines. Metabolic profiling of high-grade serious ovarian cancer (HGSOC) also identified NAA as a metabolite that was correlated with reduced survival of patients when high (13). In addition, these investigators also studied open access RNA Seq data from The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov/) and found that high *Nat8l* expression was associated with worse overall survival of patients with melanoma, renal cell, breast, colon, and uterine cancer proposing a general role for NAA in cancer. Similar to observations in lung cancer cells, Nat8l silencing reduced cancer proliferation in ovarian cancer cell lines which could interestingly be rescued by NAA supplementation (13). They also found that Nat8l-silencing in orthotopic mouse models for ovarian cancer and melanoma significantly reduced tumor growth. Zand et al. also suggested that silencing of Nat8l expression downregulates the antiapoptotic pathway mediated through FOXM1; however, the mechanism how NAA regulates FOXM1 expression was not revealed (13). Another mechanism was proposed in SUM 149 cells, the primary model for IBC. Wynn et al. found out that silencing of the oncogene RhoC, a driver of metastatic potential, strongly reduced Nat8l expression and NAA content in SUM149 cells. Notably, Aspa expression was not detected in this cancer cell model further arguing for a role of NAA distinct from its catabolism in cancer (14). Also, no correlation of Aspa expression with tumor NAA levels was found in ovarian cancer samples (13). Finally, according to the TCGA database, *Aspa* expression is downregulated in several cancers arguing that NAA itself and not its breakdown products (aspartate or acetate) might be important for cancers. Although there is no evidence yet to prove that cancers do not consume NAA, well-controlled metabolic tracing experiments could conclude the fate of NAA in proliferating cells. Considering NAA is not consumed by tumors would bring up the intriguing question why cancer cells would excrete a metabolite that could very well contribute to biosynthetic and energetic needs of proliferation. Thus, also further investigations are required to provide a direct role for NAA function independent from its catabolism. It is also important to note that potential interactions between NAA (secreted by cancer cells) and the host organism (e.g., immune system) have not yet been investigated and may reveal novel roles for NAA.

OUTLOOK/FUTURE ASPECTS

Many questions remain open when it comes to the functional role of NAA in cancer, adipose tissue energy metabolism and lipid-associated disorders also as in the latter there is a discrepancy about the levels of NAA in urine and adipose tissue Nat8l expression (10, 33, 34, 36). Future studies will also have to dissect whether NAA *per se* or the catabolism of NAA by providing acetate and not to forget aspartate [as its availability correlates with cell proliferation (37–39)] for further usage are responsible for the effects associated with either NAA accumulation or depletion in diverse malignancies. Without debate, NAA plays a crucial role

in physiological conditions and in the development of several pathological conditions also outside the CNS. Thus, investigating the underlying mechanism might pave the way for therapeutic targeting of a variety of diseases correlated with deviant NAA concentrations.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Macropinocytosis: A Metabolic Adaptation to Nutrient Stress in Cancer

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Oncogenic mutations, such as Ras mutations, drive not only enhanced proliferation but also the metabolic adaptations that confer to cancer cells the ability to sustain cell growth in a harsh tumor microenvironment. These adaptations might represent metabolic vulnerabilities that can be exploited to develop novel and more efficient cancer therapies. Macropinocytosis is an evolutionarily conserved endocytic pathway that permits the internalization of extracellular fluid *via* large endocytic vesicles known as macropinosomes. Recently, macropinocytosis has been determined to function as a nutrient-scavenging pathway in Ras-driven cancer cells. Macropinocytic uptake of extracellular proteins, and their further degradation within endolysosomes, provides the much-needed amino acids that fuel cancer cell metabolism and tumor growth. Here, we review the molecular mechanisms that govern the process of macropinocytosis, as well as discuss recent work that provides evidence of the important role of macropinocytosis as a nutrient supply pathway in cancer cells.

Keywords: macropinocytosis, Ras, growth factors, nutrient uptake, cancer metabolism

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INTRODUCTION

Sustained rapid proliferation represents a major metabolic hurdle for cancer cells. The challenge lies in balancing the towering energy and nutrient demands required for biomass production with the harsh nutrient-depleted conditions of the tumor microenvironment. Hence, it is not surprising that tumors have evolved the capacity to employ the same oncogenic signaling pathways [e.g., RAS, MYC, PI3-kinase (PI3K)] that trigger aberrant growth to also control the metabolic rewiring that is necessary to adapt to a nutrient-deprived ecosystem (1).

Metabolic reprogramming is now recognized as one of the hallmarks of cancer cells (2) and the topic has become of increasing interest in recent years. Importantly, a better understanding of the molecular mechanisms and metabolic adaptations that confer growth and survival advantages to cancer cells could lead to the discovery of novel therapeutic opportunities. One of the many adaptive strategies that cancer cells use to fulfill their metabolic demands is the ability to exploit alternative nutrient acquisition pathways (3). Among them, macropinocytosis is an evolutionarily conserved form of bulk endocytosis by which cells incorporate extracellular fluid into large, irregularly shaped vesicles called macropinosomes (4). Macropinocytosis was first observed microscopically in malignant cells in the 1930s (5), and since then, it has been extensively studied in different cell types and in varying contexts. For example, the amoeboid organism *Dictyostelium discoideum* utilizes macropinocytic uptake to engulf fluid and nutrients during axenic growth (6). In antigen presentation

that occurs in the mammalian immune system, macrophages and dendritic cells employ macropinocytosis to internalize and process extracellular antigenic proteins (7). Macropinocytosis can occur at basal rates, as it occurs spontaneously in many cells, or it can be dramatically induced by receptor tyrosine kinase (RTK) activation or by oncogenes such as Ras (8) and v-Src (9). Although having been observed in transformed cells for nearly 30 years, the biological relevance of macropinocytosis in cancer has only recently been elucidated (10). In cancer cells harboring oncogenic Ras mutations, macropinocytosis serves as a nutrient uptake pathway by which extracellular protein is internalized and degraded to supply the much-needed amino acids that support cellular growth (10–12). This novel function of macropinocytosis as a feeding mechanism in tumors has provided a new perspective for cancer metabolism research and has positioned this ancient mechanism as a promising target for therapeutic intervention.

In this review, we aim to summarize the mechanisms that govern macropinocytosis regulation in cancer cells, as well as to provide the latest findings supporting its important role as a nutrient supply pathway that enables tumor cell proliferation and survival.

REGULATION OF MACROPINOCYTOSIS IN CANCER CELLS

Macropinocytosis is a clathrin-independent endocytic process driven by actin. In contrast to the closely related phagocytosis pathway, macropinocytic uptake is non-selective and not controlled by its cargo (13). Other distinctive features that define macropinocytosis are the fact that it can be stimulated by growth factors, and that it is suppressed by ion exchange inhibitors such as amiloride and its derivatives, which specifically inhibit macropinocytosis as opposed to other endocytic pathways (4, 14). Another definitive property is that macropinosomes are larger than other endocytic vesicles and can be specifically labeled by high molecular weight dextrans (15), which are incorporated into discrete vesicles larger than 0.2 μm in diameter.

Macropinocytosis is intimately linked to actin cytoskeleton dynamics. Protrusions of the plasma membrane, known as membrane ruffles, are formed *via* actin polymerization. Nascent macropinosomes arise at the cell surface of ruffling cells when these membrane protrusions spontaneously form cup-shaped ruffles that close, leading to fission of the nascent macropinosome from the plasma membrane and the internalization of extracellular fluid (13). Two types of membrane ruffles have been described and both can lead to macropinocytosis: planar ruffles, which are derived from the cell edges, and circular dorsal ruffles, which occur at the apical cell surface (16).

Both membrane ruffling and macropinocytosis depend heavily on actin cytoskeleton remodeling, as evidenced by their complete abrogation by inhibitors that disrupt actin polymerization, like cytochalasin D (17, 18). Moreover, many key regulators of actin polymerization, such as members of the Ras superfamily of small guanosine triphosphatases (GTPases), Ras, Rac, Cdc42, Arf6, and Rab5, among others, have been associated with ruffle formation and macropinocytic activity (19). Additionally, membrane phospholipids, in particular phosphatidylinositol (PI), PI4P, PI5P, PI(4,5)P_2, and PI(3,4,5)P_3, and the phospholipid kinases and phosphatases that interconvert them, are important players in the spatiotemporal regulation of macropinocytosis (13). For instance, inhibition of PI3K by either wortmannin or LY294002 has been shown to abolish macropinocytosis in several cell types including cancer cells, fibroblasts, and macrophages (18, 20, 21). Stage-specific enrichment of each type of PI during macropinosome maturation allows for the sequential recruitment and activation of specific enzymes and adapter proteins, including small GTPases and other proteins necessary for actin polymerization and membrane trafficking, such as Scar/Wave, Wasp, and Arp2/3 complexes, as well as sorting nexins (22–24).

These orchestrated rearrangements of the actin cytoskeleton, as well as the production and turnover of phospholipids necessary for macropinocytosis, can be initiated by growth factor-dependent activation of RTKs (**Figure 1**). Induction of macropinocytosis by growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and macrophage colony-stimulating factor, has been well studied and relies on the capacity of growth factors to stimulate membrane ruffling through the activation of the small GTPases Ras and Rac (14, 25–27). Several studies have demonstrated that Ras activation, either by growth factor stimulation or through oncogenic mutation, leads to increased membrane ruffling and macropinocytosis. First observations by Bar-Sagi and Feramisco showed that microinjection of oncogenic Hras into rat embryo fibroblasts rapidly induced ruffles and fluid-phase uptake of high molecular weight dextran (8). Similarly, Kras-transformed Rat-1 fibroblasts showed increased macropinocytosis that was dependent on PI3K and phospholipase C activity (18). More recently, it was shown that human bladder and pancreatic cancer cells that harbor oncogenic *HRAS* or *KRAS* mutations, respectively, display an enhancement of macropinocytosis relative to cancer cells of the same tissue type that express wild-type *HRAS* or *KRAS* (10). Furthermore, macropinocytic activity was also observed *in vivo* in a *Kras*-mutant mouse model of pancreatic ductal adenocarcinoma (PDAC) (10, 28), as well as in human primary PDAC specimens (11).

Ras activation leads to the stimulation of a plethora of different signal transduction pathways, including Rac, Cdc42, PI3K, and Raf/Erk activation. Activation of Rac by Ras has been shown to have an important role in inducing both membrane ruffling and macropinocytosis in different cell types (26). Both Rac1 transient activation and subsequent deactivation are required for complete closure and maturation of macropinosomes (29). Interestingly, Rac1 and Cdc42 are necessary and sufficient to induce macropinocytosis uptake in bladder cancer cells, as demonstrated by employing dominant negative and constitutively active forms of these small GTPases (30). In addition, it was recently demonstrated that Dock1, a Rac-specific guanine nucleotide exchange factor, is required for oncogenic Ras-induced macropinocytosis in several cancer cells (31).

Rac and Cdc42 can activate actin polymerization *via* p21-activated kinase 1 (Pak1), which has been shown to

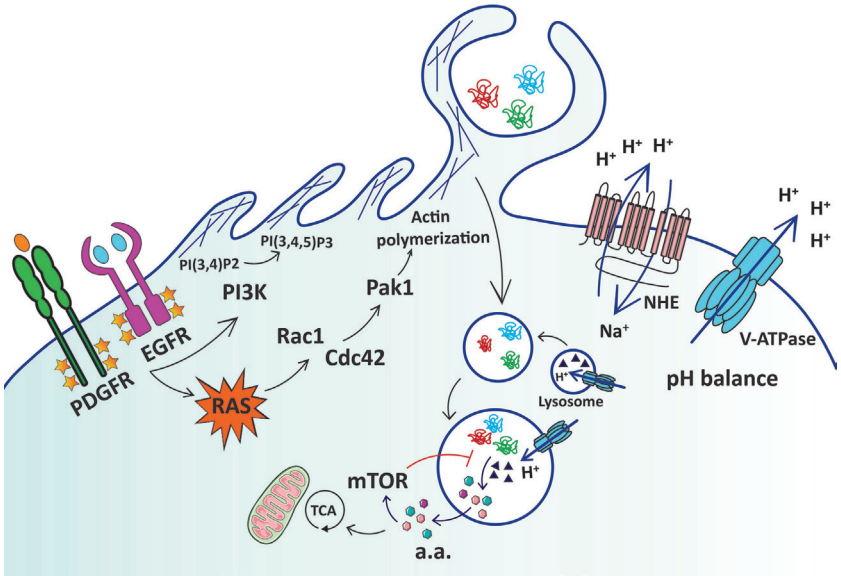

FIGURE 1 | Schematic representation of extracellular protein uptake *via* macropinocytosis in cancer cells. Ras activation, either by growth factor stimulation or through oncogenic mutation, leads to increased membrane ruffling and macropinocytosis *via* activation of Rac1 and Cdc42, which in turn stimulate p21-activated kinase 1 (Pak1) to induce actin polymerization. Activation of Rac1 and Cdc42 is sensitive to changes in submembranous pH, and the activity of Na+/H+ exchangers (NHEs) and vacuolar H+-ATPase (V-ATPases) is crucial to maintaining pH homeostasis. Conversion of membrane phosphoinositides by PI3-kinase (PI3K) is also necessary for macropinocytosis. Macropinosomes containing extracellular proteins such as albumin and collagen are internalized and subsequently fuse with lysosomes. Lysosomal proteases (▲) allow the catabolism of extracellular proteins into free amino acids (a.a.) that can fuel the TCA cycle to promote cell growth and survival. mTORC1 finely regulates the utilization of extracellular protein-derived amino acids by inhibiting macropinocytosed protein catabolism when free a.a. are abundant. Yellow stars represent phosphorylation of growth factor receptors.

co-localize with macropinosomes in 3T3 fibroblasts, and can drive macropinocytosis through circular dorsal ruffles when expressed in a constitutively active form (32, 33). Pak1 activity is also important for closure of macropinocytic cups through phosphorylation of CtBP1/BARS, a protein involved in membrane fission in the context of EGF-induced macropinocytosis (34). Signaling through other members of the Rab family of small GTPases, such as Rab5 and its effector Rabankyrin-5, have been shown to play a role in macropinosome formation and maturation. Specifically, both have been shown to be associated with membrane ruffles in epithelial cells (35, 36) and expression of a Rab5 dominant negative form inhibits PDGF-stimulated circular ruffles in MEFs (37).

While Ras, Rac, and Cdc42 signaling are critical for the early steps of macropinocytosis (i.e., membrane ruffling and formation of macropinocytic cups), PI3K activity seems to be specifically required for macropinosome closure in tumor cells and macrophages (18, 20, 21). Studies in the epithelial carcinoid cell line A431 have shown that PI3K inhibitors do not affect EGFR-induced membrane ruffling, but they inhibit macropinocytosis (21). These studies showed that as the macropinocytic cups close, PI(3,4)P2 is depleted from the cup while PI(3,4,5)P3 production by PI3K increases, and these highly regulated kinetics may be required for coordinated actin remodeling that allows macropinosome closure. It should be noted that these observations may be cell and/or stimuli specific, as other studies have demonstrated that PI3K can control plasma membrane ruffling caused by either oncogenic v-Src expression or PDGF stimulation (18, 38). The regulation of membrane ruffling by PI3K in these contexts may be mediated by PI3K-dependent activation of Rac1 (39).

The fate of macropinosomes after internalization varies depending on the cell type, as they can be recycled to the cell membrane as is the case in A431 cells (40) or they can adopt degradative properties by fusing with lysosomes and undergoing a lysosome-dependent acidification, as is the case in macrophages (41) and Ras-transformed cancer cells (10). Although the mechanisms underlying macropinosome maturation remain to be explored, a switch from Rab5 to Rab7 accumulation on the macropinosome (42) and the recruitment of specific septins to the maturing macropinosome, seem to regulate fusion events with late endosomal/lysosomal compartments (43). Although much work has been done in past years to elucidate the mechanisms that control macropinocytosis, more studies are necessary to further identify the cell- and tissue-specific pathways that regulate this process, especially in cancer cells where it can represent a therapeutic targeting strategy.

MACROPINOCYTOSIS AND pH HOMEOSTASIS

As discussed above, macropinocytic induction arises as a result of the coordinated interactions among small GTPases, actin filaments, and membrane phosphoinositides in restricted areas of the plasma membrane known as membrane ruffles. Because

of the electrostatic origin of some of these interactions, they are susceptible to alterations in the charge balance across the plasma membrane. Accordingly, quite soon after the discovery of growth factor-induced macropinocytosis, it was shown that conditions that acidify the cytosol, such as the addition of NH_4^+, or the blockade of the Na^+/H^+ exchangers (NHEs) by amiloride, dramatically inhibit macropinocytosis (14). Given that NHE inhibition selectively blocks macropinocytosis, leaving coated vesicles intact, sensitivity to amiloride and its analogs, like 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) and HOE-694, has been used as a distinctive feature of macropinocytosis (4). Nevertheless, the functional association between Na^+/H^+ exchange and macropinocytosis and the mechanisms mediating amiloride/EIPA inhibition remained unknown for many years.

Koivusalo et al. described that targeting of NHEs, by amiloride and HOE-694, abrogates EGF-induced macropinocytosis by lowering the submembranous pH (44). Interestingly, these changes in pH (from pH = 7.8 to 6.8) seemed to exclusively affect the recruitment and activation of Rac1 and Cdc42 to membrane ruffles. Consequently, the recruitment of their downstream effectors, Pak1 and Arp2/3, was also abrogated, thus inhibiting ruffle formation without affecting EGFR phosphorylation or PI3K activation. The authors also showed that EGF could stimulate Na^+/H^+ exchange with a concomitant alkalinization of the cytoplasm at sites of nascent macropinosome formation. Inhibition of the NHEs resulted in an accumulation of acidic equivalents, which are thought to occur due to a boost of metabolic activity driven by EGF stimulation. The outcome was an overall acidification of the cytoplasm, with a more pronounced effect in the vicinity of the plasma membrane where macropinocytosis was taking place (44). Although this study only addressed NHE inhibition of EGF-induced macropinocytosis in A431 cells, it is likely that similar mechanisms account for amiloride/EIPA inhibition in the setting of other growth factors and in Ras-induced macropinocytosis, where H^+ accumulation caused by increased metabolic activity and actin polymerization also occurs, especially in cancer cells where the high metabolic rate is well known to promote acidification of the cell and the tumor microenvironment (45–47).

The most commonly used macropinocytosis inhibitors, amiloride and EIPA, broadly target the SLC9A gene family of NHEs, which includes 11 isoforms reported to date (48). Whether specific NHE isoforms differentially regulate macropinocytosis remains an open question. Although NHE1 is highly expressed in several cancer cell lines (49) and is the most widely studied isoform, gene expression analyses indicate that NHE6, 7, and 8 are expressed at levels comparable to NHE1 in PDAC cells (50); therefore, it would be useful to conduct further studies aimed at identifying the contribution of specific NHE isoforms to macropinocytosis.

Underscoring the importance of NHE-dependent macropinocytosis in tumor growth, EIPA treatment of mice bearing MIA-PaCa2-derived xenograft tumors showed a suppression of intratumoral macropinocytosis that was accompanied by a reduction in tumor size relative to control mice (10). Moreover, EIPA treatment was effective only in tumors with high macropinocytic activity, as tumor growth rate was not affected in tumors derived from BxPC3 cells, which display low levels of macropinocytosis. These results indicated that enhanced macropinocytic activity in particular tumors might represent a metabolic vulnerability that can be specifically harnessed to restrain tumor growth.

In addition to nascent macropinosome formation, later stages of macropinosome maturation where the degradation of the macropinocytosed cargo occurs are also dependent on pH regulation. Macropinosome maturation includes fusion with lysosomes, which facilitates the delivery of the machinery necessary for compartmental acidification and the lysosomal proteases that are responsible for protein catabolism (41). Vacuolar H^+-ATPases (V-ATPases) function to maintain the acidic pH of different intracellular organelles such as late endosomes and lysosomes and these proton pumps play a critical role in vesicular trafficking and protein degradation (51). Concordantly, inhibition of V-ATPases by bafilomycin A1 has been shown to impair downstream events in the macropinocytosis pathway (**Figure 1**). For instance, bafilomycin A1 inhibits degradation of LDL causing its accumulation in macropinocytic vesicles in macrophages (52). Furthermore, protein degradation of macropinocytosed albumin was prevented by treatment with bafilomycin A1 in *KRAS*-mutant PDAC cells (10). Interestingly, bafilomycin A1 also inhibits the initial stages of macropinocytosis in *HRAS*-mutant T24 bladder cells (53), as well as in *KRAS*-mutant A549 lung cells (54). These effects of bafilomycin on nascent macropinosome formation may be a result of either (1) perturbations of cytosolic pH due to impaired pumping of protons into lysosomes or (2) alterations of submembranous pH due to inhibition of proton pumping to the extracellular space. Supporting the second possibility, several studies have demonstrated that V-ATPases can reside at the plasma membrane in *KRAS*-mutant PDAC and breast tumor cells and contribute to pH regulation at the plasma membrane (55, 56).

Altogether, these results suggest that pH homeostasis, both at the cellular and organellular level, is vital to properly execute the macropinocytosis program. Therefore, from the perspective of the pathological state, pH homeostasis is at the center of cancer cell metabolism and is critical to the role of macropinocytosis as a vital nutrient supply route that supports cancer cell growth and survival.

MACROPINOCYTOSIS AS A SURVIVAL STRATEGY IN TUMORS

Oncogenic Ras triggers a myriad of cellular adaptations to promote the metabolic rewiring that allows cancer cells to sustain unrestrained proliferation [reviewed in Ref. (57)]. Such rewiring includes enhanced glucose uptake and glycolytic activity, shifts in glutamine metabolism and redox balance (58, 59), increased flux of glucose to anabolic pathways such as hexosamines and ribose-5-phosphate (60), as well as upregulation of the major nutrient-scavenging mechanisms: autophagy (61–63) and macropinocytosis (10). It was recently demonstrated that extracellular amino acids and lipids rather than glucose contribute to the majority of cell biomass in proliferating cancer cells (64). These findings underscore the relevance of nutrient-scavenging

pathway exploitation by cancer cells to support tumor growth and survival.

Unlike autophagy, which generates nutrients from a limited supply of intracellular organelles and cytosolic proteins, macropinocytosis serves as a feeding mechanism by internalizing and degrading extracellular proteins. In this way, the resulting protein-derived amino acids can be utilized by the tumor to fuel central carbon metabolism, in addition to other metabolic pathways. This function has been proven to be particularly relevant to sustaining tumor growth in nutrient-deprived environments both *in vitro* and *in vivo*. For instance, mutant *KRAS*-driven pancreatic cancer cells, which rely on glutamine metabolism to support their growth, can maintain proliferation under glutamine-limiting conditions if supplied with extracellular serum albumin. Importantly, this escape from the deleterious effects of glutamine deprivation is suppressed by the inhibition of macropinocytosis (10). Similarly, extracellular serum albumin has the ability to reverse a proliferation arrest in PDAC cells grown in the absence of essential amino acids, such as leucine (11, 12). Tracing experiments with labeled extracellular protein showed that protein-derived amino acids are indeed incorporated into TCA cycle metabolites, supporting the growth-promoting role of macropinocytosis in Ras-transformed cells (10). Thus, macropinocytosis is necessary to support cell growth under nutrient-deprived conditions. Whether the extent of macropinocytosis can be dialed up or down depending on nutritional status remains to be elucidated.

The potential importance of macropinocytosis in human cancer is underscored by the observation that PDAC tumor tissues from Whipple procedure patients display enhanced macropinocytic uptake relative to adjacent non-neoplastic regions (11). Such PDAC tumors are hypovascularized and are depleted of amino acids (11); therefore, extracellular protein scavenging represents an attractive alternative for nutrient acquisition in these tumors as opposed to the import of free, circulating amino acids, which would depend on adequate perfusion. In agreement with this, extracellular protein uptake and catabolism *via* macropinocytosis has been directly evidenced *in vivo* in murine PDAC tumors (28). Moreover, inhibition of macropinocytosis *via* EIPA treatment suppressed tumor growth in xenograft tumors (10). Although the majority of the studies on the utilization of extracellular protein as a nutrient source have been performed in pancreatic cancer cells and animal models, similar results have also been reported in Ras-driven cancer cells of different tissue origins, such as bladder, lung, sarcoma, and colon cancer (10, 31, 65). The cell growth effects mediated by macropinocytosis under glutamine-deprived conditions were suppressed in *KRAS*-mutant lung, sarcoma, and colon cells, when the Rac1 activator Dock1 was inhibited either pharmacologically or by genetic ablation. Moreover, treatment with a Dock1 inhibitor decreased tumor growth and metastasis in mice (31). In addition to tumor cells, macropinocytosis was also observed in the stromal compartment of PDAC tumors (11). Further studies are necessary to evaluate the potential contribution of macropinocytosis to survival and growth of these cells.

The intracellular degradation of extracellular proteins acquired *via* macropinocytosis is dependent upon lysosomes, which are tightly controlled by the mammalian target of rapamycin complex (mTORC1), a key regulator of cell growth that responds to nutrient availability (66). Lysosomal catabolism of extracellular proteins has been shown to activate mTORC1 in Ras-transformed cells (12), and furthermore, macropinocytosis-derived lysosomal amino acids are required for rapid activation of mTORC1 in response to growth factor stimulation to promote cell growth (67). On the other hand, mTORC1 activation seems to minimize lysosomal degradation of macropinocytosed proteins. Suppression of mTORC1 by rapamycin or torin1 enhances protein catabolism and proliferation in amino acid-starved PDAC cells *in vitro*, and in hypovascularized, nutrient-poor regions of PDAC tumors (12). Thus, although it seems that mTORC1 does not directly regulate the early steps of macropinocytosis, it does play a pivotal role in regulating the degradation of proteins that are internalized by macropinosomes, allowing for the coordination of protein catabolism in response to nutrient availability. Hence, when free amino acids are plentiful, mTORC1 activation could suppress the catabolism of macropinocytosed proteins and conversely, as amino acid levels decrease upon consumption, mTORC1 suppression could allow for enhanced protein degradation. It is also conceivable that as protein-derived amino acids are produced, they, in turn, activate mTORC1, establishing a feedback regulatory loop that serves to shift between nutrient acquisition pathways depending on nutrient availability (**Figure 1**).

Finally, molecules other than serum albumin are also internalized *via* macropinocytosis, and uptake of these molecules might also contribute to tumor metabolism and growth. For example, tumor cells can internalize and catabolize extracellular matrix molecules, such as fibronectin and collagen (28, 68). Like serum albumin, internalization of these matrix molecules serves to produce protein-derived amino acids that can support tumor cell growth. In addition to extracellular proteins, *KRAS*-mutant A549 lung cancer cells can also macropinocytose extracellular ATP in order to increase the intracellular ATP pool (69). Ras-driven cancer cells are also known to have increased lipid scavenging to support tumor metabolism (70, 71), and although the mechanisms remain to be elucidated, it is reasonable to hypothesize that serum lipids bound to albumin are taken up *via* macropinocytosis.

CONCLUDING REMARKS

The critical role that metabolic reprogramming and adaptation strategies play in supporting the growth of tumors is now widely recognized. By inducing the internalization of extracellular proteins, and other macromolecules, that can be further processed and utilized to fuel different metabolic pathways, macropinocytosis provides not only a survival mechanism under nutrient-scarce conditions but also the potential for unrestricted tumor growth in an adverse tumor microenvironment. For this reason, targeting macropinocytosis has emerged as a novel therapeutic strategy that requires further investigation. Understanding the molecular events that drive macropinocytosis in the context of different cancers might inform the design of more specific and potent inhibitors. Given that macropinocytosis is crucial to

sustaining tumor growth under nutrient-deprived conditions, it is possible that macropinocytosis inhibition would be particularly beneficial in patients suffering from severely hypoxic or hypovascularized tumors, such as PDAC. Moreover, studies focused on combination therapies employing macropinocytosis inhibitors in conjunction with other metabolic pathway inhibitors could pave the way for improved therapeutic outcomes.

AUTHOR CONTRIBUTIONS

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Obesity and Breast Cancer: Current Insights on the Role of Fatty Acids and Lipid Metabolism in Promoting Breast Cancer Growth and Progression

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Obesity and excess accumulation of adipose tissue are known risk factors for several types of cancer, including breast cancer. With the incidence of obesity constantly rising worldwide, understanding the molecular details of the interaction between adipose tissue and breast tumors, the most common tumors in women, becomes an urgent task. In terms of lipid metabolism, most of the studies conducted so far focused on upregulated *de novo* lipid synthesis in cancer cells. More recently, the use of extracellular lipids as source of energy came into focus. Especially in obesity, associated dysfunctional adipose tissue releases increased amounts of fatty acids, but also dietary lipids can be involved in promoting tumor growth and progression. In addition, it was shown that breast cancer cells and adipocytes, which are a major component of the stroma of breast tumors, are able to directly interact with each other. Breast cancer cells and adjacent adipocytes exchange molecules such as growth factors, chemokines, and interleukins in a reciprocal manner. Moreover, it was shown that breast cancer cells can access and utilize fatty acids produced by neighboring adipocytes. Thus adipocytes, and especially hypertrophic adipocytes, can act as providers of lipids, which can be used as a source of energy for fatty acid oxidation and as building blocks for tumor cell growth.

Keywords: breast cancer, obesity, adipose tissue, lipid metabolism, free fatty acids

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INTRODUCTION

Breast cancer is the most abundant malignant tumor and the leading cause of death from cancer in women worldwide (1, 2). Established risk factors for breast cancer are a woman's age, own or familial history of breast cancer or of precancerous lesions, genetic configuration, pregnancies and reproductive treatment, consumption of alcohol, and exposure to ionizing radiation (3). In addition, overweight and obesity are now regarded as promoting factors for breast cancer development and progression. This perception is based on numerous recent epidemiological and experimental studies with following observations: several population studies demonstrated that obesity and associated excess accumulation of adipose tissue are associated with an elevated risk for breast cancer, especially

in post-menopausal women (4–6) and are independent negative prognostic factors for mammary tumors (7–10). On a molecular level, several studies showed that adipocytes, which are a major component of the stromal environment of mammary tumors, exert tumor-promoting effects on breast cancer cells. Several hypotheses about how adipose tissue and adipocytes promote tumorigenesis have been described, but the molecular mechanisms that underly this interaction are yet to be defined in more detail.

Signaling molecules and metabolites secreted by adipose tissue and adipocytes, especially in the obese state, are now recognized as important factors for cancer progression as they directly or indirectly stimulate anti-apoptotic effects, cell growth, angiogenesis, and migration (11, 12). Mature adipocytes are the major cell type of white adipose tissue and are primarily responsible for the metabolic homeostasis of the body. Lipids are stored here in the form of triacylglycerol (TAG) and released as free fatty acids (FFA) in times of demand. Besides energy storage, adipocytes also play an active role in endocrine signaling to other tissues of the body, by secreting hormones, adipokines, cytokines, and growth factors (13, 14). An elevated intake of calories and a largely sedentary lifestyle can lead to obesity, which often results in dysfunctional adipose tissue. In particular, adipocytes become hypertrophic and store elevated amounts of TAGs along with higher secretion of adipokines and pro-inflammatory cytokines, such as tumor necrosis factor-α, IL-6, IL-8, and PAI-1 (**Figure 1**). These molecules are chemoattractants for macrophages, monocytes, and other immune cells, which induce a chronic low-grade inflammation within the adipose tissue. As a result, lipolysis is initiated and adipocytes release elevated amounts of FFAs, which adversely affects lipid homeostasis of the entire organism and leads to subsequent metabolic diseases (12). The release of higher amounts of fatty acids could be a direct mechanism through which adiposity may promote cancer progression by delivering building blocks for the production of pro-tumorigenic signaling lipids (14).

Visceral obesity and increased adipose tissue mass are often accompanied by low levels of plasma high-density-lipoprotein cholesterol (HDL-C), which has been associated with breast cancer risk in some studies (15, 16). However, evidence for the relationship between plasma HDL-C and breast cancer risk

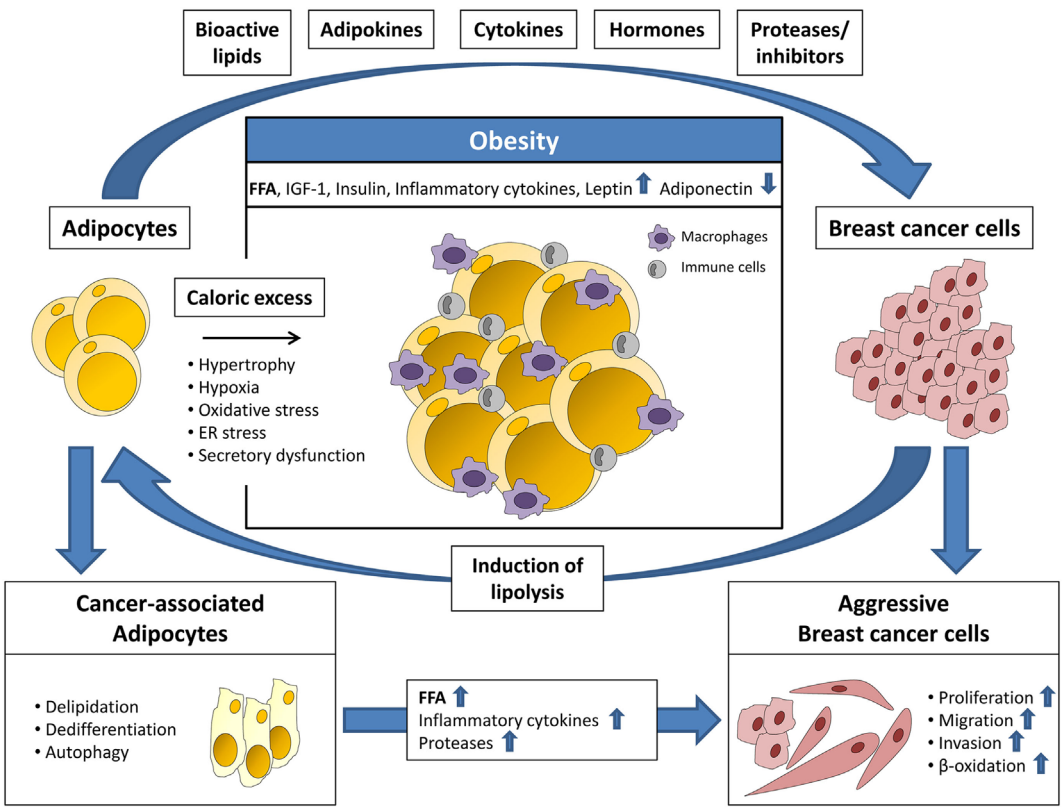

FIGURE 1 | Adipocytes and breast cancer cells interact *via* several secreted factors. Adipocytes secrete bioactive lipids, adipokines, cytokines, hormones, and proteases/protease inhibitors priming breast cancer cells for a more aggressive phenotype. This includes increased proliferation, migration, invasion, and β-oxidation. Breast cancer cells induce adipocyte lipolysis resulting in the formation of cancer-associated adipocytes (CAAs), which are characterized by delipidation, dedifferentiation, autophagy, and altered secretion. In turn, the increased release of free fatty acids (FFA), inflammatory cytokines, and proteases from CAAs promotes breast cancer progression. In obesity, the adipose tissue is characterized by hypertrophy and increased infiltration of macrophages and other immune cells. Furthermore, adipocyte function is impaired due to hypoxia, oxidative, and ER stress leading to secretory dysfunction. The resulting elevated release of FFA, insulin-like growth factor-1 (IGF-1), insulin, inflammatory cytokines, and leptin, and decreased secretion of adiponectin, enhances the tumor-promoting effects of adipose tissue.

remains equivocal (17) and it is not clear whether low HDL-C causally affects tumorigenesis or merely serves as a biomarker for poor lifestyle and dietary habits.

Epidemiologic studies have also investigated the relationship between elevated plasma low-density-lipoprotein cholesterol or total cholesterol and cancer occurrence and prognosis. In terms of breast cancer onset, these studies yielded contradictory findings (17, 18). However, in several recent studies, elevated plasma cholesterol levels were associated with a poor prognosis and the use of cholesterol-lowering medication (statins) increased recurrence-free survival of breast cancer patients (19–21). Large-scale prospective studies, adequately controlled for confounding factors, are necessary to substantiate the potential beneficial effects of statins and other cholesterol-lowering drugs in breast cancer patients.

Regarding intracellular lipid metabolism, it is well known that several tumor cells show a hyperactivation of various lipid synthesis pathways, including breast cancer. Breast cancer cells show an increased activity of fatty acid synthase (FASN), an enzyme used for *de novo* fatty acid synthesis. In addition, breast cancer cells also show an upregulation of monoacylglycerol lipase (MAGL). The MAGL pathway controls the intracellular release of fatty acids and its hyperactivation is often associated with the aggressiveness of a tumor. Together, FASN and MAGL very likely promote cancer progression by synthesizing and mobilizing intracellular lipids, which in turn promote tumor growth (14, 22, 23). Interestingly, lipidomic analyses demonstrated that the incorporation of endogenous fatty acids into membrane phospholipids is enhanced in mammary carcinomas as compared to normal human breast tissue. Furthermore, these changes in membrane lipid composition correlated with tumor progression, hormone receptor status, and patient survival, with the concentration of these lipids being the highest in ER-negative and grade 3 tumors (24). Moreover, another study showed that the ratios of specific monounsaturated fatty acid phosphatidylcholines compared to saturated fatty acid phosphatidylcholines are significantly higher in cancerous tissue in comparison to healthy reference sections (25). A different aspect of the role of lipid metabolism in cancer is seen in patients with late-stage cancers, who often suffer from cachexia. This phenomenon is characterized by the loss of both muscle and fat mass through catabolic mechanisms. This process is triggered by a marked upregulation of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), which break triglycerides into diglycerides and diglycerides into fatty acids, respectively. The resulting elevated levels of circulating FFA can be used as building blocks for cancer cell growth or tumorigenic signaling lipids (26). Thus, cancer cells are able to utilize FFA not just from *de novo* lipogenesis but also from exogenous fat sources. Intriguingly, a few recent articles described that breast cancer cells can access and directly use lipids from neighboring adipocytes (27, 28). One study even demonstrated that the predominant source of *de novo* lipid synthesis by breast cancer cells is extracellular lipids, not just glucose and glutamine (27). Together, these studies indicate that breast cancer cells are metabolically very flexible and fit the current notion that metabolic reprogramming is an emerging hallmark of cancer cells. However, in contrast to endocrine and paracrine effects of adipose tissue in obesity, the role of extracellular fatty acids in breast cancer metabolism is a relatively new area of research and warrants further elucidation.

In this review, we will focus on the role of lipids from excess adipose tissue in obesity, from tumor-associated adipocytes or dietary lipids, and discuss how these extracellular fatty acids drive tumor growth and progression.

LIPIDS DELIVERED TO BREAST CANCER CELLS FUEL TUMOR GROWTH

Direct Interaction of Adipocytes and Breast Cancer Cells

The tumor microenvironment plays an important role for its growth and progression since non-malignant cells of the stroma, such as endothelial cells, immune cells, tumor-associated macrophages and tumor-associated fibroblasts, deliver tumor-promoting molecules, including chemokines, interleukins, and growth factors (29). In breast cancer, the interaction of breast tumor cells with surrounding fibroblasts, immune, endothelial, and mesenchymal cells is well studied. By contrast, the crosstalk of breast tumor cells with associated adipocytes has been addressed only recently. In fact, adipocytes are a major component of the microenvironment of mammary tumors. During early tumor cell invasion, breast cancer cells invade the mammary fat pad and exist in direct conjunction with neighboring adipocytes (30). Several recent studies demonstrated that this direct interaction with adipocytes has tumor-promoting effects (**Figure 1**). Breast cancer cells secrete, among other factors, cytokines and lypolytic enzymes, which affect adipocytes. In a reciprocal manner, associated adipocytes secrete adipokines, growth factors, proteases, and fatty acids, which stimulate tumor growth and survival (31, 32). In addition, a study by Dirat et al. showed that breast cancer cells induce lipolysis together with a phenotypic change in neighboring adipocytes. These fat cells, termed cancer-associated adipocytes (CAAs), are characterized by a fibroblast-like morphology, a significant decrease in number and size of intracellular lipid droplets and loss of terminal adipocyte differentiation markers, such as leptin or FABP2 (**Figure 1**). Functionally, CAAs secrete increased amounts of proteases and interleukins, such as PAI-1, IL-6, and IL-1β, which promote tumor aggressiveness. In addition, CAAs were shown to deliver fatty acids, important building blocks for tumor proliferation (30). Using a co-culture model of ovarian cancer cells and omental adipocytes, Nieman and co-workers showed that cancer cells have the ability to take up and utilize fatty acids from surrounding fat cells (28). This co-cultivation induced lipolysis within the adipocytes and enabled a direct transfer of lipids to the cancer cells together with enhanced lipid storage and mitochondrial oxidation. Analogous co-cultivation of omental adipocytes with MCF-7 and MDA-MB-231 breast tumor cells also resulted in lipid droplet accumulation in the cancer cells (28). The impact of adipocyte-derived fatty acids on breast cancer cell progression was underscored by work conducted by Balaban et al. showing that MCF-7 and MDA-MB-231 breast cancer cells induced HSL/ATGL-dependent lypolysis in co-cultured adipocytes which resulted in increased cancer cell proliferation and migration. This effect was even more enhanced

when adipocytes were loaded with a mixture of oleate, palmitate, and linoleate beforehand of co-cultivation to mimic "obese" adipocytes and thereby demonstrated that an increased availability of fatty acids for mitochondrial oxidation promotes breast cancer cell progression (27). Together, these studies suggest a metabolic shift of cancer cells in adaption to the availability of metabolic substrates in the microenvironment. This metabolic shift activates alternative pathways to support tumor growth and survival. To date, most of the studies examining breast cancer cell lipid metabolism focused on glucose and glutamine metabolism as precursors for *de novo* lipogenesis. The data described above clearly point out that extracellular lipids are an important source for breast cancer cell lipid synthesis and fatty acid oxidation. The translational relevance of these findings is substantiated by a recent study by Camarda et al. (33). The authors demonstrate that highly aggressive triple-negative breast cancer cells, which overexpress the oncogenic transcription factor MYC, show significantly increased rates of fatty acid oxidation. Pharmacological inhibition of fatty acid oxidation dramatically decreased energy metabolism and, therefore, cell and tumor growth *in vitro* and *in vivo* in a MYC-dependent manner. Together, these data highlight that targeting lipid metabolism and lipid uptake should be considered for the development of novel therapeutic strategies in breast cancer.

Fatty Acids Released by Adipose Tissue in Obesity

Obesity is described as excess fat storage and accumulation of adipose tissue, which becomes deregulated. Dysfunctional adipocytes release increased amounts of fatty acids which accumulate in non-adipose tissues, such as liver, heart, or muscle. Intermediates of intracellular fatty acid metabolism, such as ceramides or diacylglycerols (DAGs), can ultimately induce lipotoxicity (34). Lipotoxicity is characterized by cell cycle and mitochondrial deregulation, autophagy, and apoptosis. Several recent studies have shown that an over-production of ceramides or DAGs induces growth arrest and apoptosis in various cancer cells (35, 36). These discoveries open interesting inroads for the development of new lipid-based cancer treatment options. On the other hand, elevated levels of fatty acids can be utilized by cancer cells as source of energy or as building blocks for oncogenic lipid signaling molecules, such as lysophosphatidic acid (LPA), prostaglandins and sphingosine-1-phosphate (S1P) (**Figure 1**) (14). In the past few years, several studies addressed the cellular and molecular mechanisms linking fatty acids and cancer using cell culture experiments and animal models. For example, oleate, which is the most abundant fatty acid esterified to triglycerides in adipose tissue, has been explored for its potential role in cancer progression (37–39). A recent *in vitro* study points in the direction that breast cancer cells use exogenous lipids, such as oleate, to regulate lipid metabolism, in addition to *de novo* fatty acid synthesis (40). Moreover, the authors show that a proliferative effect of oleate on breast cancer cells is dependent on the fatty acid translocase/CD36, as silencing of CD36 mRNA expression significantly decreased exogenous fatty acid uptake, which turns CD36 into an interesting candidate for novel treatment strategies (40). Also recently, Shen et al. demonstrated that oleate induces the expression of angiopoietin-like 4 (*ANGPTL4*) in head and neck squamous cell carcinoma resulting in anoikis resistance and metastasis *via* upregulation of fibronectin (41). Notably, palmitate and linoleate also induced *ANGPTL4* gene expression in these cancer cells. Moreover, the induction of *ANGPTL4* expression by oleate was also detected in other cancer cell types, including breast cancer cells (41). This suggests an interesting link since Angptl4 has been described to promote breast cancer cell invasion and metastasis to the lung *in vitro* and *in vivo*, respectively (42–44). The role of oleate was also studied with respect to metabolic adaptions in highly aggressive cancer cells. An *in vitro* study by Li and co-workers showed that AMPK is activated in highly metastatic gastric and breast cancer cells treated with oleate (45). AMPK promoted the rates of fatty acid oxidation and ATP synthesis in these cells, enabling increased cell growth and cell migration. In low metastatic cancer cells, oleate reduced cell proliferation and migration, indicating a selective tumor-promoting function of oleate on highly metastatic cancer cells (45). The pro-tumorigenic effect of oleate was also demonstrated by an independent study showing that the treatment with oleate promoted cell invasion in highly metastatic breast cancer cells, but not in low metastatic cancer cells (38). Addressing the potential underlying mechanism, Hardy et al. showed that oleate enhanced cell proliferation *via* activation of G protein-coupled receptor 40 in highly aggressive breast cancer cells (46). Moreover, oleate treatment of breast cancer cells resulted in long-term survival in serum-free media, which was associated with enhanced intracellular lipid droplet formation and upregulation of lipolysis (47). In contrast to the tumor-promoting effects of oleate, palmitate, which is the most abundant circulating saturated fatty acid in the human circulation, exhibited inhibitory effects in *in vitro* studies (48, 49). For example, the treatment of breast cancer cells with palmitate mediated the inhibition of cell proliferation and induction of apoptosis. Interestingly, oleate antagonized the proapoptotic function of palmitate in these experiments (49).

Together, these data indicate that the effects of fatty acids on breast cancer progression are complex and depend on the fatty acid subtype, the combination thereof, and the specific breast cancer subtype. More future studies are warranted to uncover the detailed link between obesity, fatty acids, fatty acid metabolism intermediates, and breast cancer progression.

Cholesterol Metabolism and Breast Cancer

Changes in cholesterol and lipid metabolism (often due to poor diet or obesity) have been extensively studied as risk factors for various malignancies, including breast cancer. Several epidemiological studies investigated the relationship between cholesterol and the risk of breast cancer, with inconsistent results (17). However, Li and co-workers demonstrated in a more recent meta-analysis study that dietary cholesterol was associated with an increased risk of breast cancer (50). Evidence for the role of elevated plasma cholesterol in promoting breast cancer was also obtained in recent experimental studies. The induction of hypercholesterolemia in mice resulted in enhanced breast cancer

growth, suggesting tumor-promoting effects of hypercholesterolemia (51, 52). Moreover, the primary oxysterol metabolite of cholesterol, 27-hydroxycholesterol (27-OHC), was identified to promote growth and metastasis *in vivo* (53, 54). Higher levels of 27-OHC were also detected in human estrogen receptor-positive breast tumors as compared to adjacent normal breast tissue (55). In addition, 27-OHC was also described to play a crucial role in mediating resistance of estrogen receptor-positive breast cancer to specific endocrine therapies (56, 57). Together the data show that alterations in lipid and cholesterol metabolism might be important factors in promoting breast cancer progression. To fully understand how obesity and associated changes in lipid metabolism affect breast cancer biology is going to be one of the demanding but irremissible tasks in battling breast cancer.

The Role of Omega-3 and Omega-6 Polyunsaturated Fatty Acids (PUFAs) in Breast Cancer

The impact of FFA and specific components, such as saturated, monounsaturated, and PUFAs, were studied in several human diseases, including cancer. Much of the data implicate that saturated fatty acids and monounsaturated fatty acids elevate cancer risk, whereas specific PUFAs (omega-3 PUFAs) exhibit anticancer effects (58–61). Still, since not all of the studies conducted so far showed consistent results, more detailed analyses are warranted. Particularly with regard to breast cancer, the contribution of dietary fatty acids depends on diverse factors, e.g., breast cancer subtype, a woman's menopausal status, fatty acid species, and intake ratios (62).

The two major groups of PUFAs, omega-3 and omega-6 PUFAs, are essential fatty acids, which must be ingested as part of a diet. Omega-3 PUFAs, such as eicosapentaenoic acid and docosahexaenoic acid are precursors for the production of anti-inflammatory eicosanoids and inflammation resolving derivates, such as resolvins and protectins (63). On the other hand, eicosanoids resulting from the omega-6 PUFA–arachidonic acid (AA) axis are predominantly involved in the initiation and maintenance of inflammation (63). In recent years, epidemiologic studies have explored the role of omega-3 and omega-6 PUFAs on cancer risk and reported that consumption of western diets with a low omega-3:omega-6 ratio is associated with a higher risk of several cancer types (64). Notably, an elevated intake of omega-3 PUFAs as well as a higher dietary intake ratio of omega-3:omega-6 PUFAs correlated with reduced breast cancer risk in obese women, but there was no such association in overweight or normal weight women (65). Thus, this study suggests a link between obesity, omega-3-PUFAs intake, and breast cancer risk. Several mechanisms have been proposed for the anti-tumor effects of omega-3 PUFAs, including the alteration of the cell plasma membrane composition, the inhibition of AA-derived synthesis of inflammatory eicosanoids, and alteration of gene expression of genes known to be involved in cell proliferation and apoptosis (62). Especially in connection with obesity, omega-3 PUFAs might be a useful tool in reducing obesity-associated inflammation and related tumor risk (66, 67).

In conclusion, these studies support the interesting notion that PUFAs, especially omega-3 PUFAs, are linked to reduced breast cancer risk, in particular by decreasing pro-tumorigenic inflammation. However, more clinical studies are needed to fully understand the role of omega-3 and omega-6 PUFAs in obesity-associated breast cancer.

SUMMARY

Obesity is now recognized as an important risk factor for breast cancer development and progression. Several mechanisms have been suggested to explain this association, including inflammatory signaling, chemokines, adipokines, and insulin. In addition, more recent studies demonstrated that extracellular lipids play an important role in promoting breast cancer growth and progression by serving as substrates for activated fatty acid oxidation or as building blocks for oncogenic lipid signaling molecules. Breast cancer cells may obtain extracellular lipids through deregulated adipose tissue, by dietary intake or by directly interacting with adipocytes of the tumoral stroma. Emerging evidence clearly indicates that breast tumor cells are able to adapt to their metabolic environment in a very flexible manner. Targeting the utilization of extracellular lipids in breast tumor cells may open up new avenues for breast cancer treatment.

AUTHOR CONTRIBUTIONS

CB and SCS conceived the review, critically analyzed the current literature, and wrote and revised the manuscript.

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OPINION
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Is There a Therapeutic Window for Metabolism-Based Cancer Therapies?

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Cells that have undergone an oncogenic transformation have an altered metabolism compared to the cells they originate from (1). This observation led to the addition of "a deregulated metabolism" to the hallmarks of cancer (2). Accordingly, it has been extensively demonstrated that many of the observed alterations in the metabolism of cancer cells are important for their proliferation (1, 3, 4). However, a metabolic alteration that is important for cancer cell proliferation is not automatically a good target for treatment, as treatments also have to be selective toward cancer cells. Since almost all of the cancer-induced metabolic changes are not caused by gain of function mutations in specific enzymes, metabolism-based drug have to be developed against the naturally occurring enzymes. Thus, the valid question arises whether there is a therapeutic window for targeting the deregulated metabolism of cancer cells. In the following, I would like to describe the challenges and advocate the opportunities for metabolic drug targets in cancer treatment. In the first section, I will address the question whether there is in general a therapeutic window for metabolism-based cancer treatment, while in the second section, I will discuss new concepts that can refine metabolism-based anticancer strategies.

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THERAPEUTIC WINDOW

Is there a therapeutic window for metabolism-based cancer treatment? A major challenge for metabolic drugs in cancer treatment is that metabolism is a universal cellular process and, with a few exceptions (such as gain of function mutations in metabolic enzymes), the metabolic alterations found in cancer cells are present in similar form in some non-transformed cell; i.e., while cells that undergo an oncogenic transformation will always change their metabolism, there is no single metabolic change that unifies all cancer cells and separates them from all non-transformed cells. Based on this fact, one could argue that targeting the metabolism of cancer cells is challenging, since it is not selective. However, an opportunity for treatment arises based on the fact that many metabolic changes in cancer cells support cell proliferation, while the majority of the non-transformed cells are in a differentiated and low proliferative state. Thus, metabolic drugs that impair cellular proliferation preferentially target cancer cells. The validity of this reasoning is supported by the fact that many of the first chemotherapeutic agents that are still used in the clinics are targeting the metabolism of proliferating cancer cells (5). Examples are the antifolate methotrexate and the nucleoside analog 5-fluorouracil. Despite the fact that these agents target any highly proliferating cell rather than only cancer cells, their usage has revolutionized cancer treatment and the benefits still justify the side effects arising from their moderate selectivity. Thus, metabolism-based treatments are feasible, currently used in the clinics, and a patient benefit at least in the scope of a typical standard of care chemotherapeutic agent can be expected.

Yet, is it possible to refine metabolism-based cancer therapies by increasing efficacy and selectivity and thus broaden the treatment window that arises from the metabolic changes that occur in

cancers? In the following, I will focus on three concepts that aim to refine metabolism-based anticancer drugs.

METABOLIC VULNERABILITIES ARISING FROM THE CANCER-SPECIFIC GENETIC LANDSCAPE

One of the earliest approaches to refine metabolism-based anticancer drugs has focused on metabolic vulnerabilities that arise due to the genetic loss of tumor suppressors or hyperactivation of oncogenes. The rationale for this approach is that many tumor suppressors and oncogenes regulate metabolic genes and consequently loss or hyperactivation of this regulation creates dependencies on specific metabolic pathways (6). This approach led to the identification of an oncogene specific and targetable metabolism in cultured cancer cells. Yet, recent *in vivo* data show that the organ microenvironment and the cell origin can redefine the oncogene-imposed metabolic dependencies of cancer cells and thus can lead to impaired *in vivo* efficacy of metabolic drugs (7–11). A solution to this challenge is the integration of oncogene profiles with the cell origin and the organ microenvironment. An example for the validity of this concept is the finding that cancers with $Kras^{G12D/+}$; $Trp53^{-/-}$ background originating and growing in the lung are susceptible to branched chain amino acid metabolism inhibition, while this is not the case for cancers with the same genetic background but originating and growing in the pancreas (10). Thus, the cancer-specific oncogene and tumor suppressor landscape can be exploited to increase the efficacy of metabolic drugs in the context of the cell origin and the organ microenvironment.

Another concept that builds on the genetic landscape of cancers to increase the selectivity of metabolic drugs focuses on the metabolic vulnerabilities arising from a mutation in or gene loss of a metabolic enzyme (**Figure 1A**). The rationale of this concept is that normal cells have the metabolic flexibility to cope with drugs that (partially) inhibit an enzyme, while cancer cells fail to have this flexibility due to a mutation or loss in an enzyme concomitant to the enzyme targeted by the drug. An example for this concept are cancers with homozygous loss of p16/CDKN2A resulting in the passenger deletion of the enzyme methylthioadenosine phosphorylase (MTAP) (which is found in ~15% of all cancers and>50% of glioblastoma multiforme) and inhibition of the enzyme arginine methyltransferase (PRMT5) (12–14). Mechanistically, loss of MTAP results in the accumulation of its metabolite substrate methylthioadenosine, which partially inhibits PRMT5 activity. Consequently, cancers with loss of MTAP and therefore already impaired PRMT5 activity are hypersensitive toward PRMT5 inhibitors (12–14). Another example for this concept is demonstrated by the effectiveness of a pyruvate carboxylase (PC) knockdown to impair the proliferation of paraganglioma with mutation in succinate dehydrogenase (SDH) (15, 16). Mechanistically, mutations in SDH result in a truncated tricarboxylic acid cycle and therefore impaired glutamine anaplerosis (17), which is a process that supports aspartate production required for nucleotide biosynthesis. Consequently, SDH mutant tumors switch to PC-dependent anaplerosis to sustain nucleotide biosynthesis. In turn, SDH mutant tumors are hypersensitive toward PC knockdown, while non-transformed cells have the flexibility to use either path of tricarboxylic acid cycle anaplerosis. Thus, combining the genetic loss of an enzyme with a metabolic drug creates hypersensitivity specifically in cancer cells. Taken together, identifying the metabolic vulnerabilities that arise from the cancer-specific genetic landscape can be conceptualized to increase the selectivity and efficacy of metabolic drugs.

CANCER CELL PHENOTYPES BEYOND PROLIFERATION

A recent concept to refine metabolic drugs is focused on understanding the metabolic vulnerabilities of metastasizing rather than proliferating cancer cells. As described in the first section, most metabolism-based anticancer drugs inhibit the proliferation of cancer cells (1, 18). Unquestionable, this is a very important aspect of cancer therapy. However, this focus on proliferation contributes to the moderate selectivity of metabolism-based drugs (and many other drugs that target cancer cell proliferation), since some non-transformed cells also proliferate. A solution to this challenge is the concept to go beyond the proliferative phenotype of cancer cells and target their single cell survival and colonization capacity (**Figure 1B**). These latter phenotypes are less frequently found in normal cells compared to the proliferation phenotype. Moreover, they are particularly important for cancer progression toward metastasis formation, which results in up to 90% of the patient mortality. Thus, considering phenotypes beyond proliferation can increase selectivity of metabolic drugs and advance their application toward inhibition of metastasis formation. An example for this concept is the recent discovery that inhibition of proline catabolism impairs metastasis formation by breast cancer cells without apparent adverse effects on normal cells and organ function (19). Mechanistically, metastasizing cancer cells rely on proline catabolism to fuel their increased energy need during the colonization of distant organs. Consequently, targeting proline metabolism does not affect primary cancer growth or non-transformed cells, but impairs metastasis formation in distant organs (19). Another example for this concept is the finding that the survival of metastasizing cancer cells in the circulation depends on their antioxidants metabolism (20). Consequently, targeting one carbon metabolism that contributes *via* NADPH production to the cellular antioxidants response [e.g., by inhibiting methylenetetrahydrofolate dehydrogenase (MTHFD1)] decreases the survival of cancer cells in the circulation and subsequently metastasis formation in distant organs (20). Taken together, targeting cancer cell phenotypes beyond proliferation refines metabolic drugs and extends their application toward anti-metastatic agents.

EXTENSION TO STROMAL AND IMMUNE CELLS

An additional concept to refine the use of metabolic drugs in cancer treatment is targeting the entire cellular composition of

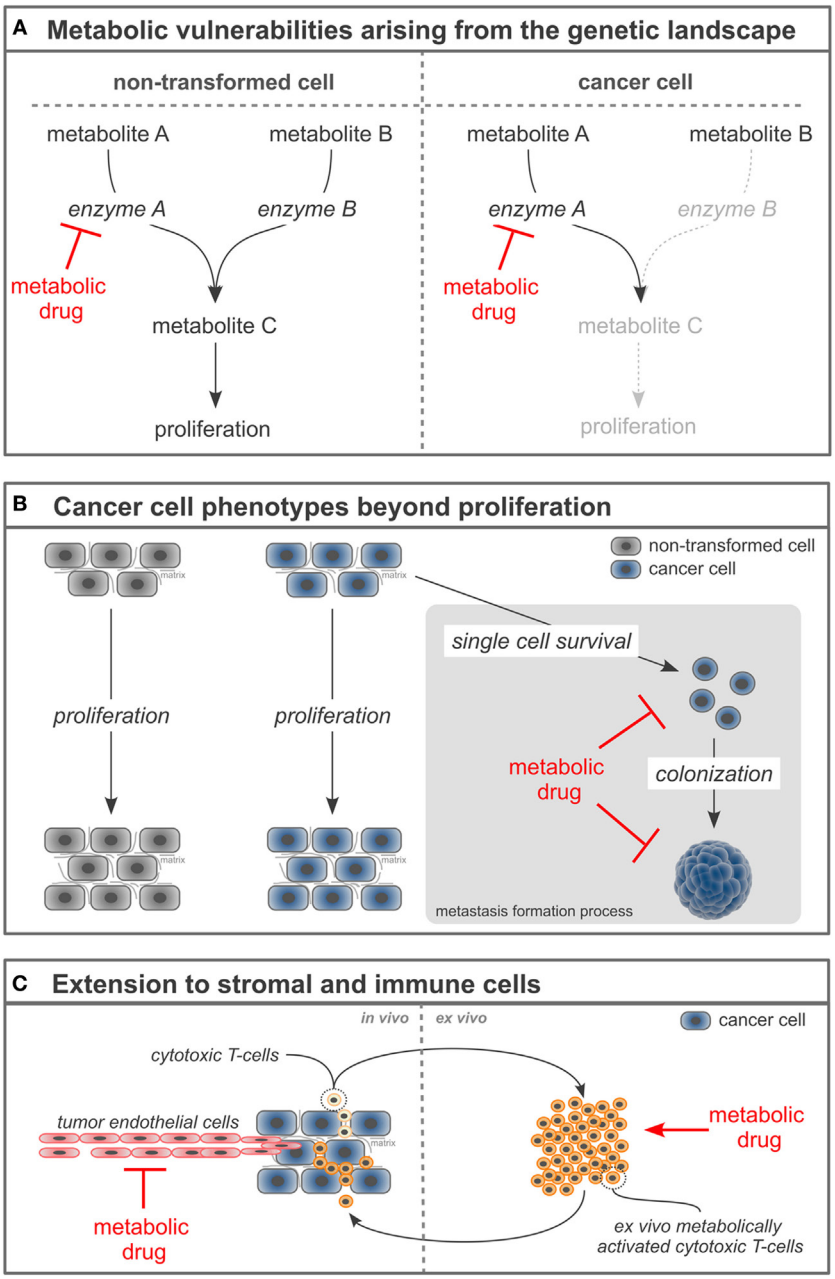

FIGURE 1 | Novel concepts to refine metabolism-based cancer therapies. **(A)** Loss or mutation of enzymes in cancer cells can create hypersensitivity of the cancer cells toward the inhibition of a concomitant enzyme. **(B)** Targeting cancer cell phenotypes beyond proliferation such as single cell survival and colonization can increase the selectivity of metabolism-based drugs and broaden their application toward metastases prevention and treatment. **(C)** Manipulating the cellular tumor composition by targeting stromal and immune cells with metabolism-based drugs can enable a comprehensive cancer therapy.

a cancer, which includes stromal and immune cells. Classically, metabolism-based drugs have been developed against cancer cells. However, within the tumor, not only cancer cells but also stromal and immune cells are found. Many stromal and some immune cells (such as tumor-associated macrophages) are reprogrammed to support the development and progression of cancer, while other immune cells within the tumor (such as cytotoxic T-cells) counteract cancer development and progression. Thus, targeting stromal and/or immune cells along with the cancer cells can be a comprehensive treatment concept (**Figure 1C**). The effectiveness of this concept has been shown for stromal cells: tumor endothelial cells display an aberrant activation (in form of proliferation and migration), which leads to tumor vascularization, but also vascular permeability. This aberrant activation is at least in part driven by high glycolytic rates (21). Consequently, downregulating glycolysis in tumor

endothelial cells can normalize the tumor vasculature, which has been shown to result in increased efficacy of chemotherapy and decreased metastasis formation (21). Both effects relied on a tightened vascular barrier that resulted in improved delivery of chemotherapeutic agents to the cancer and decreased success of cancer cell intravasation to the vasculature. Thus, targeting tumor-associated stromal cells and cancer cells at the same time can provide a synergistic anticancer efficacy.

Targeting the metabolism of immune cells emerges to be more complex, since the different subclasses of immune cells exhibit either pro- or antitumor capacities (22, 23). Therefore, any metabolism-based therapy targeting immune cells needs to either hamper the fitness of immune cells with protumor capacity or boost the fitness of immune cells with antitumor capacity. To achieve such selectivity, an increased understanding of the metabolism of immune cells is needed. An approach to circumvent the above-described complexity is to stimulate the metabolic fitness of antitumor immune cells *ex vivo* and combine it with a consecutive adoptive transfer. For example, it has been shown that the *ex vivo* treatment of cytotoxic T-cells with the metabolite S-2-hydroxyglutarate (not to be confused with the oncometabolite R-2-hydroxyglutarate) results (after adoptive transfer) in enhanced *in vivo* proliferation, survival, and antitumor capacity of the treated cytotoxic T-cells (24). Mechanistically, S-2-hydroxyglutarate treatment induced changes in histone and DNA methylation as well as the activation of HIF-1α-dependent transcriptional programs (24). Thus, while approaches targeting the metabolism of immune cells *in vivo* require further research, *ex vivo* approaches show promising results. Taken together, targeting the metabolism of stromal and immune cells can refine cancer treatment.

In conclusion, metabolism-based drugs are important contributors to cancer treatment. Novel concepts such as targeting metabolic vulnerabilities of cancer cells arising from their genetic landscape, metabolic requirements of metastasizing cancer cells, and stromal and immune cells have the potential to refine metabolism-based anticancer therapies. Moreover, combining current and future metabolism-based drugs with targeted delivery such as nanobodies (25) and magnetic nanoparticles (26) can further advance their use in cancer treatment. Thus, my answer to the question "Is there a therapeutic window for metabolism-based cancer therapies?" is *yes*.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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