THE WARBURG EFFECT REGULATION UNDER SIEGE: THE INTERTWINED PATHWAYS IN HEALTH AND DISEASE

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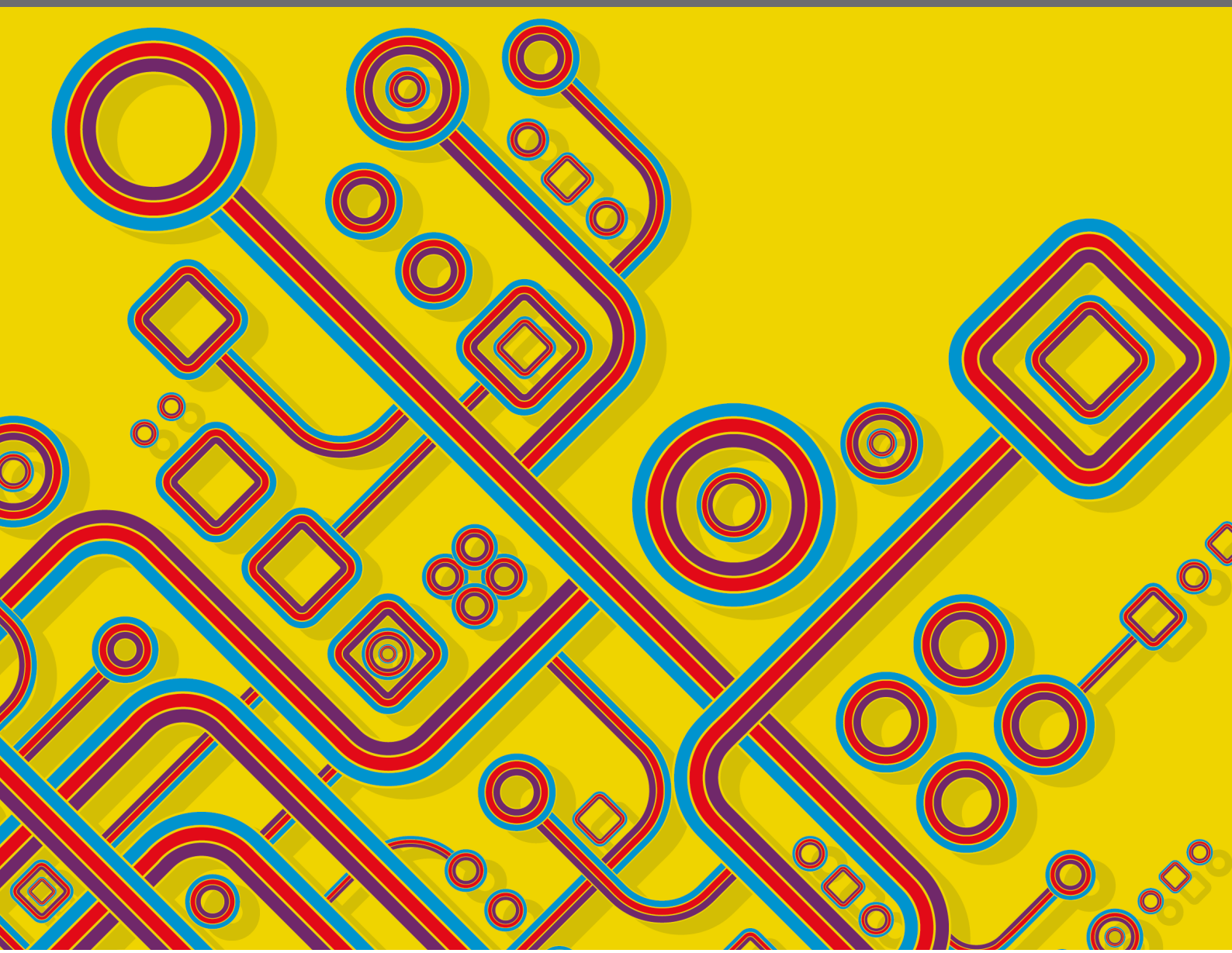

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THE WARBURG EFFECT REGULATION UNDER SIEGE: THE INTERTWINED PATHWAYS IN HEALTH AND DISEASE

Topic Editors:
Concetta Bubici, Brunel University London, United Kingdom
Salvatore Papa, University of Leeds, United Kingdom

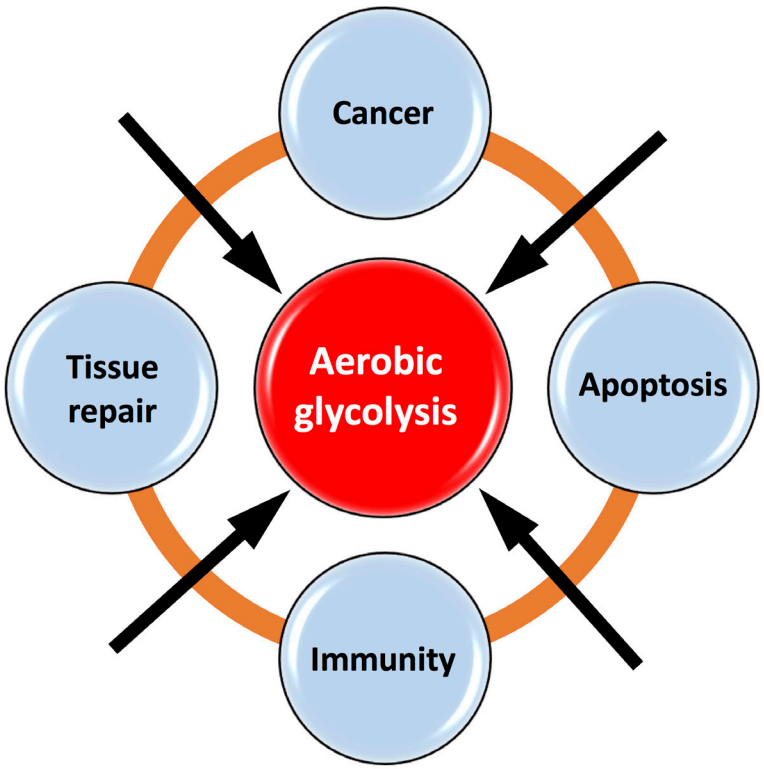

Image by Salvatore Papa and Concetta Bubici.
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Many cells, including immune, neuronal, cancer and stem cells, become dependent on aerobic glycolysis to escape apoptosis and accommodate their bioenergetics needs. How this metabolic change, also known as the Warburg effect, is regulated remains largely unknown. The Warburg effect has been widely investigated in cancer cells where it was first observed with the aim of decoding the molecular networks controlling its activation for therapeutic purposes.

This Research Topic aimed to discuss and review all the intracellular signaling regulating the Warburg effect in cancerous and normal non-cancerous cells though original research articles, mini reviews and reviews.

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EDITORIAL
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Editorial: The Warburg Effect Regulation Under Siege: the Intertwined Pathways in Health and Disease

Concetta Bubici[1]* *and Salvatore Papa*[2]*

[1] Division of Biosciences Institute of Environment, Health and Societies, Department of Life Sciences, College of Health and Life Sciences, Brunel University London, Uxbridge, United Kingdom, [2] Cell Signaling and Cancer Laboratory, Leeds Institute of Cancer and Pathology, Faculty of Medicine and Health, University of Leeds, St. James' University Hospital, Leeds, United Kingdom

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Editorial on the Research Topic

The Warburg Effect Regulation Under Siege: the Intertwined Pathways in Health and Disease

In the 1920s, the biochemist Otto Warburg observed that, unlike normal cells, cancer cells catabolize glucose into lactate under aerobic conditions (hence the name "The Warburg Effect" or aerobic glycolysis) (Warburg et al., 1927). For eight decades, the Warburg's observation was almost ignored, as only limited evidence indicated that aerobic glycolysis is characteristic of nearly all types of cancer. Over the last decade, with the development of modern analytical tools, we have witnessed a resurgence of interest in the study of the Warburg effect and the mechanistic basis of its occurrence in cancer. Not surprising, Hanahan and Weinberg (2011) in their revised list of "Hallmarks of Cancer" have included the Warburg effect as an emerging hallmark of cancer. More recently, however, a plethora of detailed investigations have also revealed crucial roles for the Warburg effect in a number of homeostatic processes, including high cell turnover and proliferation, immune responses, and brain development. To highlight the importance of the Warburg effect in health and disease, we are delighted to introduce our Research Topic containing a diversity of Review and Research Articles that will discuss the ground-breaking discoveries and advances in the field.

The Research Topic covers progress in understanding the role of the Warburg effect in cancer, immunity, inflammation, atherosclerosis, angiogenesis, and tissue homeostasis. The collection of articles in this Research Topic represents a valuable platform where the Warburg Effect is discussed from different perspectives, including how intrinsic and extrinsic regulatory pathways control aerobic glycolysis in normal and diseased conditions, and appropriately discusses the molecular basis for its inhibition in an array of human diseases.

The opening article of our Series is the detailed review by Wiese and Hitosugi that summarizes the complex role and regulation the key driver of Warburg effect, the pyruvate kinase M2 isoform (PKM2), in different cell types. The pyruvate kinase (PK) is a homotetrameric glycolytic enzyme that exists in mammals as four isoforms (PKL, PKR, PKM1, and PKM2) with different expression patterns. Its enzymatic activity catalyses the conversion of phosphoenolpyruvate (PEP) to pyruvate. The authors highlight the paradox that unlike other glycolytic enzymes, for which it is required an elevated expression and activity, PKM2 catalytic activity has to be maintained down to promote the Warburg effect in cancer cells. Indeed, it has been proposed that low PKM2 activity allows PEP and upstream glycolytic intermediate to accumulate and flow to anabolic pathways; when its activity

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Edited and reviewed by:
Ana Cuenda,
Spanish National Research Council (CSIC), Spain

***Correspondence:**
Concetta Bubici
concetta.bubici@brunel.ac.uk
Salvatore Papa
s.papa@leeds.ac.uk

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is elevated the upstream glycolytic intermediate flow to form pyruvate, which is then converted to lactate or enters mitochondria for complete oxidation. Other than the allosteric regulation by glycolytic intermediates, post-translational modifications alter the protein localization, enzymatic activity, and stability of PKM2 to promote the Warburg Effect. Above all, tyrosine phosphorylation of PKM2 (i.e., Tyr105) by Tyrosine Kinases results in decreased PKM2 activity, and this decrease promotes the Warburg Effect (Hitosugi et al., 2009; Wiese and Hitosugi). On the other hand, it has been shown that threonine phosphorylation (i.e., Thr365) via c-Jun N-terminal Kinase (JNK) results in increased PKM2 activity and consequential apoptosis of cancer cells (Iansante et al., 2015; Papa et al., 2019), highlighting the necessity for highly proliferating cancer cells to preserve low PKM2 activity. The authors highlight the complex role of PKM2 with anti- and pro-tumorigenic function in cancer development depending on cancer type.

By employing population and single cell time-lapse imaging approaches, Lucantoni et al. demonstrate that the dual inhibition of glycolysis and mitochondrial respiration increase cell death and decrease clonogenic capacity of breast cancer cells. Importantly, it was shown that the treatment of breast cancer cells with the mitochondrial fission inhibitor MDIVI-1 alone increases aerobic glycolysis while had no effect on mitochondrial morphology. Suppressing the MDIVI-1-mediated increased glycolysis with 2-deoxy-D-glucose (2-DG)—a derivative of glucose that serves as a glycolytic inhibitor—resulted in a synthetical lethal effect in breast cancer cells.

In another research article focusing on liver cancer, Lee et al. show that glycolytic enzymes involved in glycolysis and mitochondrial oxidative metabolism (OXPHOS) are highly expressed in livers of patients with hepatocellular carcinoma (HCC) compared to healthy livers, suggesting that glycolysis cooperates with OXPHOS to sustain fast cellular proliferation in HCC. The authors also show that, like HCC, aerobic glycolysis is increased in livers of patients with cirrhosis, a chronic inflammatory liver condition predisposing to the development of HCC. However, in contrast to a general increase in expression levels of genes involved in glycolysis, OXPHOS genes remained at the same level to healthy livers in cirrhotic livers. This suggests aerobic glycolysis may represent a marker for early detection and chemoprevention of HCC. The authors also highlight the fact that aerobic glycolysis is not only a distinctive phenotype of tumors but is involved in pre-malignant conditions. A review article by Hou and Syn provides an in-depth view of the metabolic changes that occur during hepatic fibrogenesis, highlighting the impact of the Warburg effect in the activation of hepatic stellate cells such as the transdifferentiation of quiescent perisinusoidal liver resident cells into proliferative, contractile, and fibrogenic cells that is the core of liver fibrosis, which drives the progression of chronic liver diseases toward liver cirrhosis and hepatic failure.

Changes in glycolytic and mitochondrial metabolism have an impact on the skeletal muscle fiber composition and are reviewed by Julien et al. in this issue. The authors describe the metabolic characteristic of muscle fibers and evaluate the metabolite-dependent intracellular pathways that influence fiber composition. In particular, the authors discuss that during extensive exercise a rapid increase of glycolysis occurs in skeletal muscle fibers and pyruvate is converted to lactate, reminiscent of the Warburg effect in cancer cells. This metabolic swift is under control of specific metabolite-dependent cell signaling and transcriptional programs. A better understanding of these programs may facilitate therefore the therapy progress against aging and neurodegenerative diseases like amyotrophic lateral sclerosis, Huntington's disease, and Alzheimer's disease.

We were also thrilled to read a review article by Theodorou and Boon discussing the role of glucose, fatty acid, and amino acid metabolism in endothelial cells in response to various physiological and pathological stimuli. Their report suggests that interfering with the glycolytic metabolism in endothelial cells may provide therapeutic strategy for atherosclerosis, even in the presence of risk factors such as diabetes and obesity. In support of this idea, Fitzgerald et al. review how changes in endothelial cell metabolism affect endothelial cell fate during physiological sprouting, as well as during angiogenesis. Targeting metabolism in endothelial cells may have therapeutic potential for pathologies associated with angiogenesis such as cancer.

Metabolic pathways have also been implicated in the differentiation and activation of immune cells. Salmond reviews how the mechanistic target of rapamycin (mTOR) pathway regulates the metabolic reprogramming of specialized helper T cell (Th) subsets and discusses the role of glycolytic metabolism in T cell subsets. While the differentiation and effector functions of inflammatory Th1, Th2, and Th17 cells relies on engagement of aerobic glycolysis where mTOR signals play a key role in these processes, memory T cells and Tregs are dependent on fatty acid oxidation pathways. Although it was believed that compared to resting dendritic cells (DCs), activated DCs possess only increased glycolytic metabolism, Du et al. review recent progress in understanding the metabolic changes occurring in different DC subsets and outstanding questions in the field. The review article by Sieow et al. provides an overview on how myeloid cells undergo metabolic reprogramming in the tumor microenvironment and covers implications for cancer immunotherapies.

Following these articles on the Warburg effect, Rosenzweig et al. report a very interesting review article discussing a new perspective that goes beyond the Warburg effect. In particular, they describe how the one-carbon metabolism is regulated in cancer and normal proliferating cells. One-carbon metabolism supports multiple physiological processes that include nucleotide biosynthesis, amino acid homeostasis, methylation, and redox defense. The authors argue that one-carbon metabolism is therefore an essential integrator of the nutritional status of proliferating cells that serves as building blocks for biosynthesis, epigenetic, and redox reactions, and discuss how dysregulated oncogenic signals control these metabolic pathways to support and sustain high rates of proliferation and cell survival essential for tumor growth.

As the implications of the Warburg effect in health and disease continue to emerge, we are entering into a renaissance period for metabolism research. Since the Warburg's original observation, we have learnt a lot about the effect of signal

transduction on metabolic pathways and how cells rapidly reprogram their metabolism although much remains to be discovered.

This Research Topic has attracted two original research articles and a considerable number of remarkable review articles providing a comprehensive view of the current knowledge on the molecular pathways and regulation of the Warburg effect and its implications in homeostasis and pathology and relevance in translational research.

We thank all authors and referees for their contributions in producing this special collection. Last but not least, we thank the Frontiers Editorial Office and the Journal Development Team for their dedication and work that made this Research Topic possible.

AUTHOR CONTRIBUTIONS

The guest editors conceived the research topic and wrote the editorial.

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REFERENCES

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*. 144, 646–674. doi: 10.1016/j.cell.2011.02.013

Hitosugi, T., Kang, S., Vander Heiden, M. G., Chung, T. W., Elf, S., Lythgoe, K., et al. (2009). Tyrosine phosphorylation inhibits PKM2 to promote the Warburg effect and tumor growth. *Sci. Signal.* 2:ra73. doi: 10.1126/scisignal.2000431

Iansante, V., Choy, P. M., Fung, S. W., Liu, Y., Chai, J. G., Dyson, J., et al. (2015). PARP14 promotes the Warburg effect in hepatocellular carcinoma by inhibiting JNK1-dependent PKM2 phosphorylation and activation. *Nat. Commun*. 6:7882. doi: 10.1038/ncomms8882

Papa, S., Choy, P. M., and Bubici, C. (2019). The ERK and JNK pathways in the regulation of metabolic reprogramming. *Oncogene* 38, 2223–2240. doi: 10.1038/s41388-018-0582-8

Warburg, O., Wind, F., and Negelein, E. (1927). The metabolism of tumors in the body. *J. Gen. Physiol*. 8, 519–530. doi: 10.1085/jgp.8.6.51

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MINI REVIEW
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Tyrosine Kinase Signaling in Cancer Metabolism: PKM2 Paradox in the Warburg Effect

Elizabeth K. Wiese[1,2] *and Taro Hitosugi*[1,3]*

[1] Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Rochester, MN, United States, [2] Molecular Pharmacology and Experimental Therapeutics Graduate Program, Mayo Clinic Graduate School of Biomedical Sciences, Mayo Clinic, Rochester, MN, United States, [3] Division of Oncology Research, Mayo Clinic, Rochester, MN, United States

The Warburg Effect, or aerobic glycolysis, is one of the major metabolic alterations observed in cancer. Hypothesized to increase a cell's proliferative capacity via regenerating NAD^+, increasing the pool of glycolytic biosynthetic intermediates, and increasing lactate production that affects the tumor microenvironment, the Warburg Effect is important for the growth and proliferation of tumor cells. The mechanisms by which a cell acquires the Warburg Effect phenotype are regulated by the expression of numerous oncogenes, including oncogenic tyrosine kinases. Oncogenic tyrosine kinases play a significant role in phosphorylating and regulating the activity of numerous metabolic enzymes. Tyrosine phosphorylation of glycolytic enzymes increases the activities of a majority of glycolytic enzymes, thus promoting increased glycolytic rate and tumor cell proliferation. Paradoxically however, tyrosine phosphorylation of pyruvate kinase M2 isoform (PKM2) results in decreased PKM2 activity, and this decrease in PKM2 activity promotes the Warburg Effect. Furthermore, recent studies have shown that PKM2 is also able to act as a protein kinase using phosphoenolpyruvate (PEP) as a substrate to promote tumorigenesis. Therefore, numerous recent studies have investigated both the role of the classical and non-canonical activity of PKM2 in promoting the Warburg Effect and tumor growth, which raise further interesting questions. In this review, we will summarize these recent advances revealing the importance of tyrosine kinases in the regulation of the Warburg Effect as well as the role of PKM2 in the promotion of tumor growth.

Keywords: the Warburg Effect, tyrosine kinases, PKM2, lactate, pyruvate kinase

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Edited by:
Salvatore Papa,
University of Leeds, United Kingdom

Reviewed by:
Brandon Faubert,
University of Texas Southwestern Medical Center, United States
Raju V. S. Rajala,
University of Oklahoma Health Sciences Center, United States

***Correspondence:**
Taro Hitosugi
hitosugi.taro@mayo.edu

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INTRODUCTION

Approximately 90 years ago, Otto Warburg described the phenotype he observed in cancer cells where he noted that cancer cells display increased glucose consumption and increased lactate production regardless of oxygen availability (Warburg, 1956). This upregulation of glycolysis, coined the Warburg Effect or aerobic glycolysis, is a common phenotype in cancer; approximately 70% of 2,000,000 cancer tissues examined display high expression of the genes related to the Warburg Effect as compared to the more than 2,000,000 examined normal tissues (Altenberg and Greulich, 2004). Cancer cells possess the ability to proliferate rapidly, survive under hypoxic conditions, avoid immune surveillance, and metastasize; alterations in cellular metabolism are

necessary to promote each of these characteristics (Hanahan and Weinberg, 2011). While it may seem counterintuitive for cancer to upregulate a less ATP and energy producing pathway as compared to the mitochondrial oxidative phosphorylation pathway, there are numerous advantages to the Warburg Effect. The Warburg Effect allows for rapid regeneration of NAD^+ from NADH by lactate dehydrogenase A (LDHA), the ability to survive in hypoxic environments due to decreased dependence on oxidative phosphorylation, and increased glycolytic biosynthetic intermediates to support macromolecule biosynthesis (Gatenby and Gillies, 2004; Lunt and Vander Heiden, 2011). The increase in lactate production has also been proposed to aid in avoiding immune surveillance as well as acidifying the tumor microenvironment to aid in metastasis (Gillies et al., 2002; Gottfried et al., 2006; Fischer et al., 2007). Thus, there is a growth and proliferative advantage for cancer cells that display the Warburg Effect, and additional mechanisms by which a cell acquires this metabolic phenotype continue to be the focus of numerous studies.

Aberrant oncogene expression that drives oncogenesis also alters cellular metabolism and can promote the Warburg Effect. One mechanism by which oncogenes promote the Warburg Effect is via transcriptional regulation of glycolytic enzymes. Numerous genes coding for glycolytic enzymes contain consensus motifs for the binding of HIF-1 or c-myc (Kim et al., 2004). Therefore, overexpression of c-myc or HIF-1 results in increased transcription and increased gene expression of multiple glycolytic enzymes and, therefore, the subsequent increase in glycolytic activity observed with the Warburg Effect.

In addition to transcriptional regulation, post-translational modifications also alter the protein localization, enzymatic activity, or stability of glycolytic enzymes to promote the Warburg Effect. Aberrant kinase activity is one of the well-known drivers of oncogenesis. Overexpression and constitutively activated kinase signaling results in continuous phosphorylation and activation of signaling pathways well known to contribute to cell growth and proliferation. Constitutive activation of tyrosine kinase signaling is present in numerous types of cancer; overexpression or mutation of at least 30 different tyrosine kinases has been associated with cancer (Blume-Jensen and Hunter, 2001). Tyrosine kinases phosphorylate many glycolytic enzymes as well as components of the pyruvate dehydrogenase complex, promoting the Warburg Effect, increased lactate production and increased tumor growth (Hitosugi et al., 2009, 2011, 2013; Fan et al., 2011, 2014; Shan et al., 2014). In this review, we will summarize the importance of tyrosine phosphorylation of glycolytic enzymes, including phosphoglycerate mutase 1 (PGAM1), lactate dehydrogenase A (LDHA), and pyruvate kinase M2 isoform (PKM2). Tyrosine phosphorylation of PKM2 results in an interesting, paradoxical effect, where phosphorylation decreases PKM2 activity, and this decrease in activity promotes increased glycolytic flux and lactate production in cancer (Hitosugi et al., 2009). In addition to discussing the role of tyrosine kinases in the regulation of the Warburg Effect, we will also summarize the recent studies examining the importance of PKM2 in promoting tumor cell proliferation and tumor growth.

TYROSINE KINASE SIGNALING IN THE WARBURG EFFECT

Aberrant tyrosine kinase signaling is a key driver of oncogenesis and tumor growth in numerous different cancers, including both blood cancers and solid tumors. BCR-ABL fusion, TEL-PDGFRβ fusion, FLT3 internal tandem duplication mutation, and JAK2 V617F mutation are all known to contribute to leukemia while FGFR3 mutations are frequently observed in multiple myeloma (Blume-Jensen and Hunter, 2001; Levis and Small, 2003; Renneville et al., 2008). In solid tumors, ErbB2/HER2 overexpression is a well-known driver of breast cancer as well as colon cancer, and EGFR overexpression is commonly observed in lung cancer and head and neck cancers (Blume-Jensen and Hunter, 2001; Baselga, 2006). In addition to the roles of aberrant tyrosine kinase signaling in regulating pathways that promote cell growth and proliferation, tyrosine kinase signaling also influences cellular metabolism (Blume-Jensen and Hunter, 2001; Hitosugi and Chen, 2014). Tyrosine kinase signaling in cancer metabolism functions to enhance the Warburg Effect via increasing glycolysis and lactate production (Hitosugi and Chen, 2014). Glycolytic targets of tyrosine kinase signaling include PGAM1, PKM2, and LDHA where phosphorylation of each of these enzymes promotes increased glycolytic rate and increased tumor cell proliferation (Hitosugi et al., 2009, 2013; Fan et al., 2011).

Tyrosine Phosphorylation of PGAM1

Frequently, tyrosine phosphorylation increases the activity of glycolytic enzymes in cancer. PGAM1 is one such target of tyrosine phosphorylation that displays increased activity upon phosphorylation (Hitosugi et al., 2013). PGAM1 catalyzes the conversion of 3-phosphoglycerate (3-PG) to 2-phosphoglycerate (2-PG) upon binding of the cofactor 2,3-bisphoshoglycerate (2,3-BPG) (Grisolia and Cleland, 1968), and it displays increased expression in hepatocarcinoma and leukemia (Ren et al., 2010; Hitosugi et al., 2012). Consistent with increased expression in promoting tumor growth, phosphorylation of PGAM1 at Y26 by FGFR1 and other tyrosine kinases increases the binding of the cofactor 2,3-BPG to enhance PGAM1 activity and subsequently increasing tumor growth (**Figure 1**; Hitosugi et al., 2013).

PGAM1 is an important step in the regulation of not only glycolysis, but also branching pathways from glycolysis such as the pentose phosphate pathway (PPP) and the serine biosynthesis pathway. It has been shown that the PGAM1 substrate 3-PG binds to and inhibits 6-phosphogluconate dehydrogenase (6-PGD) in the PPP while the PGAM1 product 2-PG activates 3-phosphoglycerate dehydrogenase (PHGDH) in the serine biosynthesis pathway (**Figure 1**; Hitosugi et al., 2012). Therefore, PGAM1 inhibition, which increases 3-PG levels and decreases 2-PG levels, decreases PPP and serine biosynthesis fluxes, respectively (**Figure 1**; Hitosugi et al., 2012). These studies highlight the importance of PGAM1 and its tyrosine phosphorylation in the regulation of glycolysis as well as flux through anabolic biosynthetic pathways to support cancer cell proliferation and tumor growth.

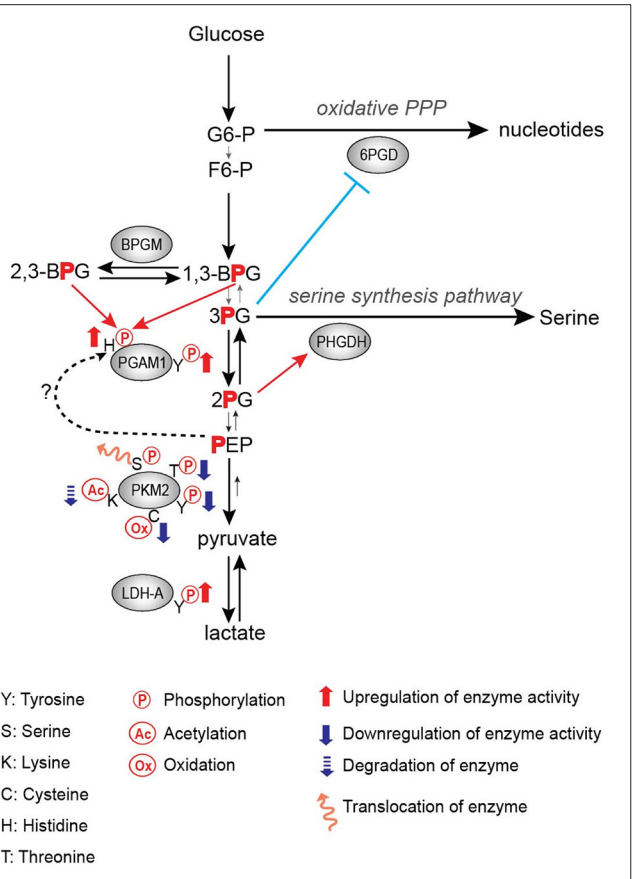

FIGURE 1 | Regulation of glycolytic enzymes by post-translational modifications in the Warburg Effect.

Tyrosine Phosphorylation of LDHA

LDHA catalyzes the conversion of pyruvate to lactate while also regenerating NAD^+ from NADH. Lactate has numerous proposed roles in promoting tumor growth, including acidifying the tumor microenvironment to promote metastasis and immune invasion, being an energy source for tumor cells, and altering gene expression through its role in regulating transcription factors such as HIF-1 (Chen et al., 2016; Faubert et al., 2017; Brooks, 2018). Thus, the importance of LDHA expression in cancer has been demonstrated in numerous studies, where knockdown or inhibition of LDHA impedes tumor growth (Fantin et al., 2006; Xian et al., 2015; Boudreau et al., 2016). Additionally, LDHA expression is increased in multiple types of cancer, likely driven by c-myc and HIF-1 overexpression (Shim et al., 1997; Kim et al., 2004). Fan et al. showed that tyrosine phosphorylation of LDHA is an additional approach by which oncogenes upregulate LDHA activity to promote tumor growth in non-small cell lung carcinoma (NSCLC) H1299 cell line xenograft model, where phosphorylation of LDHA at Y10 promotes the formation of the highly active tetrameric conformation of LDHA while phosphorylation of Y83 promotes increased binding affinity of LDHA for the cofactor NADH (**Figure 1**; Fan et al., 2011).

However, contrary to the proposed importance of increased LDHA activity in cancer, LDHA has also been identified as dispensable for tumor growth in lymphoma and brain tumor models (Nilsson et al., 2012; Sundstrom et al., 2015). Additionally, cells can acquire resistance to LDHA inhibition mediated through the AMPK-S6K pathway and an increased ability to utilize oxidative phosphorylation (Boudreau et al., 2016). Thus, the importance of LDHA and tyrosine phosphorylation of LDHA in promoting tumor growth appears to be dependent on the cellular context.

Tyrosine Phosphorylation of PKM2

Activation of glycolytic enzymes via phosphorylation by tyrosine kinases, as observed with PGAM1 and LDHA, logically contributes to increased glycolytic flux and lactate production. However, contrary to the activating effects of tyrosine phosphorylation on PGAM1 and LDHA, phosphorylation of PKM2 results in decreased activity (Hitosugi et al., 2009). PKM2 catalyzes the formation of pyruvate and ATP from phosphoenolpyruvate (PEP) and ADP. Phosphorylation of PKM2 Y105 by tyrosine kinases such as FGFR1, BCR-ABL, and Jak2 inhibits the formation of the highly active tetrameric conformation, thus resulting in decreased PKM2 enzymatic activity (**Figure 1**; Hitosugi et al., 2009). Inhibition of FGFR1 by the FGFR1 inhibitor TKI258 results in decreased PKM2 Y105 phosphorylation in H1299 cells expressing FGFR1 and in KG-1a cells expressing FOP-FGFR1 fusion (Hitosugi et al., 2009). Paradoxically, this decrease in PKM2 activity promotes increased lactate production and tumor growth (Hitosugi et al., 2009). Thus, PKM2 has continued to be an area of active research to further understand its role in tumorigenesis and cancer cell proliferation.

PKM2 PARADOX IN THE WARBURG EFFECT

PKM2 Regulation

As one of the irreversible enzymes of glycolysis (K_{eq} approximately 10^4), pyruvate kinase is thought to be one of the rate limiting steps of glycolysis and thus important in regulating glycolytic activity (Mellati et al., 1992; Christofk et al., 2008b; Nelson et al., 2008). However, whether pyruvate kinase is a rate limiting step in cancer remains under debate (Xie et al., 2016). PKM2 is one of the four pyruvate kinase isoforms. The four pyruvate kinase isoforms are (1) PKL which is primarily expressed in the liver and kidneys, (2) PKR which is exclusively expressed in red blood cells, (3) PKM1 which is highly expressed in differentiated tissues with high energetic demands, and (4) PKM2 which is highly expressed in undifferentiated tissues as well as rapidly proliferating tissues including cancer (Jurica et al., 1998). PKM mRNA is alternatively spliced to produce PKM1 or PKM2, where mutually exclusive selection of exon 9 or 10 results in the expression of PKM1 or PKM2, respectively (David et al., 2010). C-myc drives the expression of polypyrimidine tract binding protein (PTB), heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) and A2 (hnRNPA2) which function to inhibit the inclusion of exon 9, thus promoting the inclusion of exon

10 and subsequent PKM2 expression (David et al., 2010). SRSF3, a splicing factor that is overexpressed in numerous different cancers, also is capable of binding the PKM transcript and promoting the inclusion of exon 10 to promote PKM2 expression (Wang et al., 2012).

Low Pyruvate Kinase Activity Is Important for Tumor Growth

The relationship between PKM2 expression and activity in cancer has been the focus of numerous studies seeking to elucidate the role of pyruvate kinase activity in regulating tumorigenesis and tumor growth. Because PKM2 has displayed both tumor promoting and tumor suppressive effects, and its activity is in general downregulated in cancer, we have categorized these studies, based on whether decreased pyruvate kinase activity promotes tumor growth or not (**Table 1**). Eigenbrodt et al. first described the decrease in pyruvate kinase activity with PKM2 expression in transformed cells, noting the paradox between decreased PKM2 activity yet increased glycolysis in the Warburg Effect (Eigenbrodt and Glossmann, 1980) Almost 30 years later, Christofk et al. demonstrated that in H1299 cells, which predominantly express PKM2, stable expression of PKM1 in place of PKM2 resulted in increased pyruvate kinase activity and increased oxidative phosphorylation, yet decreased lactate production and decreased tumor growth (Christofk et al., 2008a). Additional studies also identified PKM2 as a phosphotyrosine binding protein, where binding of phosphotyrosine residues results in decreased pyruvate kinase activity, increased tumor growth, and increased lactate production (Christofk et al., 2008b). These studies show consistent evidence of the PKM2 paradox: decreased pyruvate kinase activity supports increased glycolytic activity and tumor growth.

In addition to the oncogenic drivers that regulate the expression of PKM2, PKM2 activity is regulated via post-translational modifications (**Figure 1**; Hitosugi et al., 2009; Anastasiou et al., 2011; Lv et al., 2011; Yu et al., 2013; Iansante et al., 2015). Unlike PKM1 which exists in a stable, highly active tetrameric conformation, PKM2 is allosterically regulated by the binding of fructose-1,6-bisphosphate (FBP). The binding of FBP to the low activity dimer confirmation promotes the tetramerization of PKM2, resulting in the formation of the highly active conformation (Jurica et al., 1998). Multiple residues of PKM2 are capable of being post-translationally modified, including tyrosine phosphorylation, serine/threonine phosphorylation, cysteine oxidation, and lysine acetylation (Prakasam et al., 2018). Tyrosine phosphorylation of PKM2 Y105 disrupts FBP binding to inhibit the formation of the highly active tetramer conformation, thus decreasing its enzymatic activity (Hitosugi et al., 2009). This phosphorylation is negatively regulated by protein tyrosine phosphatase 1B (PTP1B), where decreased PTP1B activity results in increased PKM2 Y105 phosphorylation and decreased PKM2 activity (Bettaieb et al., 2013). Threonine phosphorylation of PKM2 T454 by PIM2 functions to inhibit the enzymatic activity of PKM2. Similarly to the observed consequences of Y105 phosphorylation, T454 phosphorylation promotes increased glucose consumption, increased lactate production, and increased cell proliferation (Yu et al., 2013). Cysteine oxidation of C358 upon elevated levels of reactive oxygen species (ROS) also functions to block the formation of the highly active tetramer conformation and inhibit PKM2 activity to promote tumor growth (Anastasiou et al., 2011). Lysine acetylation of PKM2 inhibits PKM2 activity by both decreasing the affinity for the substrate PEP as well as decreasing PKM2 protein stability, which again contributes to tumor growth (Lv et al., 2011). Phosphorylation of PKM2 at T365 by JNK1 results in increased PKM2 activity by increasing the affinity of PKM2 for the substrates PEP and ADP; however, in cancer JNK1 is inactivated by PARP14, thus maintaining the dephosphorylated T365 PKM2 and low activity (Iansante et al., 2015). Again, the expression of PARP14, and subsequent decrease in PKM2 T365 phosphorylation and activity promotes increased glucose consumption and increased lactate production to promote the Warburg Effect (Iansante et al., 2015). Despite the different mechanisms of action in reducing PKM2 activity, each study showed that decreased PKM2 activity via post-translational modification supported the Warburg Effect phenotype and increased tumor proliferation (**Table 1**; Hitosugi et al., 2009; Anastasiou et al., 2011; Lv et al., 2011; Yu et al., 2013).

Because of the observed importance of decreased PKM2 activity on tumor proliferation, PKM2 activators have been developed as an approach to target cancer. The small molecule PKM2 activators DASA-58 and TEPP-46 were shown to activate PKM2 by promoting the formation of tetrameric PKM2 (Anastasiou et al., 2012). When tested in mouse xenograft models, TEPP-46 treatment resulted in a significant decrease in tumor growth at concentrations that did not cause any major toxicities (Anastasiou et al., 2012). In the clinic, these activators have been tested for the treatment of diseases related to pyruvate kinase deficiency. However, no cancer clinical trials have been completed due to difficulties in selecting the appropriate patient population, as the role of pyruvate kinase activity in cancer is heavily context and tumor dependent.

To further examine the role of PKM2 in promoting cancer cell proliferation, numerous mouse models have been constructed. These models have illustrated an interesting and complicated relationship between PKM2 activity and tumor growth. Some PKM2 deletion models have shown that decreased pyruvate kinase activity increases tumorigenesis. Using a $Brca1^{fl/fl}$ MMTV-Cre $Trp53^{+/-}$ breast cancer model to assess the role of PKM2 in breast cancer tumorigenesis, Israelsen et al. showed that PKM2 specific knockout promoted more rapid breast cancer development (Israelsen et al., 2013). This model allowed for the continued transcription of PKM1 from the PKM gene, and low levels of PKM1 expression were observed in PKM2 $^{\Delta/\Delta}$ cells. Despite the low expression of PKM1, the authors concluded that low pyruvate kinase activity was maintained, and this low pyruvate kinase activity is beneficial for tumor growth (Israelsen et al., 2013). This observation is consistent with the published *in vitro* cell line studies (Christofk et al., 2008a; Hitosugi et al., 2009; Anastasiou et al., 2011; Lv et al., 2011). Similar results were observed with a germline PKM2 deletion as well as a medulloblastoma model with PKM2 deletion, where PKM2 $^{-/-}$ mice displayed increased incidence of hepatocellular carcinoma

TABLE 1 | Effects of altered PKM2 activity or expression on tumor growth.

Cancer tissue	Oncogenic driver	Pyruvate kinase model	Tumor growth
H1299 NCSLC (Christofk et al., 2008a)		mPKM2 as compared to mPKM1 (Low PK activity)	Increased tumor growth
H1299 NCSLC (Hitosugi et al., 2009)	FGFR1	pY105 PKM2 (Low PK activity)	Increased tumor growth
A549 NSCLC (Anastasiou et al., 2011)	ROS	oxC358 PKM2 (Low PK activity)	Increased tumor growth
A549 NSCLC (Yu et al., 2013)	PIM2	pT454 PKM2 (Low PK activity)	Increased cell proliferation
H1299 NSCLC (Lv et al., 2011)		acK305 PKM2 (Degradation)	Increased tumor growth
Breast cancer (Israelsen et al., 2013)	$Brca1^{fl/fl}$ MMTV-Cre $Trp53^{+/-}$	$PKM2^{\Delta/\Delta}$	Increased tumor growth
Medulloblastoma (Tech et al., 2017)	ND2:SmoA1	$PKM2^{CKO}$	Increased tumor growth
Hepatocellular Carcinoma (Dayton et al., 2016a)		Germline $PKM2^{-/-}$	Increased tumor growth
Leukemia (Wang Y. H. et al., 2014)	BCR-ABL MLL-AF9	$PKM2^{-/-}$	Delayed tumor initiation
Sarcoma (Dayton et al., 2018)	$Kras^{LSL-G12D/+}$;$p53^{fl/fl}$	$PKM2^{-/-}$	Delayed tumor initiation but no effect on tumor growth
Colon cancer (Lau et al., 2017)	APC^{CKO}	$PKM2^{\Delta/\Delta}$	No effect on tumor growth
87-5 SCLC Lu139 SCLC (Morita et al., 2018)		mPKM2	Decreased tumor growth

and increased medulloblastoma tumor growth respectively (Dayton et al., 2016a; Tech et al., 2017). Finally, in a $PKM2^{fl/fl}$ Cre-ER MEF model, the $PKM2^{\Delta/+}$ MEFs that gained PKM1 expression displayed slower proliferation than $PKM2^{fl/+}$ MEFs (Lunt et al., 2015). These models continue to support the role of decreased PKM2 activity in supporting tumor proliferation (**Table 1**).

The physiological benefit of this decreased activity continues to be the focus of numerous studies. While it may seem counterintuitive for cancer to display decreased PKM2 activity in the Warburg Effect, it has been proposed that this decrease in PKM2 activity promotes an increase in flux of glycolytic intermediates to biosynthetic pathways including PPP for nucleotide biosynthesis as well as serine biosynthesis pathways (Eigenbrodt and Glossmann, 1980; Anastasiou et al., 2011; Lunt and Vander Heiden, 2011; Lunt et al., 2015). Decreased PKM2 activity has also been proposed to support the increase in an alternative glycolytic pathway. The increase in the levels of the PKM2 substrate PEP caused by decreased PKM2 activity leads to phosphorylation of PGAM1 at histidine 11 (H11), resulting in activation of PGAM1, and thereby further increasing glycolysis and glycolytic intermediates to support macromolecule biosynthesis (Vander Heiden et al., 2010b). In this case, the phosphate group of PEP is transferred to histidine 11 of PGAM1 by an unidentified mechanism (**Figure 1**; Vander Heiden et al., 2010b). Another recent study has shown that H11 of PGAM1 is non-enzymatically phosphorylated either by 2,3-BPG or 1,3-BPG (**Figure 1**; Oslund et al., 2017). Since 2,3-BPG levels were increased in PKM2-expressed cells as compared to PKM1-expressed cells (Vander Heiden et al., 2010b), it would be intriguing to examine whether increased H11 phosphorylation by decreased PKM2 activity is a result of a non-enzymatic reaction by increased 2,3-BPG levels or an enzymatic reaction by an unidentified histidine protein kinase (**Figure 1**). Finally, Cortes-Cros et al. showed that PKM2 knockdown supported an increase in glycolytic biosynthetic intermediates and serine synthesis (Cortes-Cros et al., 2013). They also investigated whether PKM2 regulated glutamine consumption, as glutamine is another major carbon source for anabolic synthesis, and observed that PKM2 knockdown had no effect on glutamine consumption (Cortes-Cros et al., 2013). Thus, decreased PKM2 activity is proposed to support macromolecule biosynthesis through the increased flux of glycolytic intermediates.

High Pyruvate Kinase Activity Is Important for Tumor Growth

There are also models that contradict the importance of decreased PKM2 activity in tumor proliferation. PKM2 inhibition via small molecule inhibitors, such as shikonin, showed that increasing inhibitor concentrations resulted in increased cytotoxicity (Vander Heiden et al., 2010a; Chen et al., 2011; Li et al., 2014). Additionally, curcumin, which has been frequently observed to inhibit cancer cell proliferation, was found to decrease PKM2 expression. This curcumin mediated decrease in PKM2 expression led to decreased glucose consumption, lactate production, and cell proliferation (Siddiqui et al., 2018). Using *in vivo* models, PKM2 deletion in hematopoietic cells delayed the onset of leukemia in BCR-ABL or MLL-AF9 leukemia models (Wang Y. H. et al., 2014). Interestingly, PKM2 inhibition by shikonin, decreased PKM2 expression by curcumin, and PKM2 deletion *in vivo* all resulted in decreased lactate concentrations, which contradict previous models which demonstrated that decreased pyruvate kinase activity increases lactate concentration (Chen et al., 2011; Li et al., 2014; Wang Y. H. et al., 2014; Siddiqui et al., 2018). A recent study by Dayton et al. showed that PKM2 deletion in a $Kras^{LSL-G12D}$/p53 driven sarcoma model results in delayed tumor onset, contradicting

the importance of decreased PKM2 activity in tumor initiation (Dayton et al., 2018). However, following tumor initiation, there was no difference in tumor growth between the $PKM2^{+/+}$ and PKM2 $^{-/-}$ tumors, suggesting PKM2 has no effect on tumor growth (Dayton et al., 2018). Also supporting the notion that PKM2 has no effect on tumor growth, Lau et al. observed no difference in the number of tumors between $PKM2^{+/+}$ and $PKM2^{\Delta/\Delta}$ mice in an APC^{CKO} colon cancer model (Lau et al., 2017). Finally, Morita et al. observed in small cell lung carcinoma (SCLC) that PKM1 expression, not PKM2, is the isoform responsible for promoting tumor growth (Morita et al., 2018). Thus, these studies contradict the importance of PKM2 in tumor growth (**Table 1**).

Non-canonical Activities of PKM2

One of the possible explanations to why different cancer types respond differently to changes in pyruvate kinase activity is the non-canonical activity of PKM2. PKM2 has been proposed to be involved in regulating gene transcription through its nuclear and protein kinase activity. PKM2 undergoes nuclear translocation upon interactions with PHD3, JMJD5, and EGFR activation as well as following phosphorylation at PKM2 S37 and S202 by ERK1/2 and Akt respectively (Luo et al., 2011; Yang et al., 2011, 2012b; Wang H. J. et al., 2014; Park et al., 2016). In the nucleus, PKM2 can regulate HIF-1, β-catenin, c-myc, and STAT5 activity, which regulates genes involved in glucose metabolism to promote the Warburg Effect and genes important for supporting increased cell proliferation (Luo et al., 2011; Yang et al., 2011, 2012b; Wang H. J. et al., 2014; Park et al., 2016). While phosphorylation of PKM2 at S37 is important for the translocation of PKM2 into the nucleus from the cytosol, dephosphorylation of PKM2 S37 by Cdc25a in the nucleus is required for the subsequent binding of PKM2 to β-catenin and β-catenin transactivation (Yang et al., 2012b; Liang et al., 2016). Activation of β-catenin promotes increased glucose consumption, increased lactate production, and increased cell proliferation (Liang et al., 2016). Therefore, increased Cdc25a expression and dephosphorylation of PKM2 in the nucleus is important in promoting the Warburg Effect (Liang et al., 2016).

PKM2 is also thought to act as a protein kinase where it uses PEP as a phosphate donor to phosphorylate substrate proteins to regulate gene expression and cell cycle progression (Gao et al., 2012; Yang et al., 2012a; Jiang et al., 2014a,b). However, the protein kinase activity of PKM2 has been debated, and the ability of PKM2 deletion to promote tumor growth further questions the importance of the protein kinase activity of PKM2 in certain cancer models (Israelsen et al., 2013; Hosios et al., 2015). Thus, the roles, or lack thereof, of PKM2 in promoting tumor growth are complex and appear to be heavily context and model dependent.

CONCLUSION

Metabolic reprogramming is one of the hallmarks of cancer (Hanahan and Weinberg, 2011). This upregulation of glycolysis and lactate production is a phenotype important for the growth and proliferation of many different types of cancer. Oncogenic tyrosine kinase signaling is one of the key drivers of the Warburg Effect via tyrosine phosphorylation of glycolytic enzymes. Usually, tyrosine phosphorylation results in increased activity of the glycolytic enzyme, consistent with the observed increase in glycolytic flux in the Warburg Effect (Fan et al., 2011; Hitosugi et al., 2013). However, PKM2, the final step in glycolysis, displays decreased enzymatic activity when tyrosine phosphorylated (Hitosugi et al., 2009). This is one example highlighting the PKM2 paradox in cancer: decreased PKM2 activity promotes increased lactate production and tumor growth.

However, not all tumor types or tumor models support the hypothesis that decreased PKM2 activity promotes tumor growth. In a BCR-ABL or MLL-AF9 leukemia model, PKM2 deletion correlates with delayed leukemia onset (Wang Y. H. et al., 2014). In this model, the pyruvate kinase expression level that correlates with the highest lactate concentrations displayed the greatest tumor growth (Wang Y. H. et al., 2014). Thus, despite the opposite effect regarding PKM2 activity, the Warburg Effect holds true: tumor cells display increased glycolysis and increased lactate production.

Questions still remain regarding PKM2 activity, lactate production, and the Warburg Effect. What is not yet clear is how pyruvate kinase activity regulates lactate production. The molecular mechanisms behind the paradox between decreased pyruvate kinase activity and increased lactate production, why the paradox is present in some cancer models and not others, and the role of post-translational modifications of PKM2 via oncogenic tyrosine kinases among others remain to be fully elucidated. The seemingly contradictory effects of PKM2 on cancer cell proliferation and tumor growth also need to be examined further, including whether the effects of PKM2 are dependent on tumor microenvironment, whether PKM2 promotes survival to cellular stress, and whether PKM2 plays a role in promoting metastasis (Dayton et al., 2016b). However, what is becoming increasingly clear is the complexity regarding metabolic regulation in tumor cells, where the regulation of pyruvate kinase activity and isoform expression is important for tumor growth and proliferation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Altenberg, B., and Greulich, K. O. (2004). Genes of glycolysis are ubiquitously overexpressed in 24 cancer classes. *Genomics* 84, 1014–1020. doi: 10.1016/j.ygeno.2004.08.010

Anastasiou, D., Poulogiannis, G., Asara, J. M., Boxer, M. B., Jiang, J. K., Shen, M., et al. (2011). Inhibition of pyruvate kinase M2 by reactive oxygen species contributes to cellular antioxidant responses. *Science* 334, 1278–1283. doi: 10.1126/science.1211485

Anastasiou, D., Yu, Y., Israelsen, W. J., Jiang, J. K., Boxer, M. B., Hong, B. S., et al. (2012). Pyruvate kinase M2 activators promote tetramer formation and suppress tumorigenesis. *Nat. Chem. Biol.* 8, 839–847. doi: 10.1038/nchembio.1060

Baselga, J. (2006). Targeting tyrosine kinases in cancer: the second wave. *Science* 312, 1175–1178. doi: 10.1126/science.1125951

Bettaieb, A., Bakke, J., Nagata, N., Matsuo, K., Xi, Y., Liu, S., et al. (2013). Protein tyrosine phosphatase 1B regulates pyruvate kinase M2 tyrosine phosphorylation. *J. Biol. Chem.* 288, 17360–17371. doi: 10.1074/jbc.M112.441469

Blume-Jensen, P., and Hunter, T. (2001). Oncogenic kinase signalling. *Nature* 411, 355–365. doi: 10.1038/35077225

Boudreau, A., Purkey, H. E., Hitz, A., Robarge, K., Peterson, D., Labadie, S., et al. (2016). Metabolic plasticity underpins innate and acquired resistance to LDHA inhibition. *Nat. Chem. Biol.* 12, 779–786. doi: 10.1038/nchembio.2143

Brooks, G. A. (2018). The science and translation of lactate shuttle theory. *Cell Metab.* 27, 757–785. doi: 10.1016/j.cmet.2018.03.008

Chen, J., Xie, J., Jiang, Z., Wang, B., Wang, Y., and Hu, X. (2011). Shikonin and its analogs inhibit cancer cell glycolysis by targeting tumor pyruvate kinase-M2. *Oncogene* 30, 4297–4306. doi: 10.1038/onc.2011.137

Chen, Y. J., Mahieu, N. G., Huang, X., Singh, M., Crawford, P. A., Johnson, S.L., et al. (2016). Lactate metabolism is associated with mammalian mitochondria. *Nat. Chem. Biol.* 12, 937–943. doi: 10.1038/nchembio.2172

Christofk, H. R., Vander Heiden, M. G., Harris, M. H., Ramanathan, A., Gerszten, R. E., Wei, R., et al. (2008a). The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature* 452, 230–233. doi: 10.1038/nature06734

Christofk, H. R., Vander Heiden, M. G., Wu, N., Asara, J. M., and Cantley, L. C. (2008b). Pyruvate kinase M2 is a phosphotyrosine-binding protein. *Nature* 452, 181–186. doi: 10.1038/nature06667

Cortés-Cros, M., Hemmerlin, C., Ferretti, S., Zhang, J., Gounarides, J. S., Yin, H., et al. (2013). M2 isoform of pyruvate kinase is dispensable for tumor maintenance and growth. *Proc. Natl. Acad. Sci. U.S.A.* 110, 489–494. doi: 10.1073/pnas.1212780110

David, C. J., Chen, M., Assanah, M., Canoll, P., and Manley, J. L. (2010). HnRNP proteins controlled by c-Myc deregulate pyruvate kinase mRNA splicing in cancer. *Nature* 463, 364–368. doi: 10.1038/nature08697

Dayton, T. L., Gocheva, V., Miller, K. M., Bhutkar, A., Lewis, C. A., Bronson, R. T., et al. (2018). Isoform-specific deletion of PKM2 constrains tumor initiation in a mouse model of soft tissue sarcoma. *Cancer Metab.* 6:6. doi: 10.1186/s40170-018-0179-2

Dayton, T. L., Gocheva, V., Miller, K. M., Israelsen, W. J., Bhutkar, A., Clish, C. B., et al. (2016a). Germline loss of PKM2 promotes metabolic distress and hepatocellular carcinoma. *Genes Dev.* 30, 1020–1033. doi: 10.1101/gad.278549.116

Dayton, T. L., Jacks, T., and Vander Heiden, M. G. (2016b). PKM2, cancer metabolism, and the road ahead. *EMBO Rep.* 17, 1721–1730. doi: 10.15252/embr.201643300

Eigenbrodt, E., and Glossmann, H. (1980). Glycolysis - one of the keys to cancer? *TIPS* 1, 240–245. doi: 10.1016/0165-6147(80)90009-7

Fan, J., Hitosugi, T., Chung, T. W., Xie, J., Ge, Q., Gu, T. L., et al. (2011). Tyrosine phosphorylation of lactate dehydrogenase A is important for NADH/NAD(+) redox homeostasis in cancer cells. *Mol. Cell. Biol.* 31, 4938–4950. doi: 10.1128/MCB.06120-11

Fan, J., Kang, H. B., Shan, C., Elf, S., Lin, R., Xie, J., et al. (2014). Tyr-301 phosphorylation inhibits pyruvate dehydrogenase by blocking substrate binding and promotes the Warburg effect. *J. Biol. Chem.* 289, 26533–26541. doi: 10.1074/jbc.M114.593970

Fantin, V. R., St-Pierre, J., and Leder, P. (2006). Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell* 9, 425–434. doi: 10.1016/j.ccr.2006.04.023

Faubert, B., Li, K. Y., Cai, L., Hensley, C. T., Kim, J., Zacharias, L. G., et al. (2017). Lactate metabolism in human lung tumors. *Cell* 171, 358.e9–371.e9. doi: 10.1016/j.cell.2017.09.019

Fischer, K., Hoffmann, P., Voelkl, S., Meidenbauer, N., Ammer, J., Edinger, M., et al. (2007). Inhibitory effect of tumor cell-derived lactic acid on human T cells. *Blood* 109, 3812–3819. doi: 10.1182/blood-2006-07-035972

Gao, X., Wang, H., Yang, J. J., Liu, X., and Liu, Z. R. (2012). Pyruvate kinase M2 regulates gene transcription by acting as a protein kinase. *Mol. Cell* 45, 598–609. doi: 10.1016/j.molcel.2012.01.001

Gatenby, R. A., and Gillies, R. J. (2004). Why do cancers have high aerobic glycolysis? *Nat. Rev. Cancer* 4, 891–899. doi: 10.1038/nrc1478

Gillies, R. J., Raghunand, N., Karczmar, G. S., and Bhujwalla, Z. M. (2002). MRI of the tumor microenvironment. *J. Magn. Reson. Imaging* 16, 430–450. doi: 10.1002/jmri.10181

Gottfried, E., Kunz-Schughart, L. A., Ebner, S., Mueller-Klieser, W., Hoves, S., Andreesen, R., et al. (2006). Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. *Blood* 107, 2013–2021. doi: 10.1182/blood-2005-05-1795

Grisolia, S., and Cleland, W. W. (1968). Influence of salt, substrate, and cofactor concentrations on the kinetic and mechanistic behavior of phosphogylcerate mutase. *Biochemistry* 7, 1115–1121. doi: 10.1021/bi00843a032

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674. doi: 10.1016/j.cell.2011.02.013

Hitosugi, T., and Chen, J. (2014). Post-translational modifications and the Warburg effect. *Oncogene* 33, 4279–4285. doi: 10.1038/onc.2013.406

Hitosugi, T., Fan, J., Chung, T. W., Lythgoe, K., Wang, X., Xie, J., et al. (2011). Tyrosine phosphorylation of mitochondrial pyruvate dehydrogenase kinase 1 is important for cancer metabolism. *Mol. Cell* 44, 864–877. doi: 10.1016/j.molcel.2011.10.015

Hitosugi, T., Kang, S., Vander Heiden, M. G., Chung, T. W., Elf, S., Lythgoe, K., et al. (2009). Tyrosine phosphorylation inhibits PKM2 to promote the Warburg effect and tumor growth. *Sci. Signal.* 2:ra73. doi: 10.1126/scisignal.2000431

Hitosugi, T., Zhou, L., Elf, S., Fan, J., Kang, H. B., Seo, J. H., et al. (2012). Phosphoglycerate mutase 1 coordinates glycolysis and biosynthesis to promote tumor growth. *Cancer Cell* 22, 585–600. doi: 10.1016/j.ccr.2012.09.020

Hitosugi, T., Zhou, L., Fan, J., Elf, S., Zhang, L., Xie, J., et al. (2013). Tyr26 phosphorylation of PGAM1 provides a metabolic advantage to tumours by stabilizing the active conformation. *Nat. Commun.* 4:1790. doi: 10.1038/ncomms2759

Hosios, A. M., Fiske, B. P., Gui, D. Y., and Vander Heiden, M. G. (2015). Lack of evidence for PKM2 protein kinase activity. *Mol. Cell* 59, 850–857. doi: 10.1016/j.molcel.2015.07.013

Iansante, V., Choy, P. M., Fung, S. W., Liu, Y., Chai, J. G., Dyson, J., et al. (2015). PARP14 promotes the Warburg effect in hepatocellular carcinoma by inhibiting JNK1-dependent PKM2 phosphorylation and activation. *Nat. Commun.* 6:7882. doi: 10.1038/ncomms8882

Israelsen, W. J., Dayton, T. L., Davidson, S. M., Fiske, B. P., Hosios, A. M., Bellinger, G., et al. (2013). PKM2 isoform-specific deletion reveals a differential requirement for pyruvate kinase in tumor cells. *Cell* 155, 397–409. doi: 10.1016/j.cell.2013.09.025

Jiang, Y., Li, X., Yang, W., Hawke, D. H., Zheng, Y., Xia, Y., et al. (2014a). PKM2 regulates chromosome segregation and mitosis progression of tumor cells. *Mol. Cell* 53, 75–87. doi: 10.1016/j.molcel.2013.11.001

Jiang, Y., Wang, Y., Wang, T., Hawke, D. H., Zheng, Y., Li, X., et al. (2014b). PKM2 phosphorylates MLC2 and regulates cytokinesis of tumour cells. *Nat. Commun.* 5:5566. doi: 10.1038/ncomms6566

Jurica, M. S., Mesecar, A., Heath, P. J., Shi, W., Nowak, T., and Stoddard, B. L. (1998). The allosteric regulation of pyruvate kinase by fructose-1,6-bisphosphate. *Structure* 6, 195–210. doi: 10.1016/S0969-2126(98)00021-5

Kim, J. W., Zeller, K. I., Wang, Y., Jegga, A. G., Aronow, B. J., O'Donnell, K. A., et al. (2004). Evaluation of myc E-box phylogenetic footprints in glycolytic genes by chromatin immunoprecipitation assays. *Mol. Cell. Biol.* 24, 5923–5936. doi: 10.1128/MCB.24.13.5923-5936.2004

Lau, A. N., Israelsen, W. J., Roper, J., Sinnamon, M. J., Georgeon, L., Dayton, T. L., et al. (2017). PKM2 is not required for colon cancer initiated by APC loss. *Cancer Metab.* 5:10. doi: 10.1186/s40170-017-0172-1

Levis, M., and Small, D. (2003). FLT3: itdoes matter in leukemia. *Leukemia* 17, 1738–1752. doi: 10.1038/sj.leu.2403099

Li, W., Liu, J., and Zhao, Y. (2014). PKM2 inhibitor shikonin suppresses TPA-induced mitochondrial malfunction and proliferation of skin epidermal JB6 cells. *Mol. Carcinog.* 53, 403–412. doi: 10.1002/mc.21988

Liang, J., Cao, R., Zhang, Y., Xia, Y., Zheng, Y., Li, X., et al. (2016). PKM2 dephosphorylation by Cdc25A promotes the Warburg effect and tumorigenesis. *Nat. Commun.* 7:12431. doi: 10.1038/ncomms12431

Lunt, S. Y., Muralidhar, V., Hosios, A. M., Israelsen, W. J., Gui, D. Y., Newhouse, L., et al. (2015). Pyruvate kinase isoform expression alters nucleotide synthesis to impact cell proliferation. *Mol. Cell* 57, 95–107. doi: 10.1016/j.molcel.2014.10.027

Lunt, S. Y., and Vander Heiden, M. G. (2011). Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu. Rev. Cell Dev. Biol.* 27, 441–464. doi: 10.1146/annurev-cellbio-092910-154237

Luo, W., Hu, H., Chang, R., Zhong, J., Knabel, M., O'Meally, R., et al. (2011). Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. *Cell* 145, 732–744. doi: 10.1016/j.cell.2011.03.054

Lv, L., Li, D., Zhao, D., Lin, R., Chu, Y., Zhang, H., et al. (2011). Acetylation targets the M2 isoform of pyruvate kinase for degradation through chaperone-mediated autophagy and promotes tumor growth. *Mol. Cell* 42, 719–730. doi: 10.1016/j.molcel.2011.04.025

Mellati, A. A., Yücel, M., Altinörs, N., and Gündüz, U. (1992). Regulation of M2-type pyruvate kinase from human meningioma by allosteric effectors fructose 1, 6 diphosphate and L-alanine. *Cancer Biochem. Biophys.* 13, 33–41.

Morita, M., Sato, T., Nomura, M., Sakamoto, Y., Inoue, Y., Tanaka, R., et al. (2018). PKM1 confers metabolic advantages and promotes cell-autonomous tumor cell growth. *Cancer Cell* 33, 355.e7–367.e7. doi: 10.1016/j.ccell.2018.02.004

Nelson, D. L., Lehninger, A. L., and Cox, M. M. (2008). *Lehninger Principles of Biochemistry.* New York, NY: W.H. Freeman & Company.

Nilsson, L. M., Forshell, T. Z., Rimpi, S., Kreutzer, C., Pretsch, W., Bornkamm, G. W., et al. (2012). Mouse genetics suggests cell-context dependency for Myc-regulated metabolic enzymes during tumorigenesis. *PLoS Genet.* 8:e1002573. doi: 10.1371/journal.pgen.1002573

Oslund, R. C., Su, X., Haugbro, M., Kee, J. M., Esposito, M., David, Y., et al. (2017). Bisphosphoglycerate mutase controls serine pathway flux via 3-phosphoglycerate. *Nat. Chem. Biol.* 13, 1081–1087. doi: 10.1038/nchembio.2453

Park, Y. S., Kim, D. J., Koo, H., Jang, S. H., You, Y. M., Cho, J. H., et al. (2016). AKT-induced PKM2 phosphorylation signals for IGF-1 stimulated cancer cell growth. *Oncotarget* 7, 48155–48167. doi: 10.18632/oncotarget.10179

Prakasam, G., Iqbal, M. A., Bamezai, R. N. K., and Mazurek, S. (2018). Posttranslational modifications of pyruvate kinase m2: tweaks that benefit cancer. *Front. Oncol.* 8:22. doi: 10.3389/fonc.2018.00022

Ren, F., Wu, H., Lei, Y., Zhang, H., Liu, R., Zhao, Y., et al. (2010). Quantitative proteomics identification of phosphoglycerate mutase 1 as a novel therapeutic target in hepatocellular carcinoma. *Mol. Cancer* 9:81. doi: 10.1186/1476-4598-9-81

Renneville, A., Roumier, C., Biggio, V., Nibourel, O., Boissel, N., Fenaux, P., et al. (2008). Cooperating gene mutations in acute myeloid leukemia: a review of the literature. *Leukemia* 22, 915–931. doi: 10.1038/leu.2008.19

Shan, C., Kang, H. B., Elf, S., Xie, J., Gu, T. L., Aguiar, M., et al. (2014). Tyr-94 phosphorylation inhibits pyruvate dehydrogenase phosphatase 1 and promotes tumor growth. *J. Biol. Chem.* 289, 21413–21422. doi: 10.1074/jbc.M114.581124

Shim, H., Dolde, C., Lewis, B. C., Wu, C. S., Dang, G., Jungmann, R. A., et al. (1997). c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. *Proc. Natl. Acad. Sci. U.S.A.* 94, 6658–6663. doi: 10.1073/pnas.94.13.6658

Siddiqui, F. A., Prakasam, G., Chattopadhyay, S., Rehman, A. U., Padder, R. A., Ansari, M. A., et al. (2018). Curcumin decreases Warburg effect in cancer cells by down-regulating pyruvate kinase M2 via mTOR-HIF1α inhibition. *Sci. Rep.* 8:8323. doi: 10.1038/s41598-018-25524-3

Sundstrøm, T., Espedal, H., Harter, P. N., Fasmer, K. E., Skaftnesmo, K. O., Horn, S., et al. (2015). Melanoma brain metastasis is independent of lactate dehydrogenase A expression. *Neuro Oncol.* 17, 1374–1385. doi: 10.1093/neuonc/nov040

Tech, K., Tikunov, A. P., Farooq, H., Morrissy, A. S., Meidinger, J., Fish, T., et al. (2017). Pyruvate kinase inhibits proliferation during postnatal cerebellar neurogenesis and suppresses medulloblastoma formation. *Cancer Res.* 77, 3217–3230. doi: 10.1158/0008-5472.CAN-16-3304

Vander Heiden, M. G., Christofk, H. R., Schuman, E., Subtelny, A. O., Sharfi, H., Harlow, E. E., et al. (2010a). Identification of small molecule inhibitors of pyruvate kinase M2. *Biochem. Pharmacol.* 79, 1118–1124. doi: 10.1016/j.bcp.2009.12.003

Vander Heiden, M. G., Locasale, J. W., Swanson, K. D., Sharfi, H., Heffron, G. J., Amador-Noguez, D., et al. (2010b). Evidence for an alternative glycolytic pathway in rapidly proliferating cells. *Science* 329, 1492–1499. doi: 10.1126/science.1188015

Wang, H. J., Hsieh, Y. J., Cheng, W. C., Lin, C. P., Lin, Y. S., Yang, S. F., et al. (2014). JMJD5 regulates PKM2 nuclear translocation and reprograms HIF-1α-mediated glucose metabolism. *Proc. Natl. Acad. Sci. U.S.A.* 111, 279–284. doi: 10.1073/pnas.1311249111

Wang, Y. H., Israelsen, W. J., Lee, D., Yu, V. W. C., Jeanson, N. T., Clish, C. B., et al. (2014). Cell-state-specific metabolic dependency in hematopoiesis and leukemogenesis. *Cell* 158, 1309–1323. doi: 10.1016/j.cell.2014.07.048

Wang, Z., Chatterjee, D., Jeon, H. Y., Akerman, M., Vander Heiden, M. G., Cantley, L. C., et al. (2012). Exon-centric regulation of pyruvate kinase M alternative splicing via mutually exclusive exons. *J. Mol. Cell Biol.* 4, 79–87. doi: 10.1093/jmcb/mjr030

Warburg, O. (1956). On the origin of cancer cells. *Science* 123, 309–314. doi: 10.1126/science.123.3191.309

Xian, Z. Y., Liu, J. M., Chen, Q. K., Chen, H. Z., Ye, C. J., Xue, J., et al. (2015). Inhibition of LDHA suppresses tumor progression in prostate cancer. *Tumour Biol.* 36, 8093–8100. doi: 10.1007/s13277-015-3540-x

Xie, J., Dai, C., and Hu, X. (2016). Evidence that does not support pyruvate kinase M2 (PKM2)-catalyzed reaction as a rate-limiting step in cancer cell glycolysis. *J. Biol. Chem.* 291, 8987–8999. doi: 10.1074/jbc.M115.704825

Yang, W., Xia, Y., Hawke, D., Li, X., Liang, J., Xing, D., et al. (2012a). PKM2 phosphorylates histone H3 and promotes gene transcription and tumorigenesis. *Cell* 150, 685–696. doi: 10.1016/j.cell.2012.07.018

Yang, W., Xia, Y., Ji, H., Zheng, Y., Liang, J., Huang, W., et al. (2011). Nuclear PKM2 regulates beta-catenin transactivation upon EGFR activation. *Nature* 480, 118–122. doi: 10.1038/nature10598

Yang, W., Zheng, Y., Xia, Y., Ji, H., Chen, X., Guo, F., et al. (2012b). ERK1/2-dependent phosphorylation and nuclear translocation of PKM2 promotes the Warburg effect. *Nat. Cell Biol.* 14, 1295–1304. doi: 10.1038/ncb2629

Yu, Z., Zhao, X., Huang, L., Zhang, T., Yang, F., Xie, L., et al. (2013). Proviral insertion in murine lymphomas 2 (PIM2) oncogene phosphorylates pyruvate kinase M2 (PKM2) and promotes glycolysis in cancer cells. *J. Biol. Chem.* 288, 35406–35416. doi: 10.1074/jbc.M113.508226

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Metabolic Targeting of Breast Cancer Cells With the 2-Deoxy-D-Glucose and the Mitochondrial Bioenergetics Inhibitor MDIVI-1

Federico Lucantoni[1,2], *Heiko Dussmann*[1,2] *and Jochen H. M. Prehn*[1,2]*

[1] Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, Dublin, Ireland, [2] Centre for Systems Medicine, Royal College of Surgeons in Ireland, Dublin, Ireland

Breast cancer cells have different requirements on metabolic pathways in order to sustain their growth. Triple negative breast cancer (TNBC), an aggressive breast cancer subtype relies mainly on glycolysis, while estrogen receptor positive (ER+) breast cancer cells possess higher mitochondrial oxidative phosphorylation (OXPHOS) levels. However, breast cancer cells generally employ both pathways to sustain their metabolic needs and to compete with the surrounding environment. In this study, we demonstrate that the mitochondrial fission inhibitor MDIVI-1 alters mitochondrial bioenergetics, at concentrations that do not affect mitochondrial morphology. We show that this effect is accompanied by an increase in glycolysis consumption. Dual targeting of glycolysis with 2-deoxy-D-glucose (2DG) and mitochondrial bioenergetics with MDIVI-1 reduced cellular bioenergetics, increased cell death and decreased clonogenic activity of MCF7 and HDQ-P1 breast cancer cells. In conclusion, we have explored a novel and effective combinatorial regimen for the treatment of breast cancer.

Keywords: Warburg effect, breast Cancer, MDIVI-1, cell death, bioenergetics, OXPHOS

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Concetta Bubici,
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Pedro A. Lazo,
Instituto de Biología Molecular y Celular del Cancer (IBMCC), Spain

***Correspondence:**
Jochen H. M. Prehn
jprehn@rcsi.ie

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INTRODUCTION

Metabolic rewiring in breast cancer cells critically contributes to disease progression (Long et al., 2016). In general, this malignancy displays a highly lipogenic phenotype with glucose and glutamine playing a central role in sustaining cell growth (Mishra and Ambs, 2015). Breast cancer cells possess a highly versatile metabolism and can use different energy sources and metabolic pathways for their energetic and anabolic requirements. Metabolic rewiring also allows breast cancer cells to adapt to different nutrient available in the surrounding environment and to switch metabolic pathways to adapt to limiting growth conditions (Vander Heiden and DeBerardinis, 2017). It is widely accepted that glucose plays an important role in cancer progression. In 1920, the German scientist Otto Warburg, observed that tumors take up increased amount of glucose, compared to the surrounding cells, with the subsequent fermentation to lactate (Warburg et al., 1927). Of note, breast cancer is a heterogeneous disease that can be divided into several molecular subtypes, each of them characterized by the presence of distinct metabolic alterations. In line with

Abbreviations: 2DG, 2-deoxy-D-glucose; ER, estrogen receptor; FRET, foerster resonance energy transfer; KB, krebs buffer; OXPHOS, mitochondrial oxidative phosphorylation; TMRM, tetramethylrhodamine methyl ester; TNBC, triple negative breast cancer.

the Warburg effect, it has been shown that triple negative breast cancer (TNBC) and HER2 positive breast cancer possess higher levels of glycolytic activity than estrogen receptor positive (ER+) breast cancer cells (Choi et al., 2013; Pelicano et al., 2014; Lanning et al., 2017). In TNBC, it was reported that EGF signaling is responsible for the activation of the first step in glycolysis (Lim et al., 2016), and that c-Myc regulate this metabolic feature by suppressing the expression of thioredoxin-interacting protein (Shen et al., 2015). Glutamine is also an important mediator of breast cancer metabolism. Indeed, both TNBC and HER2+ subtypes possess increased glutamine consumption and glutaminolysis levels (Kung et al., 2011; Kim et al., 2013; Lampa et al., 2017). In contrast, ER+ breast cancer cells have been found to be more dependent on OXPHOS, even when glycolysis is functioning (Rodriguez-Enriquez et al., 2010; Lanning et al., 2017).

In a recent study, we have recently shown that breast cancer bioenergetics are also controlled by members of the BCL-2 protein family, a family of proteins originally shown to be primarily involved in apoptosis regulation (Czabotar et al., 2014). However, it is now becoming increasingly evident, that BCL-2 family proteins also regulate mitochondrial fusion and fission dynamics and may regulate mitochondrial respiratory chain activity (Chen and Pervaiz, 2007; Alavian et al., 2011; Hardwick and Soane, 2013; Gross, 2016; Williams et al., 2016). BCL2 and BCL(X)L selective inhibitors (Venetoclax and WEHI-539, respectively) were able to decrease mitochondrial bioenergetics and ATP production (Lucantoni et al., 2018). Of note, this metabolic inhibition observed was independent of apoptosis induction. Additionally, we have also shown that these inhibitors were able to decrease mitochondrial morphology and fusion/fission dynamics (Lucantoni et al., 2018). We then highlighted that dual targeting of glycolysis, with 2-deoxy-D-glucose, and mitochondrial metabolism, using BCL2 inhibitors can be used as a potential strategy to stop the progression of both ER+ and TN breast cancer (Lucantoni et al., 2018).

In the present study, we wanted to explore the effect of mitochondrial dynamics interference on bioenergetics and cell survival. Mitochondria are tightly regulated organelles that provides for different cellular functions, apart from their role of producing high amount of energy (Friedman and Nunnari, 2014). The complex mitochondrial structure is deeply linked to the bioenergetics function performed by this organelle at the physiological level (Galloway et al., 2012). The highly mitochondrial plasticity is regulated by enzymatic processes (Westermann, 2010). Mitochondrial fusion and fission are important in maintaining a healthy pool of mitochondria, since structural abnormalities lead to bioenergetics defects (Chen et al., 2005), compromised autophagy control systems (Twig and Shirihai, 2011) and accumulation of mitochondrial DNA damage (Chen et al., 2010). These membrane remodeling activities are mediated by dynamin-related proteins 1 (Drp1), mitofusin (Mfn) 1 and 2 and Opa1. Drp1 is the key regulator of mitochondrial fission, the splitting of one mitochondrion in two or more smaller mitochondria. When recruited on the outer mitochondrial membrane by human fission protein 1 (Fis1), Drp1, together with other structural bending proteins, forms oligomeric rings around the membrane to fragment it (Detmer and Chan, 2007; McBride and Soubannier, 2010). Due to the importance of mitochondrial dynamics, an effort has been made to develop chemical tools to alter these processes. In fact, a selective Drp1 inhibitor, MDIVI-1, has been discovered through a screening of a chemical library (Cassidy-Stone et al., 2008). Nonetheless, a recent work, shed light on MDIVI-1 selectivity and mechanism of action, as this compound was reported to inhibit reversibly mitochondrial complex I-dependent O_2 consumption (Bordt et al., 2017).

Here, we show, by employing population and single cell time-lapse imaging approaches, how the dual targeting of mitochondrial bioenergetics, with the fission and complex I inhibitor MDIVI-1, and glycolysis inhibition, can be used as a potential strategy for the treatment of breast cancer.

MATERIALS AND METHODS

Materials and Reagents

Fetal bovine serum, RPMI 1640 medium, Thiazolyl Blue Tetrazolium Bromide (MTT), dimethyl sulfoxide (DMSO), sodium pyruvate, D-glucose, 2-deoxy-D-glucose and MDIVI-1 came from Sigma-Aldrich (Dublin, Ireland). Tetramethylrhodamine methyl ester (TMRM) was from Invitrogen (Biosciences, Ireland).

Cell Lines

MCF7 and HDQ-P1 cells were cultured in RPMI-1640 supplemented with 10% FBS, 1% L-Glutamine and 1% Penicilin/Streptomycin. All cell lines were incubated at 37°C in humidified atmosphere with 5% of CO_2. Cell lines were authenticated by STR typing from Source Bioscience (Nottingham, United Kindom).

MTT Assay

The MTT assay was used to determine mitochondrial activity following combination treatment of MDIVI-1 and 2DG. MCF7 cells and HDQ-P1 cells were seeded at a density of 1.5×10^4 cells for well on 96-well plates, kept at 5% CO_2 and 37°C and treated with increasing concentration of MDIVI-1 (from 0.1 to 10 μM) in combination with 2DG (from 0.3 to 30 mM). After 72 h, 20 μL of 5 mg/mL MTT in 1X PBS was added to each well and the plate incubated at 37°C for 4 h. Consequently medium was removed and crystals were suspended in 100 μL DMSO. Absorbance at 570 nm was recorded on a Clariostar reader (BMG Labtech, Ireland). Experiments were repeated three times on cultures from different platings; each treatment was performed in triplicate during every experiment.

Live Cell Time-Lapse Imaging of Mitochondrial and Cytosolic ATeam FRET Probe, TMRM Dye, and Glucose FRET Probe

Cells were seeded at a concentration of 2×10^3 in sterile Willco dishes and let to adhere over-night. Then, the plasmid with the mitochondrial targeted Ateam construct (Imamura et al., 2009)

was transfected into MCF7 cells with lipofectamine 2000 for 4 h. On the day of the experiment, adherent cells were washed twice with krebs-hepes buffer (KB, 140 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 15 mM HEPES) and the medium replaced with 1 mL of KB containing 30 nM TMRM, 2 mM sodium pyruvate and 2.5 mM $CaCl_2$. Mineral oil was added on top of the KB to prevent evaporation and the dishes transferred to a heated stage above a 63×/1.4 NA Plan-Apochromat oil immersion objective lens on an inverted confocal laser-scanning microscopes (LSM 710, Zeiss). Mitochondrial ATP kinetics measurements were carried out using a lasers of 405, 488, and 561 nm for excitation of FRET/CFP, YFP and TMRM respectively with a pixel dwell time of 2.55 μs and images taken every minute. Detection ranges were set to 445–513 nm and 513–562 nm for CFP and FRET/YFP, while 562–710 nm was used for TMRM with pinholes set to 2 μm optical sectioning (FWHM). Cells were treated with 0.1, 1, and 10 μM MDIVI-1, with a time window of 20 min before each addition.

The FLII^{12}Pglu-700μδ6 "glucose-FRET" probe (plasmid #17866, Addgene) was used to detect intracellular glucose concentration with a linear response range between 0.05 and 9.6 mM. Glucose binds to the glucose–galactose-binding protein with the subsequent probe conformational change and increase in FRET signal. This plasmid has been optimized to reduce pH sensitivity [enhanced yellow fluorescent protein (YFP) replaced with citrine protein] and other potential artifacts (Fehr et al., 2003; Takanaga et al., 2008). Again, cells were seeded in sterile Willco dishes and transfected as described above. On the day of the experiment KB with 5 mM glucose was added on top of the cells, with mineral oil to prevent evaporation. The dishes were transferred to a heated stage above a 40×/1.3 Numerical Aperture (NA) Plan-Neofluar oil immersion objective lenses on a inverted epifluorescence microscope (Axiovert 200M, Zeiss), used with selected polychroic mirror and filter wheel settings. Experiments were carried out using 0.09% of a HBO 100 mercury short-arc lamp for excitation with a band pass of 438/24 nm (center wavelength and band width) for FRET/CFP (cyan fluorescent protein) and a band pass filter with 500/24 nm YFP (yellow fluorescent protein) with exposure time of 20 ms, and 531/40 nm for TMRM with an exposure time of 10 ms. Baseline levels were measured for 20 min, then 1 μM MDIVI-1 was added for 30 min. Subsequently we added 5 mM 2DG for 30 min and 20 mM glucose for the last 20 min. Same setup and settings were used for cytosolic ATP reporter imaging using ATeam1.03-nD/nA/pcDNA3 (plasmid #51958, Addgene). For this experiment, we measured 20 min baseline followed by 5 mM 2DG treatment for 30 min. Then we added 3 μM MDIVI-1 or vehicle for 2 h, and 20 mM glucose for the final 30 min.

Images were processed using ImageJ2 (National Institutes of Health, Bethesda, MD, United States) and Metamorph 7.5 (Universal Imaging Co., Westchester, PA, United States). Time-lapse sequences were imported into ImageJ and background was first subtracted from each image. After creating combined images of the three fields of views for each channel sequence, a median filter with a radius of one pixel was applied. The combined images were then processed using Metamorph. Mitochondria, cytosolic glucose and ATP signals within cells were segmented from background using the YFP time lapse images. The segmented mitochondrial or cytosolic areas were converted into a mask used to remove background values from any further analysis of the FRET/CFP stack. To this end the FRET image stack was first multiplied by the YFP-mask and divided by CFP image stack, and regions of interest were then selected for analysis. A custom made Metamorph journal was used to obtain the average intensity signal from all regions, and an excel macro was then applied to sort the values and to converted them to percentage normalized to the baseline. All experiments were performed at least three times independently of each other.

Live Cell Time-Lapse Imaging of Caspase DEVD FRET Probe

MCF7-DEVD cells (Rehm et al., 2002) where plated on glass bottom dishes (Willco Wells, Netherlands). Time lapse experiments where performed on LSM 710 or a home build epifluorescence live cell imaging setup both equipped with stage incubator set to 37°C and 5% CO_2. Drugs were added as described in the figures (10 mM 2DG after 30 min and 3 μM MDIVI-1 after 60 min) and cells were imaged in intervals of 2–5 min for 48 h. On the LSM 710 and the epifluorescence settings were used as described above in order to image the CFP and YFP intensities to determine a disruption of FRET monitoring caspase-3 activation. Single cell CFP/FRET kinetics were analyzed after background subtraction in all YFP positive areas using ImageJ (1.51 k, by Wayne Rasband, NIH) and plotted in MS-Excel. An increase in the CFP/FRET ratio is indicative of DEVD substrate cleavage. Treatment and control experiments were performed three times each.

Synergy Calculations

MTT was employed to measure mitochondrial activity, while phenol red absorbance was used to obtain pH values of the nutrient medium covering the live cells. After 72 h treatment MTT protocol was utilized as previously described. An excel template was used to calculate the mitochondrial activity after normalization to vehicle treated cells and Combination index, using Webb's fractional product method (Webb, 1963). An excel template was used to calculate the fraction affected from MTT data and the results were analyzed with the web version of Chalice Analyzer (Horizon Discovery) to calculate isobologram. pH was recorded before the addition of MTT through the measurement of phenol red absorbance spectra. The wavelength range was 350–650 nm with a step width of 5 nm and a bidirectional mode was employed for the reading. The path length correction, considering the volume (200 μL) and the thickness of the plate was taken into account, using appropriate options on the ClarioStar reader. An excel template was utilized to calculate the 560/440 nm ratio and the formula $\log\left[\frac{\frac{560\ nm}{440\ nm}}{0.0002}\right]/1.18$ was employed to obtain pH values. Each treatment was performed in duplicate; experiments were repeated three times on cultures from different platings.

Clonogenic Assay

A 1000 cells were seeded in a 6-well plate. After 72 h treatment with 3 μM of MDIVI-1 alone and in combination with 10 mM 2DG, fresh medium was added in each well and colonies were growth for 7 days. Cells were then fixed in 4% PFA for 10 min at room temperature and stained with crystal violet (0.5% in 1× PBS). Plates were scanned on a CanoScan LiDE 80 (Canon) at a resolution of 1200 dpi. Images were then cropped with ImageJ and analyzed with OpenCFU software (Geissmann, 2013). Experiments were repeated three times on cultures from different platings; each treatment was performed in triplicate during every experiment.

Flow Cytometry

Cells were seeded on a 24 well plate at a density of $6^{*}10^{4}$ cells for well and treated with vehicle, 10 mM 2DG, 3 μM MDIVI-1, and combination treatments. After incubation time (72 h) cells were collected by tripsinization and stained with Annexin V-FITC and PI (Biovision) for 20 min at room temperature in dark condition and analyzed using a CyFlow ML (Partec) flow cytometer and FloMax software. A minimum of 10,000 events were recorded for each sample. Each treatment was performed in triplicate; experiments were repeated three independent times.

Statistical Analysis

Data are given as means ± SD (standard deviation). Correlations were assessed using Spearman's rank correlation analysis. For statistical comparison, two-way analysis of variance (ANOVA) or one-way analysis followed by Tukey's *post hoc* test were employed. $P < 0.05$ were considered to be statistically significant.

RESULTS

Mitochondrial Fission and Complex I Inhibitor MDIVI-1 Decrease Mitochondrial ATP Production and Bioenergetics

MCF7 cells were transfected with a mitochondrial ATeam expression vector, a FRET based sensor that enables the measurement of ATP production/consumption kinetics in living cells (Imamura et al., 2009). Cells were placed in Krebs buffer (KB) in the presence of 2 mM pyruvate to supply mitochondrial respiration. Following measurement of baseline kinetics for mitochondrial ATP and membrane potential, MDIVI-1 was titrated by adding increasing concentrations of this inhibitor, every 20 min (0.1, 1, and 10 μM). As highlighted in **Figures 1A,B** sequential additions of MDIVI-1 decreased mitochondrial ATP levels over the time-course, with 1 and 10 μM showing the highest effect. We analyzed the FRET/CFP ratio and found a significant decrease in the mitochondrial ATP production at all concentration used (**Figure 1B**). Interestingly, we also observed an increase in mitochondrial membrane potential when 0.1 and 1 μM of MDIVI-1 was used (**Figures 1A–C**). This is in line with a previous work that highlighted mitochondrial hyperpolarization following complex I inhibition with rotenone (Forkink et al., 2014). However, we recorded a high heterogeneity of changes in the membrane potential when 10 μM of the inhibitor was added, implying disruption of mitochondrial activity at different levels (**Figures 1A,B,D**).

MDIVI-1 Treatment Decrease Intracellular Glucose Concentration

In order to have a better understanding of the bioenergetics status of the cell, we also studied the kinetics of glucose consumption, using a glucose sensitive FRET probe (Takanaga et al., 2008). In this case, cells where placed in 5 mM glucose in order to mimic a more physiological tumor environment. After measurement of baseline kinetics, we added 1 μM of MDIVI-1 and followed the intracellular glucose levels. As shown in **Figures 2A–C** addition of 1 μM MDIVI-1 significantly decreased cytosolic glucose kinetics. In order to confirm that the effects observed were dependent on glycolysis, we used the glycolytic inhibitor 2DG. We found that the addition of 5 mM 2DG increased cellular glucose concentration (**Figure 2B**). The further addition of 20 mM glucose caused an increment in the signal (**Figure 2C**). Again, we observed an increase in TMRM signal after 1 μM MDIVI-1 treatment that was maintained following 2DG addition. Intriguingly, addition of 20 mM glucose decreased membrane potential level to normal (**Figures 2A,B,D**). These findings suggested that upon induced mitochondrial dysfunction, in the presence of oxygen, the Warburg effect sustains cancer cells and also reveals a fast response and adaptability of breast cancer cells to changes in the surrounding environment.

The Dual Inhibition of Glycolysis and OXPHOS as a Potential Treatment Strategy for Breast Cancer

Targeting the Warburg effect with 2DG has been proposed as a promising treatment strategy for a variety of cancer types (Aft et al., 2002; Zhang et al., 2006, 2014). 2DG is a glucose analog in which an atom of hydrogen replaced the 2-hydroxyl group. Upon cellular uptake, 2DG is phosphorylated by HKII with the formation of 2DG-P, which is not further converted in fructose-6-phosphate by phosphohexose isomerase. This ultimately leads to raised 2DG-P levels, hexokinase II inhibition and decreased cytosolic ATP (Maher et al., 2004). Due to observed decrease in mitochondrial ATP production and increase in glucose consumption following MDIVI-1, we further explored the idea of combining this treatment with glycolysis inhibition through 2DG.

When breast cancer cells were treated with combination of 2DG and MDIVI-1, we recorded altered bioenergetics responses (**Figure 3**). We performed a 6 × 6 dose matrix format to test for any synergistic activity between MDIVI-1 and 2DG in either ER+ MCF7 or TNBC HDQ-P1 cancer cells. After 72 h treatment with increasing concentrations of 2DG in combination with increasing concentrations of MDIVI-1, mitochondrial activity was evaluated and data analyzed using Webb fractional product method (Webb, 1963) and isobologram analysis for evaluation of drug interactions. Additionally, we also measured the medium pH through phenol red absorbance as a read-out of lactate

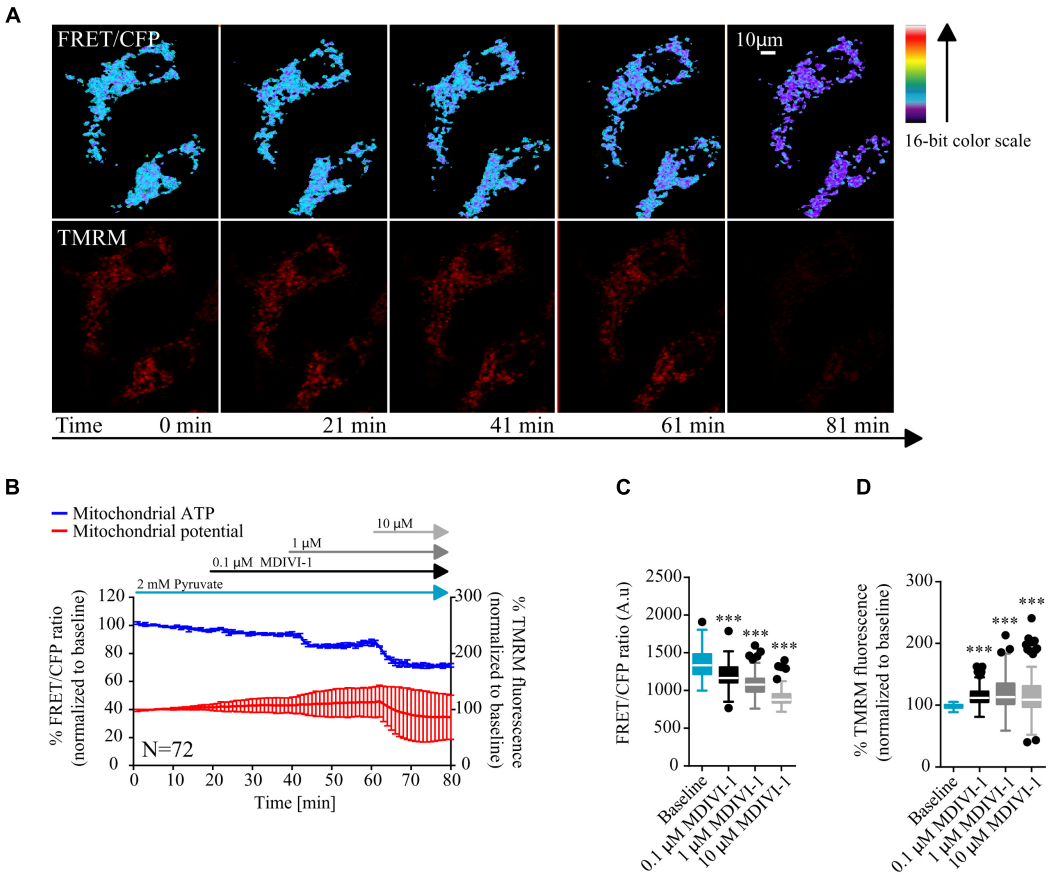

FIGURE 1 | MDIVI-1 treatment decrease mitochondrial ATP production. **(A)** Representative images of the FRET/CFP ratio in MCF7 cells transfected with the mito ATeam FRET probe in MDIVI-1 titration experiments. TMRM was used to measure mitochondrial membrane potential changes. **(B)** Mitochondrial ATP and membrane potential kinetics in MCF7 during MDIVI-1 titration. FRET/CFP ratio and TMRM fluorescence kinetics were recorded simultaneously. Baseline was recorded for 20 min, after which 0.1, 1, and 10 µM of MDIVI-1 was added to the medium with intervals of 20 min. All data represent mean ± SD from $n = 3$ independent experiments and both signals are normalized to the baseline levels. **(C)** The absolute FRET/CFP ratio was analyzed by taking the minimal value reached by the probe in each cell after each MDIVI-1 addition into account. Values were evaluated by one-way ANOVA with Tukey post-test for multiple comparison (* indicates a p-value < 0.05, ** indicates a p-value < 0.01, and *** indicates a p-value < 0.001). **(D)** TMRM intensity values, normalized to the baseline levels, were analyzed by taking the maximal value reached during MDIVI treatments into account and statistical analysis was performed as described in **(C)**.

production during glycolysis. Higher concentrations of 2DG (10 and 30 mM) in combination with 0.3–10 µM MDIVI-1 induced a significant decrease in MTT absorbance in MCF7 cells (**Figure 3A**). Similar results were obtained for HDQ-P1 cells (**Figure 3B**), starting at lower concentrations (3–30 mM 2DG in combination with 0.3–10 µM MDIVI-1). We then analyzed the synergistic interactions between the treatments, and found that 10 and 30 mM 2DG highlighted synergistic combination index values (CI, **Figures 3A,B**, black boxes) in combination with 1–10 µM (MCF7 cells) and 0.3–10 µM MDIVI-1 (HDQ-P1). CI is an indicator of synergy (CI < 1), additivity (CI = 1) or antagonism (CI > 1). When using the isolobogram analysis, we were able to validate synergy between the two compounds in MCF7 and HDQ-P1 cells (**Figure 3E**). Moreover, the synergy interaction was more efficient in HDQ-P1 cells when compared to MCF7 (**Figure 3F**), as CI values were significantly lower.

As shown in **Figures 3C,D**, higher concentrations of 2DG (10 and 30 mM) increased the medium pH to 7 in both MCF7 and HDQ-P1 cells, respectively. Treatment with 10 and 30 mM 2DG in combination with MDIVI-1 (0.1–10 µM) also increased the pH at 7, indicating that cells started to take up lactate from the medium. Lower concentrations of 2DG (0.3–1 mM) in combination with MDIVI-1 (0.1–10 µM) did not change the pH compared to control conditions (**Figures 3C,D**).

Furthermore, we also took advantage of the cytosolic version of the ATP FRET reporter to study the bioenergetics response of MCF7 cell treated with the combination of 2DG and MDIVI-1. Cells were placed in 5 mM glucose and treated with 5 mM 2DG for 30 min after recording baseline values. As expected, addition of 2DG decreased the cytosolic ATP production (**Figures 4A–D**). When 3 µM MDIVI-1 was added to the medium, a further reduction of cytosolic ATP was observed when compared to vehicle treated cells (**Figure 4E**). The final addition of 20 mM glucose partially recovered ATP production; interestingly, this recovery was less pronounced in the combination treatment

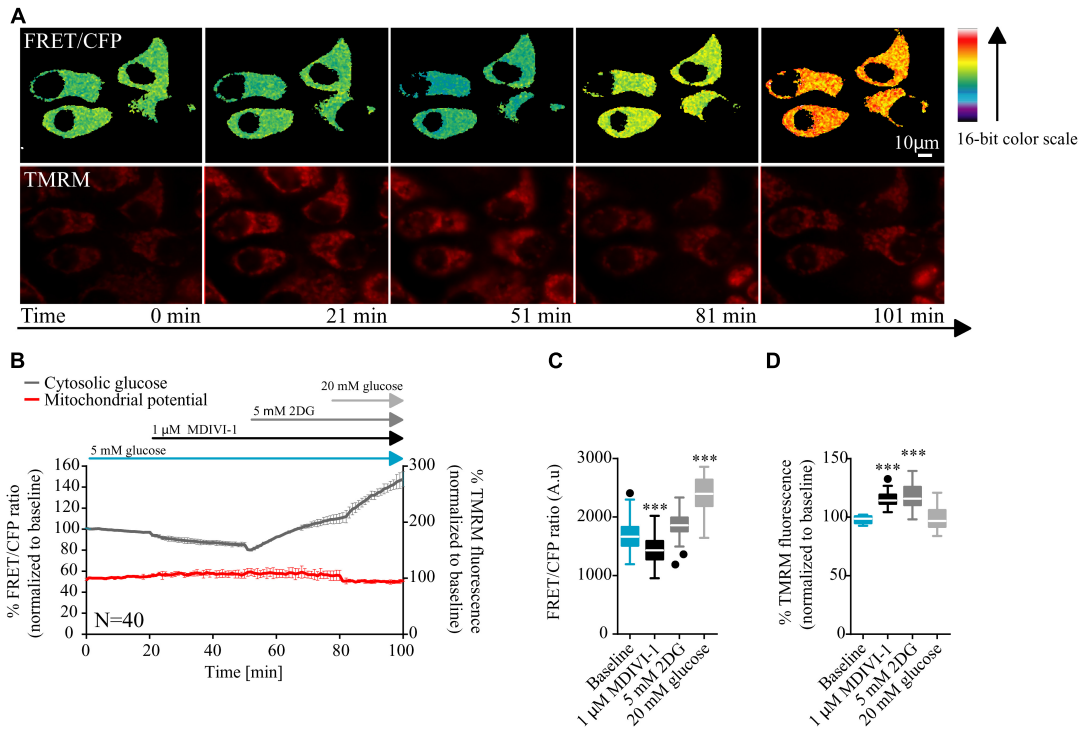

FIGURE 2 | MDIVI-1 treatment decrease glucose consumption. **(A)** Representative images of the FRET/CFP ratio of MCF7 cells transfected with the glucose FRET probe. **(B)** Cytosolic glucose and membrane potential kinetics in MCF7 during MDIVI-1 treatment. FRET/CFP ratio kinetics and TMRM fluorescence were recorded simultaneously in MCF7 cells. Baseline was recorded for 20 min, after which 1 μM of MDIVI-1 was added to the medium. After 30 min 5 mM 2DG was added for a further 30 min, followed by the addition of 20 mM glucose for 20 min. All data represent mean ± SD from $n = 3$ independent experiments and both signals are normalized to the baseline levels. **(C)** The absolute FRET/CFP ratio was analyzed by taking the minimal value reached by the probe in each cell after each MDIVI-1 addition and the maximal value after 2DG and glucose treatment into account. Values were evaluated by one-way ANOVA with Tukey post-test for multiple comparison (* indicates a p-value < 0.05, ** indicates a p-value < 0.01 and *** indicates a p-value < 0.001). **(D)** TMRM intensity values, normalized to the baseline levels, were analyzed by taking the maximal value reached during MDIVI-1 treatments into account and statistical analysis was performed as described in **(C)**.

when compared to vehicle (**Figure 4E**). We also analyzed the slope of ATP consumption and found that MDIVI-1 treated cells showed faster ATP consumption kinetics when compared to vehicle treated cells (**Figure 4G**). Moreover 2DG increased the TMRM signal, while MDIVI-1 addition slightly decreased TMRM fluorescence when compared to vehicle (**Figure 4F**). Similar results were obtained when 20 mM glucose was added to cells under combination treatment (**Figure 4F**).

This suggested that combination treatments of 2DG and MDIVI-1 induced metabolic stress with an associated inhibition of glycolytic activity and mitochondrial respiration.

2DG in Combination With MDIVI-1 Decrease Clonogenic Potential and Increase Cell Death in Breast Cancer Cells

We next asked whether combination treatment would affect clonogenic potential and cell death levels of breast cancer cells. One of the optimal synergistic concentrations (10 mM 2DG in combination with 3 μM MDIVI-1) was subsequently selected to perform the experiments. Treatment of MCF7 cells with 10 mM 2DG induced a 30% decrease in colony formation, when compared to vehicle treated cells (**Figures 4A,B**). On the other hand, treatment with MDIVI-1 alone did not induce any change in colony formation (**Figures 4A,B**). Interestingly, 2DG/MDIVI-1 combination, showed a pronounced inhibition of colony formation, with a decrease to 7% of colonies when compared to vehicle or 2DG alone (**Figures 4A,B**). Similar results were observed in the TNBC cell lines where 2DG decreased the number of colonies to 70% and combination treatments to 5–10% when compared to either vehicle or 2DG treated cells (**Figures 4A,B**).

Furthermore, we employed flow cytometry to assess the levels of Annexin V/PI levels after treatments. Following 72 h, addition of 10 mM 2DG slightly decreased surviving cells to 80% and increased apoptotic cell levels to 10–15%, while 3 μM MDIVI-1 treatment did not have any effect (**Figure 4C**). Interestingly, combination treatment decreased surviving cell levels to around 50–60% and increased apoptotic cells to 50–60% in both MCF7 and HDQ-P1 (**Figure 4C**). Finally, we took advantage of MCF7 cells overexpressing a caspase activity FRET reporter constituted by the cleavage sequence DEVD (Rehm et al., 2002). Cells were treated with vehicle or combination treatment of 10 mM 2DG and 3 μMDIVI-1. We found that during combination treatment, caspase activity increased following

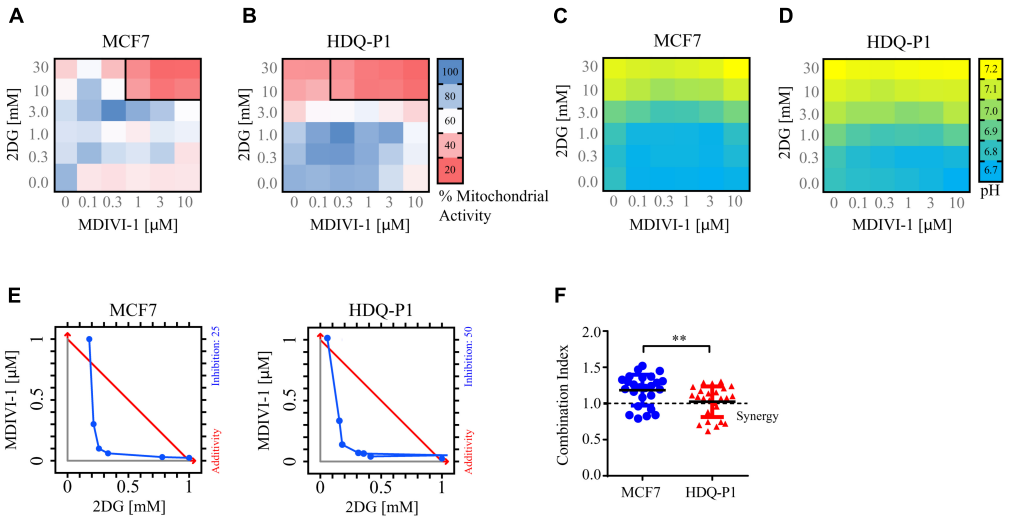

FIGURE 3 | Combination treatment of 2DG with MDIVI-1 synergistically alters mitochondrial activity and pH levels in breast cancer cell lines. A 6 × 6 dose matrix assay was performed treating MCF7 and HDQ-P1 cells with increasing concentration of 2DG in combination with increasing concentration of MDIVI-1 for 72 h. Mitochondrial activity was measured by MTT assay, while the extracellular pH was measured by phenol red absorbance. Data are represented as a heatmap. **(A,B)** Mitochondrial activity values for 2DG in combination with MDIVI-1 in MCF7 and HDQ-P1 cells, respectively. **(C,D)** pH data for 2DG in combination with MDIVI-1 in MCF7 and HDQ-P1 cells, respectively. All values represent mean from $n = 3$ independent experiments. Each experimental treatment was performed in duplicate. **(E)** Isobologram analysis for fraction affected in % calculated from MTT, results for MCF7 and HDQ-P1 cells. **(F)** CI values were calculated using Webb's fractional product method and analyzed with one-way ANOVA with Tukey post-test to test significance in MCF7 and HDQ-P1 cells, respectively (** indicates a p-value < 0.05). A CI value lower than 1 means synergy while a CI lower than 0.3 is classified as strong synergy; CI values > 1 are considered as antagonistic. Results represent means ± SD.

different kinetics (**Figures 5E,F**) when compared to control experiment (**Figures 5D,F**).

DISCUSSION

In this work, we demonstrated that the mitochondrial complex I and fission inhibitor MDIVI-1 inhibits mitochondrial ATP production, and increases glucose consumption in cancer cells. Furthermore, we highlighted that this inhibitor is synthetically lethal in breast cancer cells when combined with the glycolysis inhibitor 2DG.

We first determined the activity of MDIVI-1 on mitochondrial ATP production in breast cancer cells, by using a single-cell time lapse imaging approach. As previously stated, MDIVI-1 was found to inhibit OXPHOS complex I (Bordt et al., 2017). Thus, in line with these results, we found that titration of this inhibitor profoundly affected mitochondrial bioenergetics. Importantly, this effect occurred at much lower concentrations compared to the ones used to inhibit mitochondrial fission and complex I (25–100 μM) (Bordt et al., 2017). In conjunction with mitochondrial ATP experiments we also looked into the effect of this inhibitor on glucose consumption. Our data suggest that the decrease in mitochondrial ATP level and bioenergetics are accompanied by an activation of glycolysis as a compensatory mechanism to provide for the energetics need of breast cancer cells. Indeed, it has been previously shown that upon complex I inhibition with rotenone, breast cancer cells increase their glucose uptake and switch to a more glycolytic phenotype (Xu et al., 2015). Similar results were obtained with the gene-silencing of a mitochondrial complex I subunit (Suhane et al., 2013). Another independent study has shown that upon complex I inhibition with Metformin, glycolysis activates and promote cellular growth (Menendez et al., 2012).

Our study also demonstrates the importance of glycolysis inhibition as a strategy for the treatment of breast cancer. Glycolysis is an important metabolic route; in addition to function as a rapid source of ATP, it has been shown to be involved in other important metabolic pathways, such as pentose phosphate, hexosamine and glycogen synthesis (Hay, 2016). Cancer cells increase their glucose uptake by modulating the expression of hexokinase II (HKII), which, in turn, phosphorylates glucose and blocks its transport to the extracellular compartment mediated by specific transporters (Mathupala et al., 2001; Patra et al., 2013). HKII is associated with the mitochondrial outer membrane through the interaction with voltage-dependent anion-selective channel (VDAC). VDAC transfers the ATP produced by the mitochondria to HKII to catalyze the glucose phosphorylation reaction (Mathupala et al., 2006). A second regulation step is the conversion of phosphoenolpyruvate into pyruvate mediated by pyruvate kinases. In order to reroute metabolites to different pathways and support cell growth, low affinity pyruvate kinase M2 isoform is exploited by cancers to decrease this reaction (Israelsen and Vander Heiden, 2015). In conjunction, pyruvate is converted to lactate to maintain NAD^+ levels. It has also been observed that acidification of tumor microenvironment by extracellular lactate may improve tumor invasion (Gatenby et al., 2006).

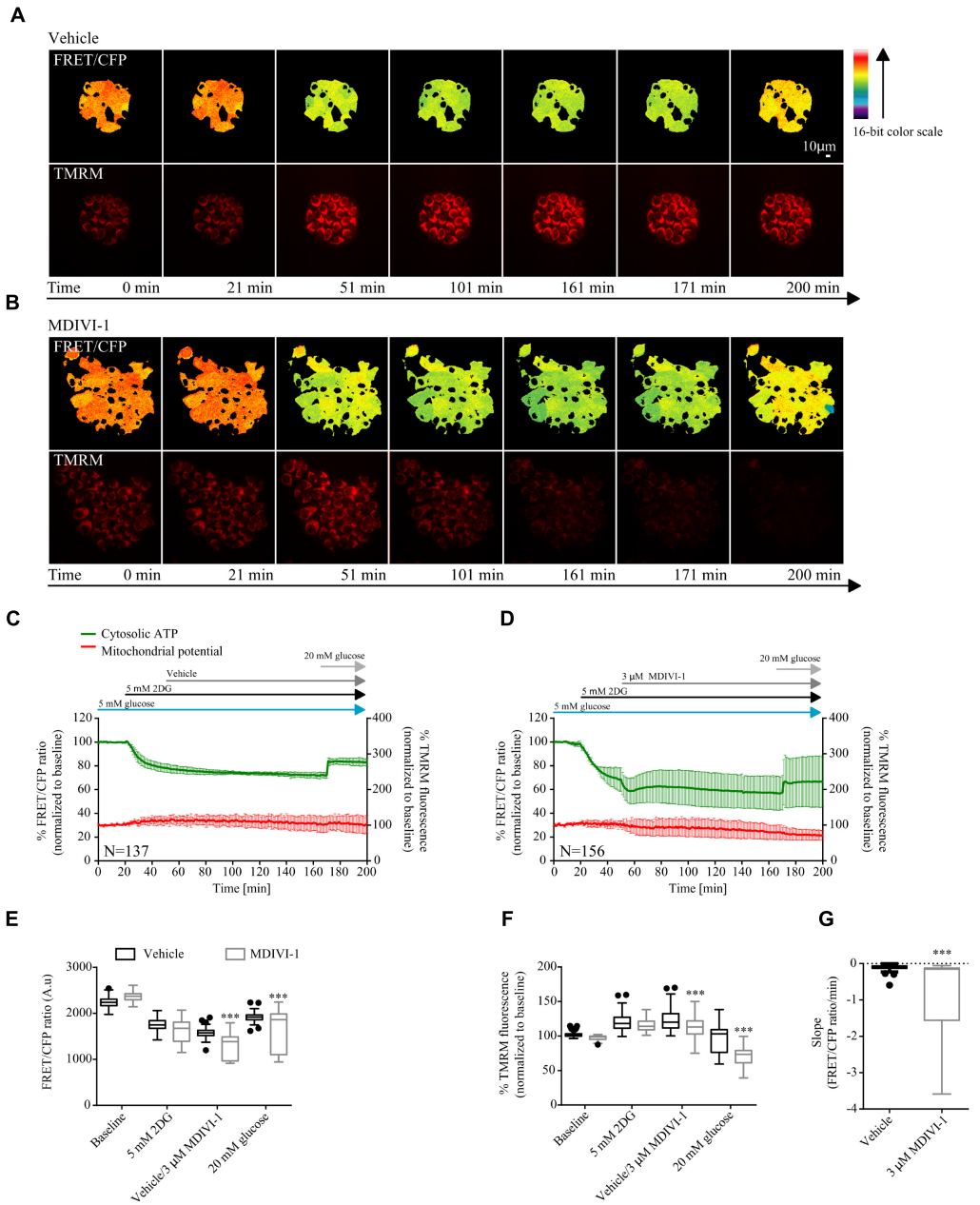

FIGURE 4 | Combination treatment of 2DG with MDIVI-1 decrease ATP bioenergetics. **(A,B)** Representative images for FRET/CFP ratio from cytosolic ATP FRET probe and TMRM for vehicle and MDIVI-1 in combination with 2DG, respectively. **(C,D)** Cytosolic ATP and membrane potential traces in MCF7 during vehicle and MDIVI-1 treatment in combination with 2DG, respectively. FRET/CFP ratio kinetics and TMRM fluorescence were recorded simultaneously in MCF7 cells. Cells were placed in KB with 5 mM glucose and baseline was recorded for 20 min, after which 5 mM 2DG was added to the medium. After 30 min vehicle or 3 μM MDIVI-1 was added. After recording of the signal for 2 h 20 mM glucose was added for 30 min. All data represent mean ± SD from $n = 3$ independent experiments and both signals are normalized to the baseline levels. **(E)** The absolute FRET/CFP ratio was analyzed by taking the minimal value reached by the probe in each cell after 2DG and MDIVI-1 addition and the maximal value after 20 mM glucose treatment into account. Values were evaluated by one-way ANOVA with Tukey post-test for multiple comparison (* indicates a p-value < 0.05, ** indicates a p-value < 0.01 and *** indicates a p-value < 0.001). **(F)** TMRM intensity values, normalized to the baseline levels, were analyzed by taking the max and min values after each treatment into account and statistical analysis was performed as described in **(E)**. **(G)** Slope values were assessed by dividing the minimal FRET/CFP ratio to the Δtime (time offset – time onset). Values were analyzed using Mann-Whitney test to show significance (*** indicates a p-value < 0.001).

Additionally, it has been highlighted that the microenvironment acidification and the competition for the available glucose by tumors, restricts the activity of the immune system (Chang et al., 2015; Ho et al., 2015). As previously stated, cancer cells have the ability to reroute metabolic pathways, hence this might contribute to attenuate the outcome of 2DG-based therapy.

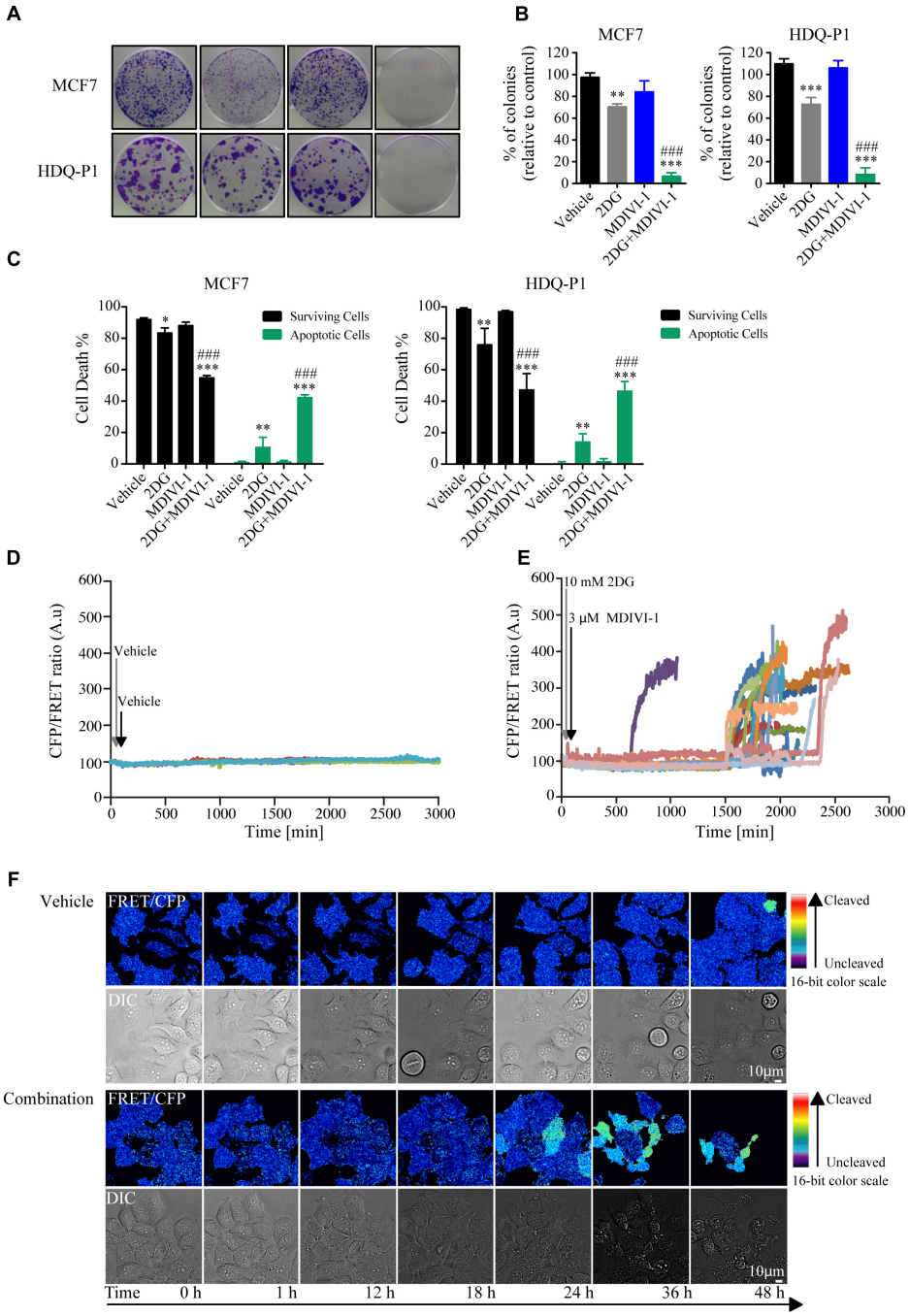

FIGURE 5 | Combination treatment of 2DG with MDIVI-1 decrease clonogenic potential and surviving cell levels in MCF7 and HDQ-P1 cells. **(A,B)** Clonogenic assay of cells treated with vehicle, 10 mM 2DG, 3 μM of MDIVI-1 and combination treatment of 10 mM 2DG with 3 μM of MDIVI-1 in MCF7 and HDQ-P1 cells, respectively. After 72 h treatment medium was changed and clonogenic capability assayed after 7 days in culture. Images were cropped using ImageJ and colonies were counted automatically with Open CFU software and the change in colony growth was normalized to vehicle-treated cells. Bars represent means ± SD from three independent experiments. One-way ANOVA with Tukey post-test was used to assess significance comparison (* indicates a p-value < 0.05, ** indicates a p-value < 0.01, and *** indicates a p-value < 0.001). **(C)** Percentages of surviving (AnnV-/PI-) and apoptotic (Ann V+/PI− plus AnnV+/PI+ fraction) after vehicle, 10 mM 2DG or 3 μM MDIVI-1 alone and in combination in MCF7 and HDQ-P1 cells, respectively. Significance was assayed with a two-way ANOVA and Tukey post-test comparison (* indicates a p-value < 0.05, ** indicates a p-value < 0.01, and *** indicates a p-value < 0.001). Each column represents mean ± SD for $n = 3$ experiments. In all experiments, asterisk was used to indicate significance between treated conditions and vehicle control, while hash was used for significance between MDIVI-1 and 2DG. **(D,E)** Kinetics of caspase cleavage following treatment of MCF7-DEVD cells with vehicle or 10 mM 2DG in combination with 3 μM MDIVI-1. Cells were imaged for 48 h and treatment were added after the first 30 min (10 mM 2DG) and 60 min (3 μM MDIVI-1) of the experiment. An increase in the CFP/FRET ratio is indicative of caspase cleavage. **(F)** Representative images of caspase cleavage in MCF7-DEVD cells following vehicle or combination treatments.

During glucose starvation or energetic stress, in order to survive, cancer cells switch to different sources of energy and carbon, through activation of AMPK signaling (Faubert et al., 2015). Furthermore, cancer cells might engage OXPHOS to compensate for decreased glycolysis or use alternative carbon sources. It has been shown that fatty acid oxidation increases upon glucose withdrawal to sustain ATP generation (Wolfe, 1998; Jelluma et al., 2006). Additionally, when glucose is removed and substituted by pyruvate, an increase in TCA cycle metabolites, alanine, and aspartate was observed (Oppermann et al., 2016). An alternative energy provider for cancer cells is glutamine, which is used to fuel TCA cycle or support nucleotide, protein, and lipid synthesis (De Vitto et al., 2016). Glucose starvation/deprivation might also activate glutaminolysis. Glutamine is initially deaminated by glutaminase with the production of ammonia and glutamate. Glutamate dehydrogenase converts this last one in α-ketoglutarate, to enter TCA cycle and produce ATP. In this context, it has been observed that glucose starvation increased activity of glutamate dehydrogenase (Jin et al., 2016). Hence the combined treatment of 2DG with an agent that inhibits mitochondrial respiration, as performed in this study, represents an attractive treatment approach.

2DG also activates autophagy, a conserved mechanism that recycles intracellular components, such as misfolded proteins or damaged organelles, and that has been proven to sustain cancer growth (Giammarioli et al., 2012; White, 2015). It has been proposed that autophagy activation upon 2DG treatment, involved ER stress and elicited a protective mechanism (Xi et al., 2011).

It has been also highlighted that 2DG-P accumulation in the cells increases the carbon flow into citrate production, impairing ADP phosphorylation, with a decrease in glycolysis (Pietzke et al., 2014). 2DG also acts on protein glycosylation and induces ER stress (Kang and Hwang, 2006; Kurtoglu et al., 2007). Hence other mechanisms beyond bioenergetics inhibition may contribute to the synergistic activity of 2DG and MDIVI-1 observed in this study. It has been demonstrated that 2DG sensitizes cancer cells to both chemotherapy and radiation therapy, showing that patients could benefit from this combination treatment (El Mjiyad et al., 2011). This glycolytic inhibitor has been tested in a variety of clinical trials, alone or in combination with chemotherapy (Raez et al., 2005; Goldberg et al., 2012; Sborov et al., 2015). It has been shown that 2DG is well tolerated up to a concentration of 200 mg/kg followed by whole brain irradiation (Mohanti et al., 1996). Hence combination treatments with 2DG represent a viable and promising strategy.

Indeed 2DG, as a single agent, has not yet been successfully translated to the clinic, due to poor efficiency recorded in clinical trials (Bost et al., 2016). As an example it has been shown that 2DG alone was not able to effectively remove cancer cells in an *in vivo* model of human osteosarcoma and non-small cell lung cancers (Maschek et al., 2004). In line with previous literature, we also found that only a small population of breast cancer cells treated with 2DG underwent cell death (**Figure 4C**). As pointed out in a review by Zhang et al. 2DG may have a cyto-protective effect, as ATP is crucial for the development of both intrinsic and extrinsic apoptosis (Zhang et al., 2014). When cells were treated with MDIVI-1 alone, cell death was also absent in ER+ (MCF7) and TN (HDQ-P1) breast cancer cells (**Figure 4C**), and only the combination was effective. Previous studies have also suggested that combining 2DG with agents that target OXPHOS represents a viable treatment strategy. Metformin inhibits complex I activity and increases glycolysis and lactate production (Chaube et al., 2015). Thus blocking lactate production was found to be deleterious for metabolism and synthetically lethal in melanoma (Chaube et al., 2015). Moreover, another group has reported a synthetically lethality of complex I inhibition with metformin and glucose withdrawal (Menendez et al., 2012). Of note, MDIVI-1 was effective at much lower concentrations and may have multiple mechanisms of action to inhibit mitochondrial respiration as a consequence of this dual targeting of complex I and fission inhibition and potentially other processes in mitochondria (**Figure 1**).

Metabolism and cellular bioenergetics are being recognized as important hallmark in different cancers pathways, such as formation of metastasis, tumor microenvironment, and treatment resistance (Pavlova and Thompson, 2016). To date, the current therapeutic landscape against this important module of cancer cells is lacking of options. Therefore, more targeted approaches that act on different metabolic/bioenergetics-related modules indeed need to be developed. This is especially important in light of the recent interest in the metabolic adaptation of immune cells in cancer progression (Xing et al., 2015). The integration of both fields has been shown to have a key role in cancer treatment (Renner et al., 2017).

Our experimental results also demonstrate, for the first time, that MDIVI-1 decrease mitochondrial bioenergetics at a concentration much lower of the one reported to inhibit fission. We also reported for the first time that the targeting of glycolysis and OXPHOS employing a combination of 2DG and MDIVI-1 can be applied to both ER+ and TNBC breast cancer subtypes. More importantly, it has to be pointed, that MDIVI-1 was found to possess low or no toxicity *in vivo* (Rappold et al., 2014). Unraveling new combination treatments and new drugs to target the "engines" of cancer cells have the potential to be critical in future investigations and to treat patients with therapy resistant cancer.

AUTHOR CONTRIBUTIONS

FL conceived and designed the study. FL and HD acquired the data. FL, HD, and JP wrote, reviewed, and/or revised the manuscript. JP supervised the study.

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REFERENCES

Aft, R. L., Zhang, F. W., and Gius, D. (2002). Evaluation of 2-deoxy-D-glucose as a chemotherapeutic agent: mechanism of cell death. *Br. J. Cancer* 87, 805–812. doi: 10.1038/sj.bjc.6600547

Alavian, K. N., Li, H., Collis, L., Bonanni, L., Zeng, L., Sacchetti, S., et al. (2011). Bcl-xL regulates metabolic efficiency of neurons through interaction with the mitochondrial F1FO ATP synthase. *Nat. Cell Biol.* 13, 1224–1233.

Bordt, E. A., Clerc, P., Roelofs, B. A., Saladino, A. J., Tretter, L., Adam-Vizi, V., et al. (2017). The putative drp1 inhibitor mdivi-1 is a reversible mitochondrial complex i inhibitor that modulates reactive oxygen species. *Dev. Cell* 40, 583.e–594.e. doi: 10.1016/j.devcel.2017.02.020

Bost, F., Decoux-Poullot, A. G., Tanti, J. F., and Clavel, S. (2016). Energy disruptors: rising stars in anticancer therapy? *Oncogenesis* 5:e188. doi: 10.1038/oncsis.2015.46

Cassidy-Stone, A., Chipuk, J. E., Ingerman, E., Song, C., Yoo, C., Kuwana, T., et al. (2008). Chemical inhibition of the mitochondrial division dynamin reveals its role in Bax/Bak-dependent mitochondrial outer membrane permeabilization. *Dev. Cell* 14, 193–204. doi: 10.1016/j.devcel.2007.11.019

Chang, C.-H., Qiu, J., O'Sullivan, D., Buck, Michael, D., Noguchi, T., et al. (2015). Metabolic competition in the tumor microenvironment is a driver of cancer progression. *Cell* 162, 1229–1241. doi: 10.1016/j.cell.2015.08.016

Chaube, B., Malvi, P., Singh, S. V., Mohammad, N., Meena, A. S., and Bhat, M. K. (2015). Targeting metabolic flexibility by simultaneously inhibiting respiratory complex I and lactate generation retards melanoma progression. *Oncotarget* 6, 37281–37299.

Chen, H., Chomyn, A., and Chan, D. C. (2005). Disruption of fusion results in mitochondrial heterogeneity and dysfunction. *J. Biol. Chem.* 280, 26185–26192. doi: 10.1074/jbc.M503062200

Chen, H., Vermulst, M., Wang, Y. E., Chomyn, A., Prolla, T. A., McCaffery, J. M., et al. (2010). Mitochondrial fusion is required for mtDNA stability in skeletal muscle and tolerance of mtDNA mutations. *Cell* 141, 280–289. doi: 10.1016/j.cell.2010.02.026

Chen, Z. X., and Pervaiz, S. (2007). Bcl-2 induces pro-oxidant state by engaging mitochondrial respiration in tumor cells. *Cell Death. Differ.* 14, 1617–1627. doi: 10.1038/sj.cdd.4402165

Choi, J., Jung, W. H., and Koo, J. S. (2013). Metabolism-related proteins are differentially expressed according to the molecular subtype of invasive breast cancer defined by surrogate immunohistochemistry. *Pathobiology* 80, 41–52. doi: 10.1159/000339513

Czabotar, P. E., Lessene, G., Strasser, A., and Adams, J. M. (2014). Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat. Rev. Mol. Cell Biol.* 15, 49–63. doi: 10.1038/nrm3722

De Vitto, H., Perez-Valencia, J., and Radosevich, J. A. (2016). Glutamine at focus: versatile roles in cancer. *Tumour Biol.* 37, 1541–1558. doi: 10.1007/s13277-015-4671-9

Detmer, S. A., and Chan, D. C. (2007). Functions and dysfunctions of mitochondrial dynamics. *Nat. Rev. Mol. Cell Biol.* 8, 870–879. doi: 10.1038/nrm2275

El Mjiyad, N., Caro-Maldonado, A., Ramirez-Peinado, S., and Munoz-Pinedo, C. (2011). Sugar-free approaches to cancer cell killing. *Oncogene* 30, 253–264.

Faubert, B., Vincent, E. E., Poffenberger, M. C., and Jones, R. G. (2015). The AMP-activated protein kinase (AMPK) and cancer: many faces of a metabolic regulator. *Cancer Lett.* 356(2 Pt A), 165–170. doi: 10.1016/j.canlet.2014.01.018

Fehr, M., Lalonde, S., Lager, I., Wolff, M. W., and Frommer, W. B. (2003). In vivo imaging of the dynamics of glucose uptake in the cytosol of COS-7 cells by fluorescent nanosensors. *J. Biol. Chem.* 278, 19127–19133. doi: 10.1074/jbc.M301333200

Forkink, M., Manjeri, G. R., Liemburg-Apers, D. C., Nibbeling, E., Blanchard, M., Wojtala, A., et al. (2014). Mitochondrial hyperpolarization during chronic complex I inhibition is sustained by low activity of complex II, III, IV and V. *Biochim. Biophys. Acta (BBA) – Bioenerget.* 1837, 1247–1256. doi: 10.1016/j.bbabio.2014.04.008

Friedman, J. R., and Nunnari, J. (2014). Mitochondrial form and function. *Nature* 505, 335–343. doi: 10.1038/nature12985

Galloway, C.A., Lee, H., and Yoon, Y. (2012). Mitochondrial morphology – Emerging role in bioenergetics. *Free Radical Biol. Med.* 53, 2218–2228.

Gatenby, R. A., Gawlinski, E. T., Gmitro, A. F., Kaylor, B., and Gillies, R. J. (2006). Acid-mediated tumor invasion: a multidisciplinary study. *Cancer Res.* 66, 5216–5223. doi: 10.1158/0008-5472.can-05-4193

Geissmann, Q. (2013). OpenCFU, a new free and open-source software to count cell colonies and other circular objects. *PLoS One* 8:e54072. doi: 10.1371/journal.pone.0054072

Giammarioli, A. M., Gambardella, L., Barbati, C., Pietraforte, D., Tinari, A., Alberton, M., et al. (2012). Differential effects of the glycolysis inhibitor 2-deoxy-D-glucose on the activity of pro-apoptotic agents in metastatic melanoma cells, and induction of a cytoprotective autophagic response. *Int. J. Cancer* 131, E337–E347. doi: 10.1002/ijc.26420

Goldberg, L., Israeli, R., and Kloog, Y. (2012). FTS and 2-DG induce pancreatic cancer cell death and tumor shrinkage in mice. *Cell Death Dis.* 3:e284.

Gross, A. (2016). BCL-2 family proteins as regulators of mitochondria metabolism. *Biochim. Biophys. Acta (BBA) – Bioenerget.* 1857, 1243–1246. doi: 10.1016/j.bbabio.2016.01.017

Hardwick, J. M., and Soane, L. (2013). Multiple functions of BCL-2 family proteins. *Cold Spring Harb. Perspect. Biol.* 5:a008722.

Hay, N. (2016). Reprogramming glucose metabolism in cancer: can it be exploited for cancer therapy? *Nat. Rev. Cancer* 16, 635–649. doi: 10.1038/nrc.2016.77

Ho, P. C., Bihuniak, J. D., Macintyre, A. N., Staron, M., Liu, X., Amezquita, R., et al. (2015). Phosphoenolpyruvate is a metabolic checkpoint of anti-tumor T cell responses. *Cell* 162, 1217–1228. doi: 10.1016/j.cell.2015.08.012

Imamura, H., Huynh Nhat, K. P., Togawa, H., Saito, K., Iino, R., Kato-Yamada, Y., et al. (2009). Visualization of ATP levels inside single living cells with fluorescence resonance energy transfer-based genetically encoded indicators. *Proc. Natl. Acad. Sci. U.S.A.* 106, 15651–15656. doi: 10.1073/pnas.0904764106

Israelsen, W. J., and Vander Heiden, M. G. (2015). Pyruvate kinase: function, regulation and role in cancer. *Semin. Cell Dev. Biol.* 43, 43–51. doi: 10.1016/j.semcdb.2015.08.004

Jelluma, N., Yang, X., Stokoe, D., Evan, G. I., Dansen, T. B., and Haas-Kogan, D. A. (2006). Glucose withdrawal induces oxidative stress followed by apoptosis in glioblastoma cells but not in normal human astrocytes. *Mol. Cancer Res.* 4, 319–330. doi: 10.1158/1541-7786.MCR-05-0061

Jin, L., Alesi, G. N., and Kang, S. (2016). Glutaminolysis as a target for cancer therapy. *Oncogene* 35, 3619–3625. doi: 10.1038/onc.2015.447

Kang, H. T., and Hwang, E. S. (2006). 2-Deoxyglucose: an anticancer and antiviral therapeutic, but not any more a low glucose mimetic. *Life Sci.* 78, 1392–1399. doi: 10.1016/j.lfs.2005.07.001

Kim, S., Kim, D. H., Jung, W.-H., and Koo, J. S. (2013). Expression of glutamine metabolism-related proteins according to molecular subtype of breast cancer. *Endocr. Relat. Cancer* 20, 339–348. doi: 10.1530/erc-12-0398

Kung, H.-N., Marks, J. R., and Chi, J.-T. (2011). Glutamine synthetase is a genetic determinant of cell type–specific glutamine independence in breast epithelia. *PLoS Genet.* 7:e1002229. doi: 10.1371/journal.pgen.1002229

Kurtoglu, M., Gao, N., Shang, J., Maher, J. C., Lehrman, M. A., Wangpaichitr, M., et al. (2007). Under normoxia, 2-deoxy-D-glucose elicits cell death in select tumor types not by inhibition of glycolysis but by interfering with N-linked glycosylation. *Mol. Cancer Ther.* 6, 3049–3058. doi: 10.1158/1535-7163.mct-07-0310

Lampa, M., Arlt, H., He, T., Ospina, B., Reeves, J., Zhang, B., et al. (2017). Glutaminase is essential for the growth of triple-negative breast cancer cells with a deregulated glutamine metabolism pathway and its suppression synergizes with mTOR inhibition. *PLoS One* 12:e0185092. doi: 10.1371/journal.pone.0185092

Lanning, N. J., Castle, J. P., Singh, S. J., Leon, A. N., Tovar, E. A., Sanghera, A., et al. (2017). Metabolic profiling of triple-negative breast cancer cells reveals metabolic vulnerabilities. *Cancer Metab.* 5:6. doi: 10.1186/s40170-017-0168-x

Lim, S.-O., Li, C.-W., Xia, W., Lee, H.-H., Chang, S.-S., Shen, J., et al. (2016). EGFR signaling enhances aerobic glycolysis in triple-negative breast cancer cells to promote tumor growth and immune escape. *Cancer Res.* 76, 1284–1296. doi: 10.1158/0008-5472.can-15-2478

Long, J.-P., Li, X.-N., and Zhang, F. (2016). Targeting metabolism in breast cancer: how far we can go? *World J. Clin. Oncol.* 7, 122–130. doi: 10.5306/wjco.v7.i1.122

Lucantoni, F., Düssmann, H., Llorente-Folch, I., and Prehn, J. H. M. (2018). BCL2 and BCL(X)L selective inhibitors decrease mitochondrial ATP production in breast cancer cells and are synthetically lethal when combined with 2-deoxy-D-glucose. *Oncotarget* 9, 26046–26063. doi: 10.18632/oncotarget.25433

Maher, J. C., Krishan, A., and Lampidis, T. J. (2004). Greater cell cycle inhibition and cytotoxicity induced by 2-deoxy-D-glucose in tumor cells treated under hypoxic vs aerobic conditions. *Cancer Chemother. Pharmacol.* 53, 116–122. doi: 10.1007/s00280-003-0724-7

Maschek, G., Savaraj, N., Priebe, W., Braunschweiger, P., Hamilton, K., Tidmarsh, G. F., et al. (2004). 2-Deoxy-d-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers in vivo. *Cancer Res.* 64, 31–34. doi: 10.1158/0008-5472.can-03-3294

Mathupala, S. P., Ko, Y. H., and Pedersen, P. L. (2006). Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria. *Oncogene* 25, 4777–4786. doi: 10.1038/sj.onc.1209603

Mathupala, S. P., Rempel, A., and Pedersen, P. L. (2001). Glucose catabolism in cancer cells: identification and characterization of a marked activation response of the type II hexokinase gene to hypoxic conditions. *J. Biol. Chem.* 276, 43407–43412. doi: 10.1074/jbc.M108181200

McBride, H., and Soubannier, V. (2010). Mitochondrial function: OMA1 and OPA1, the grandmasters of mitochondrial health. *Curr. Biol.* 20, R274–R276. doi: 10.1016/j.cub.2010.02.011

Menendez, J. A., Oliveras-Ferraros, C., Cufi, S., Corominas-Faja, B., Joven, J., Martin-Castillo, B., et al. (2012). Metformin is synthetically lethal with glucose withdrawal in cancer cells. *Cell Cycle* 11, 2782–2792. doi: 10.4161/cc.20948

Mishra, P., and Ambs, S. (2015). Metabolic signatures of human breast cancer. *Mol. Cell Oncol.* 2:e992217. doi: 10.4161/23723556.2014.992217

Mohanti, B. K., Rath, G. K., Anantha, N., Kannan, V., Das, B. S., Chandramouli, B. A. R., et al. (1996). Improving cancer radiotherapy with 2-deoxy-d-glucose: phase I/II clinical trials on human cerebral gliomas. *Int. J. Radiat. Oncol. Biol. Phys.* 35, 103–111. doi: 10.1016/S0360-3016(96)85017-6

Oppermann, H., Ding, Y., Sharma, J., Berndt Paetz, M., Meixensberger, J., Gaunitz, F., et al. (2016). Metabolic response of glioblastoma cells associated with glucose withdrawal and pyruvate substitution as revealed by GC-MS. *Nutr. Metab.* 13:70.

Patra, K. C., Wang, Q., Bhaskar, P. T., Miller, L., Wang, Z., Wheaton, W., et al. (2013). Hexokinase 2 is required for tumor initiation and maintenance and its systemic deletion is therapeutic in mouse models of cancer. *Cancer Cell* 24, 213–228. doi: 10.1016/j.ccr.2013.06.014

Pavlova, N. N., and Thompson, C. B. (2016). The emerging hallmarks of cancer metabolism. *Cell Metab.* 23, 27–47. doi: 10.1016/j.cmet.2015.12.006

Pelicano, H., Zhang, W., Liu, J., Hammoudi, N., Dai, J., Xu, R.-H., et al. (2014). Mitochondrial dysfunction in some triple-negative breast cancer cell lines: role of mTOR pathway and therapeutic potential. *Breast Cancer Res.* 16:434. doi: 10.1186/s13058-014-0434-6

Pietzke, M., Zasada, C., Mudrich, S., and Kempa, S. (2014). Decoding the dynamics of cellular metabolism and the action of 3-bromopyruvate and 2-deoxyglucose using pulsed stable isotope-resolved metabolomics. *Cancer Metab.* 2, 9–9. doi: 10.1186/2049-3002-2-9

Raez, L. E., Rosenblatt, J., Schlesselman, J., Langmuir, V., Tidmarsh, G., Rocha-Lima, C., et al. (2005). Combining glycolytic inhibitors with chemotherapy: phase I trial of 2-deoxyglucose and docetaxel in patients with solid tumors. *J. Clin. Oncol.* 23, 238s–238s. doi: 10.1200/jco.2005.23.16_suppl.3190

Rappold, P. M., Cui, M., Grima, J. C., Fan, R. Z., de Mesy-Bentley, K. L., Chen, L., et al. (2014). Drp1 inhibition attenuates neurotoxicity and dopamine release deficits in vivo. *Nat. Commun.* 5:5244. doi: 10.1038/ncomms6244

Rehm, M., Düssmann, H., Jänicke, R. U., Tavaré, J. M., Kögel, D., and Prehn, J. H. M. (2002). Single-cell fluorescence resonance energy transfer analysis demonstrates that caspase activation during apoptosis is a rapid process, role of caspase-3. *J. Biol. Chem.* 277, 24506–24514. doi: 10.1074/jbc.M110789200

Renner, K., Singer, K., Koehl, G. E., Geissler, E. K., Peter, K., Siska, P. J., et al. (2017). Metabolic hallmarks of tumor and immune cells in the tumor microenvironment. *Front. Immunol.* 8:248. doi: 10.3389/fimmu.2017.00248

Rodriguez-Enriquez, S., Carreno-Fuentes, L., Gallardo-Perez, J. C., Saavedra, E., Quezada, H., Vega, A., et al. (2010). Oxidative phosphorylation is impaired by prolonged hypoxia in breast and possibly in cervix carcinoma. *Int. J. Biochem. Cell Biol.* 42, 1744–1751. doi: 10.1016/j.biocel.2010.07.010

Sborov, D. W., Haverkos, B. M., and Harris, P. J. (2015). Investigational cancer drugs targeting cell metabolism in clinical development. *Exp. Opin. Investig. Drugs* 24, 79–94. doi: 10.1517/13543784.2015.960077

Shen, L., O'Shea, J. M., Kaadige, M. R., Cunha, S., Wilde, B. R., Cohen, A. L., et al. (2015). Metabolic reprogramming in triple-negative breast cancer through Myc suppression of TXNIP. *Proc. Natl. Acad. Sci. U.S.A.* 112, 5425–5430. doi: 10.1073/pnas.1501555112

Suhane, S., Kanzaki, H., Arumugaswami, V., Murali, R., and Ramanujan, V. K. (2013). Mitochondrial NDUFS3 regulates the ROS-mediated onset of metabolic switch in transformed cells. *Biol. Open* 2, 295–305. doi: 10.1242/bio.20133244

Takanaga, H., Chaudhuri, B., and Frommer, W. B. (2008). GLUT1 and GLUT9 as major contributors to glucose influx in HepG2 cells identified by a high sensitivity intramolecular FRET glucose sensor. *Biochim. Biophys. Acta* 1778, 1091–1099. doi: 10.1016/j.bbamem.2007.11.015

Twig, G., and Shirihai, O. S. (2011). The interplay between mitochondrial dynamics and mitophagy. *Antioxid. Redox Signal.* 14, 1939–1951. doi: 10.1089/ars.2010.3779

Vander Heiden, M. G., and DeBerardinis, R. J. (2017). Understanding the intersections between metabolism and cancer biology. *Cell* 168, 657–669. doi: 10.1016/j.cell.2016.12.039

Warburg, O., Wind, F., and Negelein, E. (1927). The metabolism of tumors in the body. *J. Gen. Physiol.* 8, 519–530.

Webb, J. L. (1963). Effect of more than one inhibitor. *Enzyme Metab. Inhibit.* 1, 66–79.

Westermann, B. (2010). Mitochondrial fusion and fission in cell life and death. *Nat. Rev. Mol. Cell Biol.* 11, 872–884. doi: 10.1038/nrm3013

White, E. (2015). The role for autophagy in cancer. *J. Clin. Invest.* 125, 42–46. doi: 10.1172/JCI73941

Williams, A., Hayashi, T., Wolozny, D., Yin, B., Su, T. C., Betenbaugh, M. J., et al. (2016). The non-apoptotic action of Bcl-xL: regulating Ca(2+) signaling and bioenergetics at the ER-mitochondrion interface. *J. Bioenerg. Biomembr.* 48, 211–225. doi: 10.1007/s10863-016-9664-x

Wolfe, R. R. (1998). Metabolic interactions between glucose and fatty acids in humans. *Am. J. Clin. Nutr.* 67(3 Suppl.), 519S–526S. doi: 10.1093/ajcn/67.3.519S

Xi, H., Kurtoglu, M., Liu, H., Wangpaichitr, M., You, M., Liu, X., et al. (2011). 2-Deoxy-D-glucose activates autophagy via endoplasmic reticulum stress rather than ATP depletion. *Cancer Chemother. Pharmacol.* 67, 899–910. doi: 10.1007/s00280-010-1391-0

Xing, Y., Zhao, S., Zhou, B. P., and Mi, J. (2015). Metabolic reprogramming of the tumour microenvironment. *FEBS J.* 282, 3892–3898. doi: 10.1111/febs.13402

Xu, Q., Biener-Ramanujan, E., Yang, W., and Ramanujan, V. K. (2015). Targeting metabolic plasticity in breast cancer cells via mitochondrial complex I modulation. *Breast Cancer Res. Treat.* 150, 43–56. doi: 10.1007/s10549-015-3304-8

Zhang, D., Li, J., Wang, F., Hu, J., Wang, S., and Sun, Y. (2014). 2-Deoxy-D-glucose targeting of glucose metabolism in cancer cells as a potential therapy. *Cancer Lett.* 355, 176–183. doi: 10.1016/j.canlet.2014.09.003

Zhang, X. D., Deslandes, E., Villedieu, M., Poulain, L., Duval, M., Gauduchon, P., et al. (2006). Effect of 2-deoxy-D-glucose on various malignant cell lines in vitro. *Anticancer. Res.* 26, 3561–3566.

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High Expression of Glycolytic Genes in Cirrhosis Correlates With the Risk of Developing Liver Cancer

Nathan C. W. Lee[1†], Maria Annunziata Carella[2†], Salvatore Papa[1] and Concetta Bubici[2,3*]*

[1] Cell Signaling and Cancer Laboratory, Leeds Institute of Cancer and Pathology, Faculty of Medicine and Health, University of Leeds, St James's University Hospital, Leeds, United Kingdom, [2] Division of Biosciences, Department of Life Sciences, College of Health and Life Sciences, Institute of Environment, Health and Societies, Brunel University London, Uxbridge, United Kingdom, [3] Faculty of Medicine, Imperial College London, London, United Kingdom

A marked increase in the rate of glycolysis is a key event in the pathogenesis of hepatocellular carcinoma (HCC), the main type of primary liver cancer. Liver cirrhosis is considered to be a key player in HCC pathogenesis as it precedes HCC in up to 90% of patients. Intriguingly, the biochemical events that underlie the progression of cirrhosis to HCC are not well understood. In this study, we examined the expression profile of metabolic gene transcripts in liver samples from patients with HCC and patients with cirrhosis. We found that gene expression of glycolytic enzymes is up-regulated in precancerous cirrhotic livers and significantly associated with an elevated risk for developing HCC. Surprisingly, expression levels of genes involved in mitochondrial oxidative metabolism are markedly increased in HCC compared to normal livers but remain unchanged in cirrhosis. Our findings suggest that key glycolytic enzymes such as hexokinase 2 (HK2), aldolase A (ALDOA), and pyruvate kinase M2 (PKM2) may represent potential markers and molecular targets for early detection and chemoprevention of HCC.

Keywords: glucose metabolism, aerobic glycolysis, the Warburg effect, liver, liver cancer

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***Correspondence:**
Salvatore Papa
s.papa@leeds.ac.uk
Concetta Bubici
concetta.bubici@brunel.ac.uk

† These authors have contributed equally to this work

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the main type of primary liver cancer and the second leading cause of cancer-related mortality worldwide, with more than 700,000 deaths every year (El-Serag, 2011; Jemal et al., 2011; Forner et al., 2018). The incidence of HCC has risen considerably over the last two decades, especially in United States and Europe (El-Serag, 2011; Jemal et al., 2011). At present, treatment options mainly consist of tumor resection, liver transplant, chemotherapies and radiologic intervention, all of which are limited to patients with early-stage disease. However, the large majority of HCC patients are usually diagnosed at advanced stages, which lack of curative therapies (Llovet et al., 2008; Ulahannan et al., 2014; Forner et al., 2018). Therefore, early HCC detection and prevention are required for reducing the high mortality rate. A better understanding of the molecular basis of HCC formation and the identification of markers are essential for the development of preventive therapies targeting the specific HCC-promoting factors and thereby improvement prognosis.

The development of HCC has been closely linked to cirrhosis, an inflammatory liver condition in which the normal liver tissue is replaced by scar tissue and regenerative nodules after long-term damage induced by various etiologies including hepatitis B (HBV) or C (HCV) viral infection, chronic alcohol consumption or nonalcoholic fatty liver disease (Wright, 1991; Borzio et al., 1995;

Donato et al., 2001). In fact, up to 90% of all cases of HCC develop in patients with cirrhosis, suggesting a role for this liver condition in the process of hepatocarcinogenesis (El-Serag, 2011; Jemal et al., 2011; Forner et al., 2018). At the cellular level, the occurrence of cirrhosis is tightly coupled with multiple rounds of hepatocyte death, inflammatory responses and compensatory hepatocyte proliferation, resulting in the formation of the regenerative nodules surrounded by fibrous bands characteristic of cirrhotic livers. It is now clear that the regenerative nodules consist of a mixed population of proliferative progenitor cells, newly generated hepatocytes and apoptosis-resistant (damaged) hepatocytes that, overtime, are likely to develop into dysplastic nodules, leading to the development of HCC (Borzio et al., 1995; Schuppan and Afdhal, 2008). Therefore, cirrhosis is recognized as a precancerous state and is important for the investigations of molecular markers of HCC development and preventive strategies. Although the etiology and pathological characteristics of liver cirrhosis have been reported in fully, the underlying molecular mechanisms of its progression to HCC are far less unknown.

Like many other cancers, HCC develops slowly after progressive accumulation of genetic and epigenetic alterations in liver cells accompanied by substantial changes in energy metabolism leading to unrestricted proliferation of mature hepatocytes. One of the most common metabolic changes observed in HCC cells is an increase in the rate of glycolysis with consequent lactate production (Kitamura et al., 2011; Huang et al., 2013; Iansante et al., 2015; reviewed in Hay, 2016). This metabolic phenomenon, known as the Warburg effect or aerobic glycolysis, occurs even in the presence of copious levels of oxygen and functional mitochondria (Gatenby and Gillies, 2004; Lunt and Vander Heiden, 2011; DeBerardinis and Chandel, 2016; Liberti and Locasale, 2016). Indeed, under aerobic conditions, normal differentiated hepatocytes typically metabolize glucose into pyruvate, which is further metabolized to carbon dioxide in the mitochondria through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) for ATP production, whereas under low oxygen conditions, the hepatocytes convert the pyruvate derived from glycolysis into lactate. Thus, HCC cells seem to re-adjust their energy metabolism by shifting toward glycolysis irrespective of oxygen availability in a manner similar to virtually all cancer cells (Iansante et al., 2015; Hay, 2016). This metabolic shift allows the highly proliferating HCC cells to accumulate intermediary glucose metabolites that can be channeled into biosynthetic pathways such as the pentose phosphate pathway (PPP), which generates the cellular reductant NADPH and macromolecules (nucleotides, amino acids, and fatty acids) required for the doubling of biomass, and to suppress apoptosis (Jones and Thompson, 2009; Hay, 2016; Kowalik et al., 2017). There is ample evidence that the readjustment of cell metabolism occurs as a consequence of activation of oncogenes or loss of tumor suppressors that influences the expression and activities of metabolic enzymes to stimulate glucose consumption (Hay, 2016). Moreover, an increased rate of glucose uptake, which is due to an up-regulation of the main glucose transport GLUT1, is also a remarkable feature observed in HCC cells (Hay, 2016). Nevertheless, limited data are available on altered gene expression of the enzymes involved in glycolysis and oxidative mitochondrial metabolism *in vivo*. In addition, whether the aerobic glycolytic metabolism is operative in the early stage of the hepatic carcinogenesis particularly in cirrhosis and correlates with poor patient prognosis have not been completely elucidated. Such a background prompted us to investigate the expression level of enzymes required for the glycolytic and mitochondrial metabolism in liver samples from patients with cirrhosis and patients with HCC available from six open source data sets.

MATERIALS AND METHODS

Data Sets Review

Differential gene expression analyses involving multiple clinical samples were performed using different data sets available through Gene Expression Omnibus (GEO) database (Barrett, 2013). Independent of age, gender, race, and region, we selected one HCC data set (GSE36376) and three cirrhotic data sets (GSE25097, GSE6764, and GSE14323) (accessed on March–May 2018) (Wurmbach et al., 2007; Sung et al., 2012; Lim et al., 2013; Levy et al., 2016) as they contained the largest cohort of HCC livers compared to adjacent non-tumor livers and cirrhotic livers compared to normal healthy livers, respectively. The GSE36376 data set consists of tumor and adjacent non-tumor liver tissues containing no necrosis or hemorrhage from 240 primary HCC patients who were treated with surgical resection or liver transplantation. None of the patients received preoperative chemotherapy (Lim et al., 2013). The GSE25097 data set consists of six liver specimens from healthy donors and 40 cirrhotic livers (Sung et al., 2012). The GSE6764 data set consists of 13 HCV-associated cirrhotic liver tissues compared with healthy livers of 10 patients undergoing resection: one patient for hepatic haemangioma, three for focal nodular hyperplasia, two for adenoma/cystadenoma, one for neuroendocrine tumor, and one living donor liver transplantation (Wurmbach et al., 2007). The GSE14323 data set consists of 41 HCV-associated cirrhotic liver tissues compared with healthy livers of 18 patients (Levy et al., 2016). For HCC, we also used a second independent data set from The Cancer Genome Atlas-Liver Hepatocellular Carcinoma (TCGA-LIHC) database, which contained a cohort of more than 350 HCC samples compared to 50 normal healthy livers (Ally et al., 2017). The TCGA-LIHC data set consists of surgical resection of biopsy biospecimens collected from patients diagnosed with HCC, and had not received prior treatment for their disease (ablation, chemotherapy, or radiotherapy). Each frozen primary tumor specimen had a companion normal tissue specimen (blood or blood components, including DNA extracted at the tissue source site). Pathology quality control was performed on each tumor and normal tissue specimen. Haematoxylin and eosin (H&E) stained sections from each sample were subjected to independent pathology review to confirm that the tumor specimen was histologically consistent with the allowable HCCs and the adjacent tissue specimen contained no tumor cells. Adjacent tissue with cirrhotic changes was not acceptable as a germline control (Ally et al., 2017). The data set GSE15654 was used for the HCC risk-association

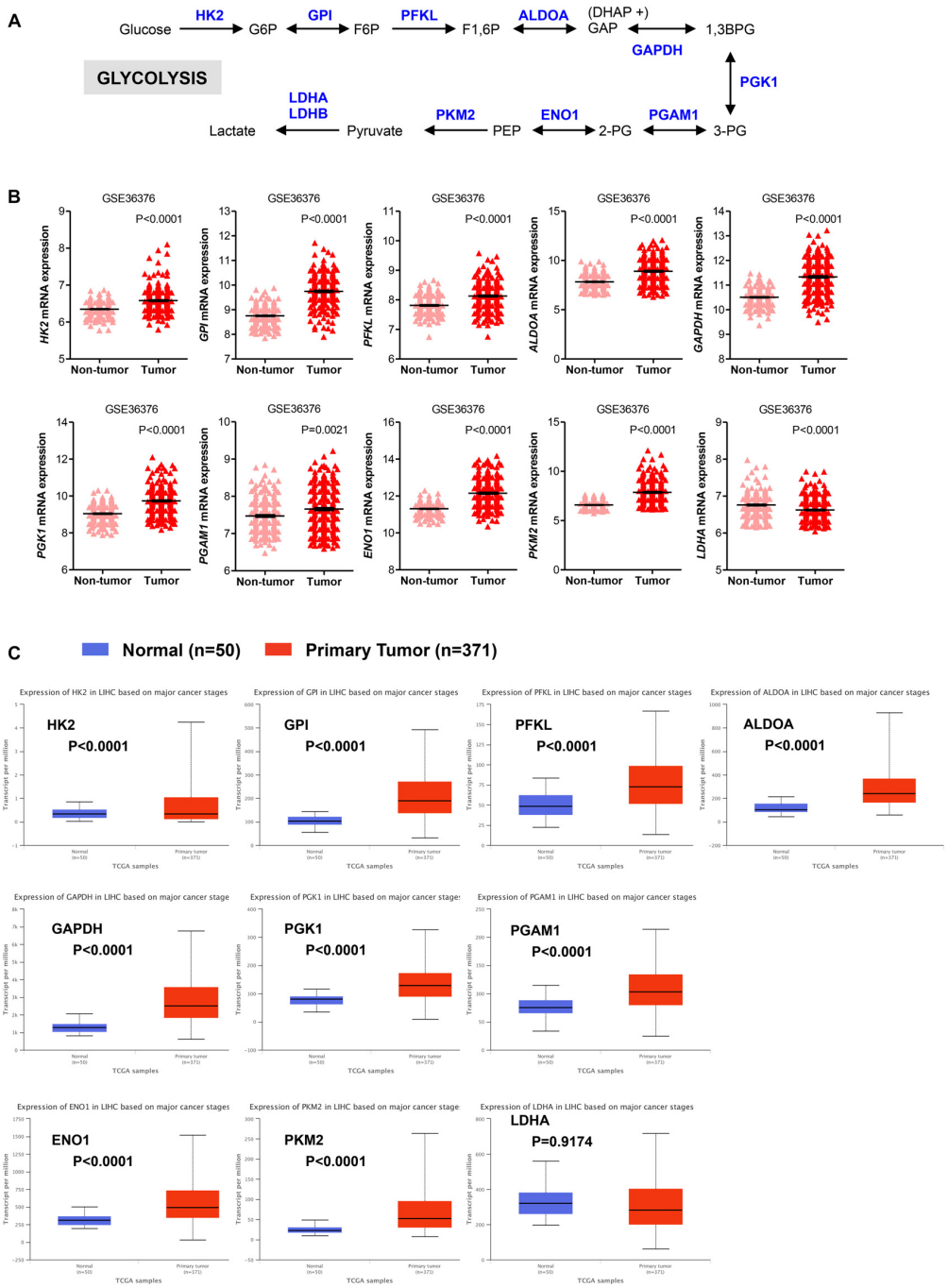

FIGURE 1 | Glycolytic genes are overexpressed in HCC. **(A)** A simplified representation depicting the glycolytic pathway in liver tumors. Abbreviations of the enzymes are as follows: hexokinase 2 (HK2), glucose-6-phosphate isomerase (GPI), phosphofructokinase liver isoform (PFKL), aldolase A (ALDOA), glyceraldehyde 3 phosphate dehydrogenase (GAPDH), phosphoglycerate kinase 1 (PGK1), phosphoglycerate mutase 1 (PGAM), enolase 1 (ENO1), and pyruvate kinase M2 (PKM2), lactate dehydrogenase (LDH). Abbreviations of the metabolites are as follow: glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose 1,6-biphosphate (F1,6BP), glyceraldehyde 3-phosphate (GAP), and dihydroxyacetone phosphate (DHAP), 1,3-biphosphoglycerate (1,3BPG), glycerol-3-phosphate (3-PG), glycerol-2-phosphate (2-PG), phosphoenolpyruvate (PEP). **(B)** Scatterplots showing the transcript levels of different glycolytic enzymes in the clinical data set GSE36376 consisting of HCC (n = 240) and adjacent non-tumor (n = 193) liver tissue (Lim et al., 2013). The horizontal lines indicate mean ± SEM P-values were calculated by non-parametric Mann–Whitney tests. **(C)** Boxplots showing differential gene expression of glycolytic enzymes among normal liver tissues (n = 50) vs. primary tumor tissues (n = 371) (TGA-LIHC samples) analyzed using the UALCAN bioinformatic tool of genomic database (Ally et al., 2017; Chandrashekar et al., 2017). Values are expressed as transcript per million. For each box plot, the whiskers represent the 2.5–97.5th percentile range of values, the lower and up boundaries denote the 25th and the 75th percentile of each data set, respectively, and the horizontal line represents the median value for each group. P-values were calculated by t-test.

of cirrhotic patients. The data set consists of 216 livers from patients with hepatitis C-related early-stage (Child-Pugh class A) cirrhosis who were prospectively followed up for a median of 10 years (Hoshida et al., 2013). Clinicopathological features of each cohort analyzed in this study are shown in the relevant cited studies.

Data Sets Analyses

For the analyses of GEO data sets, the raw data of each data set was downloaded from GEO accession links. Samples of each data set were categorized into two groups: diseased patients versus control tissue. The differential expression of genes between the two groups was calculated using a non-parametric Mann–Whitney *U* test, assigning a specific threshold (*P*-value <0.05). The analyses were performed in Prism GraphPad packages, as previously reported (Barbarulo et al., 2013).

For the differential gene expression analyses of TGCA-LIHC we used a user-friendly, interactive web resource for analyzing cancer transcriptome data accessible at http://ualcan.path.uab.edu/index.html (accessed on April/May 2018) (Chandrashekar et al., 2017). For the overall survival plots of the TGCA-LIHC data set we used GEPIA, a newly developed interactive web server available at http://gepia.cancer-pku.cn/index.html (accessed on April/May 2018) (Tang et al., 2017). KM plotter (available at http://kmplot.com/analysis/index.php?p=service&cancer=liver_rnaseq) (accessed on August 2018) was used to generate Kaplan-Meyer plots of an additional liver HCC data set (Szász et al., 2016). In both cases, the median value of gene expression was used to arrange the low and high expression groups. The overall survival significance between the two groups was calculated using Log-rank test, assigning a specific threshold (*P*-value <0.05).

RESULTS

A salient feature of HCC cells is that they adjust their metabolic profile to fulfill the bioenergetics and anabolic demands of the high rates of proliferation (Kitamura et al., 2011; Huang et al., 2013; Iansante et al., 2015; Hay, 2016). Yet, little is known about the metabolic changes at premalignant stages of disease. To have a complete overview of the metabolic genes expressed in HCC and premalignant stages of disease, we analyzed the transcription profiling of enzymes involved in glycolysis, PPP, TCA, and OXPHOS in liver samples from patients with HCC and patients with cirrhosis.

Gene Expression of Glycolysis and PPP Enzymes in HCC

The glycolytic pathway consists of ten enzymatic reactions through which glucose is converted into pyruvate. Among the multiple isoforms of enzymes that catalyze each glycolytic reaction, we evaluated mRNA expression levels of those that are predominantly expressed in the liver (**Figure 1A**; Gatenby and Gillies, 2004; Lunt and Vander Heiden, 2011; Hay, 2016). As shown in **Figure 1B**, expression of the majority of glycolytic transcripts, including the rate limiting glycolytic enzyme HK2 [hexokinase 2], ALDOA [aldolase, fructose-bisphosphate A], PFKL [6-phosphofructokinase, liver type], GAPDH [glyceraldehyde 3-phosphate dehydrogenase], PKM2 [pyruvate kinase M2]) was significantly increased in HCC livers compared to their adjacent non-tumor tissues ($P < 0.0001$). An exception was the expression of PGAM1 transcripts that was still significantly higher ($P = 0.0021$) but with a less extent compared to other enzymes. LDHA [lactate dehydrogenase A] enzyme converts pyruvate into lactate in a reaction that generates NAD+, diverting glycolysis-derived pyruvate from the mitochondrial oxidative pathway (Gatenby and Gillies, 2004; Hay, 2016). As such, the decreased expression and activity of LDHA would favor the routing of pyruvate into mitochondria where it can be further metabolized through TCA and oxidative phosphorylation. Expression of LDHA was significantly reduced in HCC samples compared to their adjacent non-tumor tissues. In contrast, mRNA expression of the other lactate dehydrogenase isoform, LDHB, showed no significant changes ($P = 0.0797$; data not shown). Similar results were also obtained in The Cancer Genome Atlas (TCGA) dataset consisting of 371 primary HCC tumors and 50 normal liver samples (LIHC cohort) (Ally et al., 2017). We observed significantly higher expression of all glycolytic enzymes in primary HCC samples compared with normal livers; the only exceptions were LDHA and LDHB (**Figure 1C** and data not shown).

We also evaluated the mRNA levels of enzymes involved in the oxidative phase of PPP. In this phase, two molecules of NADP+ are reduced to NADPH, utilizing the energy from the conversion of glucose-6-phosphate into ribulose 5-phosphate, which then enter the non-oxidative phase leading to precursors of nucleotide synthesis (**Figure 2A**; Hay, 2016; Kowalik et al., 2017). We found that in HCC samples the transcript levels of the rate limiting enzyme glucose-6-phosphate dehydrogenase (G6PD) were significantly higher than those in adjacent non-tumor ($P < 0.0001$) and normal livers ($P < 0.0001$) samples, respectively (**Figures 2B,C**). Similar to G6PD, mRNA levels of the other two enzymes involved in the oxidative phase of the PPP, 6-phosphogluconolactonase (PGLS) and 6-phosphogluconate dehydrogenase (PGD), were higher in HCC samples compared to control tissue (**Figures 2B,C**). Altogether these analyses are consistent with an increase in glycolysis and PPP pathways, leading to sustained ATP and cellular building blocks production both needed for abnormal hepatocytes proliferation (Gatenby and Gillies, 2004; Kowalik et al., 2017).

Gene Expression of Enzymes Involved in Mitochondrial Oxidative Metabolism in HCC

Once in the mitochondria, pyruvate can be converted into acetyl-CoA by the pyruvate dehydrogenase (PDH) complex. Acetyl-CoA then enters into the TCA cycle to generate NADH and FADH2, which transfer their electrons to the electron transport chain to generate ATP through OXPHOS (Lunt and Vander Heiden, 2011; Ahn and Metallo, 2015; Liberti and Locasale, 2016). In two distinct data sets, we observed that in HCC liver samples the expression of PDHA1 gene, which

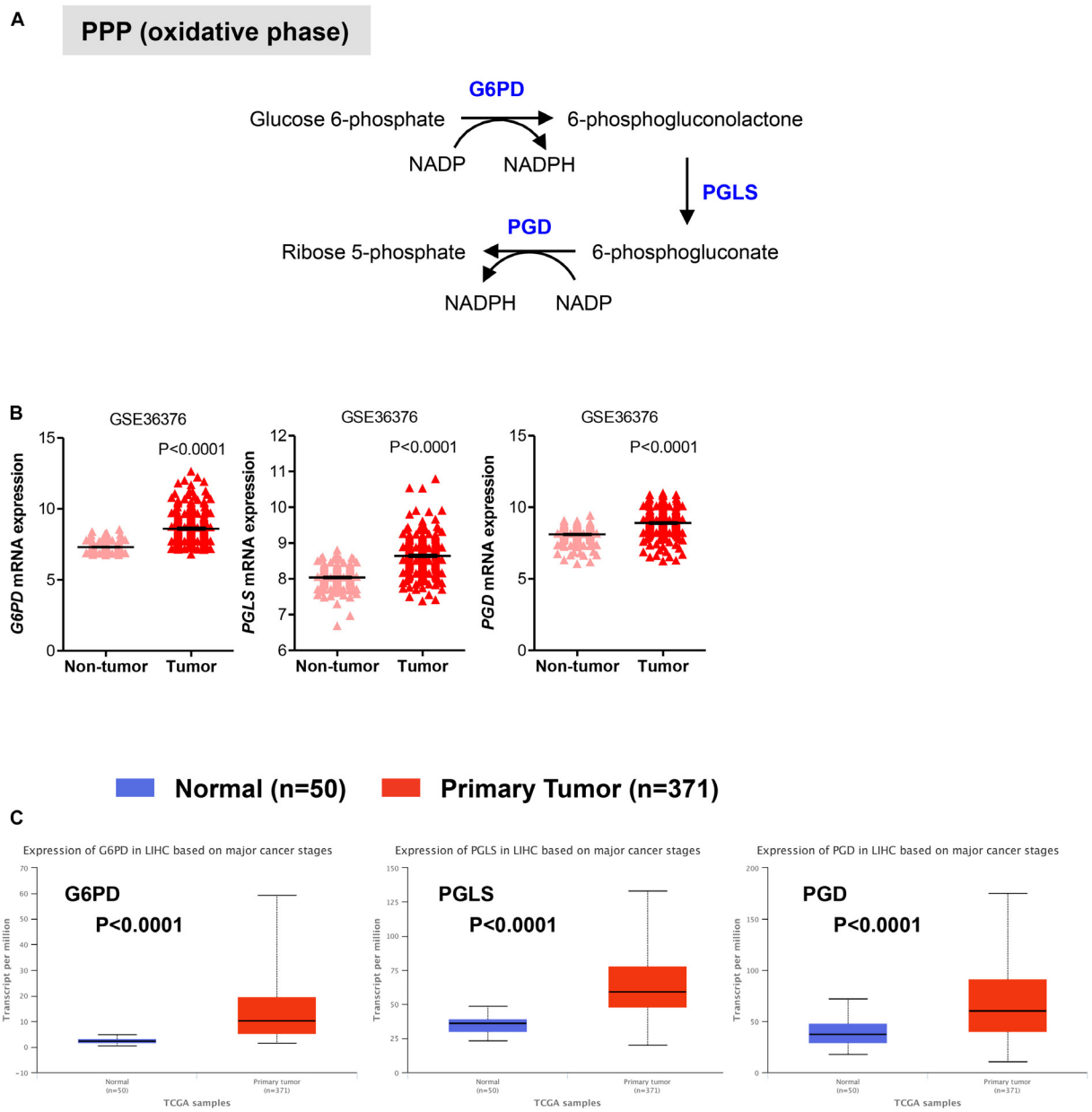

FIGURE 2 | Expression of genes in pentose phosphate pathway (PPP). **(A)** Diagram of the oxidative phase of the PPP. Abbreviations of the enzymes are as follows: glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconolactonase (PGLS), 6-phosphogluconate dehydrogenase (PGD). Activation of the two dehydrogenase enzymes, G6PD – the rate-limiting enzyme – and PGD, results in the production of NADPH, H+ ions, and ribose 5-phosphate. **(B,C)** Gene expression analyses showing enhanced expression of G6PD, PGLS, and PGD in primary HCC tumor samples compared to either adjacent non-tumor samples in GSE36376 data set **(B)** or normal liver tissues in TGA-LIHC data set **(C)**, respectively. *P*-values were calculated by nonparametric Mann–Whitney tests in **(A)** or by *t*-test **(B)**.

encodes the E1 alpha 1 subunit of the PDH complex, was significantly higher than that in non-tumor tissue ($P < 0.0001$) or normal livers ($P < 0.0001$), while in HCC samples the expression of the succinate dehydrogenase (SDHB), which converts succinate into fumarate in the TCA, was significantly lower than that in non-tumor tissue ($P < 0.0001$) or normal livers ($P < 0.0001$) (**Figures 3A,B**). These observations are consistent with a recent study showing that decreased expression levels of SDHB in HCC promote the Warburg effect (Tseng et al., 2018). Less clear was the gene expression pattern of the isocitrate dehydrogenases 2 (IDH2), which converts isocitrate to alpha-ketoglutarate in the TCA. Expression levels of IDH2 were higher in HCC samples compared to adjacent non-tumor samples ($P = 0.0010$) from the GSE36376 data set, while IDH2 expression showed no changes in the TGA-LIHC data set ($P = 0.7106$) (**Figures 3A,B**).

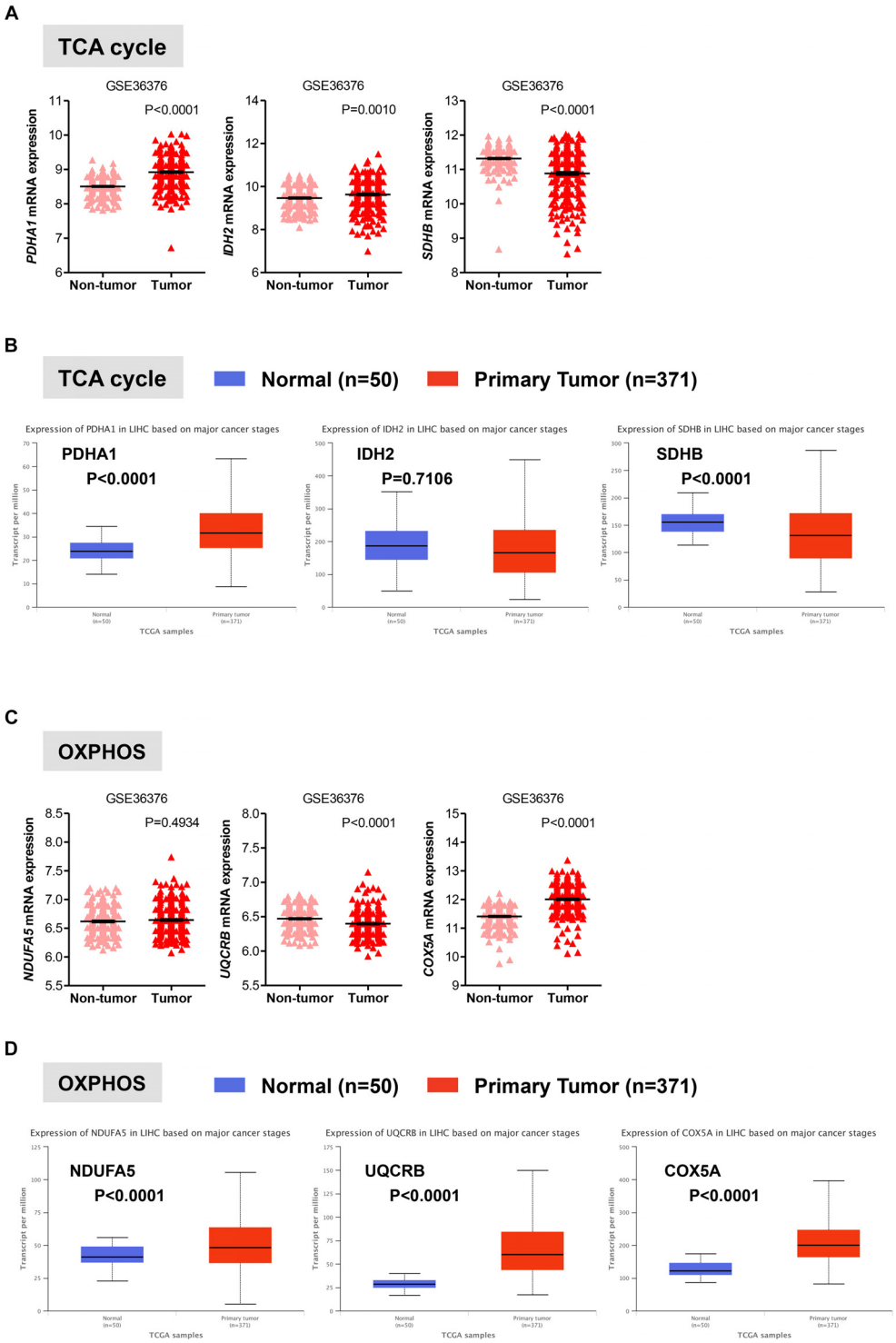

FIGURE 3 | Gene expression analysis of the oxidative mitochondrial metabolism in HCC. **(A,C)** Scatterplots showing the transcript levels of representative TCA **(A)** and OXPHOS **(C)** enzymes in the clinical data set GSE36376 consisting of HCC (n = 240) and adjacent non-tumor (n = 193) liver tissue (Lim et al., 2013). The horizontal lines indicate mean ± SEM P-values were calculated by nonparametric Mann–Whitney tests. **(B,D)** Boxplots showing differential gene expression of TCA **(B)** and OXPHOS **(D)** among normal liver tissues (n = 50) vs. primary tumor tissues (n = 371) (TGA-LIHC samples) analyzed using the UALCAN bioinformatic tool of genomic database (Ally et al., 2017; Chandrashekar et al., 2017). Values are expressed as transcript per million. For each box plot, the whiskers represent the 2.5–97.5th percentile range of values, the lower and up boundaries denote the 25th and the 75th percentile of each data set, respectively, and the horizontal line represents the median value for each group. P-values were calculated by t-test.

Oxidative phosphorylation is coupled with the electron transport, which is organized into four large membrane-embedded proteins complexes I to IV (Alberts et al., 2002), to generate ATP. As recent studies have shown that certain types of cancer rely on both glycolytic and mitochondrial metabolism for ATP production (Lunt and Vander Heiden, 2011; Liberti and Locasale, 2016; Gentric et al., 2017), we examined whether this was the case for HCC. We analyzed the expression profile of representative genes involved in the formation of the electron transport chain complexes including NDUFA5 (complex I), UQCRB (complex III), and COXA5 (complex IV). Except for COXA5, expression of NDUFA5 and UQCRB were either constant (NDUAF5; $P = 0.4934$) or significantly reduced (UQCRB; $P < 0.0001$) in HCC when compared to adjacent non-tumor tissue in the GSE36376 cohort (**Figure 3C**). However, the expression levels of all three genes showed a significant increase in HCC samples compared to normal liver samples (**Figure 3D**).

Collectively, our results indicate that in HCC livers the expression of genes involved in the glycolytic and oxidative metabolism is higher relative to normal livers, while the expression of oxidative metabolic genes is either comparable or reduced compared to surrounding non-tumor tissue (**Figures 1–3**). Because HCC develops in the settings of cirrhosis, it is likely that surrounding non-tumor tissue is indeed a cirrhotic tissue with the presence of tumor nodules nearby (Hoshida et al., 2013). Therefore, the apparent discrepancy between the results obtained from our analysis of the two distinct data sets may reflect diverse microenvironments that differentiate a normal liver from an adjacent-to-tumor liver.

Importantly, Idle and colleagues (Beyoglu et al., 2013) have measured the expression of different tissue metabolites (including glucose, glycerol-3 and 2-phosphate, malate, alanine) in a panel of 31 HCC livers and found that in HCC there is a four-fold increase in glycolysis over mitochondrial OXPHOS compared to corresponding non-tumor liver tissue. These tissue metabolomic studies are in line with our analyses whereby changes in transcript expression of glycolysis, but not OXPHOS, in HCC are consistent with a Warburg-type metabolism.

Expression of Glycolysis and PPP Enzymes Correlates With Poor Patient Survival in HCC

We next evaluated the prognostic value of key regulators of aerobic glycolysis and mitochondrial metabolism in HCC dataset (TCGA-LIHC) through the online tool GEPIA, a web server for cancer and normal gene expression profiling and interactive analyses (Tang et al., 2017). The cumulative patient survival curves were investigated using the Kaplan-Meier method, and differences in survival times were calculated according to the log-rank test. As shown in **Figure 4A**, when HCC patients were divided into two groups according to the median value of each gene transcript that is HK2, PKM2, ALDOA, and LDHA we found that the overall survival rate was significantly lower in the high expression groups in the TCGA-LIHC patient cohort ($P < 0.05$). Similar trends were also detected for other glycolytic genes with some reaching sufficient significance ($P < 0.05$; i.e., GAPDH, PGK1, ENO1) and very few not reaching significance (GPI, $P = 0.100$; PGAM1, $P = 0.052$; LDHB, $P = 0.058$) (data not shown). On the contrary, PFKL follows an inverse trend. High expression of PFKL was, indeed, inversely associated with poor overall survival, but this was not statistically significant ($P = 0.420$) (**Figure 4A**). Moreover, although in our differential gene expression analyses LDHA mRNA expression was not sufficiently higher in HCC livers compared to healthy and surrounding non-tumor livers (**Figures 1B,C**), it seems that LDHA expression has a prognostic value in HCC (**Figure 4A**). This is in agreement with previous studies demonstrating that high level of serum LDH is associated with poor patient overall survival in several malignancies, including HCC (Wulaningsih et al., 2015).

In examining the individual genes functioning in the oxidative phase of the PPP, we found that the overall survival rate was significantly lower in the group of patients that expressed high levels of G6PD, the rate-limiting enzyme of the PPP, and PGD mRNA (**Figure 4B**). Of note, although the expression of PGLS had no prognostic value ($P = 0.055$; **Figure 4B**), it seems that at least the rate-limiting enzyme G6PD of the oxidative phase of PPP has important clinical implications for patients with HCC. Surprisingly, metabolic genes involved in mitochondrial metabolism have no prognostic value in HCC as neither the expression of genes related to TCA cycle nor OXPHOS was associated with poor patient overall survival (**Figures 4C,D**) despite their significant increases in HCC samples compared to normal livers (**Figure 3**). This could indicate that TCA and OXPHOS enzymes may be subject to a strict post-translational regulation that uncouple the mRNA levels from the actual enzyme function in HCC livers.

Interestingly, similar trend was obtained by analyzing survival of HCC patients by using a distinct data set via the KM plotter web server tool (Szász et al., 2016) (**Supplementary Figure S1**). Overall these data indicate that glycolytic genes have prognostic role in HCC.

Gene Expression of Glycolysis and PPP Enzymes in Cirrhotic Livers

Liver cirrhosis is a precancerous state of HCC (Wright, 1991; Borzio et al., 1995; Donato et al., 2001; El-Serag, 2011; Jemal et al., 2011; Forner et al., 2018). Therefore, cirrhosis is a unique model for investigating markers for early detection of HCC *in vivo* (El-Serag, 2011; Forner et al., 2018; Fujiwara et al., 2018). To better understand the metabolic alterations in early liver carcinogenesis, we examined the expression of glycolytic transcripts as well as transcripts of mitochondrial metabolism in three independent clinical data sets totalising 94 cirrhotic livers and 34 healthy livers. We surveyed the transcripts levels of representative glycolysis enzymes. As shown in **Figure 5A**, statistically significant differences in mRNA levels were detected between cirrhotic and normal livers for HK2, ALDOA, and PKM2 in the three data sets analyzed. As LDHA probe was not found in the GSE25097 cohort, we analyzed the expression of LDHB and found that (like the other glycolytic enzymes)

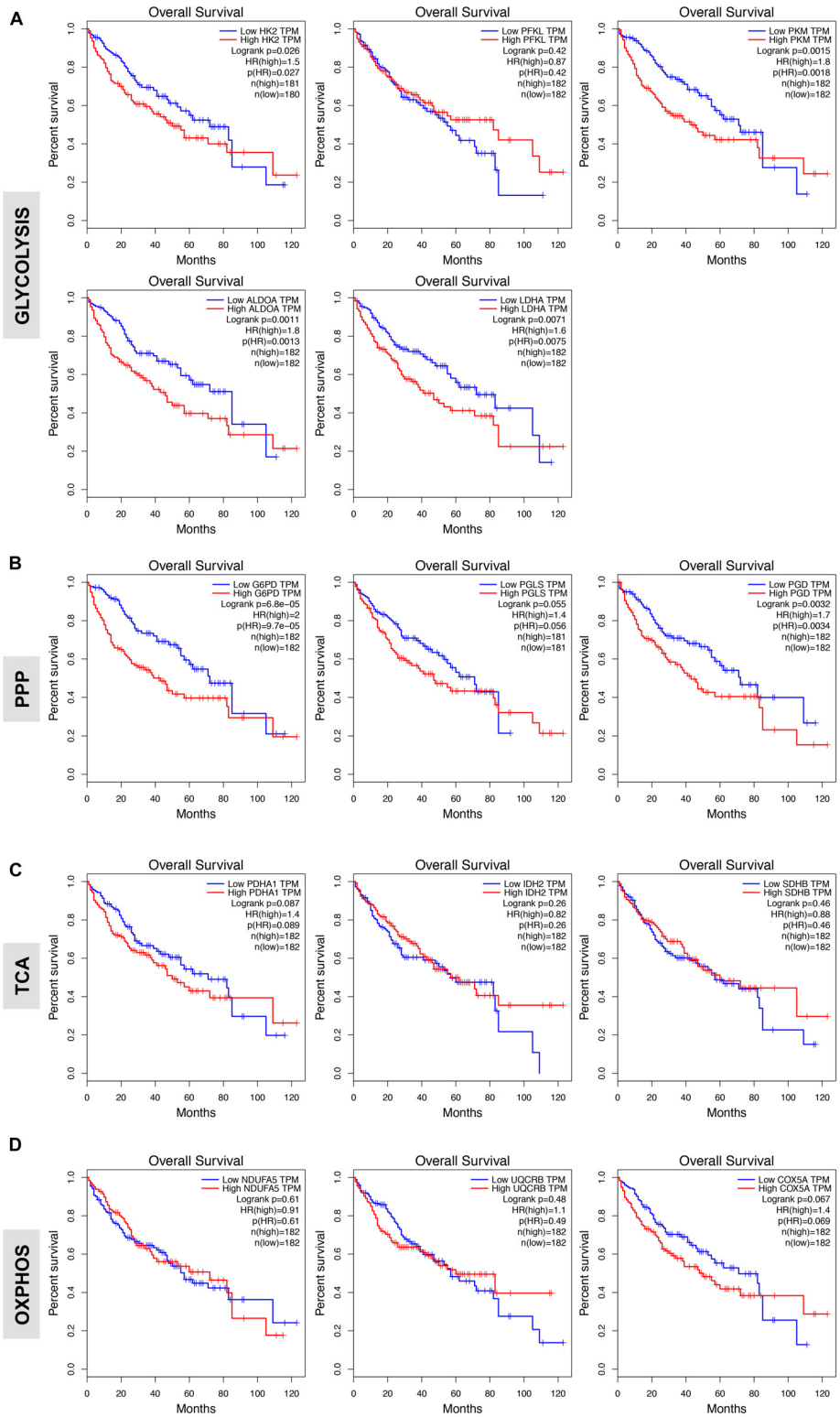

FIGURE 4 | Glycolytic gene expression is associated with poor patient prognosis. **(A,B)** High expression of genes associated with glycolysis and oxidative phase of PPP significantly correlates with poor overall patients' survival. Shown are the Kaplan-Meier overall survival curves of HCC patients according to the designated gene expression levels above or below the median value based on TGA-LIHC data set and analyzed with the GEPIA bioinformatic tool of genomic database (Ally et al., 2017; Tang et al., 2017). **(C,D)** Kaplan-Meier overall survival curves of HCC patients in TGA-LIHC data set showing homogenous prognostic significance of TCA and OXPHOS genes.

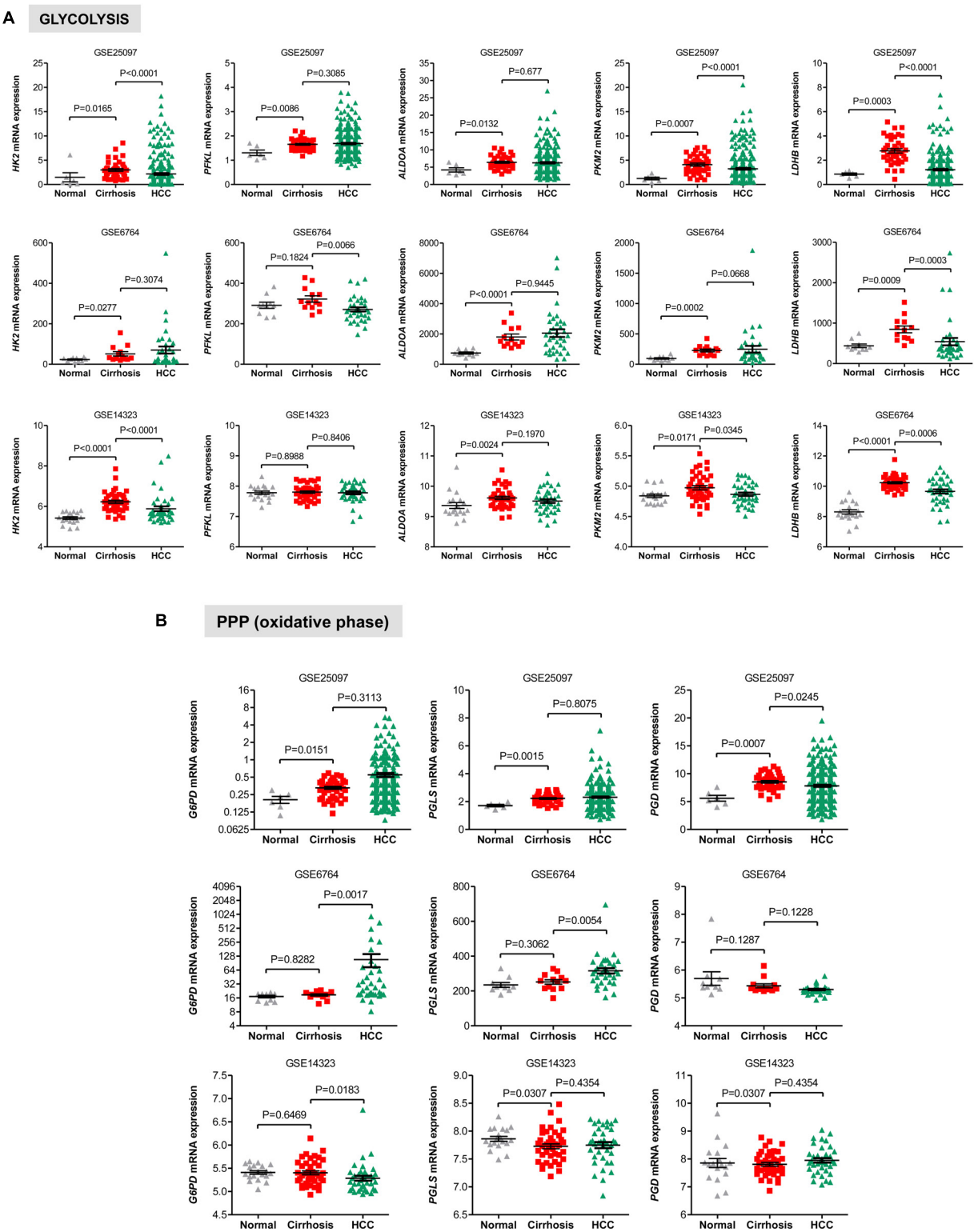

FIGURE 5 | Gene expression of glycolysis and PPP enzymes in cirrhotic and HCC livers. **(A)** Scatterplots showing the increased expression of transcript levels of representative glycolytic enzymes in cirrhotic livers compared to HCC livers and normal healthy liver tissues in three independent clinical data sets GSE25097, GSE6764, and GSE14323 (Wurmbach et al., 2007; Sung et al., 2012; Levy et al., 2016). **(B)** Scatterplots showing differential gene expression of genes related to oxidative PPP among cirrhotic livers compared to HCC livers and normal healthy liver tissues in three independent clinical data sets GSE25097, GSE6764, and GSE14323 (Wurmbach et al., 2007; Sung et al., 2012; Levy et al., 2016). The horizontal lines indicate mean ± SEM *P*-values were calculated by non-parametric Mann–Whitney tests.

expression of LDHB was also increased in cirrhotic liver samples compared to normal livers (**Figure 5A**). On the contrary, expression levels of PFKL were significantly higher in cirrhotic livers compared to healthy specimens in the GSE25097 cohort (P = 0.0086) but found constant in the two other data sets (P = 0.1824 and P = 0.9888). Collectively, these results are consistent with those observed in HCC. Indeed, while the expression of HK2, ALDOA, and PKM2 transcripts is increased in HCC compared to normal livers and associated with poor overall patient survival, elevated levels of PFKL scored in HCC had no prognostic value (see **Figure 4**). This suggests that the expression of these genes in cirrhotic livers resemble the gene expression signature of HCC (**Figure 5A**).

We also evaluated the levels of transcripts encoding genes in the oxidative phase of PPP and found that while in the GSE25097 dataset there was a significant increase of the three PPP genes analyzed (including the rate limiting enzymes G6PD), no changes were observed in the other two data sets, except for PGLS, whose expression was significantly lower in cirrhotic livers (**Figure 5B**). Notably, no significant increase of glycolytic and PPP gene expression has been observed in HCC compared to cirrhotic livers (**Figures 5A,B**), suggesting that reprogramming of glucose metabolism may occur at pre-cancerous stages. For the TCA enzymes, the mRNA levels of PDHA1, IDH2, and SHDB were constant in both data sets of cirrhotic livers compared to normal livers (**Figure 6A**). Similarly, transcripts levels of representative OXPHOS genes that were highly expressed in HCC were not found differentially expressed in cirrhotic livers compared to healthy livers (**Figure 6B**). Collectively, these results suggest that, in contrast to a general increase in glycolysis genes, TCA and OXPHOS genes remained at the same level in cirrhosis. These observations suggest a shift toward aerobic glycolysis and PPP relative to oxidative phosphorylation in cirrhotic livers. This is also supported by the fact that, in contrast to HCC (**Figure 1**), cirrhotic livers exhibit elevated expression of LDHB compared to healthy livers (**Figure 5A**), suggesting that pyruvate may be diverted away from the mitochondria for ATP production.

Furthermore, as documented for HCC, Nishikawa et al. (2014) demonstrated that hepatocytes isolated from liver rats with early signs of cirrhosis shows a metabolic shift from OXPHOS to glycolysis for the production of ATP, while normal rat hepatocytes continue to use OXPHOS for ATP generation. It was also shown that expression of glycolytic genes is severely decreased in cirrhotic hepatocytes with decompensated liver function, suggesting that failing livers do not require glycolysis to overcome early sign of injury. Altogether these studies (Beyoglu et al., 2013; Nishikawa et al., 2014) are in line with our analyses whereby an increased expression of glycolysis transcripts, but not OXPHOS transcripts, is detected in both HCC and cirrhotic livers compared to normal livers.

Glycolytic Gene Expression in Cirrhotic Livers Is Associated With the Risk of Developing HCC

Next, we examined if genes involved in glycolysis, PPP, TCA cycle and OXPHOS are associated with a progression of cirrhosis to

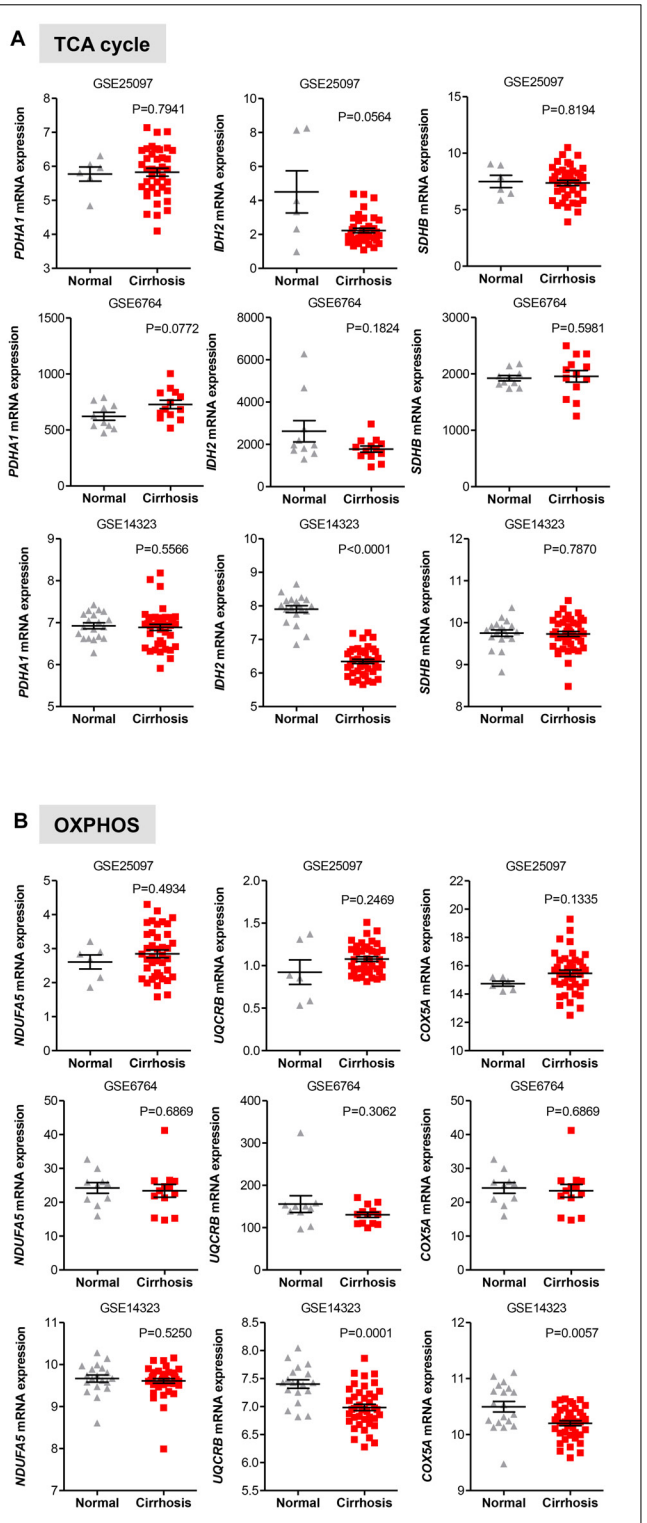

FIGURE 6 | Gene expression of TCA and OXPHOS enzymes in cirrhotic livers. **(A,B)** Scatterplots showing homogenous expression of representative TCA and OXPHOS biomarkers in cirrhotic livers compared to normal healthy liver tissues in three independent clinical data sets GSE25097, GSE6764, and GSE14323 (Wurmbach et al., 2007; Sung et al., 2012; Levy et al., 2016). The horizontal lines indicate mean ± SEM P-values were calculated by non-parametric Mann–Whitney tests.

TABLE 1 | Summary of the clinical parameters associated with each group of cirrhotic livers.

Prognosis	Low HCC risk	High HCC risk
HCC development rate	18%	42%
Patients' survival rate	85%	63%

HCC and patient survival. The clinical data set GSE15654 consists of biopsies obtained from patients with hepatitis C-related Child-Pugh A cirrhosis who were prospectively followed in an HCC surveillance program for a median of 10 years and classified as low (n = 55) and high (n = 60) HCC risk based on the rates of patient survival and incidence of developing HCC (**Table 1**; Hoshida et al., 2013). We examined the expression of HK2, PFKL, ALDOA, PKM2, and LDHB transcripts by interrogating this public gene expression database. As shown in **Figure 7A** and **Table 1**, high mRNA expression of HK2, PFKL, ALDOA, and PKM2 positively correlated with a progression of cirrhosis to HCC and a worse survival rate. An exception was LDHB, which is not differentially expressed in the two prognostic groups.

For the oxidative phase of PPP enzymes, high G6PD mRNA expression levels were found to be associated with a progression of cirrhosis to HCC and reduced survival rate (P = 0.0209), while PGLS and PGD show no differential expression ($P > 0.050$; **Figure 7B**). Finally, in line with results showed in **Figure 6**, no increase in expression of the metabolic genes in TCA cycle and OXPHOS was observed between the low and high HCC risk groups (**Figures 7C,D**). On the contrary, the expression of IDH2, SDHB, and COXA5 were inversely correlated with poor prognosis (**Figures 7C,D**). Thus, in cirrhotic livers high expression of glycolysis genes is associated with an elevated risk of developing HCC, suggesting that the glycolytic phenotype has a possible prognostic role in cirrhotic patients. Moreover, HK2, PFKL, ALDOA, PKM2, and LDHB levels show a weak differential expression in cirrhotic and HCC livers (**Figure 5A**), indicating that the acquisition of a glycolytic phenotype occurs during both initiation and maintenance of HCC. Collectively, these results suggest that glycolytic enzymes may represent potential targets for HCC chemoprevention.

DISCUSSION

In more than 80% of cases HCC arise in the settings of cirrhosis (El-Serag, 2011; Jemal et al., 2011; Forner et al., 2018; Fujiwara et al., 2018). Therefore, understanding how cirrhosis develops from normal liver and progresses to HCC is particularly relevant for identifying chemoprevention therapies. Hepatitis B (HBV) or C (HCV) viral infection, chronic alcohol consumption and nonalcoholic fatty liver disease have been reported to be the prominent causes of the development of cirrhosis (El-Serag, 2011). Nevertheless, the early biochemical and molecular events that underlie the progression of cirrhosis to HCC remain largely unclear.

Notable features of malignant hepatocytes – the predominant liver parenchymal cells – include a high proliferative potential. In order to proliferate HCC hepatocytes readjust their metabolic activities to fulfill the bioenergetic and anabolic needs for doubling mass (Gatenby and Gillies, 2004; Lunt and Vander Heiden, 2011; Hay, 2016). It is, however, unclear how and when HCC cells acquire these metabolic changes during the hepatocarcinogenetic process.

Here we report changes in transcript expression of glycolysis and PPP genes in cirrhosis consistent with a Warburg-type metabolism and show a positive correlation between glycolytic gene expression and the risk of developing HCC and poor patient survival.

Compared to normal livers, elevated mRNA expression of key metabolic genes in glycolysis, PPP, TCA, and OXPHOS were found in livers samples from patients with HCC, implying a pathogenic role of glucose metabolism for this disease. A distinctive function of normal hepatocytes is the conversion of non-carbohydrate carbon substrates, like lactate and pyruvate, into glucose through the gluconeogenesis pathway. Several gluconeogenic enzymes such as glucose-6-phosphatase (G6Pase), fructose-1,6-bisphosphatase (FBP1), and PEP carboxykinase (PEPCK) are known to bypass the first, second and third irreversible reactions of the glycolytic pathway, respectively, resulting in slowing down the rate of glycolysis in normal hepatocytes (**Figure 8**; Hay, 2016). In HCC, G6Pase, FBP1, and PEPCK, together with the low-affinity hexokinase 4 (HK4) are suppressed, while the high-affinity hexokinase HK2 is overexpressed (Wang et al., 2012; Hay, 2016). Consistent with this, we found that HK2 is not only highly expressed in HCC but also in cirrhosis as compared to normal liver samples, suggesting the occurrence of the glycolytic phenotype early in the hepatocarcinogenetic process. This hypothesis is supported by the observation that the expression levels of other key glycolytic enzymes such as ALDOA and PKM2 are also overexpressed in cirrhotic livers. Among the three known isoforms of the glycolytic enzyme aldolase (ALDOA, ALDOB, and ALDOC), which catalyzes the reversible conversion of Fructose 1,6-bisphosphate (F1,6BP) to Dihydroxyacetone phosphate (DHAP) and Glyceraldehyde 3-phosphate (G3P), ALDOA catalyzes this reaction more efficiently thus accelerating the flux through glycolysis (Castaldo et al., 2000; Hay, 2016). Indeed, ALDOA was reported to be the main isoform expressed in most cancer types (Asaka et al., 1994). Our finding that the levels of ALDOA are up regulated in cirrhotic and HCC samples suggests that a high glycolytic phenotype is a remarkable feature of both precancerous and cancerous conditions.

Recent works have established that the pyruvate kinase M2 isoform (PKM2), which catalyzes the synthesis of pyruvate and ATP, using phosphoenolpyruvate (PEP) and ADP as substrates, is a critical driver of the Warburg effect (Christofk et al., 2008; Cortés-Cros et al., 2013; Israelsen et al., 2013; reviewed in Wiese and Hitosugi, 2018). Indeed, similar to other types of cancer cells, HCC cells express PKM2, the pyruvate activity (PK) of which is maintained at low levels by pro-survival signaling pathways (reviewed in Papa and Bubici, 2016). The low activity of PKM2 allows the accumulation of glycolytic intermediates that can be diverted into the biosynthetic pathways to form amino acids, nucleic acids, and lipids (Christofk et al., 2008; Cortés-Cros et al., 2013; Israelsen et al., 2013). While

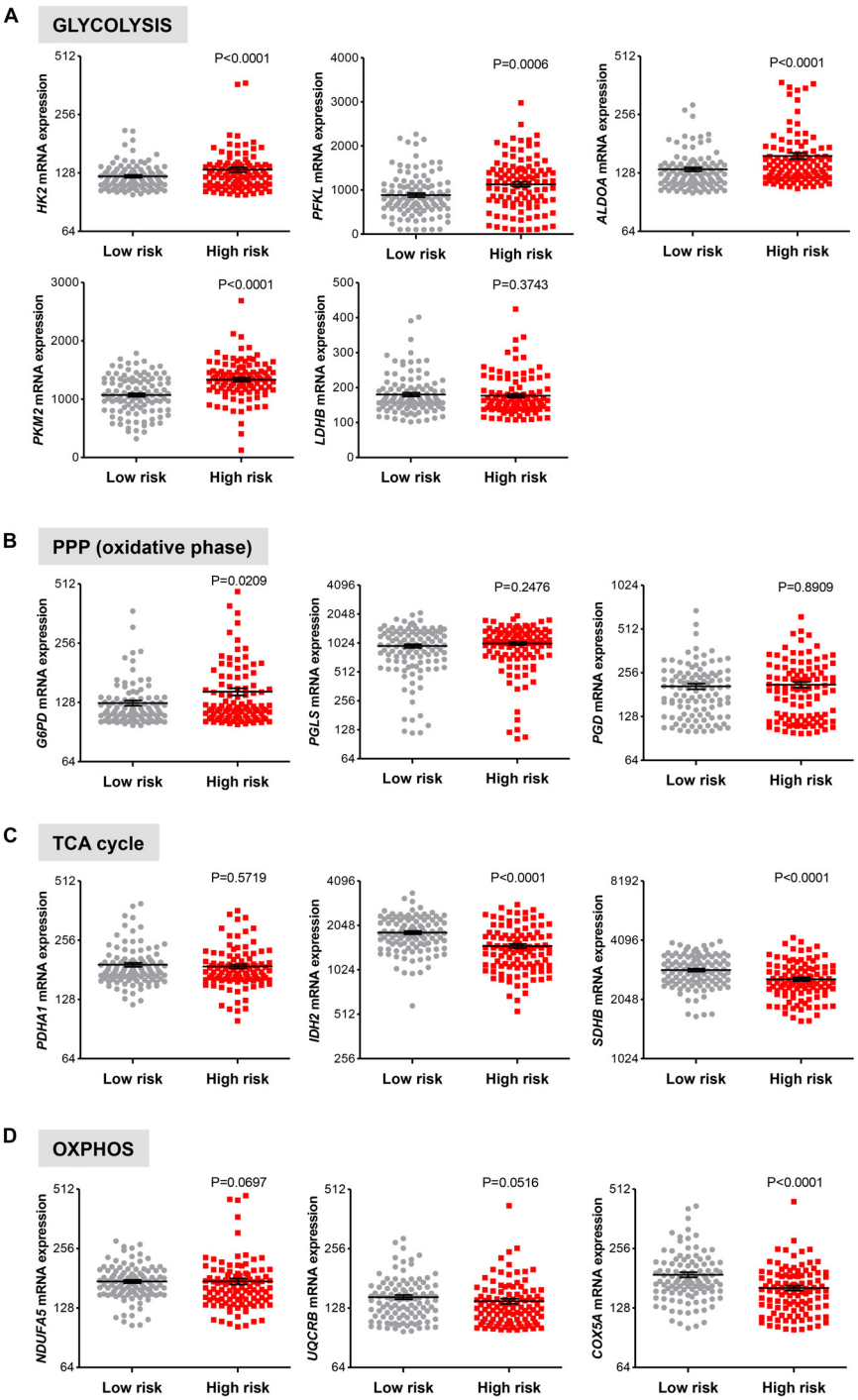

FIGURE 7 | Increased risk of developing HCC in cirrhotic patients significantly correlates with high expression of glycolytic genes. **(A–D)** Levels of transcripts in the clinical data set GSE15654 consisting of 115 patients with newly diagnosed cirrhosis who were prospectively followed up in an HCC surveillance program and classified as having low (n = 55) and high (n = 60) HCC risk based on the rates of patient survival and risk of developing HCC (Hoshida et al., 2013). The horizontal lines indicate mean ± SEM P-values were calculated by non-parametric Mann–Whitney tests.

promoting these biosynthetic pathways, low PKM2 activity also contributes to boost the levels of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and antioxidant reduced glutathione (GSH), which serves to detoxify reactive oxygen species (ROS) whose accumulation would result in apoptosis in chronically injured and tumor tissues (Papa and Bubici, 2016).

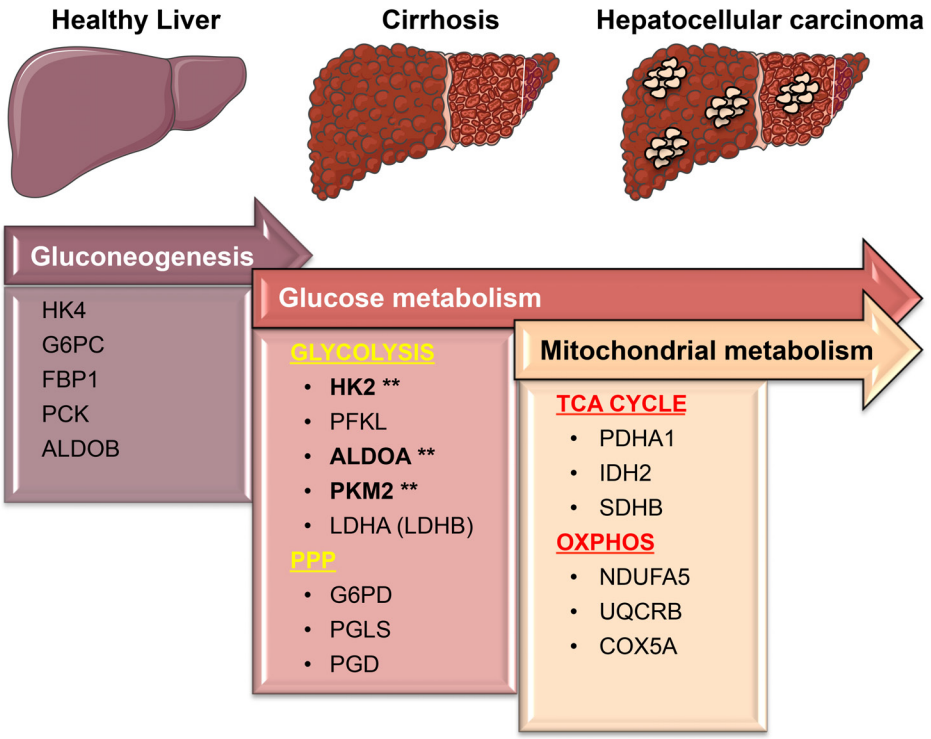

FIGURE 8 | A simplified model showing transcriptional changes in gene expression of metabolic enzymes that characterize the progression from a normal liver to cirrhosis, which culminates in HCC. One of the most pronounced transcriptional changes during the transition from normal to HCC is the suppression of HK4 and ALDOB followed by the induction of the high affinity HK2 and ALDOA. This allows glucose to enter in the cells and be converted into pyruvate. PKM2 and lactate dehydrogenase (LDHA/LDHB), the last two enzymes in the glycolytic pathway, are essentially needed to maintain the high glycolytic rate of liver carcinomas by fast removal of accumulating upstream intermediates to favor the entrance into other metabolic pathways such as the oxidative phase of PPP. Surprisingly, those metabolic changes mostly occur early on the premalignant stage (cirrhosis) during hepatocarcinogenesis. While in cirrhotic livers glycolysis provides both biosynthetic building blocks and anti-oxidant defenses needed for the survival (as opposed to apoptosis) of damaged cells, in livers with HCC glycolysis cooperates with OXPHOS and TCA cycle to sustain energy needed for fast proliferation and metastasis. Our analyses suggest that the expression of glycolytic enzymes such as HK2, ALDOA, and PKM2 (marked by asterisks) in precancerous, chronically inflamed livers may be considered as new predictive biomarkers to prevent the development of liver cancer. Figure was created modifying illustrations provided by Servier (http://smart.servier.com) under Creative Commons Attribution 3.0 Unported License.

We indeed observed remarkable higher expression levels of PKM2 in cirrhotic and HCC livers compared to normal livers. Thus, although further studies are required, it is reasonable to hypothesize that the enhanced expression of PKM2 in cirrhotic livers may contribute to the apoptosis resistance of damaged (injured) hepatocytes that continue to survive, proliferate and accumulate until becoming malignant HCCs. Of note is also the observation that the expression of LDHA (or LDHB) (the glycolytic enzymes converting pyruvate in lactate) have no HCC risk associated in cirrhotic patients (**Figure 7**), suggesting that glycolytic-derived pyruvate may not be all converted in lactate and is therefore channeled to other metabolic pathways (i.e., mevalonate pathway; discussed below).

While it was long believed that the aerobic glycolytic phenotype is associated with an impaired mitochondrial oxidative metabolism (Lunt and Vander Heiden, 2011), recent research in the field have demonstrated that glycolytic and mitochondrial metabolism are both used by cancer cells for ATP production and macromolecule synthesis (Weinberg et al., 2010; Ahn and Metallo, 2015; Gentric et al., 2017; Takahashi et al., 2018). In agreement with these studies, our analysis in HCC samples show high expression levels of genes related to mitochondrial metabolism compared to normal livers. The fact that in cirrhosis the expression levels of TCA and OXPHOS genes are lower than those in normal livers or remain unchanged suggests the occurrence of both glycolytic and mitochondrial metabolism in HCC only. By contrast, cirrhotic livers have high expression of HK2, ALDOA, PKM2, and LDHB as well as display a significant increase in G6PD expression. It appears that metabolic readjustments (that is, glycolysis shift) occur in cirrhosis.

This may actually be explained by the fact that chronically injured tissues such as cirrhotic livers have a more natural tendency to trigger protective programs against ROS-inflicted cell death (via necrosis or apoptosis), rather than mitochondrial metabolism that contributes to the generation of ROS (Papa et al., 2009; Luedde et al., 2014; DeBerardinis and Chandel, 2016).

Indeed, it has been suggested that in cancer cells the production of ROS by complex I, II, and III of the electron transport chain contributes to DNA damage and is required for cancer cell survival, Kras-induced cellular transformation and cancer metastasis (Weinberg et al., 2010; Takahashi et al., 2018;

reviewed in Lunt and Vander Heiden, 2011; Reczek and Chandel, 2015; DeBerardinis and Chandel, 2016). Thus, it appears that in inflamed livers the transcriptional regulation of genes involved in glycolysis operates to trigger cellular protective programs that serve to counterbalance the chronic injury. Besides transcriptional regulation, metabolic enzymes are known to be tightly regulated by post-transcriptional modifications including phosphorylation, acetylation, glycosylation which affect the metabolic cellular activities and have not been investigated in this study. Of note, hypoxia (via activation of HIF-1α transcription factor) and other intracellular signaling pathways (i.e., PI3K/AKT, JNK, ERK, PTEN, p53) have also been shown to regulate and drive the metabolic shift toward aerobic glycolysis of HCC cells (Iansante et al., 2015; Cassim et al., 2018). It should be instructive to evaluate the differential signature of these metabolic regulators in normal and cirrhotic livers and test whether these pathways are activated in cirrhotic livers. Further studies such as protein expression analysis are therefore required to understand the specific mechanisms underlying the metabolic changes observed in HCC and cirrhosis, although it might be difficult due to shortage of human cirrhotic samples available.

Prevention of HCC is an unmet medical need (El-Serag, 2011; Jemal et al., 2011; Forner et al., 2018). Interestingly, HK2, ALDOA and PKM2 expression levels appear to have important clinical implication for patients with cirrhosis, as analysis of cirrhotic livers from patients followed up during a span of 10 years showed a positive correlation between high expression of glycolysis genes and progression of cirrhosis to HCC. This suggests that expression of these glycolytic enzymes could be used as a new biomarker for the risk of developing HCC. By contrast, neither TCA nor OXPHOS gene expression analyzed in this study was associated with an increased risk of HCC.

Although there are potential limitations associated with the nature of the analyses carried out in this study, we sought to detect an early sign of metabolic changes in the development of HCC and show that HK2, ALDOA and PKM2 expression levels in cirrhotic livers could be used as new predictive biomarkers for HCC development. Early studies demonstrated that rat cirrhotic livers show a metabolic shift from OXPHOS to glycolysis, while normal rat hepatocytes continue to use OXPHOS (Chen et al., 2012; Beyoglu et al., 2013; Nishikawa et al., 2014; Gao et al., 2015). Further support to our observations comes from ongoing clinical trials for prevention of HCC development and recurrence. Randomized clinical trials are currently ongoing to explore the use of statins (i.e., Simvastatin, Atorvastatin) to prevent either HCC development in cirrhotic (precancerous) patients or HCC recurrence in HCC-free patients surgically treated with curative ablation or hepatectomy (Goodman, 2016; Chen, 2017; Fujiwara et al., 2018). Statins are a class of lipid-lowering medications that target the rate-controlling enzyme (NADH/NADPH-dependent) 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase of the mevalonate pathway in which cholesterol and other isoprenoids are produced (Clendening and Penn, 2012; Fujiwara et al., 2018). The mevalonate pathway begins with acetyl-CoA and ends with the production of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), precursors of isoprenoids. Importantly, it has been shown that aerobic glycolysis positively regulates the mevalonate pathway by producing pyruvate, which is needed for the initial formation of acetyl-CoA (Clendening and Penn, 2012). Thus, it is very likely that by blocking the mevalonate pathway via inhibition of HMG-CoA reductase, statins may contribute to the suppression of aerobic glycolysis through a negative feedback (Clendening and Penn, 2012). If these clinical trials would be beneficial to reduce HCC risk, further studies would be necessary to directly link the mevalonate pathways to aerobic glycolysis in liver cirrhosis.

ETHICS STATEMENT

The study involves the analyses of public available data sets using human subjects. Therefore, approval for the use of human subject has been granted to third parties that have deposited the public data sets, which have been properly cited in the text.

AUTHOR CONTRIBUTIONS

NL, MC, CB, and SP performed the experiments. CB and SP are both senior authors, conceived the idea, chiefly carried out data analysis and interpretation, coordinate the study, and wrote the manuscript, which was commented by all the authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2018.00138/full#supplementary-material

REFERENCES

Ahn, C. S. and Metallo, C. M. (2015). Mitochondria as biosynthetic factories for cancer proliferation. *Cancer Metab.* 3:1. doi: 10.1186/s40170-015-0128-2

Alberts, B., Johnson, A., Lewis, J., et al. (2002) "Electron-transport chains and their proton pumps," in *Molecular Biology of the Cell*, 4th Edn, (New York, NY: Garland Science).

Ally, A., Balasundaram, M., Carlsen, R., Chuah, E., Clarke, A., Dhalla, N. et al. (2017). Comprehensive and integrative genomic characterization

of hepatocellular carcinoma. *Cell* 169, 1327–1341. doi: 10.1016/j.cell.2017.05.046

Asaka, M., Kimura, T., Meguro, T., Kato, M., Kudo, M., Miyazaki, T., et al. (1994). Alteration of aldolase isozymes in serum and tissues of patients with cancer and other diseases. *J. Clin. Lab. Anal.* 8, 144–148. doi: 10.1002/jcla.1860080306

Barbarulo, A., Iansante, V., Chaidos, A., Naresh, K., Rahemtulla, A., Franzoso, G., et al. (2013). Poly(ADP-ribose) polymerase family member 14 (PARP14) is a novel effector of the JNK2-dependent pro-survival signal in multiple myeloma. *Oncogene* 32, 4231–4242. doi: 10.1038/onc.2012.448

Barrett, T. (2013). "Gene Expression Omnibus (GEO)," in *The NCBI Handbook,* 2nd Edn, (Bethesda, MD: National Center for Biotechnology Information).

Beyoglu, D., Imbeaud, S., Maurhofer, O., Bioulac-Sage, P., Zucman-Rossi, J., Dufour, J. F., et al. (2013). Tissue metabolomics of hepatocellular carcinoma: tumor energy metabolism and the role of transcriptomic classification. *Hepatology* 58, 229–238. doi: 10.1002/hep.26350

Borzio, M., Bruno, S., Roncalli, M., Mels, G. C., Ramella, G., Borzio, F., et al. (1995). Liver cell dysplasia is a major risk factor for hepatocellular carcinoma in cirrhosis: a prospective study. *Gastroenterology* 108, 812–817. doi: 10.1016/0016-5085(95)90455-7

Castaldo, G., Calcagno, G., Sibillo, R., Cuomo, R., Nardone, G., Castellano, L., et al. (2000). Quantitative analysis of aldolase A mRNA in liver discriminates between hepatocellular carcinoma and cirrhosis. *Clin. Chem.* 46, 901–906.

Cassim, S., Raymond, V. A., Lacoste, B., Lapierre, P., and Bilodeau, M. (2018). Metabolite profiling identifies a signature of tumorigenicity in hepatocellular carcinoma. *Oncotarget* 9, 26868–26883. doi: 10.18632/oncotarget.25525

Chandrashekar, D. S., Bashel, B., Balasubramanya, S. A. H., Creighton, C. J., Ponce-Rodriguez I., Chakravarthi, B. V. S. K., et al. (2017). UALCAN: a portal for facilitating tumor subgroup gene expression and survival analyses. *Neoplasia* 19, 649–658. doi: 10.1016/j.neo.2017.05.002

Chen PY. (2017). *Statin for Preventing Hepatocellular Carcinoma Recurrence after Curative Treatment (SHOT). ClinicalTrials.gov Identifier: NCT03024684*. Available at: https://clinicaltrials.gov/ct2/show/NCT03024684

Chen, Y., Choi, S. S., Michelotti, G. A., Chan, I. S., Swiderska-Syn, M., Karaca, G. F., et al. (2012). Hedgehog controls hepatic stellate cell fate by regulating metabolism. *Gastroenterology* 143:1319–1329. doi: 10.1053/j.gastro.2012.07.115 controls hepatic stellate cell fate by regulating metabolism. Gastroenterology 143:1319Ű1329. doi: 10.1053/j.gastro.2012.07.115

Christofk, H. R., Vander Heiden, M. G., Harris, M. H., Ramanathan, A., Gerszten, R. E., Wei, R., et al. (2008). The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature* 452, 230–233. doi: 10.1038/nature06734

Clendening, J. W., Penn, L. Z. (2012). Targeting tumor cell metabolism with statins. *Oncogene* 31, 4967–4978. doi: 10.1038/onc.2012.6

Cortés-Cros, M., Hemmerlin, C., Ferretti, S., Zhang, J., Gounarides, J. S., Yin, H., et al. (2013). M2 isoform of pyruvate kinase is dispensable for tumor maintenance and growth. *Proc. Natl. Acad. Sci. U.S.A.* 110, 489–494. doi: 10.1073/pnas.1212780110

DeBerardinis, R. J., and Chandel, N. S. (2016). Fundamentals of cancer metabolism. *Sci. Adv.* 2:e1600200. doi: 10.1126/sciadv.1600200

Donato, M. F., Arosio, E., Del Ninno, E., Ronchi, G., Lampertico, P., Morabito, A., et al. (2001). High rates of hepatocellular carcinoma in cirrhotic patients with high liver cell proliferative activity. *Hepatology* 34, 523–528. doi: 10.1053/jhep.2001.26820

El-Serag, H. B. (2011). Hepatocellular carcinoma. *N. Engl. J. Med.* 365, 1118–1127. doi: 10.1056/NEJMra1001683

Forner, A., Reig, M., and Bruix J. (2018). Hepatocellular carcinoma. *Lancet* 391, 1301–1314. doi: 10.1016/S0140-6736(18)30010-2

Fujiwara, N., Friedman, S. L., Goossens, N., Hoshida, Y. (2018). Risk factors and prevention of hepatocellular carcinoma in the era of precision medicine. *J. Hepatol.* 68, 526–549. doi: 10.1016/j.jhep.2017.09.016

Gao, R., Cheng, J., Fan, C., Shi, X., Cao, Y., Sun, B., et al. (2015). Serum metabolomics to identify the liver disease-specific biomarkers for the progression of hepatitis to hepatocellular carcinoma. *Sci. Rep.* 5:18175. doi: 10.1038/srep18175

Gatenby, R. A., and Gillies, R. J. (2004). Why do cancers have high aerobic glycolysis? *Nat. Rev. Cancer* 4, 891–899. doi: 10.1038/nrc1478

Gentric, G., Mieulet, V., and Mechta-Grigoriou, F. (2017). Heterogeneity in cancer metabolism: new concepts in an old field. *Antioxid. Redox Signal.* 26, 462–485. doi: 10.1089/ars.2016.6750

Goodman, M. T. (2016). *Simvastatin In Preventing Liver Cancer in Patients with Liver Cirrhosis. ClinicalTrials.gov Identifier: NCT02968810*. Available at: https://clinicaltrials.gov/ct2/show/study/NCT02968810

Hay, N. (2016). Reprogramming glucose metabolism in cancer: can it be exploited for cancer therapy? *Nat. Rev. Cancer* 16, 635–649. doi: 10.1038/nrc.2016.77

Hoshida, Y., Villanueva, A., Sangiovanni, A., Sole, M., Hur, C., Andersson, K. L., et al. (2013). Prognostic gene expression signature for patients with hepatitis C-related early-stage cirrhosis. *Gastroenterology* 144, 1024–1030. doi: 10.1053/j.gastro.2013.01.021

Huang, Q., Tan, Y., Yin, P., Ye, G., Gao, P., Lu, X., Wang, H., Xu, G. (2013). Metabolic characterization of hepatocellular carcinoma using nontargeted tissue metabolomics. *Cancer Res.* 73, 4992–5002. doi: 10.1158/0008-5472.CAN-13-0308

Iansante, V., Choy, P. M., Fung, S. W., Liu, Y., Chai, J. G., Dyson, J., et al. (2015). PARP14 promotes the Warburg effect in hepatocellular carcinoma by inhibiting JNK1-dependent PKM2 phosphorylation and activation. *Nat. Commun.* 6:7882. doi: 10.1038/ncomms8882

Israelsen, W. J., Dayton, T. L., Davidson, S. M., Fiske, B. P., Hosios, A. M., Bellinger, G., et al. (2013). PKM2 isoform-specific deletion reveals a differential requirement for pyruvate kinase in tumor cells. *Cell* 155, 397–409. doi: 10.1016/j.cell.2013.09.025

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., and Forman, D. (2011). Global cancer statistics. *CA Cancer J. Clin.* 61, 69–90. doi: 10.3322/caac.20107

Jones, R. G., and Thompson, C. B. (2009). Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes Dev.* 23, 537–548. doi: 10.1101/gad.1756509

Kitamura, K., Hatano, E., Higashi, T., Narita, M., Seo, S., Nakamoto, Y., et al. (2011). Proliferative activity in hepatocellular carcinoma is closely correlated with glucose metabolism but not angiogenesis. *J. Hepatol.* 55, 846–857. doi: 10.1016/j.jhep.2011.01.038

Kowalik, M. A., Columbano, A., and Perra, A. (2017). Emerging role of the pentose phosphate pathway in hepatocellular carcinoma. *Front. Oncol.* 7:87. doi: 10.3389/fonc.2017.00087

Levy, G., Habib, N., Guzzardi, M. A., Kitsberg, D., Bomze, D., Ezra, E., et al. (2016). Nuclear receptors control pro-viral and antiviral metabolic responses to hepatitis C virus infection. *Nat. Chem. Biol.* 12, 1037–1045. doi: 10.1038/nchembio.2193

Liberti, M. V., and Locasale, J. W. (2016). The Warburg effect: how does it benefit cancer cells? *Trends Biochem. Sci.* 41, 211–218. doi: 10.1016/j.tibs.2015.12.001

Lim, H. Y., Sohn, I., Deng, S., Lee, J., Jung, S. H., Mao, M., et al. (2013). Prediction of disease-free survival in hepatocellular carcinoma by gene expression profiling. *Ann. Surg. Oncol.* 20, 3747–3753. doi: 10.1245/s10434-013-3070-y

Llovet, J. M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J. F., et al. (2008). Sorafenib in advanced hepatocellular carcinoma. *N. Engl. J. Med.* 359, 378–390. doi: 10.1056/NEJMoa0708857

Luedde, T., Kaplowitz, N., and Schwabe, R. F. (2014). Cell death and cell death responses in liver disease: mechanisms and clinical relevance. *Gastroenterology* 147, 765–783. doi: 10.1053/j.gastro.2014.07.018

Lunt, S. Y., and Vander Heiden, M. G. (2011). Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu. Rev. Cell Dev. Biol.* 27, 441–464. doi: 10.1146/annurev-cellbio-092910-154237

Nishikawa, T., Bellance, N., Damm, A., Bing, H., Zhu, Z., Handa, K., et al. (2014). A switch in the source of ATP production and a loss in capacity to perform glycolysis are hallmarks of hepatocyte failure in advance liver disease. *J. Hepatol.* 60, 1203–1211. doi: 10.1016/j.jhep.2014.02.014

Papa, S., and Bubici, C. (2016). Linking apoptosis to cancer metabolism: another missing piece of JuNK. *Mol. Cell. Oncol.* 3:e1103398. doi: 10.1080/23723556.2015.1103398

Papa, S., Bubici, C., Zazzeroni, F., and Franzoso, G. (2009). Mechanisms of liver disease: cross-talk between the NF-kappaB and JNK pathways. *Biol. Chem.* 390, 965–976. doi: 10.1515/BC.2009.111

Reczek, C. R., and Chandel, N. S. (2015). ROS-dependent signal transduction. *Curr. Opin. Cell Biol.* 33, 8–13. doi: 10.1016/j.ceb.2014.09.010

Schuppan, D., and Afdhal, N. H. (2008). Liver cirrhosis. *Lancet* 371, 838–851. doi: 10.1016/S0140-6736(08)60383-9

Sung, W. K., Zheng, H., Li, S., Chen, R., Liu, X., Li, Y., et al. (2012). Genome-wide survey of recurrent HBV integration in hepatocellular carcinoma. *Nat. Genet.* 44, 765–769. doi: 10.1038/ng.2295

Szász, A. M., Lánczky, A., Nagy, Á., Förster, S., Hark, K., Green, J. E., Boussioutas, A., et al. (2016). Cross-validation of survival associated biomarkers in gastric cancer using transcriptomic data of 1,065 patients. *Oncotarget* 7, 49322–49333. doi: 10.18632/oncotarget.10337

Takahashi, N., Chen, H. Y., Harris, I. S., Stover, D. G., Selfors, L. M., Bronson, R. T., et al. (2018). Cancer cells co-opt the neuronal redox-sensing channel TRPA1 to promote oxidative-stress tolerance. *Cancer Cell* 33, 985–1003. doi: 10.1016/j.ccell.2018.05.001

Tang, Z., Li, C., Kang, B., Gao, G., Li, C., Zhang, Z. (2017). GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Res.* 45, W98–W102. doi: 10.1093/nar/gkx247

Tseng, P. L., Wu, W. H., Hu, T. H., Chen, C. W., Cheng, H. C., Li, C. F., et al. (2018). Decreased succinate dehydrogenase B in human hepatocellular carcinoma accelerates tumor malignancy by inducing the Warburg effect. *Sci. Rep.* 8:3081. doi: 10.1038/s41598-018-21361-6

Ulahannan, S. V., Duffy, A. G., McNeel, T. S., Kish, J. K., Dickie, L. A., Rahma, O. E., et al. (2014). Earlier presentation and application of curative treatments in hepatocellular carcinoma. *Hepatology* 60, 1637–1644. doi: 10.1002/hep.27288

Wang, B., Hsu, S. H., Frankel, W., Ghoshal, K., and Jacob, S. T. (2012). Stat3-mediated activation of microRNA-23a suppresses gluconeogenesis in hepatocellular carcinoma by down-regulating glucose-6-phosphatase and peroxisome proliferator-activated receptor gamma, coactivator 1 alpha. *Hepatology* 56, 186–197. doi: 10.1002/hep.25632

Weinberg, F., Hamanaka, R., Wheaton, W. W., Weinberg, S., Joseph, J., Lopez, M., et al. (2010). Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8788–8793. doi: 10.1073/pnas.1003428107

Wiese, E. K., and Hitosugi, T. (2018). Tyrosine kinase signaling in cancer metabolism: PKM2 paradox in the warburg effect. *Front. Cell Dev. Biol.* 6:79. doi: 10.3389/fcell.2018.00079

Wright, T. L. (1991). Regenerating nodules–are they premalignant lesions? *Hepatology* 13, 1254–1255. doi: 10.1016/0270-9139(91)92498-W

Wulaningsih, W., Holmberg, L., Garmo, H., Malmstrom, H., Lambe, M., Hammar, N., et al. (2015). Serum lactate dehydrogenase and survival following cancer diagnosis. *Br. J. Cancer* 113, 1389–1396. doi: 10.1038/bjc.2015.361

Wurmbach, E., Chen, Y. B., Khitrov, G., Zhang, W., Roayaie, S., Schwartz, M., et al. (2007). Genome-wide molecular profiles of HCV-induced dysplasia and hepatocellular carcinoma. *Hepatology* 45, 938–947. doi: 10.1002/hep.21622

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MINI REVIEW
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Role of Metabolism in Hepatic Stellate Cell Activation and Fibrogenesis

Wei Hou[1,2] *and Wing-Kin Syn*[2,3]*

[1] *Tianjin Second People's Hospital and Tianjin Institute of Hepatology, Tianjin, China,* [2] *Division of Gastroenterology and Hepatology, Department of Medicine, Medical University of South Carolina, Charleston, SC, United States,* [3] *Section of Gastroenterology, Ralph H. Johnson Veterans Affairs Medical Center, Charleston, SC, United States*

Activation of hepatic stellate cell (HSC) involves the transition from a quiescent to a proliferative, migratory, and fibrogenic phenotype (i.e., myofibroblast), which is characteristic of liver fibrogenesis. Multiple cellular and molecular signals which contribute to HSC activation have been identified. This review specially focuses on the metabolic changes which impact on HSC activation and fibrogenesis.

Keywords: fibroblast, glycolysis, glutaminolysis, liver fibrosis, metabolic

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University of Nebraska-Lincoln,
United States

***Correspondence:**
Wing-Kin Syn
synw@musc.edu

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INTRODUCTION

Activation of hepatic stellate cells (HSCs) involves the transition from a quiescent to a proliferative, migratory and fibrogenic phenotype (i.e., myofibroblast) which is characteristic of liver fibrogenesis. To date, multiple cell-surface, cytoplasmic and nuclear molecular signals and pathways have been reported to modulate HSC activation, including cytokines (Syn et al., 2011, 2012); adipocytokines (Saxena and Anania, 2015; Coombes et al., 2016); Toll-like receptors (TLRs) (Chou et al., 2012; Seo et al., 2016); Interleukins (ILs) (Jiao et al., 2016); collagen receptors (Liu et al., 2017); nuclear receptors (Beaven et al., 2011; Ding et al., 2013; Li et al., 2014; Palumbo-Zerr et al., 2015; Duran et al., 2016); G protein-coupled receptors (GPCRs) (Li et al., 2015, 2016a; Le et al., 2018); autophagy (Thoen et al., 2011, 2012; Hernández-Gea and Friedman, 2012; Hernández-Gea et al., 2012); endoplasmic reticulum stress (Hernández-Gea et al., 2013; Koo et al., 2016); oxidative stress (Lan et al., 2015; Ou et al., 2018); epigenetics (Coll et al., 2015; Hyun et al., 2016; Kweon et al., 2016; Huang et al., 2018; Zheng et al., 2018); cell metabolism (Nwosu et al., 2016; Du et al., 2018; Franko et al., 2018; Zhang et al., 2018), etc. In addition, extracellular/paracrine signals from resident and inflammatory cells including hepatocytes (Zhan et al., 2006), macrophages (Pradere et al., 2013), natural killer cells (Glässner et al., 2012), natural killer T cells (Wehr et al., 2013), liver sinusoidal endothelial cells (LSECs) (Xie et al., 2012), platelets (Kurokawa et al., 2016), and B cells (Thapa et al., 2015) further promote HSC activation.

In this review, we provide a focused update on the impact of cellular metabolism on HSC activation and fibrogenesis. A detailed discussion on other signals and pathways is beyond the scope of this article and has been reviewed elsewhere (Weiskirchen and Tacke, 2014; Lee et al., 2015;

Wallace et al., 2015; Yang and Seki, 2015; El Taghdouini and van Grunsven, 2016; Hyun and Jung, 2016; Nwosu et al., 2016; Schumacher and Guo, 2016; de Oliveira da Silva et al., 2017; Higashi et al., 2017; Huang et al., 2017; Jiang et al., 2017; Kisseleva, 2017; Tsuchida and Friedman, 2017; Mortezaee, 2018; Ni et al., 2018; Wang J. N. et al., 2018).

AEROBIC GLYCOLYSIS: WARBURG EFFECT

Proliferative cells are often glycolytic, similar to the Warburg state that has been described in cancer cells. Diehl and colleagues first reported that reprogramming of quiescent hepatic stellate cell (Q-HSC) into myofibroblastic hepatic stellate cell (MF-HSC) is dependent upon induction of aerobic glycolysis (Chen et al., 2012). Compared with Q-HSC, MF-HSC express higher levels of glycolytic enzymes including hexokinase 2 (HK2), phosphofructokinase platelet (PFKP), pyruvate kinase M2 (PKM2) and glucose transporter 1 (GLUT1), monocarboxylate transporter 4 (MCT4), but downregulate key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PCK1) and fructose bisphosphatase (FBP1). During HSC activation, glycolysis occurs which lead to accumulation of intracellular lactate (**Figure 1**). Conversely, inhibition of conversion of pyruvate to lactate in MF-HSC with a pharmacologic inhibitor of lactate dehydrogenase A (LDHA) led to the decrease in lactate/pyruvate ratio, inhibition of proliferation, suppression of MF-genes expression, reduction of lipid accumulation and upregulation of genes involved in lipogenesis. Mechanistically, these investigators showed that activation of the Hedgehog (Hh) pathway upregulates expression of hypoxia inducible factor 1α (HIF1α), a key modulator of the expression and activity of glycolytic enzymes, directs glycolytic reprogramming, and controls the fate of HSC. By contrast, the inhibition of Hh signaling, HIF1α expression, glycolysis, or lactate accumulation results in the reversal of MF-HSC to a Q-HSC phenotype. These cellular changes are recapitulated *in vivo*: diseased livers of animals and patients accumulate an increasing number of glycolytic stromal cells that correlates with severity of liver fibrosis. In aggregate, these findings indicate that cellular metabolism plays a central role in the fibrogenic response, and imply that targeting cellular metabolism may be a novel antifibrotic strategy.

Despite these preliminary findings, the exact mechanisms that aerobic glycolysis modulates HSC activation and fibrogenesis remain largely unknown. For example, why (and how) do HSCs switch to aerobic glycolysis even when oxygen is abundant (**Figure 1**)? What are the key mediators to trigger the switch from oxidative phosphorylation to aerobic glycolysis? While glycolysis generates only two ATPs for each molecule of glucose, the oxidative phosphorylation produces up to 38 ATPs for each molecule of glucose that is consumed. Why should a cell utilize a less efficient metabolism system (at least in terms of ATP production) to promote HSC activation? Future studies will be needed to better understand the potential roles of lactate and lactate dehydrogenases (LDHs) in metabolic reprogramming. The current data to date, however, suggest that metabolites generated by aerobic glycolysis may have a more important role in the regulation of cellular functions then simply energy production.

GLUTAMINOLYSIS: ANAPLEUROSIS

Glutaminolysis is the conversion of glutamine (Gln) in α-ketoglutarate (α-KG) and consists of two reactions: the first reaction is catalyzed by the glutaminase (GLS), which converts Gln into glutamate (Glu) by loosing an amino group; the second step consists of the conversion of Glu to α-KG and is catalyzed by glutamate dehydrogenase or aminotransferases (**Figure 2**). Glutaminolysis could be involved in the mechanism for regulating HSC activation because glutaminolytic activity might fuel anapleurosis to meet the elevated demands of bioenergetic and biosynthetic pathways needed for the myofibroblastic phenotype.

In a recent study, Du et al. (2018) demonstrated that glutaminolysis could enable the transdifferentiation of HSCs into MF-HSCs. MF-HSCs, like highly proliferative cancer cells, are also highly dependent on glutamine *in vitro*. Glutamine is critical not only for MF-HSC growth but also for acquiring and maintaining a myofibroblastic phenotype. Their results show that α-ketoglutarate (α-KG), the end-product of glutaminolysis, helps to replenish the TCA cycle to satisfy the high bioenergetic and biosynthetic demands of MF-HSCs. Similar to aerobic glycolysis, investigators reported that Hh-mediated pathways also induce glutaminolysis to increase the production of energy and anabolic substrates needed to satisfy their increased demands when Q-HSC transdifferentiate to become MF-HSC. Interestingly, Yes-associated protein 1 (YAP) was identified as a downstream mediator of Hh-directed regulator of glutaminolytic enzymes during HSC transdifferentiation, and was shown to work in concert with its realted transcriptional regulator TAZ through TEAD binding sites to regulate glutaminase 1 (Gls1) expression in HSCs.

Similar findings were reported by Li et al. (2017). They showed that *culture*- as well as *in vivo*-activated HSCs demonstrate increased utilization of glutamine and related genes expression in glutamine metabolism, including glutaminase (GLS), aspartate transaminase (GOT1) and glutamate dehydrogenase (GLUD1). In addition to Hh signaling, TGF-β1, c-Myc, and Ras signaling have also been identified as major regulators of glutamine metabolism. In sum, these data indicate that increased glutamine metabolism not only meets an increasing energy demand but also functions as a key early regulator of HSC activation and fibrogenesis.

In support of its role in liver fibrogenesis, recent data also suggest that glutaminolysis regulates MF differentiation and play a critical role in other tissues. As an example, glutaminolysis was found to be a critical factor in the metabolic reprogramming of MF differentiation in lung tissues (Bernard et al., 2018), that TGF-β1 driven GLS1 expression is dependent upon both SMAD family member 3 (SMAD3) and mitogen activated protein kinase p38 (p38MAPK) activation.

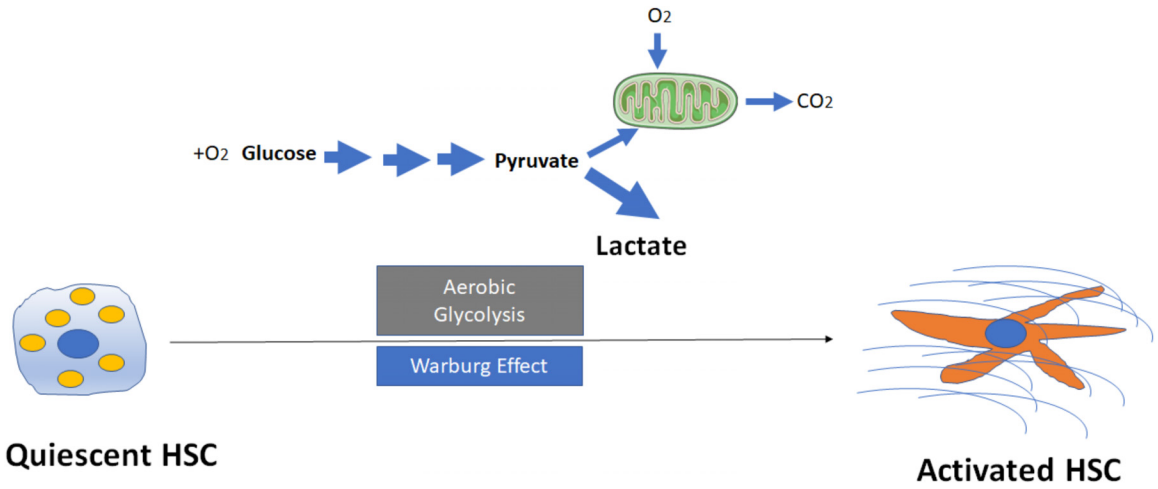

FIGURE 1 | Activation of hepatic stellate cells (HSCs) through induction of aerobic glycolysis (Warburg effect). The transformation of glucose to lactate during HSC activation even when amounts of oxygen are available, leads to accumulation of intracellular lactate. Mitochondria may remain functional and some oxidative phosphorylation continue in cells. Aerobic glycolysis is less efficient than oxidative phosphorylation for generating adenosine 5′-triphosphate (ATP), which suggests that metabolites (for example, lactate) generated by aerobic glycolysis may have a more important role in the regulation of cellular functions than simply energy production during HSC activation.

Despite compelling data, further investigations are still needed to reveal the downstream components of the Hh-Yap-glutaminase axis, and identify alternative signaling pathways which regulate glutaminolysis in HSCs. It is also unclear if changes in glutaminolysis or other anaplerotic pathways, those catalyzed by pyruvate carboxylase (PC) as an example, can also modulate other regenerative programs and/or liver cells during fibrogenesis (Harvey and Chan, 2018).

LIPID DROPLETS (LDs)

Q-HSCs are lipid-storing cells with the presence of large lipid droplets (LDs). During activation, HSCs lose their LDs (Friedman et al., 1993). LDs exist as a hydrophobic core of neutral lipids, surrounded by a phospholipid monolayer (Onal et al., 2017). In HSCs, the LDs contain in addition to neutral lipids consisting of triacylglycerols (TAG) and cholesterol esters, also retinyl esters (RE) with majority comprising of retinol/vitamin A. The exact mechanism of LD loss and its role in HSCs activation is unclear but has been recently studied. Cumulative data (Testerink et al., 2012; Tuohetahuntila et al., 2015, 2016, 2017; Ajat et al., 2017; Molenaar et al., 2017) show that LDs degrade during HSC activation in two distinct phases: (a) upon HSCs activation, the size of LDs was reduced while the number was increased during the first 7 days in culture; (b) disappearance of the LDs. During the prime stage of HSC activation there is a rapid decrease of REs, whereas the TAG content increases transiently, predominantly due to an abundant increase in polyunsaturated fatty acid (PUFA)-containing triacylglycerol, which is mediated by the increase in the ratio of the PUFA-specific fatty acid CoA synthase 4 (ACSL4) to the non-specific ASCLs, such as ASCL1.

Two pools of LDs are thought to exist in HSC: a preexisting ("original"/"old") and a dynamic ("new") pool of LDs (Molenaar et al., 2017; Tuohetahuntila et al., 2017; **Figure 3**). The preexisting LD pool, located predominately round the nucleus, containing predominantly TAGs and REs, as well as retinol acyltransferase (LRAT). During activation, lysosomal acid lipase (LAL/Lipa) is involved in the degradation of the preexisting LDs in the lysosome. The dynamic LDs, smaller than preexisting LDs, containing less REs but enriched in TAGs containing one or more PUFAs, are located in the periphery of the HSC. Diacylglycerol *O*-acyltransferase 1 (DGAT1) and adipose triglyceride lipase (ATGL), also known as patatin like phospholipase domain containing 2 (PNPLA2), are involved in the synthesis and breakdown of these newly synthesized TAGs, respectively.

Despite these new findings, the mechanism by which one pool is targeted for lipophagy and the other for lipolysis by ATGL remains elusive. It is also unclear why "old" types of LDs are degraded and "new" types of LDs formed. What triggers the replacement of retinyl esters by PUFAs? What kind of roles do the incorporated PUFAs play in contributing to HSC activation?

FREE CHOLESTEROL (FC)

Recent studies (Schwabe and Maher, 2012; Teratani et al., 2012; Tomita et al., 2014a,b; Furuhashi et al., 2017; **Figure 4**) suggest that free cholesterol (FC) also mediates HSCs activation and fibrogenesis. FC accumulation in HSCs increases Toll-like receptor 4 protein (TLR4) levels by suppressing the endosomal-lysosomal degradation pathway of TLR4, and thereby sensitizes the cells to TGF-β-induced activation through down-regulating the expression of TGFβ-pseudoreceptor Bambi (bone morphogenetic protein and activing membrane-bound inhibitor). Along with HSC activation, subsequent upregulation of both sterol regulatory element-binding protein 2 (SREBP2)

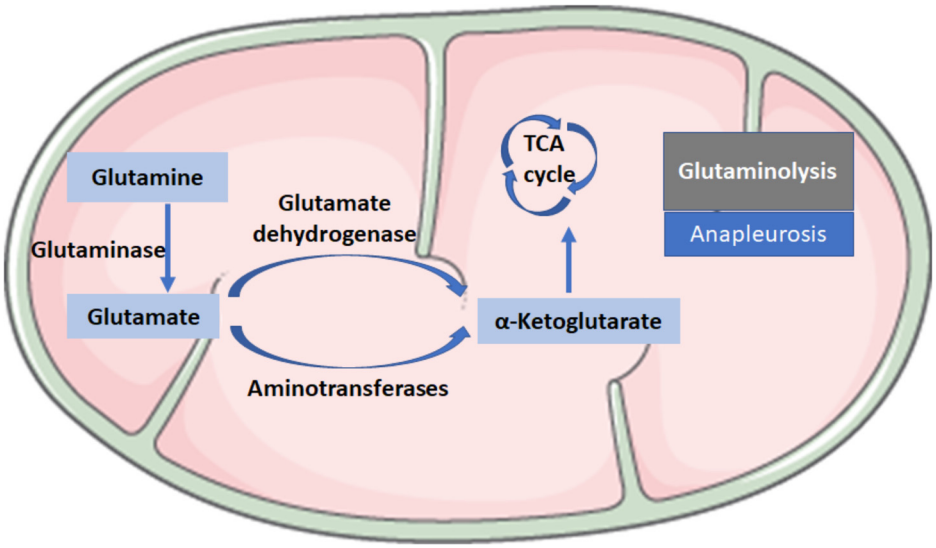

FIGURE 2 | Biochemical reactions in glutaminolysis. Glutaminolysis is the conversion of glutamine (Gln) to α-ketoglutarate (α-KG) and consists of two reactions: the first reaction is catalyzed by the glutaminase (GLS), which converts Gln into glutamate (Glu) by losing an amino group; the second step consists of the conversion of Glu to α-KG, a critical intermediate in the tricarboxylic acid (TCA) cycle, which is catalyzed by glutamate dehydrogenase or aminotransferases.

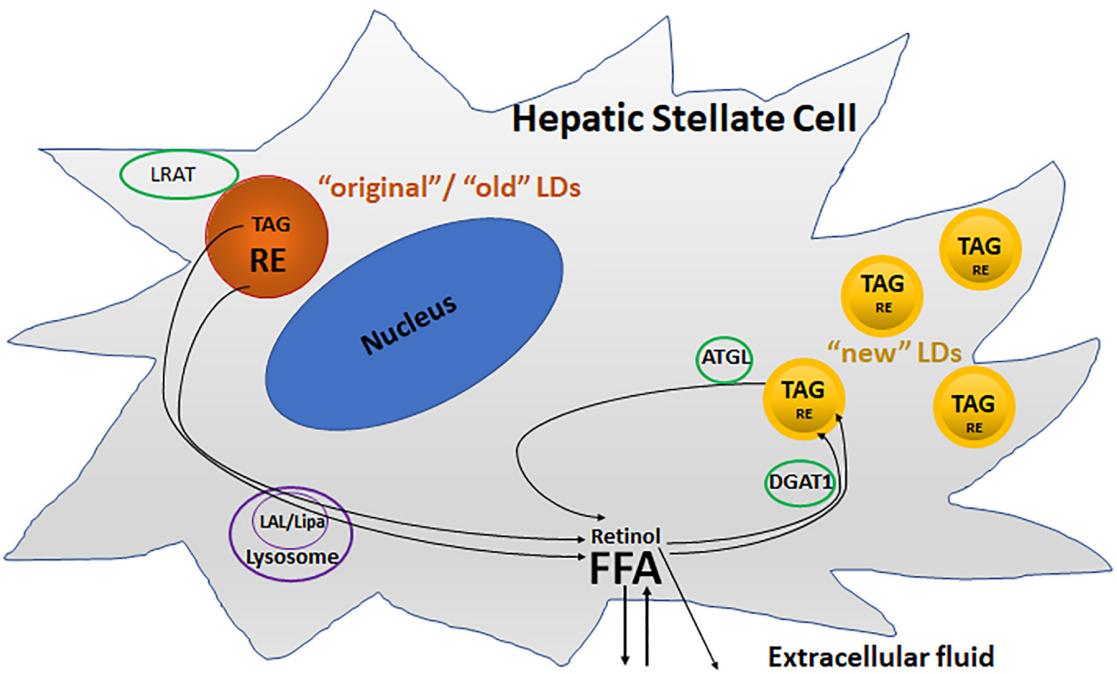

FIGURE 3 | Two different metabolic pools of lipid droplets (LDs) in activated HSCs. The "original"/"old" LDs (depicted in brown), are located predominately round the nucleus, and contains predominantly triacylglycerol (TAG) and retinyl ester (RE), as well as retinol acyltransferase (LRAT). Lysosomal acid lipase (LAL/Lipa) is involved in the degradation of the "original"/"old" LDs in the lysosome during activation. The "new" LDs (depicted in yellow) which are smaller than "old" LDs, contain less REs but are enriched in TAGs, and are located in the periphery of the cells. Diacylglycerol O-acyltransferase 1 (DGAT1) and adipose triglyceride lipase (ATGL) are involved in the synthesis and breakdown of these newly synthesized TAGs, respectively.

and miR-33a signaling through the suppression of PPARγ signaling, as well as disruption of the SREBP2-mediated cholesterol-feedback system in HSCs, which was characterized by a high SREBP cleavage-activating protein (Scap)-to-insulin-induced gene (Insig) ratio and exaggerated by the down-regulation of Insig-1 through the suppression of PPARc signaling, led to further FC accumulation and enhancing liver fibrosis in a positive feedforward loop. Notably, in a mouse model

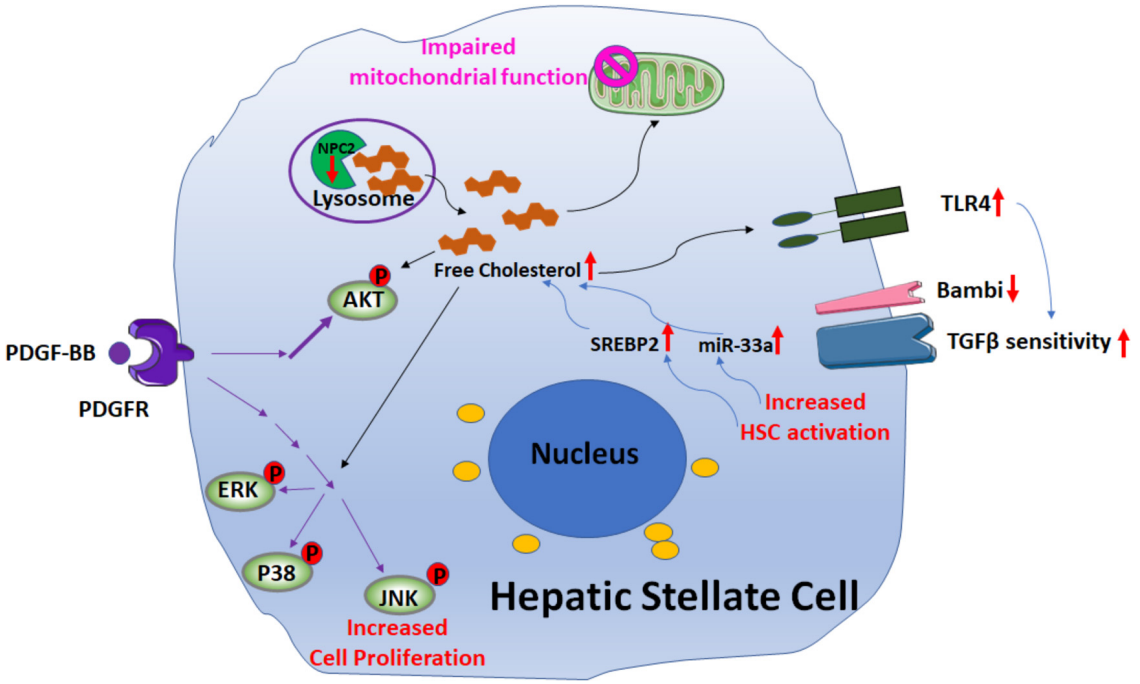

FIGURE 4 | Signaling pathways involved in free cholesterol (FC) accumulation mediated HSC activation. Downregulation of Niemann–Pick type C2 protein (NPC2) results in FC accumulation and enhances platelet-derived growth factor BB (PDGF-BB)-induced HSC proliferation by extracellular signal-regulated kinases (ERKs), p38, c-Jun N-terminal kinases (JNK), and protein kinase B (AKT) phosphorylation. In addition, the mitochondrial respiration function is impaired. FC accumulation also increases Toll-like receptor 4 protein (TLR4) expression, thereby sensitizing cells to TGF-β-induced activation through down-regulation of TGFβ-pseudoreceptor Bambi. Along with HSC activation, subsequent upregulation of both sterol regulatory element-binding protein 2 (SREBP2) and miR-33a signaling leads to further FC accumulation and exaggerates liver fibrosis in a positive feedforward loop.

of liver fibrosis it was shown that reduction of FC accumulation in activated HSCs downregulated TLR4 signaling; this resulted in an increase of Bambi expression, which was associated with a reduction of liver fibrosis (Furuhashi et al., 2017).

Further support for the role of FC in liver fibrosis was demonstrated by studies on the Niemann–Pick type C2 protein (NPC2) (Twu et al., 2016; Wang Y. H. et al., 2018; **Figure 4**). NPC2 regulates intracellular cholesterol trafficking and homeostasis by directly binding with FC and expression of NPC2 is down-regulated in CCl4- and thioacetamide (TAA)-induced liver fibrosis tissues. The loss of NPC2 enhances the accumulation of FC in HSCs and exaggerates HSC response to TGF-β1 treatment. Gene depletion of NPC2 resulted in activation of extracellular signal-regulated kinases (ERKs), p38, c-Jun N-terminal kinases (JNK), and protein kinase B (AKT) phosphorylation which all contributed to increase the HSC proliferation induced by platelet-derived growth factor BB (PDGF-BB). In addition, the mitochondrial respiration function was also impaired.

Despite accumulating data on the role of FC on HSC phenotype, little is known of the roles of individual enzymes of cholesterol biosynthesis pathway in the fibrogenic response. Future studies will be needed to understand whether enzymes such as 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), mevalonate kinase (MVK), phosphomevalonate kinase (PMVK), diphosphomevalonate decarboxylase (MVD), farnesyl diphosphate synthase (FDPS), farnesyl-diphosphate farnesyltransferase 1 (FDFT1), squalene epoxidase (SQLE), 7-dehydrocholesterol reductase (DHCR7), or related metabolites may be involved in modulating HSC biology.

TRICARBOXYLIC ACID (TCA) CYCLE

The tricarboxylic acid cycle (TCA cycle), also called Krebs cycle and citric acid cycle, which was proposed by Hans Adolf Krebs in 1937, is the final common pathway for oxidative catabolism of carbohydrates, fatty acids and amino acids, providing precursors for multiple biosynthetic pathways and plays a critical role in gluconeogenesis, transamination, deamination, and lipogenesis.

In brief, eight steps are involved in the TCA cycle, which is catalyzed by eight different enzymes including citrate synthase, aconitase, isocitrate dehydrogenase, ketoglutarate dehydrogenase, succinyl-CoA synthase, succinate dehydrogenase, fumarase, malate dehydrogenase. The TCA cycle starts with the convertion of the pyruvate into acetyl CoA, which is then converted in citrate by the combination with oxaloacetate. In a multi-steps reaction citrate is next converted in isocitrate to form then α-ketoglutarate. α-ketoglutarate loses a molecule of carbon dioxide and is oxidized to form succinyl CoA, which is then converted to succinate that is oxidized to

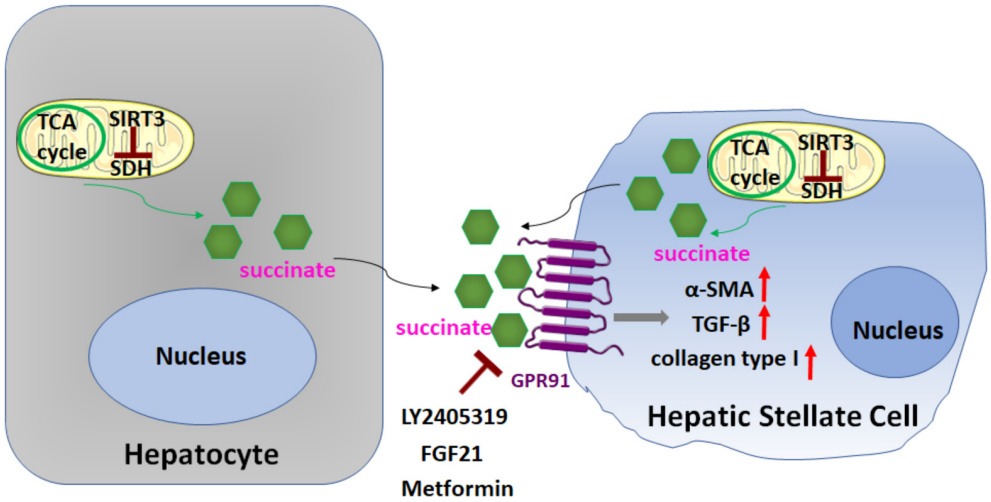

FIGURE 5 | Role of succinate in HSC activation. Succinate, an intermediate in the TCA cycle, functions as a paracrine signal between hepatocytes and HSCs, through binding and activation of its cognate G protein-coupled receptor 91 (GPR91), which resulted in upregulation of fibrogenic markers alpha-smooth muscle actin (α-SMA), transforming growth factor β (TGF-β), and collagen type I. Sirtuin 3 (SIRT3), a NAD+-dependent protein deacetylase, predominantly localized in the mitochondrial matrix, is a key regulator of dehydrogenase (SDH) activity. The SIRT3-SDH-GPR91 axis regulates HSC activation. Repression of succinate-GPR91 signaling by LY2405319, an analog of fibroblast growth factor 21 (FGF21), as well as metformin inhibits HSC activation.

form fumarate. At the end of the cycle fumarate is hydrolyzed to produce malate which is then oxidized to generate oxaloacetate. For each complete cycle there is the regeneration of oxaloacetate and the formation of two molecules of carbon dioxide.

In a recent study Li et al. (2015, 2016a; **Figure 5**) demonstrated the importance of succinate (an intermediate in the TCA cycle) in HSC activation, through binding and activation of its cognate G protein-coupled receptor 91 (GPR91). When cultured HSCs were treated directly with succinate or with inhibitors of succinate dehydrogenase (SDH) (malonate, palmitate/choline, and methionine-choline deficient media), these resulted in the induction of GPR91, and upregulation of fibrogenic markers alpha-smooth muscle actin (α-SMA), transforming growth factor β (TGF-β), and collagen type I. Conversely, transfection of siRNA against GPR91 abrogated succinate-induced increases in the expression of α-SMA. Similar findings were observed when HSCs were isolated from methionine choline deficient diet-fed mice: HSC expressed higher levels of succinate, GPR91, and α-SMA. Taken together, these findings support a key role for succinate-GPR91 in HSC activation and fibrogenesis.

Sirtuin 3 (SIRT3), a NAD^+-dependent protein deacetylase, predominantly localized in the mitochondrial matrix, is a key regulator of SDH activity. Recently, Li et al. (2016a; **Figure 5**) further found that the SIRT3-SDH-GPR91 axis regulated HSC activation, and proposed that succinate functions as a paracrine signal between hepatocytes and HSCs. Significantly, the repression of succinate-GPR91 signaling by LY2405319, an analog of the fibroblast growth factor 21 (FGF21), inhibited HSC activation. These observations suggest that the succinate-GPR91 pathway might be a potential therapeutic target in the treatment of liver fibrosis (Cho, 2018; Le et al., 2018; **Figure 5**).

A ROLE FOR DIRECT METABOLISM-TARGETED ANTIFIBROTIC STRATEGY

Since both aerobic glycolysis (a target of the hedgehog pathway) and glutaminolysis (a process strongly regulated by Yap) are necessary to fulfill inherent metabolic requirements of the MF state and safely satisfies the bioenergetic and biosynthetic demands of highly proliferative cells, combining inhibitors of glycolysis and inhibitors of glutaminolysis which restrict both metabolic adaptations might be a physiologic and practical approaches to limit accumulation of MF-HSCs during liver injury.

Curcumin, a natural occurring principal curcuminoid of turmeric, has been reported to inhibit hedgehog signaling, decrease the accumulation of ATP and lactate, and downregulate the expressions and activities of hexokinase (HK) and phosphofructokinase-2 (PFK2) within HSCs. The glucose transporter Glut4 and lactate transporter MCT4 are also concomitantly downregulated (Lian et al., 2015). Thus, curcumin exhibits inhibitory effects on multiple steps of the glycolysis pathway and regulates metabolic reprogramming in activated HSCs (Lian et al., 2015), which is consistent with the report from Diehl and colleagues which showed that Hh signaling regulates metabolism in activated HSCs (Chen et al., 2012). In addition, as shown in a recent study (She et al., 2018), curcumin could also inhibit HSC activation via suppression of succinate-associated hypoxia-inducible transcription factor-1α (HIF-1α) induction.

Statins, are drugs known to lowering the levels of cholesterol and suppressing the cholesterol biosynthesis through the competitive inhibition of 3-hydroxy-3-methylglutaryl Co-enzyme A reductase (HMGCR) and subsequent blockade of

the conversion of HMGCoA into mevalonate. Multiple studies have reported the potential antifibrotic roles of atorvastatin (Trebicka et al., 2010; Klein et al., 2012; El-Ashmawy et al., 2015; Ying et al., 2017), fluvastatin (Chong et al., 2015; Cheng et al., 2018), pitavastatin (Yang et al., 2010) and simvastatin (Wang et al., 2013; Jang et al., 2018), and recent data further reveal that these antifibrotic effects may occur via upregulation of the Krüppel-like factor 2 (KLF2) transcription factor (Marrone et al., 2013, 2015; Ray, 2015; Trebicka and Schierwagen, 2015).

Metformin, a well-known and the most widely used anti-diabetic drug, inhibiting hepatic gluconeogenesis in the liver, has been recently shown to suppress α-SMA expression via inhibition of succinate-GPR91 signaling in activated LX-2 cells (Nguyen et al., 2018; **Figure 5**). Interestingly, metformin can also attenuate activation of HSCs by activating the AMP-activated protein kinase (AMPK) pathway (Li et al., 2018; Nguyen et al., 2018). AMPK, recognized as an energy sensor with three heterotrimeric subunits (α, β, and γ), is an evolutionary conserved and ubiquitously expressed serine/threonine kinase playing a central role in the coordination of energy homeostasis. In a bleomycin model of lung fibrosis (Rangarajan et al., 2018), metformin therapeutically accelerates the resolution of well-established fibrosis in an AMPK-dependent manner through enhancing mitochondrial biogenesis and normalizing sensitivity to apoptosis. Metformin has emerged as novel antifibrotic strategies for the treatment of fibrotic diseases (Dos Santos et al., 2018; Li et al., 2018; Nguyen et al., 2018; Rangarajan et al., 2018).

CONCLUSION AND SPECULATION/HYPOTHESIS

Cells constantly reprogram their metabolic pathways through direct or indirect mechanisms. Mounting evidences have shown the cross talk between signaling pathways and metabolic control in HSCs, and the complex interplay between metabolism and fibrogenesis is an exciting area of HSC research. Although recent data have shed light on the roles of some metabolic pathways in HSC biology, many more have yet to be described. A better understanding of the roles of cellular metabolism in HSC activation and fibrogenesis will provide us with novel molecular basis for the development of antifibrotic interventions.

Some speculative hypotheses might be put forward here to broaden the horizon about the role of metabolism in HSC activation and fibrogenesis. In addition to single cell glycolysis and glutaminolysis, symbiosis may be an alternate energy metabolism model that contributes to HSC fibrogenesis. For example, some HSCs might produce lactate with ATP production by consuming glucose (Warburg effect), while a neighboring HSC might consume the secreted lactate to produce ATP via the TCA cycle and oxidative phosphorylation. In fact, lactate could be used by some cancer cells [e.g., human non-small-cell lung cancers (NSCLCs)] as a substrate for TCA intermediates through monocarboxylate transporters (MCT1/4) and also for ATP production (Faubert et al., 2017).

Many glycolytic enzymes also function as protein kinases. Although these enzymes participate in specific metabolic pathways, each metabolic enzyme is also known to catalyze a unidirectional and/or bidirectional reaction. Recent data further revealed that pyruvate kinase M2 (PKM2), phosphoglycerate kinase 1 (PGK1), ketohexokinase (KHK) isoform A (KHK-A), hexokinase (HK), and nucleoside diphosphate kinases 1 and 2 (NME1/2) can function as protein kinases and phosphorylate multiple protein substrates to regulate cellular functions (Yang and Lu, 2015; Li et al., 2016b,c). Future studies will be needed to determine whether these, hitherto unrecognized protein kinase activity (of these metabolic enzymes) might also modulate HSC phenotype.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Ajat, M., Molenaar, M., Brouwers, J. F. H. M., Vaandrager, A. B., Houweling, M., and Helms, J. B. (2017). Hepatic stellate cells retain the capacity to synthesize retinyl esters and to store neutral lipids in small lipid droplets in the absence of LRAT. *Biochim. Biophys. Acta* 1862, 176–187. doi: 10.1016/j.bbalip.2016.10.013

Beaven, S. W., Wroblewski, K., Wang, J., Hong, C., Bensinger, S., Tsukamoto, H., et al. (2011). Liver X receptor signaling is a determinant of stellate cell activation and susceptibility to fibrotic liver disease. *Gastroenterology* 140, 1052–1062. doi: 10.1053/j.gastro.2010.11.053

Bernard, K., Logsdon, N. J., Benavides, G. A., Sanders, Y., Zhang, J., Darley-Usmar, V. M., et al. (2018). Glutaminolysis is required for transforming growth factor-β1-induced myofibroblast differentiation and activation. *J. Biol. Chem.* 293, 1218–1228. doi: 10.1074/jbc.RA117.000444

Chen, Y., Choi, S. S., Michelotti, G. A., Chan, I. S., Swiderska-Syn, M., Karaca, G. F., et al. (2012). Hedgehog controls hepatic stellate cell fate by regulating metabolism. *Gastroenterology* 143, 1319.e11–1329.e11. doi: 10.1053/j.gastro.2012.07.115

Cheng, Y., Zheng, H., Wang, B., Xu, W., Xu, J., and Zhu, Y. (2018). Sorafenib and fluvastatin synergistically alleviate hepatic fibrosis via inhibiting the TGFβ1/Smad3 pathway. *Dig. Liver Dis.* 50, 381–388. doi: 10.1016/j.dld.2017.12.015

Cho, E. H. (2018). Succinate as a regulator of hepatic stellate cells in liver fibrosis. *Front. Endocrinol. (Lausanne)* 9:455. doi: 10.3389/fendo.2018.00455

Chong, L. W., Hsu, Y. C., Lee, T. F., Lin, Y., Chiu, Y. T., Yang, K. C., et al. (2015). Fluvastatin attenuates hepatic steatosis-induced fibrogenesis in rats through inhibiting paracrine effect of hepatocyte on hepatic stellate cells. *BMC Gastroenterol.* 15:22. doi: 10.1186/s12876-015-0248-8

Chou, M. H., Huang, Y. H., Lin, T. M., Du, Y. Y., Tsai, P. C., Hsieh, C. S., et al. (2012). Selective activation of Toll-like receptor 7 in activated hepatic stellate cells may modulate their profibrogenic phenotype. *Biochem. J.* 447, 25–34. doi: 10.1042/BJ20112058

Coll, M., El Taghdouini, A., Perea, L., Mannaerts, I., Vila-Casadesús, M., Blaya, D., et al. (2015). Integrative miRNA and gene expression profiling analysis of human quiescent hepatic stellate cells. *Sci. Rep.* 5:11549. doi: 10.1038/srep11549

Coombes, J. D., Choi, S. S., Swiderska-Syn, M., Manka, P., Reid, D. T., Palma, E., et al. (2016). Osteopontin is a proximal effector of leptin-mediated non-alcoholic steatohepatitis (NASH) fibrosis. *Biochim. Biophys. Acta* 1862, 135–144. doi: 10.1016/j.bbadis.2015.10.028

de Oliveira, da Silva, B., Ramos, L. F., and Moraes, K. C. M. (2017). Molecular interplays in hepatic stellate cells: apoptosis, senescence, and phenotype reversion as cellular connections that modulate liver fibrosis. *Cell Biol. Int.* 41, 946–959. doi: 10.1002/cbin.10790

Ding, N., Yu, R. T., Subramaniam, N., Sherman, M. H., Wilson, C., Rao, R., et al. (2013). A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. *Cell* 153, 601–613. doi: 10.1016/j.cell.2013.03.028

Dos Santos, C. C., Gopal, B., and Verma, S. (2018). Metformin: an old dog with a new trick? *Cell Metab.* 28, 334–336. doi: 10.1016/j.cmet.2018.08.018

Du, K., Hyun, J., Premont, R. T., Choi, S. S., Michelotti, G. A., Swiderska-Syn, M,. et al. (2018). Hedgehog-YAP signaling pathway regulates glutaminolysis to control hepatic stellate cell activation. *Gastroenterology* 154, 1465.e13–1479.e13. doi: 10.1053/j.gastro.2017.12.022

Duran, A., Hernandez, Reina-Campos, M., Castilla, E. A., Subramaniam, S., Raghunandan, S., et al. (2016). p62/SQSTM1 by binding to vitamin D receptor inhibits hepatic stellate cell activity, fibrosis, and liver cancer. *Cancer Cell* 30, 595–609. doi: 10.1016/j.ccell.2016.09.004

El Taghdouini, A., and van Grunsven, L. A. (2016). Epigenetic regulation of hepatic stellate cell activation and liver fibrosis. *Exp. Rev. Gastroenterol. Hepatol.* 10, 1397–1408. doi: 10.1080/17474124.2016.1251309

El-Ashmawy, N. E., El-Bahrawy, H. A., Shamloula, M. M., and Ibrahim, A. O. (2015). Antifibrotic effect of AT-1 blocker and statin in rats with hepatic fibrosis. *Clin. Exp. Pharmacol. Physiol.* 42, 979–987. doi: 10.1111/1440-1681.12446

Faubert, B., Li, K. Y., Cai, L., Hensley, C. T., Kim, J., Zacharias, L. G., et al. (2017). Lactate metabolism in human lung tumors. *Cell* 171, 358.e9–371.e9. doi: 10.1016/j.cell.2017.09.019

Franko, A., Kovarova, M., Feil, S., Feil, R., Wagner, R., Heni, M., et al. (2018). cGMP-dependent protein kinase I (cGKI) modulates human hepatic stellate cell activation. *Metabolism* 88, 22–30. doi: 10.1016/j.metabol.2018.09.001

Friedman, S. L., Wei, S., and Blaner, W. S. (1993). Retinol release by activated rat hepatic lipocytes: regulation by Kupffer cell-conditioned medium and PDGF. *Am. J. Physiol.* 264(5 Pt 1), G947–G952. doi: 10.1152/ajpgi.1993.264.5.G947

Furuhashi, H., Tomita, K., Teratani, T., Shimizu, M., Nishikawa, M., Higashiyama, M., et al. (2017). Vitamin A-coupled liposome system targeting free cholesterol accumulation in hepatic stellate cells offers a beneficial therapeutic strategy for liver fibrosis. *Hepatol. Res.* 48, 397–407. doi: 10.1111/hepr.13040

Glässner, A., Eisenhardt, M., Krämer, B., Körner, C., Coenen, M., Sauerbruch, T., et al. (2012). NK cells from HCV-infected patients effectively induce apoptosis of activated primary human hepatic stellate cells in a TRAIL-, FasL- and NKG2D-dependent manner. *Lab. Invest.* 92, 967–977. doi: 10.1038/labinvest.2012.54

Harvey, L. D., and Chan, S. Y. (2018). Yapping about glutaminolysis in hepatic fibrosis. *Gastroenterology* 154, 1231–1233. doi: 10.1053/j.gastro.2018.03.007

Hernández-Gea, V., and Friedman, S. L. (2012). Autophagy fuels tissue fibrogenesis. *Autophagy* 8, 849–850. doi: 10.4161/auto.19947

Hernández-Gea, V., Ghiassi-Nejad, Z., Rozenfeld, R., Gordon, R., Fiel, M. I., Yue, Z., et al. (2012). Autophagy releases lipid that promotes fibrogenesis by activated hepatic stellate cells in mice and in human tissues. *Gastroenterology* 142, 938–946. doi: 10.1053/j.gastro.2011.12.044

Hernández-Gea, V., Hilscher, M., Rozenfeld, R., Lim, M. P., Nieto, N., Werner, S., et al. (2013). Endoplasmic reticulum stress induces fibrogenic activity in hepatic stellate cells through autophagy. *J. Hepatol.* 59, 98–104. doi: 10.1016/j.jhep.2013.02.016

Higashi, T., Friedman, S. L., and Hoshida, Y. (2017). Hepatic stellate cells as key target in liver fibrosis. *Adv. Drug Deliv. Rev.* 121, 27–42. doi: 10.1016/j.addr.2017.05.007

Huang, Y., Deng, X., and Liang, J. (2017). Modulation of hepatic stellate cells and reversibility of hepatic fibrosis. *Exp. Cell Res.* 352, 420–426. doi: 10.1016/j.yexcr.2017.02.038

Huang, Y. H., Yang, Y. L., and Wang, F. S. (2018). The role of miR-29a in the regulation, function, and signaling of liver fibrosis. *Int. J. Mol. Sci.* 19:E1889. doi: 10.3390/ijms19071889

Hyun, J., and Jung, Y. (2016). MicroRNAs in liver fibrosis: focusing on the interaction with hedgehog signaling. *World J. Gastroenterol.* 22, 6652–6662. doi: 10.3748/wjg.v22.i29.6652

Hyun, J., Wang, S., Kim, J., Rao, K. M., Park, S. Y., Chung, I., et al. (2016). MicroRNA-378 limits activation of hepatic stellate cells and liver fibrosis by suppressing Gli3 expression. *Nat. Commun.* 7:10993. doi: 10.1038/ncomms10993

Jang, Y. O., Kim, S. H., Cho, M. Y., Kim, K. S., Park, K. S., Cha, S. K., et al. (2018). Synergistic effects of simvastatin and bone marrow-derived mesenchymal stem cells on hepatic fibrosis. *Biochem. Biophys. Res. Commun.* 497, 264–271. doi: 10.1016/j.bbrc.2018.02.067

Jiang, X. P., Ai, W. B., Wan, L. Y., Zhang, Y. Q., and Wu, J. F. (2017). The roles of microRNA families in hepatic fibrosis. *Cell Biosci.* 7:34. doi: 10.1186/s13578-017-0161-7

Jiao, J., Ooka, K., Fey, H., Fiel, M. I., Rahmman, A. H., Kojima, K., et al. (2016). Interleukin-15 receptor α on hepatic stellate cells regulates hepatic fibrogenesis in mice. *J. Hepatol.* 65, 344–353. doi: 10.1016/j.jhep.2016.04.020

Kisseleva, T. (2017). The origin of fibrogenic myofibroblasts in fibrotic liver. *Hepatology* 65, 1039–1043. doi: 10.1002/hep.28948

Klein, S., Klösel, J., Schierwagen, R., Körner, C., Granzow, M., Huss, S., et al. (2012). Atorvastatin inhibits proliferation and apoptosis, but induces senescence in hepatic myofibroblasts and thereby attenuates hepatic fibrosis in rats. *Lab. Invest.* 92, 1440–1450. doi: 10.1038/labinvest.2012.106

Koo, J. H., Lee, H. J., Kim, W., and Kim, S. G. (2016). Endoplasmic reticulum stress in hepatic stellate cells promotes liver fibrosis via PERK-mediated degradation of HNRNPA1 and up-regulation of SMAD2. *Gastroenterology* 150, 181.e8–193.e8. doi: 10.1053/j.gastro.2015.09.039

Kurokawa, T., Zheng, Y. W., and Ohkohchi, N. (2016). Novel functions of platelets in the liver. *J. Gastroenterol. Hepatol.* 31, 745–751. doi: 10.1111/jgh.13244

Kweon, S. M., Chi, F., Higashiyama, R., Lai, K., and Tsukamoto, H. (2016). Wnt pathway stabilizes MeCP2 protein to repress PPAR-γ in activation of hepatic stellate cells. *PLoS One* 11:e0156111. doi: 10.1371/journal.pone.0156111

Lan, T., Kisseleva, T., and Brenner, D. A. (2015). Deficiency of NOX1 or NOX4 prevents liver inflammation and fibrosis in mice through inhibition of hepatic stellate cell activation. *PLoS One* 10:e0129743. doi: 10.1371/journal.pone.0129743

Le, C. T., Nguyen, G., Park, S. Y., Choi, D. H., and Cho, E. H. (2018). LY2405319, an analog of fibroblast growth factor 21 ameliorates α-smooth muscle actin production through inhibition of the succinate-G-protein couple receptor 91 (GPR91) pathway in mice. *PLoS One* 13:e0192146. doi: 10.1371/journal.pone.0192146

Lee, Y. A., Wallace, M. C., and Friedman, S. L. (2015). Pathobiology of liver fibrosis: a translational success story. *Gut* 64, 830–841. doi: 10.1136/gutjnl-2014-306842

Li, J., Ghazwani, M., Liu, K., Huang, Y., Chang, N., Fan, J., et al. (2017). Regulation of hepatic stellate cell proliferation and activation by glutamine metabolism. *PLoS One* 12:e0182679. doi: 10.1371/journal.pone.0182679

Li, T., Eheim, A. L., Klein, S., Uschner, F. E., Smith, A. C., Brandon-Warner, E., et al. (2014). Novel role of nuclear receptor Rev-erbα in hepatic stellate cell activation: potential therapeutic target for liver injury. *Hepatology* 59, 2383–2396. doi: 10.1002/hep.27049

Li, Y. H., Choi, D. H., Lee, E. H., Seo, S. R,. Lee, S., Cho, E. H. (2016a). Sirtuin 3 (SIRT3) regulates α-smooth muscle actin (α-SMA) production through the succinate dehydrogenase-g protein-coupled receptor 91 (GPR91) pathway in hepatic stellate cells. *J. Biol. Chem.* 291, 10277–10292. doi: 10.1074/jbc.M115.692244

Li, X., Qian, X., and Lu, Z. (2016b). Fructokinase A acts as a protein kinase to promote nucleotide synthesis. *Cell Cycle* 15, 2689–2690. doi: 10.1080/15384101.2016.1204861

Li, X., Zheng, Y., and Lu, Z. (2016c). PGK1 is a new member of the protein kinome. *Cell Cycle* 15, 1803–1804. doi: 10.1080/15384101.2016.1179037

Li, Y. H., Woo, S. H., Choi, D. H., and Cho, E. H. (2015). Succinate causes α-SMA production through GPR91 activation in hepatic stellate cells. *Biochem. Biophys. Res. Commun.* 463, 853–858. doi: 10.1016/j.bbrc.2015.06.023

Li, Z., Ding, Q., Ling, L. P., Wu, Y., Meng, D. X., Li, X., et al. (2018). Metformin attenuates motility, contraction, and fibrogenic response of hepatic stellate cells in vivo and in vitro by activating AMP-activated protein kinase. *World J. Gastroenterol.* 24, 819–832. doi: 10.3748/wjg.v24.i7.819

Lian, N., Jiang, Y., Zhang, F., Jin, H., Lu, C., Wu, X., et al. (2015). Curcumin regulates cell fate and metabolism by inhibiting hedgehog signaling in hepatic stellate cells. *Lab. Invest.* 95, 790–803. doi: 10.1038/labinvest.2015.59

Liu, H., Pan, X., Cao, H., Shu, X., Sun, H., Lu, J., et al. (2017). IL-32γ promotes integrin αvβ6 expression through the activation of NF-κB in HSCs. *Exp. Ther. Med.* 14, 3880–3886. doi: 10.3892/etm.2017.4956

Marrone, G., Maeso-Díaz, R., García-Cardena, G., Abraldes, J. G., García-Pagán, J. C., Bosch, J., et al. (2015). KLF2 exerts antifibrotic and vasoprotective effects in cirrhotic rat livers: behind the molecular mechanisms of statins. *Gut* 64, 1434–1443. doi: 10.1136/gutjnl-2014-308338

Marrone, G., Russo, L., Rosado, E., Hide, D., García-Cardeña, G., García-Pagán, J. C., et al. (2013). The transcription factor KLF2 mediates hepatic endothelial protection and paracrine endothelial-stellate cell deactivation induced by statins. *J. Hepatol.* 58, 98–103. doi: 10.1016/j.jhep.2012.08.026

Molenaar, M. R., Vaandrager, A. B., and Helms, J. B. (2017). Some lipid droplets are more equal than others: different metabolic lipid droplet pools in hepatic stellate cells. *Lipid Insights* 10:1178635317747281. doi: 10.1177/1178635317747281

Mortezaee, K. (2018). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and liver fibrosis: a review. *Cell Biochem. Funct.* 36, 292–302. doi: 10.1002/cbf.3351

Nguyen, G., Park, S. Y., Le, C. T., Park, W. S., Choi, D. H., and Cho, E. H. (2018). Metformin ameliorates activation of hepatic stellate cells and hepatic fibrosis by succinate and GPR91 inhibition. *Biochem. Biophys. Res. Commun.* 495, 2649–2656. doi: 10.1016/j.bbrc.2017.12.143

Ni, M. M., Wang, Y. R., Wu, W. W., Xia, C. C., Zhang, Y. H., Xu, J., et al. (2018). Novel Insights on Notch signaling pathways in liver fibrosis. *Eur. J. Pharmacol.* 826, 66–74. doi: 10.1016/j.ejphar.2018.02.051

Nwosu, Z. C., Alborzinia, H., Wölfl, S., Dooley, S., and Liu, Y. (2016). Evolving insights on metabolism, autophagy, and epigenetics in liver myofibroblasts. *Front. Physiol.* 7:191. doi: 10.3389/fphys.2016.00191

Onal, G., Kutlu, O., Gozuacik, D., and Dokmeci Emre, S. (2017). Lipid droplets in health and disease. *Lipids Health Dis.* 16:128. doi: 10.1186/s12944-017-0521-7

Ou, Q., Weng, Y., Wang, S., Zhao, Y., Zhang, F., Zhou, J., et al. (2018). Silybin alleviates hepatic steatosis and fibrosis in NASH mice by inhibiting oxidative stress and involvement with the Nf-κB pathway. *Dig. Dis. Sci.* doi: 10.1007/s10620-018-5268-0 [Epub ahead of print].

Palumbo-Zerr, K., Zerr, P., Distler, A., Fliehr, J., Mancuso, R., Huang, J., et al. (2015). Orphan nuclear receptor NR4A1 regulates transforming growth factor-β signaling and fibrosis. *Nat. Med.* 21, 150–158. doi: 10.1038/nm.3777

Pradere, J. P., Kluwe, J., De Minicis, S., Jiao, J. J., Gwak, G. Y., Dapito, D. H., et al. (2013). Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice. *Hepatology* 58, 1461–1473. doi: 10.1002/hep.26429

Rangarajan, S., Bone, N. B., Zmijewska, A. A., Jiang, S., Park, D. W., Bernard, K., et al. (2018). Metformin reverses established lung fibrosis in a bleomycin model. *Nat. Med.* 24, 1121–1127. doi: 10.1038/s41591-018-0087-6

Ray, K. (2015). Liver: sussing out statins in cirrhosis–KLF2 is the key. *Nat. Rev. Gastroenterol. Hepatol.* 12:64. doi: 10.1038/nrgastro.2014.233

Saxena, N. K., and Anania, F. A. (2015). Adipocytokines and hepatic fibrosis. *Trends Endocrinol. Metab.* 26, 153–161. doi: 10.1016/j.tem.2015.01.002

Schumacher, J. D., and Guo, G. L. (2016). Regulation of hepatic stellate cells and fibrogenesis by fibroblast growth factors. *Biomed. Res. Int.* 2016:8323747. doi: 10.1155/2016/8323747

Schwabe, R. F., and Maher, J. J. (2012). Lipids in liver disease: looking beyond steatosis. *Gastroenterology* 142, 8–11. doi: 10.1053/j.gastro.2011.11.004

Seo, W., Eun, H. S., Kim, S. Y., Yi, H. S., Lee, Y. S., Park, S. H., et al. (2016). Exosome-mediated activation of toll-like receptor 3 in stellate cells stimulates interleukin-17 production by γδ T cells in liver fibrosis. *Hepatology*.64, 616–631. doi: 10.1002/hep.28644

She, L., Xu, D., Wang, Z., Zhang, Y., Wei, Q., Aa, J., et al. (2018). Curcumin inhibits hepatic stellate cell activation via suppression of succinate-associated HIF-1α induction. *Mol. Cell. Endocrinol.* 476, 129–138. doi: 10.1016/j.mce.2018.05.002

Syn, W. K., Agboola, K. M., Swiderska, M., Michelotti, G. A., Liaskou, E., Pang, H., et al. (2012). NKT-associated hedgehog and osteopontin drive fibrogenesis in non-alcoholic fatty liver disease. *Gut* 61, 1323–1329. doi: 10.1136/gutjnl-2011-301857

Syn, W. K., Choi, S. S., Liaskou, E., Karaca, G. F., Agboola, K. M., Oo, Y. H., et al. (2011). Osteopontin is induced by hedgehog pathway activation and promotes fibrosis progression in nonalcoholic steatohepatitis. *Hepatology* 53, 106–115. doi: 10.1002/hep.23998

Teratani, T., Tomita, K., Suzuki, T., Oshikawa, T., Yokoyama, H., Shimamura, K., et al. (2012). A high-cholesterol diet exacerbates liver fibrosis in mice via accumulation of free cholesterol in hepatic stellate cells. *Gastroenterology* 142, 152.e10–164.e10. doi: 10.1053/j.gastro.2011.09.049

Testerink, N., Ajat, M., Houweling, M., Brouwers, J. F., Pully, V. V., van Manen, H. J., et al. (2012). Replacement of retinyl esters by polyunsaturated triacylglycerol species in lipid droplets of hepatic stellate cells during activation. *PLoS One* 7:e34945. doi: 10.1371/journal.pone.0034945

Thapa, M., Chinnadurai, R., Velazquez, V. M., Tedesco, D., Elrod, E., Han, J. H., et al. (2015). Liver fibrosis occurs through dysregulation of MyD88-dependent innate B-cell activity. *Hepatology* 61, 2067–2079. doi: 10.1002/hep.27761

Thoen, L. F., Guimarães, E. L., Dollé, L., Mannaerts, I., Najimi, M., Sokal, E., et al. (2011). A role for autophagy during hepatic stellate cell activation. *J. Hepatol.* 55, 1353–1360. doi: 10.1016/j.jhep.2011.07.010

Thoen, L. F., Guimarães, E. L., and Grunsven, L. A. (2012). Autophagy: a new player in hepatic stellate cell activation. *Autophagy* 8, 126–128. doi: 10.4161/auto.8.1.18105

Tomita, K., Teratani, T., Suzuki, T., Shimizu, M., Sato, H., Narimatsu, K., et al. (2014a). Acyl-CoA: cholesterol acyltransferase 1 mediates liver fibrosis by regulating free cholesterol accumulation in hepatic stellate cells. *J. Hepatol.* 61, 98–106. doi: 10.1016/j.jhep.2014.03.018

Tomita, K., Teratani, T., Suzuki, T., Shimizu, M., Sato, H., Narimatsu, K., et al. (2014b). Free cholesterol accumulation in hepatic stellate cells: mechanism of liver fibrosis aggravation in nonalcoholic steatohepatitis in mice. *Hepatology* 59, 154–169. doi: 10.1002/hep.26604

Trebicka, J., Hennenberg, M., Odenthal, M., Shir, K., Klein, S., Granzow, M., et al. (2010). Atorvastatin attenuates hepatic fibrosis in rats after bile duct ligation via decreased turnover of hepatic stellate cells. *J. Hepatol.* 53, 702–712. doi: 10.1016/j.jhep.2010.04.025

Trebicka, J., and Schierwagen, R. (2015). Statins, rho GTPases and KLF2: new mechanistic insight into liver fibrosis and portal hypertension. *Gut* 64, 1349–1350. doi: 10.1136/gutjnl-2014-308800

Tsuchida, T., and Friedman, S. L. (2017). Mechanisms of hepatic stellate cell activation. *Nat. Rev. Gastroenterol. Hepatol.* 14, 397–411. doi: 10.1038/nrgastro.2017.38

Tuohetahuntila, M., Molenaar, M. R., Spee, B., Brouwers, J. F., Houweling, M., Vaandrager, A. B., et al. (2016). ATGL and DGAT1 are involved in the turnover of newly synthesized triacylglycerols in hepatic stellate cells. *J. Lipid Res.* 57, 1162–1174. doi: 10.1194/jlr.M066415

Tuohetahuntila, M., Molenaar, M. R., Spee, B., Brouwers, J. F., Wubbolts, R., Houweling, M., et al. (2017). Lysosome-mediated degradation of a distinct pool of lipid droplets during hepatic stellate cell activation. *J. Biol. Chem.* 292, 12436–12448. doi: 10.1074/jbc.M117.778472

Tuohetahuntila, M., Spee, B., Kruitwagen, H. S., Wubbolts, R., Brouwers, J. F., van de Lest, C. H., et al. (2015). Role of long-chain acyl-CoA synthetase 4 in formation of polyunsaturated lipid species in hepatic stellate cells. *Biochim. Biophys. Acta* 1851, 220–230. doi: 10.1016/j.bbalip.2014.12.003

Twu, Y. C., Lee, T. S., Lin, Y. L., Hsu, S. M., Wang, Y. H., Liao, C. Y., et al. (2016). Niemann-pick Type C2 protein mediates hepatic stellate cells activation by regulating free cholesterol accumulation. *Int. J. Mol. Sci.* 17:E1122. doi: 10.3390/ijms17071122

Wallace, M. C., Friedman, S. L., and Mann, D. A. (2015). Emerging and disease-specific mechanisms of hepatic stellate cell activation. *Semin. Liver Dis.* 35, 107–118. doi: 10.1055/s-0035-1550060

Wang, J. N., Li, L., Li, L. Y., Yan, Q., Li, J., and Xu, T. (2018). Emerging role, and therapeutic implication of Wnt signaling pathways in liver fibrosis. *Gene* 674, 57–69. doi: 10.1016/j.gene.2018.06.053

Wang, Y. H., Twu, Y. C., Wang, C. K., Lin, F. Z., Lee, C. Y., Liao, Y. J. (2018). Niemann-pick Type C2 protein regulates free cholesterol accumulation, and influences hepatic stellate cell proliferation, and mitochondrial respiration function. *Int. J. Mol. Sci.* 19:E1678. doi: 10.3390/ijms19061678

Wang, W., Zhao, C., Zhou, J., Zhen, Z., Wang, Y., and Shen, C. (2013). Simvastatin ameliorates liver fibrosis via mediating nitric oxide synthase in rats with non-alcoholic steatohepatitis-related liver fibrosis. *PLoS One* 8:e76538. doi: 10.1371/journal.pone.0076538

Wehr, A., Baeck, C., Heymann, F., Niemietz, P. M., Hammerich, L., Martin, C., et al. (2013). Chemokine receptor CXCR6-dependent hepatic NK T cell accumulation promotes inflammation and liver fibrosis. *J. Immunol.* 190, 5226–5236. doi: 10.4049/jimmunol.1202909

Weiskirchen, R., and Tacke, F. (2014). Cellular and molecular functions of hepatic stellate cells in inflammatory responses and liver immunology. *Hepatobil. Surg. Nutr.* 3, 344–363. doi: 10.3978/j.issn.2304-3881.2014.11.03

Xie, G., Wang, X., Wang, L., Wang, L., Atkinson, R. D., Kanel, G. C., et al. (2012). Role of differentiation of liver sinusoidal endothelial cells in progression and regression of hepatic fibrosis in rats. *Gastroenterology* 142, 918.e6–927.e6. doi: 10.1053/j.gastro.2011.12.017

Yang, J. I., Yoon, J. H., Bang, Y. J., Lee, S. H., Lee, S. M., Byun, H. J., et al. (2010). Synergistic antifibrotic efficacy of statin and protein kinase C inhibitor inhepatic fibrosis. *Am. J. Physiol. Gastrointest Liver Physiol.* 298, G126–G132. doi: 10.1152/ajpgi.00299.2009

Yang, W., and Lu, Z. (2015). Pyruvate kinase M2 at a glance. *J. Cell Sci.* 128, 1655–1660. doi: 10.1242/jcs.166629

Yang, Y. M., and Seki, E. (2015). TNFα in liver fibrosis. *Curr. Pathobiol. Rep.* 3, 253–261. doi: 10.1007/s40139-015-0093-z

Ying, L., Yan, F., Zhao, Y., Gao, H., Williams, B. R., Hu, Y., et al. (2017). (-)-Epigallocatechin-3-gallate and atorvastatin treatment down-regulates liver fibrosis-related genes in non-alcoholic fatty liver disease. *Clin. Exp. Pharmacol. Physiol.* 44, 1180–1191. doi: 10.1111/1440-1681.12844

Zhan, S. S., Jiang, J. X., Wu, J., Halsted, C., Friedman, S. L., Zern, M. A., et al. (2006). Phagocytosis of apoptotic bodies by hepatic stellate cells induces NADPH oxidase and is associated with liver fibrosis in vivo. *Hepatology* 43, 435–443. doi: 10.1002/hep.21093

Zhang, Y., Yu, X., Wang, Z., Yin, M., Zhao, Z., Li, Y., et al. (2018). Pokeweed antiviral protein attenuates liver fibrosis in mice through regulating Wnt/Jnk mediated glucose metabolism. *Saudi J. Gastroenterol.* 157–164. doi: 10.4103/sjg.SJG_470_17

Zheng, J., Wang, W., Yu, F., Dong, P., Chen, B., and Zhou, M. T. (2018). MicroRNA-30a suppresses the activation of hepatic stellate cells by inhibiting epithelial-to-mesenchymal transition. *Cell Physiol. Biochem.* 46, 82–92. doi: 10.1159/000488411

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MINI REVIEW
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Metabolic Networks Influencing Skeletal Muscle Fiber Composition

Isabelle Bourdeau Julien[1,2], *Chantelle F. Sephton*[1,2] *and Paul A. Dutchak*[1,2]*

[1] Department of Psychiatry and Neuroscience, Université Laval, Quebec, QC, Canada, [2] CERVO Brain Research Centre, Quebec, QC, Canada

Advancements in metabolomic and genomic research tools are revealing new insights into how metabolic networks can influence skeletal muscle fiber composition. In this mini-review, we summarize the recent progress of metabolite-dependent signaling pathways and transcriptional regulators that control glycolytic and oxidative metabolism and ultimately influence the type of fibers in muscle depots. These mechanisms expand the role of metabolites beyond that of basic building blocks of cellular components, and illustrate how particular metabolites can take an active role in regulating metabolic homeostasis and fiber adaptation. As new metabolite-dependent mechanisms emerge, ongoing metabolomic studies have begun to help explain why distinct metabolic pathways are used in different biological contexts and widen the view of seminal observations like the Warburg effect.

Keywords: mTORC1, GATOR1, nuclear hormone receptor (NHR), metabolism, warbug effect, muscle physiology

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Università degli Studi di Roma La Sapienza, Italy

***Correspondence:**
Paul A. Dutchak
paul.dutchak.1@ulaval.ca

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INTRODUCTION

The metabolic properties of skeletal muscle fibers are intimately related to the biological function of the tissues they comprise. Investigation of the mechanisms that control fiber composition in skeletal muscle is an active area of research, partially due to the intriguing ability of fibers to adapt to nutritional or physiological challenges at a molecular and phenotypic level (Green et al., 1992; Adams et al., 1993; Widrick et al., 2002; Eshima et al., 2017). The adaptive process is associated with changes in intracellular metabolism, gene expression, and contractility of the fibers, which can broadly affect the health of an individual. The contribution of specific metabolites to the regulation of fiber composition has largely remained unknown due to the integrative nature of metabolism in complex physiological systems. Recent studies, combining genetic and metabolomic approaches, have begun to reveal novel relationships between the metabolite-regulated pathways that can influence muscle fiber composition and the ability to undergo metabolic switching from oxidative phosphorylation to aerobic glycolysis, a process known as the Warburg effect in cancer cells. In this mini-review, we discuss the metabolic properties of skeletal muscle fiber types and highlight metabolite-dependent pathways that can influence fiber composition.

METABOLIC PROPERTIES OF MUSCLE FIBERS

Skeletal muscle depots are composed of heterogeneous populations of muscle fibers that permit a broad range of functions. Extensive research has helped define distinct types of muscle fibers that are categorized as slow-twitch (type I) and fast-twitch (type II), which contribute to long-term endurance or powerful bursts of movement, respectively (Szent-Györgyi, 2004). Slow-twitch fibers are dense in mitochondria to allow high oxidative capacity and sustain long-term energy demands;

whereas fast-twitch fibers are subdivided into fast-oxidative (type IIa) or fast-glycolytic (type IIb/x), which correlate with their mitochondrial density. While the quantity of mitochondria is distinct between these fiber types, studies have also shown differences in the metabolism and structural characteristics of the mitochondria between these fiber types (Anderson and Neufer, 2006; Picard et al., 2008; Mishra et al., 2015). The energy requirements of muscle fibers often correlate with the expression of major myosin heavy chain (MHC) isoforms, which determine the rate of cross-bridge cycling with type I being the slowest, type IIa intermediate, and IIX/b the fastest. The MHC isoforms are encoded by *Myh7, Myh2, Myh1*, and *Myh4*, which are expressed in type I, IIa, IIx, and IIb fibers, respectively (Schiaffino and Reggiani, 2011).

There is a tight regulation of glycolytic and oxidative pathways in muscle fibers to ensure ATP production meets the demand of the tissue. This is necessary because ATP turnover rates can increase over 100-fold in active muscle compared to rest (Gaitanos et al., 1993). When there is an immediate energy demand (i.e., sprinting), a rapid increase of glycolytic metabolism occurs and pyruvate is converted to lactate, reminiscent of the Warburg effect (Warburg et al., 1927). The importance of switching from oxidative phosphorylation to aerobic glycolysis, which is less efficient in generating ATP, is to increase metabolites like NAD+ that are needed to accommodate continued glycolysis and other aspects of cellular metabolism and growth. It is in this manner that skeletal muscle metabolism can recapitulate metabolic hallmarks observed in cancer cells (Lunt and Vander Heiden, 2011). Intriguingly, regulatory pathways that control the metabolic flexibility of skeletal muscle fibers are frequently associated with cancer cell metabolism, including the mTORC1 pathway that stimulates aerobic glycolysis when activated in muscle. Overtime, changes in the metabolic environment within different fiber types can activate cell signaling and transcriptional mechanisms that stimulate an adaptive process that causes phenotypic changes of the fibers, a process called fiber type switching.

METABOLITE-DEPENDENT SIGNALING MECHANISMS REGULATING FIBER COMPOSITION

Cell signaling pathways control the homeostatic and adaptive properties of skeletal muscle fibers (Egan and Zierath, 2013). Several of these pathways are regulated by secondary messengers like cyclic adenosine monophosphate (Berdeaux and Stewart, 2012) or Ca^{+2} (Chin et al., 1998), whereas others are dependent on intracellular metabolites. Important metabolite-dependent pathways that effect skeletal muscle fiber composition and cellular metabolism in accordance with nutrient availability include the mammalian Target of Rapamycin (mTORC1) and AMP-activated protein kinase (AMPK) pathways (Fryer et al., 2002; Jørgensen et al., 2006; Philp et al., 2011) (**Figure 1A**). Cross-talk signaling between these pathways, beyond the scope of this review, antagonistically control the size of muscle cells (Mounier et al., 2011).

mTORC1 is a protein kinase that controls cellular metabolism and growth, in part, by stimulatory effects on protein translation (Düvel et al., 2010; Saxton and Sabatini, 2017). While the regulation of mTORC1 signaling is dependent on cell type and proliferative state (Laplante and Sabatini, 2012), it is also effected by metabolic stress in muscle fibers (Goodman et al., 2012). mTORC1 is activated by recruitment to the lysosomal surface where it interacts with small GTP-binding proteins called Rags or RHEB, which control mTORC1 activity as a function of amino acid availability or growth factor signaling, respectively (**Figure 1A**). The GTP-bound state of RHEB is controlled by the phosphatidylinositol 3-kinase/AKT signaling pathway, which inhibits the guanosine triphosphatase-activating protein (GAP) function of TSC1/2 toward RHEB, to permit mTORC1 activation (Inoki et al., 2002; Tee et al., 2002). In contrast, intracellular amino acids can regulate the GTP binding state of the Rag proteins by affecting the GAP activity of GATOR1. GATOR1 is an evolutionarily conserved complex comprised of three requisite proteins called nitrogen permease regulator-like 2 (NPRL2), nitrogen permease regulator-like 3 (NPRL3) and DEP domain containing protein 5 (DEPDC5) (Dokudovskaya et al., 2011; Wu and Tu, 2011). Low concentrations of intracellular amino acids cause GATOR1 dissociation from its negative regulatory complex called GATOR2, permitting GATOR1 to catalyze GTP-Rag hydrolysis to GDP-Rag and impair mTORC1 activity (**Figure 1A**) (Bar-Peled et al., 2013). The ability of GATOR2 to repress GATOR1 function is controlled by other proteins that respond to particular amino acids or their derivatives, including: Sestrin (leucine) (Parmigiani et al., 2014), CASTOR (arginine) (Chantranupong et al., 2016), and SAMTOR (S-adenosyl methionine) (Gu et al., 2017). The *in vivo* contribution of these upstream regulators of GATOR1 and their impact on skeletal muscle biology remains to be examined.

While each component of GATOR1 is necessary for embryonic development (Kowalczyk et al., 2012; Dutchak et al., 2015; Hughes et al., 2017), our recent studies show that loss of NPRL2 in skeletal muscle causes constitutive activation of mTORC1, aerobic glycolysis, and increased fast-twitch (type II) fibers in soleus muscle (Dutchak et al., 2018). Our observations, and others, indicate that mTORC1 regulates mitochondrial metabolism and controls mitochondrial-dependent synthesis of aspartate and glutamine for the generation of nitrogen-containing metabolites required for growth, while stimulating aerobic glycolysis to meet the cellular demands of ATP (Laxman et al., 2014; Birsoy et al., 2015; Chen et al., 2017; Dutchak et al., 2018). Importantly, the amino acids that activate mTORC1 can function as anaplerotic substrates in the mitochondria, consistent with these metabolites having an active role in regulating cellular homeostasis. During growth and proliferative stages, oxaloacetate, and α-ketoglutarate are converted to aspartate and glutamine in order to promote protein and nucleotide biosynthesis, rather than being used for oxidative metabolism (Dibble and Manning, 2013). If they are consumed for biosynthesis, they are no longer available for the generation of ATP by the mitochondria, and so the cells must upregulate

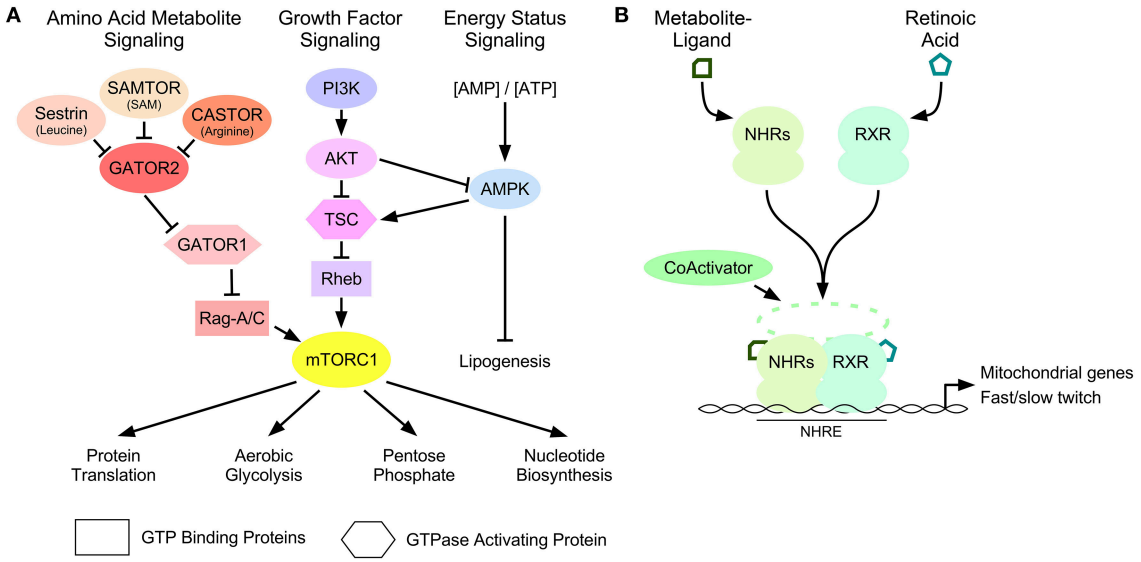

FIGURE 1 | Metabolite-dependent mechanisms that influence muscle fiber composition. **(A)** The mTORC1 signal transduction pathway is controlled by amino acid-, growth factor- and energy-dependent signaling mechanisms. Amino acid-dependent signaling through Sestrin, CASTOR, and SAMTOR, repress GATOR2-dependent inhibition of GATOR1 GAP activity toward Rag GTP-binding proteins. Growth factor signaling through the phosphatidylinositol 3 kinase (PI3K): AKT pathway inhibits the GAP activity of the tuberous sclerosis complex (TSC) toward Rheb. These pathways lead to the stimulation of mTORC1 activity. Cellular energy status controls mTORC1 through a regulatory loop between the growth factor signaling pathway and the sensor of AMP called AMPK. Downstream targets of mTORC1 control protein translation and metabolic pathways that provide the substrates required for growth. **(B)** Ligand activation of nuclear hormone receptors (NHRs), including PPARδ and TRα, and form a heterodimer with retinoic acid receptor (RXR) that recruits coactivator proteins to the nuclear hormone response elements (NHRE) of the promoters of genes involved in mitochondrial gene expression and skeletal muscle fiber-type switching.

glycolysis, leading to the Warburg effect. The selective nature of GATOR1 to respond to particular amino acids and subsequently influence carbohydrate and amino acid metabolism in the mitochondria highlights a fundamental and conserved aspect of metabolic homeostasis. It will be of interest for future studies to examine the contribution of individual amino acids toward regulating the glycolytic and oxidative pathways in different phases of skeletal muscle development.

METABOLITE-DEPENDENT TRANSCRIPTIONAL REGULATORS OF FIBER COMPOSITION

Long-term changes in skeletal muscle fiber composition are controlled by transcriptional mechanisms that regulate particular genetic programs important to each fiber type (Braun and Gautel, 2011). Early studies helped define signal transduction cascades as the major regulatory mechanism of transcription factors that contributes to fiber composition, like the calcineurin-dependent regulation of nuclear factor of activated T cells (NFAT) and myocyte enhancer factor 2 (MEF2) transcription factors, involved in the fast-to-slow twitch fiber transformation initiated by muscle contraction (Sreter et al., 1987; Kubis et al., 1997; Chin et al., 1998; Anderson et al., 2015). More recently, nuclear hormone receptor transcription factors have been shown to influence the type of fibers expressed in skeletal muscle. This class of transcription factors provide a direct link between intracellular metabolites and genomic expression as their ability to regulate gene transcription is dependent on ligand-activation by small molecules, or metabolites (**Figure 1B**) (Evans and Mangelsdorf, 2014).

A well categorized group of nuclear hormone receptors known as peroxisome-proliferating activated receptors (PPARs) are able to control cellular differentiation and metabolism when bound to their lipid-ligands. In skeletal muscle, PPARδ regulates genes important for fatty acid transport and oxidation, increasing lipid catabolism for energy production (Tanaka et al., 2003). Transgenic expression of lipid-activated PPARδ in skeletal muscle results in "marathon mice," with increased slow-twitch oxidative muscle fibers, decreased fast-twitch fibers, and resistance to weight gain when fed high-fat diets that cause normal mice to become obese (Wang et al., 2004). Further transcriptional studies using pharmacological ligands to activate PPARδ showed it can induce the expression of its transcriptional co-activator called peroxisome proliferator activated-receptor-gamma co-activator-1 (PGC-1α) in muscle, which regulates mitochondrial gene expression (Lin et al., 2005; Hondares et al., 2007).

PGC1α is a co-activator of nuclear hormone receptors that can drive the formation of oxidative fiber-types and regulate the expression of specific genes important for oxidative metabolism (Lin et al., 2002; Olesen et al., 2010; Fernandez-Marcos and Auwerx, 2011). Overexpression of PGC-1α, using the muscle creatine kinase promoter, cause type II fibers to exhibit characteristics of type I fibers, with more myoglobin, troponin

I (slow) and resistance to electrically stimulated fatigue (Lin et al., 2002). In an opposite manner, PGC-1α skeletal muscle knockout mice show a shift from type I and type Ia, to type IIx and IIb fibers (Handschin et al., 2007). PGC-1α activation is controlled by post-translational modification, including: (1) cell signaling networks, including AMPK, p38, PKA, AKT, (2) acetylation/deacetylation by GCN5 and SIRT1, respectively, and (3) methylation by PRMT1 (Fernandez-Marcos and Auwerx, 2011). Recently, studies of PGC-1α have identified multiple splice isoforms of the gene, and shown that novel variant called PGC-1α4 is increased with strength training (Ruas et al., 2012; Ydfors et al., 2013; Chan and Arany, 2014). Isoform PGC-1α4 regulates targets involved in two major signaling pathways, IGF1 signaling and myostatin, to promote strength and size of skeletal muscle (Ruas et al., 2012), in contrast to PGC-1α isoform 1 that promotes oxidative fibers (Lin et al., 2002).

The thyroid hormone nuclear receptor is activated by triiodothyronine (T3), a product of tyrosine metabolism, and contributes to skeletal muscle energy metabolism by regulating the transcription of mitochondrial genes and stimulating fiber type switching (Brent, 2012; De Andrade et al., 2015). T3 regulates the transcription of both nuclear and mitochondrial genes by binding to nuclear thyroid hormone receptors (TRα and TRβ) or a truncated forms of TRα called p43, located in the mitochondrial matrix (Brent, 2012; Lombardi et al., 2015). In mitochondrial matrix, T3 binding to p43 promotes transcription of mitochondrial genes involved in slow-twitch fiber metabolism, whereas p43 depletion has been shown to induce a switch to fast-twitch fibers and cause muscle hypertrophy (Pessemesse et al., 2011).

These studies highlight the importance of ligand-activated transcription factors and their co-activators in regulating mitochondrial biogenesis and fiber composition in muscle depots. Future approaches to refine the complex transcriptional networks involved in skeletal muscles physiology will benefit by using tissue specific models, as above, because confounding metabolic phenotypes can occur with whole body-knockout and transgenic expression systems.

FUTURE PERSPECTIVES

The metabolic contribution to skeletal muscle fiber type composition is an important consideration for human health and disease. By combining scientific observations from exercise physiology to biochemistry, we are beginning to understand the logical basis of the intertwined nature of metabolism and skeletal muscle physiology. Important breakthroughs in metabolite-dependent cell signaling and transcriptional programs have highlighted new functions for metabolites in the muscle, well beyond that of the basic building blocks of cellular components. The mechanisms that drive these metabolite-dependent pathways to regulate short-term changes in glycolytic and oxidative metabolism in accordance with longer-term changes in fiber type composition will be important for future investigation. The phenotypic differences in skeletal muscle fiber composition that are caused by genetic alterations of these metabolite-dependent regulatory pathways, detailed above, suggest that different fiber type phenotypes ultimately emerge from differences in these metabolic effector networks. It will also be of interest to characterize the initial differences in metabolic-regulatory pathways in fiber types before switching occurs, to help determine why certain fiber types and muscle depots are more responsive to adaptation. How cells cope with transient fluctuations in metabolites is an important consideration that must be viewed in the context of the proliferation and growth of the cells.

Future progress to elucidate the fiber type specific mechanisms that regulate energy metabolism and control growth will help our understanding of how heterogeneous tissues can respond to their ever changing environment. In particular, metabolic mechanisms that regulate specific muscle fiber types will have important consequences for understanding human health and metabolism-related muscular atrophies, including cancer cachexia, sepsis, and diabetes. New discoveries in muscle may also further our understanding of oncogenic events, aging and neurodegenerative diseases like amyotrophic lateral sclerosis, Huntington's disease and Alzheimer's disease.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Adams, G. R., Hather, B. M., Baldwin, K. M., and Dudley, G. A. (1993). Skeletal muscle myosin heavy chain composition and resistance training. *J. Appl. Physiol.* 74, 911–915. doi: 10.1152/jappl.1993.74.2.911

Anderson, C. M., Hu, J., Barnes, R. M., Heidt, A. B., Cornelissen, I., and Black, B. L. (2015). Myocyte enhancer factor 2C function in skeletal muscle is required for normal growth and glucose metabolism in mice. *Skele. Muscle* 5:7. doi: 10.1186/s13395-015-0031-0

Anderson, E. J., and Neufer, P. D. (2006). Type II skeletal myofibers possess unique properties that potentiate mitochondrial H(2)O(2) generation. *Am. J. Physiol. Cell Physiol.* 290, C844–C851. doi: 10.1152/ajpcell.00402.2005

Bar-Peled, L., Chantranupong, L., Cherniack, A. D., Chen, W. W., Ottina, K. A., Grabiner, B. C., et al. (2013). A tumor suppressor complex with GAP activity for the Rag GTPases that signal amino acid sufficiency to mTORC1. *Science* 340, 1100–1106. doi: 10.1126/science.1232044

Berdeaux, R., and Stewart, R. (2012). cAMP signaling in skeletal muscle adaptation: hypertrophy, metabolism, and regeneration. *Am. J. Physiol. Endocrinol. Metabol.* 303, E1–E17. doi: 10.1152/ajpendo.00555.2011

Birsoy, K., Wang, T., Chen, W. W., Freinkman, E., Abu-Remaileh, M., and Sabatini, D. M. (2015). An essential role of the mitochondrial electron transport chain in cell proliferation is to enable aspartate synthesis. *Cell* 162, 540–551. doi: 10.1016/j.cell.2015.07.016

Braun, T., and Gautel, M. (2011). Transcriptional mechanisms regulating skeletal muscle differentiation, growth and homeostasis. *Nat. Rev. Mole. Cell Biol.* 12, 349–361. doi: 10.1038/nrm3118

Brent, G. A. (2012). Mechanisms of thyroid hormone action. *J. Clin. Invest.* 122, 3035–3043. doi: 10.1172/JCI60047

Chan, M. C., and Arany, Z. (2014). The many roles of PGC-1alpha in muscle–recent developments. *Metabol. Clin. Exp.* 63, 441–451. doi: 10.1016/j.metabol.2014.01.006

Chantranupong, L., Scaria, S. M., Saxton, R. A., Gygi, M. P., Shen, K., Wyant, G. A., et al. (2016). The CASTOR proteins are arginine sensors for the mTORC1 Pathway. *Cell* 165, 153–164. doi: 10.1016/j.cell.2016.02.035

Chen, J., Sutter, B. M., Shi, L., and Tu, B. P. (2017). GATOR1 regulates nitrogenic cataplerotic reactions of the mitochondrial TCA cycle. *Nat. Chem. Biol.* 13, 1179–1186. doi: 10.1038/nchembio.2478

Chin, E. R., Olson, E. N., Richardson, J. A., Yang, Q., Humphries, C., Shelton, J. M., et al. (1998). A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. *Gene. Develop.* 12, 2499–2509. doi: 10.1101/gad.12.16.2499

De Andrade, P. B., Neff, L. A., Strosova, M. K., Arsenijevic, D., Patthey-Vuadens, O., Scapozza, L., et al. (2015). Caloric restriction induces energy-sparing alterations in skeletal muscle contraction, fiber composition and local thyroid hormone metabolism that persist during catch-up fat upon refeeding. *Front. Physiol.* 6:254. doi: 10.3389/fphys.2015.00254

Dibble, C. C., and Manning, B. D. (2013). Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. *Nat. Cell Biol.* 15, 555–564. doi: 10.1038/ncb2763

Dokudovskaya, S., Waharte, F., Schlessinger, A., Pieper, U., Devos, D. P., Cristea, I. M. et al. (2011). A conserved coatomer-related complex containing Sec13 and Seh1 dynamically associates with the vacuole in *Saccharomyces cerevisiae*. *Mole. Cell. Proteomics* 10, M110 006478. doi: 10.1074/mcp.M110.006478

Dutchak, P. A., Estill-Terpack, S. J., Plec, A. A., Zhao, X., Yang, C., Chen, J., et al. (2018). Loss of a negative regulator of mTORC1 induces aerobic glycolysis and altered fiber composition in skeletal muscle. *Cell Rep.* 23, 1907–1914. doi: 10.1016/j.celrep.2018.04.058

Dutchak, P. A., Laxman, S., Estill, S. J., Wang, C., Wang, Y., Wang, Y., et al. (2015). Regulation of hematopoiesis and methionine homeostasis by mTORC1 inhibitor NPRL2. *Cell Rep.* 12, 371–379. doi: 10.1016/j.celrep.2015.06.042

Düvel, K., Yecies, J. L., Menon, S., Raman, P., Lipovsky, A. I., Souza, A. L., et al. (2010). Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol. Cell* 39, 171–183. doi: 10.1016/j.molcel.2010.06.022

Egan, B., and Zierath, J. R. (2013). Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell Metabol.* 17, 162–184. doi: 10.1016/j.cmet.2012.12.012

Eshima, H., Tamura, Y., Kakehi, S., Kurebayashi, N., Murayama, T., Nakamura, K., et al. (2017). Long-term, but not short-term high-fat diet induces fiber composition changes and impaired contractile force in mouse fast-twitch skeletal muscle. *Physiol. Rep.* 5:e13250. doi: 10.14814/phy2.13250

Evans, R. M., and Mangelsdorf, D. J. (2014). Nuclear receptors, RXR, and the big bang. *Cell* 157, 255–266. doi: 10.1016/j.cell.2014.03.012

Fernandez-Marcos, P. J., and Auwerx, J. (2011). Regulation of PGC-1alpha, a nodal regulator of mitochondrial biogenesis. *Am. J. Clin. Nutr.* 93, 884S—890. doi: 10.3945/ajcn.110.001917

Fryer, L. G., Foufelle, F., Barnes, K., Baldwin, S. A., Woods, A., and Carling, D. (2002). Characterization of the role of the AMP-activated protein kinase in the stimulation of glucose transport in skeletal muscle cells. *Biochem. J.* 363, 167–174. doi: 10.1042/bj3630167

Gaitanos, G. C., Williams, C., Boobis, L. H., and Brooks, S. (1993). Human muscle metabolism during intermittent maximal exercise. *J. Appl. Physiol.* 75, 712–719. doi: 10.1152/jappl.1993.75.2.712

Goodman, C. A., Kotecki, J. A., Jacobs, B. L., and Hornberger, T. A. (2012). Muscle fiber type-dependent differences in the regulation of protein synthesis. *PLoS ONE* 7:e37890. doi: 10.1371/journal.pone.0037890

Green, H. J., Helyar, R., Ball-Burnett, M., Kowalchuk, N., Symon, S., and Farrance, B. (1992). Metabolic adaptations to training precede changes in muscle mitochondrial capacity. *J. Appl. Physiol.* 72, 484–491. doi: 10.1152/jappl.1992.72.2.484

Gu, X., Orozco, J. M., Saxton, R. A., Condon, K. J., Liu, G. Y., Krawczyk, P. A., et al. (2017). SAMTOR is an S-adenosylmethionine sensor for the mTORC1 pathway. *Science* 358, 813–818. doi: 10.1126/science.aao3265

Handschin, C., Chin, S., Li, P., Liu, F., Maratos-Flier, E., Lebrasseur, N. K., et al. (2007). Skeletal muscle fiber-type switching, exercise intolerance, and myopathy in PGC-1alpha muscle-specific knock-out animals. *J. Biol. Chem.* 282, 30014–30021. doi: 10.1074/jbc.M704817200

Hondares, E., Pineda-Torra, I., Iglesias, R., Staels, B., Villarroya, F., and Giralt, M. (2007). PPARdelta, but not PPARalpha, activates PGC-1alpha gene transcription in muscle. *Biochem. Biophys. Res. Commun.* 354, 1021–1027. doi: 10.1016/j.bbrc.2007.01.092

Hughes, J., Dawson, R., Tea, M., McAninch, D., Piltz, S., Jackson, D., et al. (2017). Knockout of the epilepsy gene Depdc5 in mice causes severe embryonic dysmorphology with hyperactivity of mTORC1 signalling. *Sci. Rep.* 7:12618. doi: 10.1038/s41598-017-12574-2

Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K. L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat. Cell Biol.* 4, 648–657. doi: 10.1038/ncb839

Jørgensen, S. B., Richter, E. A., and Wojtaszewski, J. F. (2006). Role of AMPK in skeletal muscle metabolic regulation and adaptation in relation to exercise. *J. Physiol.* 574, 17–31. doi: 10.1113/jphysiol.2006.109942

Kowalczyk, M. S., Hughes, J. R., Babbs, C., Sanchez-Pulido, L., Szumska, D., Sharpe, J. A., et al. (2012). Nprl3 is required for normal development of the cardiovascular system. *Mamm. Genome* 23, 404–415. doi: 10.1007/s00335-012-9398-y

Kubis, H. P., Haller, E. A., Wetzel, P., and Gros, G. (1997). Adult fast myosin pattern and Ca2+-induced slow myosin pattern in primary skeletal muscle culture. *Proc. Nat. Acad. Sci. U.S.A.* 94, 4205–4210. doi: 10.1073/pnas.94.8.4205

Laplante, M., and Sabatini, D. M. (2012). mTOR signaling in growth control and disease. *Cell* 149, 274–293. doi: 10.1016/j.cell.2012.03.017

Laxman, S., Sutter, B. M., Shi, L., and Tu, B. P. (2014). Npr2 inhibits TORC1 to prevent inappropriate utilization of glutamine for biosynthesis of nitrogen-containing metabolites. *Sci. Signal.* 7:ra120. doi: 10.1126/scisignal.2005948

Lin, J., Handschin, C., and Spiegelman, B. M. (2005). Metabolic control through the PGC-1 family of transcription coactivators. *Cell Metabol.* 1, 361–370. doi: 10.1016/j.cmet.2005.05.004

Lin, J., Wu, H., Tarr, P. T., Zhang, C.-Y., Wu, Z., Boss, O., et al. (2002). Transcriptional co-activator PGC-1α drives the formation of slow-twitch muscle fibres. *Nature* 418, 797–801. doi: 10.1038/nature00904

Lombardi, A., Moreno, M., De Lange, P., Iossa, S., Busiello, R. A., and Goglia, F. (2015). Regulation of skeletal muscle mitochondrial activity by thyroid hormones: focus on the "old" triiodothyronine and the "emerging" 3,5-diiodothyronine. *Front. Physiol.* 6:237. doi: 10.3389/fphys.2015.00237

Lunt, S. Y., and Vander Heiden, M. G. (2011). Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu. Rev. Cell Dev. Biol.* 27, 441–464. doi: 10.1146/annurev-cellbio-092910-154237

Mishra, P., Varuzhanyan, G., Pham, A. H., and Chan, D. C. (2015). Mitochondrial dynamics is a distinguishing feature of skeletal muscle fiber types and regulates organellar compartmentalization. *Cell Metabol.* 22, 1033–1044. doi: 10.1016/j.cmet.2015.09.027

Mounier, R., Lantier, L., Leclerc, J., Sotiropoulos, A., Foretz, M., and Viollet, B. (2011). Antagonistic control of muscle cell size by AMPK and mTORC1. *Cell Cycle* 10, 2640–2646. doi: 10.4161/cc.10.16.17102

Olesen, J., Kiilerich, K., and Pilegaard, H. (2010). PGC-1α-mediated adaptations in skeletal muscle. *Pflügers Arch.* 460, 153–162. doi: 10.1007/s00424-010-0834-0

Parmigiani, A., Nourbakhsh, A., Ding, B., Wang, W., Kim, Y. C., Akopiants, K., et al. (2014). Sestrins inhibit mTORC1 kinase activation through the GATOR complex. *Cell Rep.* 9, 1281–1291. doi: 10.1016/j.celrep.2014.10.019

Pessemesse, L., Schlernitzauer, A., Sar, C., Levin, J., Grandemange, S., Seyer, P., et al. (2011). Depletion of the p43 mitochondrial T3 receptor in mice affects skeletal muscle development and activity. *FASEB J.* 26, 748–756. doi: 10.1096/fj.11-195933

Philp, A., Hamilton, D. L., and Baar, K. (2011). Signals mediating skeletal muscle remodeling by resistance exercise: PI3-kinase independent activation of mTORC1. *J. Appl. Physiol.* 110, 561–568. doi: 10.1152/japplphysiol.00941.2010

Picard, M., Csukly, K., Robillard, M. E., Godin, R., Ascah, A., Bourcier-Lucas, C., et al. (2008). Resistance to Ca2+-induced opening of the permeability transition pore differs in mitochondria from glycolytic and oxidative muscles. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 295, R659–R668. doi: 10.1152/ajpregu.90357.2008

Ruas, J. L., White, J. P., Rao, R. R., Kleiner, S., Brannan, K. T., Harrison, B. C., et al. (2012). A PGC-1α isoform induced by resistance training regulates skeletal muscle hypertrophy. *Cell* 151, 1319–1331. doi: 10.1016/j.cell.2012.10.050

Saxton, R. A., and Sabatini, D. M. (2017). mTOR signaling in growth, metabolism, and disease. *Cell* 169, 361–371. doi: 10.1016/j.cell.2017.03.035

Schiaffino, S., and Reggiani, C. (2011). Fiber types in mammalian skeletal muscles. *Physiol. Rev.* 91, 1447–1531. doi: 10.1152/physrev.00031.2010

Sreter, F. A., Lopez, J. R., Alamo, L., Mabuchi, K., and Gergely, J. (1987). Changes in intracellular ionized Ca concentration associated with muscle fiber type transformation. *Am. J. Physiol.* 253, C296–C300. doi: 10.1152/ajpcell.1987.253.2.C296

Szent-Györgyi, A. G. (2004). The early history of the biochemistry of muscle contraction. *J. Gen. Physiol.* 123, 631–641. doi: 10.1085/jgp.200409091

Tanaka, T., Yamamoto, J., Iwasaki, S., Asaba, H., Hamura, H., Ikeda, Y., et al. (2003). Activation of peroxisome proliferator-activated receptor δ induces fatty acid β-oxidation in skeletal muscle and attenuates metabolic syndrome. *Proc. Nat. Acad. Sci. U.S.A.* 100, 15924–15929. doi: 10.1073/pnas.0306981100

Tee, A. R., Fingar, D. C., Manning, B. D., Kwiatkowski, D. J., Cantley, L. C., and Blenis, J. (2002). Tuberous sclerosis complex-1 and−2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. *Proc. Nat. Acad. Sci. U.S.A.* 99, 13571–13576. doi: 10.1073/pnas.202476899

Wang, Y. X., Zhang, C. L., Yu, R. T., Cho, H. K., Nelson, M. C., Bayuga-Ocampo, C. R., et al. (2004). Regulation of muscle fiber type and running endurance by PPARdelta. *PLoS Biol.* 2:e294. doi: 10.1371/journal.pbio.0020294

Warburg, O., Wind, F., and Negelein, E. (1927). The metabolism of tumors in the body. *J. Gen. Physiol.* 8, 519–530. doi: 10.1085/jgp.8.6.519

Widrick, J. J., Stelzer, J. E., Shoepe, T. C., and Garner, D. P. (2002). Functional properties of human muscle fibers after short-term resistance exercise training. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 283, R408–R416. doi: 10.1152/ajpregu.00120.2002

Wu, X., and Tu, B. P. (2011). Selective regulation of autophagy by the Iml1-Npr2-Npr3 complex in the absence of nitrogen starvation. *Mole. Biol. Cell* 22, 4124–4133. doi: 10.1091/mbc.e11-06-0525

Ydfors, M., Fischer, H., Mascher, H., Blomstrand, E., Norrbom, J., and Gustafsson, T. (2013). The truncated splice variants, NT-PGC-1alpha and PGC-1alpha4, increase with both endurance and resistance exercise in human skeletal muscle. *Physiol. Rep.* 1:e00140. doi: 10.1002/phy2.140

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MINI REVIEW
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Endothelial Cell Metabolism in Atherosclerosis

Kosta Theodorou[1] *and Reinier A. Boon*[1,2,3]*

[1] Centre of Molecular Medicine, Institute of Cardiovascular Regeneration, Goethe-University, Frankfurt am Main, Germany, [2] German Center for Cardiovascular Research DZHK, Partner Site Rhine-Main, Berlin, Germany, [3] Department of Physiology, Amsterdam Cardiovascular Sciences, VU University Medical Center, Amsterdam, Netherlands

Atherosclerosis and its sequelae, such as myocardial infarction and stroke, are the leading cause of death worldwide. Vascular endothelial cells (EC) play a critical role in vascular homeostasis and disease. Atherosclerosis as well as its independent risk factors including diabetes, obesity, and aging, are hallmarked by endothelial activation and dysfunction. Metabolic pathways have emerged as key regulators of many EC functions, including angiogenesis, inflammation, and barrier function, processes which are deregulated during atherogenesis. In this review, we highlight the role of glucose, fatty acid, and amino acid metabolism in EC functions during physiological and pathological states, specifically atherosclerosis, diabetes, obesity and aging.

Keywords: endothelial cells, metabolism, atherosclerosis, inflammation, hyperglycemia, hyperlipidemia, aging

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Centro Nacional de Investigaciones Cardiovasculares (CNIC), Spain

***Correspondence:**
Reinier A. Boon
boon@med.uni-frankfurt.de

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INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality worldwide (World Health Organization, 2014). The underlying pathology of the majority of cardiovascular-related deaths is atherosclerosis, which is a lipid-driven chronic inflammatory disease of the medium- and large-sized arteries (Lusis, 2000). One of the earliest detectable changes in the development of atherosclerosis is endothelial cell (EC) activation and dysfunction at lesion-prone areas of the arterial vasculature (Hajra et al., 2000). These areas, particularly at bends and branch points, are characterized by ECs that display a pro-inflammatory, pro-thrombotic phenotype with a reduced barrier function, which is prompted by disturbed blood flow dynamics (Tabas et al., 2015). Moreover, subendothelial retention and modification of apolipoprotein B-containing lipoproteins, such as low-density lipoproteins (LDL), further activate ECs. This triggers an inflammatory response, leading to the recruitment of monocytes into the intima where they differentiate into macrophages and engulf modified lipoproteins to become foam cells. Progressed atherosclerotic lesions are characterized by a fibrous cap overlaying a lipid rich, necrotic core, and an accumulation of leukocytes in the lateral edges, which promote plaque instability by modulating EC phenotype and through proteolytic degradation of extracellular matrix (ECM) components. Unstable lesions may, upon rupture, lead to an atherothrombotic event, causing clinical events such as myocardial infarction or stroke. Alternatively, superficial intimal erosions, as a consequence of EC apoptosis and detachment, may also trigger an atherothrombotic event and its clinical sequelae (Quillard et al., 2015; Tabas et al., 2015).

Although initially believed to be an inert, semi-permeable barrier between blood constituents, and the underlying subendothelial tissues, the endothelium is now viewed as a metabolically active organ that plays a crucial role in vascular homeostasis and throughout the life history of atherosclerosis. In this review, we will focus on EC metabolism in health and atherosclerosis development. First, we will briefly review the three major metabolic pathways [i.e., glucose, fatty acids (FA) and amino acids (AA)] in ECs, followed by the role of EC metabolism in atherosclerosis.

ENDOTHELIAL CELL METABOLISM IN HEALTH

To date, studies investigating EC metabolism have been limited to using cultured ECs, whose metabolism may be rewired because of *in vitro* conditions that do not fully recapitulate the *in vivo* environment (Hensley et al., 2016; Cantor et al., 2017). Nevertheless, great strides have been made in dissecting the roles of metabolism in EC functions by conditional targeting of key metabolic genes *in vivo*.

Glucose Metabolism

Glycolysis is the main energy supplier in cultured ECs, accounting for ∼75–85% of the total ATP production (Krützfeldt et al., 1990; De Bock et al., 2013). It has been estimated that isolated coronary microvascular ECs metabolize ∼99% of glucose into lactate under aerobic conditions, while only 0.04% is oxidized in the tricarboxylic acid (TCA) cycle (Krützfeldt et al., 1990). Indeed, inhibiting glycolysis using 2-deoxy-D-glucose induces EC cytotoxicity, indicating that glucose is essential for proper EC functioning and maintenance (Merchan et al., 2010).

Upon stimulation with pro-angiogenic signals, like vascular endothelial growth factor (VEGF) and fibroblast growth factor 2, ECs increase their glycolytic flux to support proliferation and migration (De Bock et al., 2013; Yu et al., 2017). In contrast, limiting glycolysis by pharmacological inhibition or genetic silencing of phosphofructokinase-2/fructose-2,6-biphosphatase 3 (PFKFB3) or silencing of hexokinase (HK)2 impairs EC proliferation, migration and vascular sprouting (De Bock et al., 2013; Schoors et al., 2014; Yu et al., 2017). Moreover, endothelial-specific PFKFB3 or HK2 deficiency causes vascular hypobranching in mice (De Bock et al., 2013; Yu et al., 2017).

Apart from using glucose for energy production, there are also alternative fates for glucose in ECs (Lunt and Vander Heiden, 2011; **Figure 1**). After glucose is taken up by ECs through facilitated diffusion by the glucose transporters (GLUT), primarily by GLUT1, glucose is phosphorylated by HK to glucose-6-phosphate (G6P) (Mann et al., 2003; Lunt and Vander Heiden, 2011). G6P can be converted to and stored as glycogen or processed in the oxidative branch of the pentose phosphate pathway (oxPPP) to yield nicotinamide adenine dinucleotide phosphate (NADPH) and ribose-5-phosphate (R5P), which are used for antioxidant defense and nucleotide biosynthesis, respectively (Lunt and Vander Heiden, 2011; Adeva-Andany et al., 2016). Silencing of the G6P dehydrogenase (G6PD), the rate-limiting enzymes of oxPPP, reduces EC proliferation and migration, and increases cellular reactive oxygen species (ROS) (Leopold et al., 2003). Furthermore, ECs contain glycogen reservoirs and pharmacological inhibition of glycogen phosphorylase, the rate-limiting enzyme in glycogen degradation, impairs EC migration and viability (Vizán et al., 2009). However, how glycogen metabolism affects EC functions remains to be determined.

G6P can also be further metabolized to fructose-6-phosphate (F6P), which can be shunted into the hexosamine biosynthesis pathway (HBP) to produce UDP-N-acetylglucosamine (UDP-GlcNAc), an important substrate for protein O-linked glycosylation that is critical for a plethora of EC functions (Laczy et al., 2009). Silencing of the rate-limiting enzyme in the HBP, glutamine:fructose-6-phosphate amidotransferase 1, augments VEGF-induced vascular sprouting (Zibrova et al., 2017). Furthermore, the two glycolysis intermediates F6P and glyceraldehyde-3-phosphate (G3P) can be used in the non-oxidative branch of the pentose phosphate pathway (non-oxPPP) to generate R5P, but not NADPH, in contrast to oxPPP (Lunt and Vander Heiden, 2011). Pharmacological inhibition of transketolase, the rate-limiting enzyme in non-oxPPP, limits ECs viability, and migration (Vizán et al., 2009).

ECs have a relatively low mitochondrial content (<2–12% of the total cellular volume) compared to other cell types, such as hepatocytes (28%) and cardiomyocytes (22–37%, depending on the species; Blouin et al., 1977; Oldendorf et al., 1977; Barth et al., 1992). This is also reflected by the lower mitochondrial respiration of ECs compared to more oxidative cell types, like hepatocytes and cardiomyocytes (De Bock et al., 2013). Nevertheless, ECs have a considerable spare mitochondrial respiratory capacity (Doddaballapur et al., 2015; Wilhelm et al., 2016), which may be called upon during stress conditions like glucose deprivation to metabolize alternative substrates, such as FAs and glutamine (Krützfeldt et al., 1990; Mertens et al., 1990), however this remains to be determined. Cultured ECs derive only ∼15% of the total amount of ATP via oxidative pathways, suggesting that rather by playing a major role in energy production, mitochondria in ECs are more likely to have a signaling function (by producing ROS and maintaining intracellular Ca^{2+} homeostasis) and support biomass synthesis (by generating metabolic intermediates; Quintero et al., 2006; De Bock et al., 2013; Tang et al., 2014; Schoors et al., 2015; Huang et al., 2017).

Fatty Acid Metabolism

FAs in cultured ECs act as a carbon source for the production of the AA aspartate (a nucleotide precursor) and deoxyribonucleotides which are required for DNA synthesis, rather than supplying energy (accounting for <5% of the total amount of ATP produced) or maintaining redox homeostasis (**Figure 1**; Schoors et al., 2015). Endothelial-specific deficiency or silencing of carnitine palmitoyltransferase (CPT)1A, the rate-limiting enzyme in FA oxidation (FAO), causes vascular sprouting defects *in vivo* and *in vitro* owing to a reduction in proliferation, but not migration (Schoors et al., 2015). In addition, pharmacological inhibition of CPT1 or silencing CPT1A or CPT2 reduces FAO and enhances endothelial permeability (Patella et al., 2015; Xiong et al., 2018). Furthermore, during lymphatic EC (LEC) differentiation, LECs upregulate CPT1A to support their proliferation, but also to promote their differentiation through acetyl-coenzyme A (acetyl-CoA) production, which is used for histone acetylation of lymphatic genes (Wong et al., 2017). Moreover, FAO maintains the cellular pool of acetyl-CoA and retains the identity of vascular ECs by reducing transforming growth factor β-induced endothelial-to-mesenchymal transition (EndMT) (Xiong et al., 2018). In addition to its role in EC proliferation, differentiation and permeability, FA metabolism also modulates the lipid

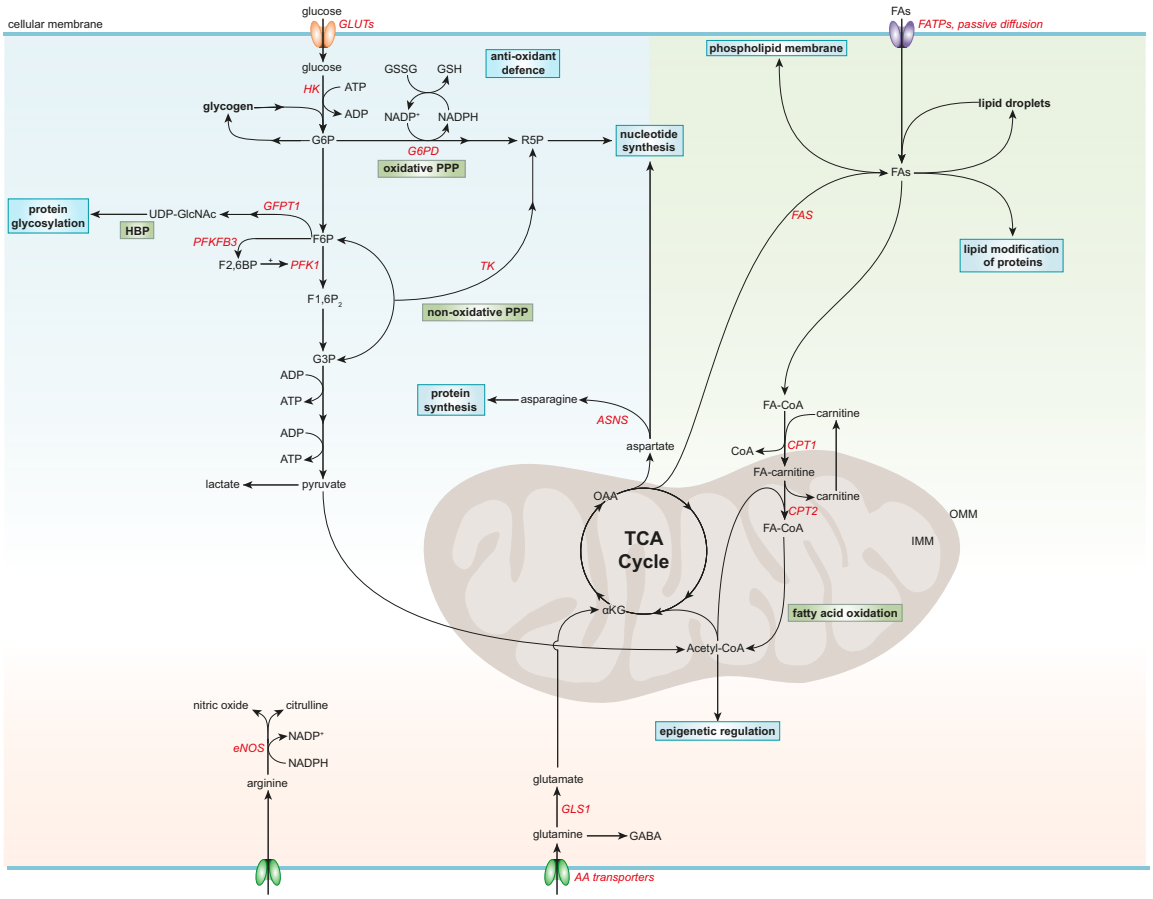

FIGURE 1 | Simplified general overview of glucose, fatty acid, and amino acid metabolism in healthy endothelial cells. Glucose enters ECs via glucose transporters (GLUTs), which is converted to glucose-6-phosphate (G6P) by hexokinase (HK) at the expense of adenosine triphosphate (ATP). G6P can be converted to glycogen for storage or used in the oxidative pentose phosphate pathway (PPP). The oxidative PPP generates reduced glutathione (GSH) and ribose-5-phosphate (R5P), which are used in the anti-oxidant defense and nucleotide synthesis, respectively. G6P can be further metabolized to fructose-6-phosphate (F6P) which is converted to uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), a substrate used for protein glycosylation, in the hexosamine biosynthesis pathway (HBP). F6P can also be converted to fructose-2,6-bisphophate (F2,6BP) by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKF3B), the main isoform in ECs. F2,6BP is a positive allosteric regulator of phosphofructokinase 1 (PFK1), which converts F6P to fructose-1,6-bisphophate ($F1,6P_2$), which is further metabolized to glyceraldehyde-3-phosphate (G3P). G3P together with F6P can be used in the non-oxidative PPP by transketolase (TK) to eventually produce R5P. G3P can also undergo several conversion steps leading to the production of ATP from adenosine diphosphate (ADP) and pyruvate, which can be further metabolized to lactate. After being taken up by fatty acid (FA) transporters (FATPs) or through passive diffusion, FAs become metabolically activated by coupling to coenzyme A (CoA). For FA oxidation (FAO) to occur, FAs have to be imported into the mitochondria, which is carried out by the acyl-carnitine shuttle system. At the outer mitochondrial membrane (OMM), carnitine palmitoyltransferase 1 (CPT1) converts FA-CoAs into FA-carnitines which facilitates the transport of FAs across the inner mitochondrial membrane (IMM) where they are converted back to FA-CoA by CPT2 (located in the IMM). FAO generates ATP and acetyl-CoA which is used in the tricarboxylic acid cycle (TCA). TCA intermediates are used for nucleotide synthesis or FA production, facilitated by fatty acid synthase (FAS). FAs can be stored in lipid droplets, converted to phospholipids to maintain the cell membrane or used for lipid modification of proteins. The amino acid (AA) glutamine is the most consumed AA in ECs. Once inside the cell, glutamine is converted to glutamate by glutaminase 1 (GLS1), which is further metabolized to α-ketoglutarate (αKG), a key intermediate in the TCA cycle. Through several metabolic steps, αKG is converted to oxaloacetic acid (OAA), which can be used for the generation of the AA aspartate. Aspartate can be used as a precursor for nucleotide synthesis or converted to the AA asparagine by asparagine synthetase (ASNS) using an ammonia group from glutamine thereby generating glutamate. Another important AA in ECs is arginine, which is used for the generation of the anti-atherogenic gaseous molecule nitric oxide using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. G6PD, glucose-6-phosphate dehydrogenase; GFPT1, glutamine:fructose-6-phosphate amidotransferase 1; GSSG, oxidized glutathione; $NADP^+$, nicotinamide adenine dinucleotide.

composition of EC membranes, thereby regulating membrane stiffness and multiple cellular functions (Caires et al., 2017; Glatzel et al., 2018; Harayama and Riezman, 2018).

Besides using FAs for their own needs, ECs regulate the transport of FAs toward metabolically active tissues, such as skeletal and cardiac muscle (Mehrotra et al., 2014). Circulating FAs, either bound to albumin or locally released from triglyceride-rich lipoproteins through lipoprotein lipase-mediated lipolysis at the luminal surface of ECs, can enter ECs via passive diffusion or by FA transporter proteins. Interestingly, ECs readily store FAs in lipid droplets as a protective measure against endoplasmic reticulum (ER) stress (Kuo et al., 2017).

Furthermore, FAs can be liberated from lipid droplets, which can be used by the ECs themselves or released to the underlying tissues (Kuo et al., 2017).

Although there is a continuous supply of FA from the blood stream that can enter cells, ECs also have the capability to synthesize FA *de novo*, since they express FA synthase (FAS; Wei et al., 2011; Hagberg et al., 2013). Silencing or genetic deletion of FAS impairs EC migration, vascular sprouting and permeability, and proper endothelial nitric oxide synthase (eNOS) functioning by reducing its palmitoylation (Wei et al., 2011).

Amino Acid Metabolism

Glutamine is the most consumed AA in ECs and is crucial for angiogenesis both *in vitro* and *in vivo* by contributing to TCA cycle anaplerosis, biomass synthesis and redox homeostasis (Huang et al., 2017; Kim B. et al., 2017). Withdrawal of glutamine, or pharmacological inhibition or knockdown of glutaminase 1 (GLS1), the rate-limiting enzyme in glutaminolysis, impairs EC proliferation, while the role of glutamine in EC migration remains controversial (Huang et al., 2017; Kim B. et al., 2017). Interestingly, supplementation of asparagine in glutamine-depleted conditions restores protein synthesis and EC function (Huang et al., 2017; Pavlova et al., 2018). Moreover, reducing glutamate-dependent asparagine synthesis by silencing asparagine synthase limits EC sprouting (Huang et al., 2017).

Arginine can be converted by eNOS to citrulline and nitric oxide (NO), an endogenous gaseous signaling molecule that has a wide variety of biological properties that maintain vascular homeostasis and are atheroprotective, such as suppression of thrombosis, inflammation and oxidative stress (Tousoulis et al., 2012).

Valine metabolism generates 3-hydroisobutyrate (3-HIB) which promotes transendothelial FA transport and skeletal muscle FA uptake and storage, however how valine and 3-HIB affect EC metabolism and function remains to be determined (Jang et al., 2016).

Furthermore, *in vitro* and *in vivo* restriction of sulfur AAs methionine and cysteine triggers an angiogenic response by promoting endothelial VEGF and hydrogen sulfide production thereby shifting EC metabolism from oxidative metabolism to glycolysis (Longchamp et al., 2018).

ENDOTHELIAL CELL METABOLISM IN ATHEROSCLEROSIS

Endothelial Cell Activation by the Atherosclerotic Microenvironment

ECs remain mostly quiescent throughout adult life, however, they can become activated in response to various physiological and pathological stimuli (Gimbrone and García-Cardeña, 2016; **Figure 2**). Disturbed blood flow dynamics are an important initiating factor of EC activation preceding atherogenesis (Hajra et al., 2000). High unidirectional laminar shear stress (LSS) activates an atheroprotective gene expression program in ECs, including the upregulation of transcription factor Krüppel-like factor 2 (KLF2; Dekker et al., 2002). KLF2 regulates a network of genes that maintain vascular barrier integrity and confer EC quiescence, resulting in an anti-inflammatory, anti-thrombotic EC phenotype (Dekker et al., 2006). Interestingly, high LSS suppresses EC glucose uptake, glycolysis and mitochondrial respiration via a KLF2-dependent mechanism (Doddaballapur et al., 2015).

In contrast, ECs at atheroprone areas of the vasculature are subject to disturbed low LSS and show activation of pro-inflammatory pathways and enhanced expression of glycolytic enzymes (Feng et al., 2017). Low LSS enhances EC glycolysis via a nuclear factor κB (NF-κB)-hypoxia inducible factor 1α–(HIF1α)-dependent axis, despite being in a highly oxygenated environment (Feng et al., 2017; Wu et al., 2017). Moreover, pro-inflammatory cytokines increase glucose uptake and glycolysis in ECs which augments cytokine-induced NF-κB activation, most likely via lactate, however this requires further investigation (Folco et al., 2011; Végran et al., 2011; Cantelmo et al., 2016). Disturbed LSS and pro-inflammatory cytokines also activate the mechanotransducers Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), which promote a pro-inflammatory EC phenotype and atherosclerosis development (Wang K. C. et al., 2016; Wang L. et al., 2016b). Similar to NF-κB, YAP/TAZ signaling promotes EC glycolysis and vice versa (Enzo et al., 2015; Bertero et al., 2016; Kim J. et al., 2017). Partially blocking glycolysis by pharmacological inhibition of PFKFB3 reduces cancer cell adhesion to ECs and improves endothelial barrier function (Cantelmo et al., 2016), which may also limit leukocyte extravasation. Together, pro-inflammatory signaling enhances glycolysis and in turn glycolysis can drive pro-inflammatory programs, thereby forming a vicious cycle resulting in sustained pro-inflammatory signaling in ECs.

Furthermore, rupture-prone human atherosclerotic lesions are characterized by the presence of intraplaque neovascularization and hemorrhage (Virmani et al., 2005). Intraplaque Hypoxia and hemoglobin:haptoglobin complexes activate HIF1α-dependent signaling in macrophages leading to enhanced VEGF secretion, which in turn increases intraplaque angiogenesis, vascular permeability and leukocyte recruitment (Sluimer et al., 2008; Guo et al., 2018).

In contrast to glycolysis, the role of FAO in EC pro-inflammatory responses is not known. On the other hand, FAO maintains EC barrier function (Patella et al., 2015; Xiong et al., 2018). Furthermore, by undergoing EndMT, ECs contribute to the pool of mesenchymal cells within atherosclerotic lesions (Evrard et al., 2016). These EC-derived mesenchymal cells may contribute to plaque instability by enhanced expression and activity of matrix degrading proteins (Chen et al., 2015; Evrard et al., 2016). FAO has been shown to inhibit EndMT (Xiong et al., 2018), however, the role of glycolysis and AA metabolism herein remains to be determined. These studies suggest that endothelial FAO may reduce atherosclerosis development.

Besides its role in glycolysis, the pro-inflammatory YAP/TAZ pathway enhances EC glutaminolysis, suggesting a pro-atherogenic role for glutamine in ECs (Bertero et al., 2016). Interestingly, pharmacological inhibition of GLS1 does not

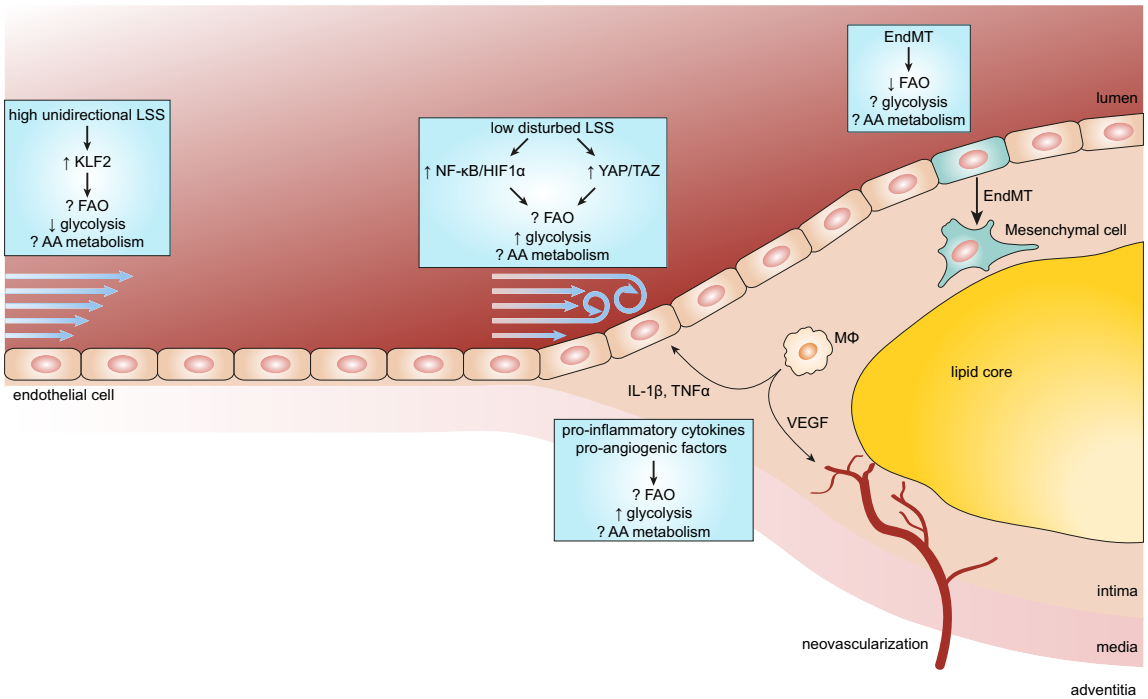

FIGURE 2 | Endothelial metabolism in atherosclerosis. Endothelial cells (ECs) exposed to high unidirectional laminar shear stress (LSS) activate atheroprotective signaling via the transcription factor Krüppel-like factor 2 (KLF2) which reduces glycolysis and maintains ECs in a quiescent state. In contrast, atheroprone regions are subjected to low disturbed LSS enhances EC glycolysis via the nuclear factor κB (NF-κB)/hypoxia inducible factor 1α (HIF1α) axis and mechanotransducers Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ). During the process of atherosclerosis, ECs are exposed to a pro-inflammatory milieu [e.g., cytokines interleukin 1β (IL-1β) and tumor necrosis factor α (TNFα)], which also enhances glycolysis in ECs. In more progressed lesions, macrophages (MΦ) start to secrete pro-angiogenic factors resulting in intraplaque neovascularization. Pro-angiogenic factors, like vascular endothelial growth factor (VEGF), enhance glycolysis in ECs to support proliferation and migration. In addition, reduced FAO in ECs predisposes them to undergo endothelial-to-mesenchymal transition (EndMT), which may affect plaque stability.

affect pro-inflammatory gene expression in or leukocyte adhesion on ECs. Glutamine starvation, however, reduces protein synthesis resulting in ER stress which stimulates pro-inflammatory signaling and apoptosis (Tabas, 2010; Huang et al., 2017). In addition, glutamine deficiency reduces nucleotide synthesis and increased ROS production, resulting in reduced proliferation and increased apoptosis, respectively (Huang et al., 2017). Moreover, ECs have been shown to synthesize the neurotransmitter γ-aminobutyric acid from glutamate, which exerts anti-inflammatory effects in ECs (Sen et al., 2016). Therefore, it remains to be determined how EC glutaminolysis affects atherosclerosis development.

Interestingly, statins, the cholesterol-lowering drugs which are the cornerstone of atherosclerosis treatment, have been shown to increase KLF2 expression, while reducing pro-inflammatory signaling by NF-κB, HIF1α, and YAP/TAZ (Dichtl et al., 2003; Sen-Banerjee et al., 2005; Wang L. et al., 2016b), suggesting that statins may also exert their anti-atherogenic effects by reprogramming EC metabolism.

Deregulated Endothelial Cell Metabolism by Risk Factors for Atherosclerosis

Risk factors, such as diabetes, obesity, and aging, have been shown to accelerate atherosclerosis development and are hallmarked by endothelial dysfunction and deregulated EC metabolism (Kanter et al., 2007; Wang and Bennett, 2012).

Diabetes is characterized by high glucose levels in the circulation which increases endothelial ROS production through auto-oxidation of glucose, NADPH-oxidases, eNOS uncoupling, and mitochondrial dysfunction, leading to DNA damage and subsequent activation of poly(ADP-ribose) polymerase 1 (PARP1) (Du et al., 2003; Forrester et al., 2018). ADP-ribosylation by PARP1 inhibits the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) resulting in the accumulation of glycolytic intermediates upstream of GAPDH and their redistribution to side branches of the glycolytic pathway, leading to (1) uncontrolled protein glycation via HBP, (2) production of advanced glycation end products (AGEs) through the polyol and methylglyoxal pathways, and (3) increased protein kinase C (PKC) activation through de novo synthesis of diacylglycerol from G3P (Du et al., 2003; Shah and Brownlee, 2016). Glycation of eNOS inhibits its activation, while PKC inhibits insulin-mediated activation of eNOS and increases the expression of the vasoconstrictor endothelin-1 (Du et al., 2001; Li et al., 2013). Furthermore, AGEs induce EC dysfunction through modification of proteins and ECM components, and activation of the receptor for AGEs resulting in activation of the pro-inflammatory NF-κB signaling pathway, increased

vascular leakage and ROS production (Shah and Brownlee, 2016). Interestingly, reverting the glucose intermediates F6P and G3P toward the PPP by activating transketolase using a thiamine derivative reduces all three hyperglycemia-induced pathways described above as well as NF-κB activity (Hammes et al., 2003).

Obesity, as well as diabetes, is associated with elevated circulating concentrations of saturated FAs and triglyceride-rich lipoproteins, which can provide an additional supply of FAs (Goldberg and Bornfeldt, 2013; Nordestgaard, 2016). FAs can induce EC apoptosis and dysfunction by impairing NO-mediated vasodilation and promoting vascular permeability, oxidative and ER stress, pro-inflammatory NF-κB signaling and inflammasome activation (Inoguchi et al., 2000; Steinberg et al., 2000; Maloney et al., 2009; Tampakakis et al., 2016; Wang L. et al., 2016a). Reducing intracellular lipid levels by increasing FAO via peroxisome-proliferator-activated receptor (PPAR) β and δ-mediated upregulation of CPT1A or overexpression of PPAR-γ coactivator 1-α reduces FA-induced EC dysfunction and apoptosis, respectively (Won et al., 2010; Toral et al., 2015). Moreover, metformin, a first-line therapeutic drug against type 2 diabetes, decreases FA-induced ER stress and ROS production via a 5′ adenosine monophosphate–activated protein kinase-PPARδ-dependent axis (Cheang et al., 2014). However, why ECs do not increase FAO naturally upon lipid overload, despite having a considerable spare mitochondrial respiratory capacity, remains to be explored.

Cellular aging is a complex process characterized by the progressive loss of cellular function and is hallmarked, among others, by deregulated nutrient sensing and mitochondrial dysfunction (López-Otín et al., 2013). Indeed, ECs from aged rats are characterized by a reduction in mitochondrial mass, altered expression of mitochondrial components and an increase in mitochondrial ROS production (Ungvari et al., 2007, 2008). Furthermore, nutrient sensing pathways are fundamental to the aging process, since dietary restriction protects against the aging-mediated decline in EC function (Csiszar et al., 2014). Interestingly, endothelial-specific overexpression of the NAD^+-dependent deacetylase sirtuin 1, a sensor that detects energy availability via NAD^+, or supplementation of NAD precursors reverse the aging-associated decline in angiogenesis (Das et al., 2018).

Furthermore, additional hallmarks of aging, such as epigenetic alterations, and cellular senescence, may also affect EC metabolism. For instance, aging-induced epigenetic modifications can regulate the expression of metabolic genes and, conversely, metabolic intermediates modulate the epigenetic landscape (Brunet and Rando, 2017). Replicative senescence in ECs, achieved through consecutive *in vitro* passaging, reduced ATP levels by ∼10-fold, despite having enhanced glycolysis, the main energy supplier in ECs (Unterluggauer et al., 2008; De Bock et al., 2013). Furthermore, inhibition of glutaminolysis induces a senescent phenotype in ECs *in vitro* (Unterluggauer et al., 2008). However, it remains to be determined which metabolic pathways are modulated by aging in ECs and whether EC metabolism can be targeted to reverse the aging-associated EC dysfunction.

CONCLUDING REMARKS

Metabolic pathways have emerged as key regulators of many EC functions, including angiogenesis, inflammation, and barrier function. However, despite major advances in our understanding in EC metabolism, there still remain many unanswered questions. Although research in endothelial AA metabolism is still in its infancy, limiting glycolysis and/or stimulating FAO in ECs may be a promising therapeutic strategy against atherosclerosis, even in the presence of risk factors such as diabetes and obesity. However, the majority of the complications due to atherosclerosis occur in the aged population (Shih et al., 2011). Therefore, a better understanding of the metabolic perturbations in aged ECs may provide additional therapeutic avenues in the treatment of atherosclerosis.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

Adeva-Andany, M. M., Gonzalez-Lucan, M., Donapetry-Garcia, C., Fernandez-Fernandez, C., and Ameneiros-Rodriguez, E. (2016). Glycogen metabolism in humans. *BBA Clin.* 5, 85–100. doi: 10.1016/j.bbacli.2016.02.001

Barth, E., Stammler, G., Speiser, B., and Schaper, J. (1992). Ultrastructural quantitation of mitochondria and myofilaments in cardiac muscle from 10 different animal species including man. *J. Mol. Cell. Cardiol.* 24, 669–681. doi: 10.1016/0022-2828(92)93381-S

Bertero, T., Oldham, W. M., Cottrill, K. A., Pisano, S., Vanderpool, R. R., Yu, Q., et al. (2016). Vascular stiffness mechanoactivates YAP/TAZ-dependent glutaminolysis to drive pulmonary hypertension. *J. Clin. Invest.* 126, 3313–3335. doi: 10.1172/JCI86387

Blouin, A., Bolender, R. P., and Weibel, E. R. (1977). Distribution of organelles and membranes between hepatocytes and nonhepatocytes in the rat liver parenchyma. A stereological study. *J. Cell Biol.* 72, 441–455. doi: 10.1083/jcb.72.2.441

Brunet, A., and Rando, T. A. (2017). Interaction between epigenetic and metabolism in aging stem cells. *Curr. Opin. Cell Biol.* 45, 1–7. doi: 10.1016/j.ceb.2016.12.009

Caires, R., Sierra-Valdez, F. J., Millet, J. R. M., Herwig, J. D., Roan, E., Vasquez, V., et al. (2017). Omega-3 fatty acids modulate TRPV4 function through plasma membrane remodeling. *Cell Rep.* 21, 246–258. doi: 10.1016/j.celrep.2017.09.029

Cantelmo, A. R., Conradi, L. C., Brajic, A., Goveia, J., Kalucka, J., Pircher, A., et al. (2016). Inhibition of the glycolytic activator PFKFB3 in endothelium induces tumor vessel normalization, impairs metastasis, and improves chemotherapy. *Cancer Cell* 30, 968–985. doi: 10.1016/j.ccell.2016.10.006

Cantor, J. R., Abu-Remaileh, M., Kanarek, N., Freinkman, E., Gao, X., Louissaint, A. Jr., et al. (2017). Physiologic medium rewires cellular metabolism and reveals uric acid as an endogenous inhibitor of UMP synthase. *Cell* 169, 258.e17–272.e17. doi: 10.1016/j.cell.2017.03.023

Cheang, W. S., Tian, X. Y., Wong, W. T., Lau, C. W., Lee, S. S., Chen, Z. Y., et al. (2014). Metformin protects endothelial function in diet-induced obese mice by inhibition of endoplasmic reticulum stress through 5' adenosine monophosphate-activated protein kinase-peroxisome proliferator-activated receptor delta pathway. *Arterioscler. Thromb. Vasc. Biol.* 34, 830–836. doi: 10.1161/ATVBAHA.113.301938

Chen, P. Y., Qin, L., Baeyens, N., Li, G., Afolabi, T., Budatha, M., et al. (2015). Endothelial-to-mesenchymal transition drives atherosclerosis progression. *J. Clin. Invest.* 125, 4514–4528. doi: 10.1172/JCI82719

Csiszar, A., Gautam, T., Sosnowska, D., Tarantini, S., Banki, E., Tucsek, Z., et al. (2014). Caloric restriction confers persistent anti-oxidative, pro-angiogenic, and anti-inflammatory effects and promotes anti-aging miRNA expression profile in cerebromicrovascular endothelial cells of aged rats. *Am. J. Physiol. Heart Circ. Physiol.* 307, H292–H306. doi: 10.1152/ajpheart.00307.2014

Das, A., Huang, G. X., Bonkowski, M. S., Longchamp, A., Li, C., Schultz, M. B., et al. (2018). Impairment of an endothelial NAD(+)-H_2S signaling network is a reversible cause of vascular aging. *Cell* 173, 74.e20–89.e20. doi: 10.1016/j.cell.2018.02.008

De Bock, K., Georgiadou, M., Schoors, S., Kuchnio, A., Wong, B. W., Cantelmo, A. R., et al. (2013). Role of PFKFB3-driven glycolysis in vessel sprouting. *Cell* 154, 651–663. doi: 10.1016/j.cell.2013.06.037

Dekker, R. J., Boon, R. A., Rondaij, M. G., Kragt, A., Volger, O. L., Elderkamp, Y. W., et al. (2006). KLF2 provokes a gene expression pattern that establishes functional quiescent differentiation of the endothelium. *Blood* 107, 4354–4363. doi: 10.1182/blood-2005-08-3465

Dekker, R. J., Van Soest, S., Fontijn, R. D., Salamanca, S., De Groot, P. G., Vanbavel, E., et al. (2002). Prolonged fluid shear stress induces a distinct set of endothelial cell genes, most specifically lung Kruppel-like factor (KLF2). *Blood* 100, 1689–1698. doi: 10.1182/blood-2002-01-0046

Dichtl, W., Dulak, J., Frick, M., Alber, H. F., Schwarzacher, S. P., Ares, M. P., et al. (2003). HMG-CoA reductase inhibitors regulate inflammatory transcription factors in human endothelial and vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* 23, 58–63. doi: 10.1161/01.ATV.0000043456.48735.20

Doddaballapur, A., Michalik, K. M., Manavski, Y., Lucas, T., Houtkooper, R. H., You, X., et al. (2015). Laminar shear stress inhibits endothelial cell metabolism via KLF2-mediated repression of PFKFB3. *Arterioscler. Thromb. Vasc. Biol.* 35, 137–145. doi: 10.1161/ATVBAHA.114.304277

Du, X. L., Edelstein, D., Dimmeler, S., Ju, Q., Sui, C., and Brownlee, M. (2001). Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. *J. Clin. Invest.* 108, 1341–1348. doi: 10.1172/JCI11235

Du, X., Matsumura, T., Edelstein, D., Rossetti, L., Zsengeller, Z., Szabo, C., et al. (2003). Inhibition of GAPDH activity by poly(ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells. *J. Clin. Invest.* 112, 1049–1057. doi: 10.1172/JCI18127

Enzo, E., Santinon, G., Pocaterra, A., Aragona, M., Bresolin, S., Forcato, M., et al. (2015). Aerobic glycolysis tunes YAP/TAZ transcriptional activity. *EMBO J.* 34, 1349–1370. doi: 10.15252/embj.201490379

Evrard, S. M., Lecce, L., Michelis, K. C., Nomura-Kitabayashi, A., Pandey, G., Purushothaman, K. R., et al. (2016). Endothelial to mesenchymal transition is common in atherosclerotic lesions and is associated with plaque instability. *Nat. Commun.* 7:11853. doi: 10.1038/ncomms11853

Feng, S., Bowden, N., Fragiadaki, M., Souilhol, C., Hsiao, S., Mahmoud, M., et al. (2017). Mechanical activation of hypoxia-inducible factor 1α drives endothelial dysfunction at atheroprone sites. *Arterioscler. Thromb. Vasc. Biol.* 37, 2087–2101. doi: 10.1161/ATVBAHA.117.309249

Folco, E. J., Sheikine, Y., Rocha, V. Z., Christen, T., Shvartz, E., Sukhova, G. K., et al. (2011). Hypoxia but not inflammation augments glucose uptake in human macrophages: implications for imaging atherosclerosis with 18fluorine-labeled 2-deoxy-D-glucose positron emission tomography. *J. Am. Coll. Cardiol.* 58, 603–614. doi: 10.1016/j.jacc.2011.03.044

Forrester, S. J., Kikuchi, D. S., Hernandes, M. S., Xu, Q., and Griendling, K. K. (2018). Reactive oxygen species in metabolic and inflammatory signaling. *Circ. Res.* 122, 877–902. doi: 10.1161/CIRCRESAHA.117.311401

Gimbrone, M. A. Jr., and García-Cardeña, G. (2016). Endothelial cell dysfunction and the pathobiology of atherosclerosis. *Circ. Res.* 118, 620–636. doi: 10.1161/CIRCRESAHA.115.306301

Glatzel, D. K., Koeberle, A., Pein, H., Loser, K., Stark, A., Keksel, N., et al. (2018). Acetyl-CoA carboxylase 1 regulates endothelial cell migration by shifting the phospholipid composition. *J. Lipid Res.* 59, 298–311. doi: 10.1194/jlr.M080101

Goldberg, I. J., and Bornfeldt, K. E. (2013). Lipids and the endothelium: bidirectional interactions. *Curr. Atheroscler. Rep.* 15:365. doi: 10.1007/s11883-013-0365-1

Guo, L., Akahori, H., Harari, E., Smith, S. L., Polavarapu, R., Karmali, V., et al. (2018). CD163+ macrophages promote angiogenesis and vascular permeability accompanied by inflammation in atherosclerosis. *J. Clin. Invest.* 128, 1106–1124. doi: 10.1172/JCI93025

Hagberg, C., Mehlem, A., Falkevall, A., Muhl, L., and Eriksson, U. (2013). Endothelial fatty acid transport: role of vascular endothelial growth factor B. *Physiology* 28, 125–134. doi: 10.1152/physiol.00042.2012

Hajra, L., Evans, A. I., Chen, M., Hyduk, S. J., Collins, T., and Cybulsky, M. I. (2000). The NF- κB signal transduction pathway in aortic endothelial cells is primed for activation in regions predisposed to atherosclerotic lesion formation. *Proc. Natl. Acad. Sci. U.S.A.* 97, 9052–9057. doi: 10.1073/pnas.97.16.9052

Hammes, H. P., Du, X., Edelstein, D., Taguchi, T., Matsumura, T., Ju, Q., et al. (2003). Benfotiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retinopathy. *Nat. Med.* 9, 294–299. doi: 10.1038/nm834

Harayama, T., and Riezman, H. (2018). Understanding the diversity of membrane lipid composition. *Nat. Rev. Mol. Cell Biol.* 19, 281–296. doi: 10.1038/nrm.2017.138

Hensley, C. T., Faubert, B., Yuan, Q., Lev-Cohain, N., Jin, E., Kim, J., et al. (2016). Metabolic heterogeneity in human lung tumors. *Cell* 164, 681–694. doi: 10.1016/j.cell.2015.12.034

Huang, H., Vandekeere, S., Kalucka, J., Bierhansl, L., Zecchin, A., Bruning, U., et al. (2017). Role of glutamine and interlinked asparagine metabolism in vessel formation. *EMBO J.* 36, 2334–2352. doi: 10.15252/embj.201695518

Inoguchi, T., Li, P., Umeda, F., Yu, H. Y., Kakimoto, M., Imamura, M., et al. (2000). High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C–dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* 49, 1939–1945. doi: 10.2337/diabetes.49.11.1939

Jang, C., Oh, S. F., Wada, S., Rowe, G. C., Liu, L., Chan, M. C., et al. (2016). A branched-chain amino acid metabolite drives vascular fatty acid transport and causes insulin resistance. *Nat. Med.* 22, 421–426. doi: 10.1038/nm.4057

Kanter, J. E., Johansson, F., Leboeuf, R. C., and Bornfeldt, K. E. (2007). Do glucose and lipids exert independent effects on atherosclerotic lesion initiation or progression to advanced plaques? *Circ. Res.* 100, 769–781. doi: 10.1161/01.RES.0000259589.34348.74

Kim, B., Li, J., Jang, C., and Arany, Z. (2017). Glutamine fuels proliferation but not migration of endothelial cells. *EMBO J.* 36, 2321–2333. doi: 10.15252/embj.201796436

Kim, J., Kim, Y. H., Kim, J., Park, D. Y., Bae, H., Lee, D. H., et al. (2017). YAP/TAZ regulates sprouting angiogenesis and vascular barrier maturation. *J. Clin. Invest.* 127, 3441–3461. doi: 10.1172/JCI93825

Krützfeldt, A., Spahr, R., Mertens, S., Siegmund, B., and Piper, H. M. (1990). Metabolism of exogenous substrates by coronary endothelial cells in culture. *J. Mol. Cell. Cardiol.* 22, 1393–1404. doi: 10.1016/0022-2828(90)90984-A

Kuo, A., Lee, M. Y., and Sessa, W. C. (2017). Lipid droplet biogenesis and function in the endothelium. *Circ. Res.* 120, 1289–1297. doi: 10.1161/CIRCRESAHA.116.310498

Laczy, B., Hill, B. G., Wang, K., Paterson, A. J., White, C. R., Xing, D., et al. (2009). Protein O-GlcNAcylation: a new signaling paradigm for the cardiovascular system. *Am. J. Physiol. Heart Circ. Physiol.* 296, H13–H28. doi: 10.1152/ajpheart.01056.2008

Leopold, J. A., Walker, J., Scribner, A. W., Voetsch, B., Zhang, Y. Y., Loscalzo, A. J., et al. (2003). Glucose-6-phosphate dehydrogenase modulates vascular endothelial growth factor-mediated angiogenesis. *J. Biol. Chem.* 278, 32100–32106. doi: 10.1074/jbc.M301293200

Li, Q., Park, K., Li, C., Rask-Madsen, C., Mima, A., Qi, W., et al. (2013). Induction of vascular insulin resistance and endothelin-1 expression and acceleration of atherosclerosis by the overexpression of protein kinase C-β isoform in the endothelium. *Circ. Res.* 113, 418–427. doi: 10.1161/CIRCRESAHA.113.301074

Longchamp, A., Mirabella, T., Arduini, A., Macarthur, M. R., Das, A., Trevino-Villarreal, J. H., et al. (2018). Amino acid restriction triggers angiogenesis via gcn2/atf4 regulation of vegf and h2s production. *Cell* 173, 117.e14–129.e14. doi: 10.1016/j.cell.2018.03.001

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The hallmarks of aging. *Cell* 153, 1194–1217. doi: 10.1016/j.cell.2013.05.039

Lunt, S. Y., and Vander Heiden, M. G. (2011). Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu. Rev. Cell Dev. Biol.* 27, 441–464. doi: 10.1146/annurev-cellbio-092910-154237

Lusis, A. J. (2000). Atherosclerosis. *Nature* 407, 233–241. doi: 10.1038/35025203

Maloney, E., Sweet, I. R., Hockenbery, D. M., Pham, M., Rizzo, N. O., Tateya, S., et al. (2009). Activation of NF-kappaB by palmitate in endothelial cells: a key role for NADPH oxidase-derived superoxide in response to TLR4 activation. *Arterioscler. Thromb. Vasc. Biol.* 29, 1370–1375. doi: 10.1161/ATVBAHA.109.188813

Mann, G. E., Yudilevich, D. L., and Sobrevia, L. (2003). Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. *Physiol. Rev.* 83, 183–252. doi: 10.1152/physrev.00022.2002

Mehrotra, D., Wu, J., Papangeli, I., and Chun, H. J. (2014). Endothelium as a gatekeeper of fatty acid transport. *Trends Endocrinol. Metab.* 25, 99–106. doi: 10.1016/j.tem.2013.11.001

Merchan, J. R., Kovacs, K., Railsback, J. W., Kurtoglu, M., Jing, Y., Pina, Y., et al. (2010). Antiangiogenic activity of 2-deoxy-D-glucose. *PLoS ONE* 5:e13699. doi: 10.1371/journal.pone.0013699

Mertens, S., Noll, T., Spahr, R., Krutzfeldt, A., and Piper, H. M. (1990). Energetic response of coronary endothelial cells to hypoxia. *Am. J. Physiol.* 258, H689–H694. doi: 10.1152/ajpheart.1990.258.3.H689

Nordestgaard, B. G. (2016). Triglyceride-rich lipoproteins and atherosclerotic cardiovascular disease: new insights from epidemiology, genetics, and biology. *Circ. Res.* 118, 547–563. doi: 10.1161/CIRCRESAHA.115.306249

Oldendorf, W. H., Cornford, M. E., and Brown, W. J. (1977). The large apparent work capability of the blood-brain barrier: a study of the mitochondrial content of capillary endothelial cells in brain and other tissues of the rat. *Ann. Neurol.* 1, 409–417. doi: 10.1002/ana.410010502

Patella, F., Schug, Z. T., Persi, E., Neilson, L. J., Erami, Z., Avanzato, D., et al. (2015). Proteomics-based metabolic modeling reveals that fatty acid oxidation (FAO) controls endothelial cell (EC) permeability. *Mol. Cell. Proteomics* 14, 621–634. doi: 10.1074/mcp.M114.045575

Pavlova, N. N., Hui, S., Ghergurovich, J. M., Fan, J., Intlekofer, A. M., White, R. M., et al. (2018). As extracellular glutamine levels decline, asparagine becomes an essential amino acid. *Cell Metab.* 27, 428.e5–438.e5. doi: 10.1016/j.cmet.2017.12.006

Quillard, T., Araujo, H. A., Franck, G., Shvartz, E., Sukhova, G., and Libby, P. (2015). TLR2 and neutrophils potentiate endothelial stress, apoptosis and detachment: implications for superficial erosion. *Eur. Heart J.* 36, 1394–1404. doi: 10.1093/eurheartj/ehv044

Quintero, M., Colombo, S. L., Godfrey, A., and Moncada, S. (2006). Mitochondria as signaling organelles in the vascular endothelium. *Proc. Natl. Acad. Sci. U.S.A.* 103, 5379–5384. doi: 10.1073/pnas.0601026103

Schoors, S., Bruning, U., Missiaen, R., Queiroz, K. C., Borgers, G., Elia, I., et al. (2015). Fatty acid carbon is essential for dNTP synthesis in endothelial cells. *Nature* 520, 192–197. doi: 10.1038/nature14362

Schoors, S., De Bock, K., Cantelmo, A. R., Georgiadou, M., Ghesquiere, B., Cauwenberghs, S., et al. (2014). Partial and transient reduction of glycolysis by PFKFB3 blockade reduces pathological angiogenesis. *Cell Metab.* 19, 37–48. doi: 10.1016/j.cmet.2013.11.008

Sen, S., Roy, S., Bandyopadhyay, G., Scott, B., Xiao, D., Ramadoss, S., et al. (2016). Gamma-aminobutyric acid is synthesized and released by the endothelium: potential implications. *Circ. Res.* 119, 621–634. doi: 10.1161/CIRCRESAHA.116.308645

Sen-Banerjee, S., Mir, S., Lin, Z., Hamik, A., Atkins, G. B., Das, H., et al. (2005). Kruppel-like factor 2 as a novel mediator of statin effects in endothelial cells. *Circulation* 112, 720–726. doi: 10.1161/CIRCULATIONAHA.104.525774

Shah, M. S., and Brownlee, M. (2016). Molecular and cellular mechanisms of cardiovascular disorders in diabetes. *Circ. Res.* 118, 1808–1829. doi: 10.1161/CIRCRESAHA.116.306923

Shih, H., Lee, B., Lee, R. J., and Boyle, A. J. (2011). The aging heart and post-infarction left ventricular remodeling. *J. Am. Coll. Cardiol.* 57, 9–17. doi: 10.1016/j.jacc.2010.08.623

Sluimer, J. C., Gasc, J. M., Van Wanroij, J. L., Kisters, N., Groeneweg, M., Sollewijn Gelpke, M. D., et al. (2008). Hypoxia, hypoxia-inducible transcription factor, and macrophages in human atherosclerotic plaques are correlated with intraplaque angiogenesis. *J. Am. Coll. Cardiol.* 51, 1258–1265. doi: 10.1016/j.jacc.2007.12.025

Steinberg, H. O., Paradisi, G., Hook, G., Crowder, K., Cronin, J., and Baron, A. D. (2000). Free fatty acid elevation impairs insulin-mediated vasodilation and nitric oxide production. *Diabetes* 49, 1231–1238. doi: 10.2337/diabetes.49.7.1231

Tabas, I. (2010). The role of endoplasmic reticulum stress in the progression of atherosclerosis. *Circ. Res.* 107, 839–850. doi: 10.1161/CIRCRESAHA.110.224766

Tabas, I., Garcia-Cardena, G., and Owens, G. K. (2015). Recent insights into the cellular biology of atherosclerosis. *J. Cell Biol.* 209, 13–22. doi: 10.1083/jcb.201412052

Tampakakis, E., Tabit, C. E., Holbrook, M., Linder, E. A., Berk, B. D., Frame, A. A., et al. (2016). Intravenous lipid infusion induces endoplasmic reticulum stress in endothelial cells and blood mononuclear cells of healthy adults. *J. Am. Heart Assoc.* 5:e002574. doi: 10.1161/JAHA.115.002574

Tang, X., Luo, Y. X., Chen, H. Z., and Liu, D. P. (2014). Mitochondria, endothelial cell function, and vascular diseases. *Front. Physiol.* 5:175. doi: 10.3389/fphys.2014.00175

Toral, M., Romero, M., Jimenez, R., Mahmoud, A. M., Barroso, E., Gomez-Guzman, M., et al. (2015). Carnitine palmitoyltransferase-1 up-regulation by PPAR-beta/delta prevents lipid-induced endothelial dysfunction. *Clin. Sci.* 129, 823–837. doi: 10.1042/CS20150111

Tousoulis, D., Kampoli, A. M., Tentolouris, C., Papageorgiou, N., and Stefanadis, C. (2012). The role of nitric oxide on endothelial function. *Curr. Vasc. Pharmacol.* 10, 4–18. doi: 10.2174/157016112798829760

Ungvari, Z., Labinskyy, N., Gupte, S., Chander, P. N., Edwards, J. G., and Csiszar, A. (2008). Dysregulation of mitochondrial biogenesis in vascular endothelial and smooth muscle cells of aged rats. *Am. J. Physiol. Heart Circ. Physiol.* 294, H2121–H2128. doi: 10.1152/ajpheart.00012.2008

Ungvari, Z., Orosz, Z., Labinskyy, N., Rivera, A., Xiangmin, Z., Smith, K., et al. (2007). Increased mitochondrial H_2O_2 production promotes endothelial NF-κB activation in aged rat arteries. *Am. J. Physiol. Heart Circ. Physiol.* 293, H37–H47. doi: 10.1152/ajpheart.01346.2006

Unterluggauer, H., Mazurek, S., Lener, B., Hutter, E., Eigenbrodt, E., Zwerschke, W., et al. (2008). Premature senescence of human endothelial cells induced by inhibition of glutaminase. *Biogerontology* 9, 247–259. doi: 10.1007/s10522-008-9134-x

Végran, F., Boidot, R., Michiels, C., Sonveaux, P., and Feron, O. (2011). Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF-kappaB/IL-8 pathway that drives tumor angiogenesis. *Cancer Res.* 71, 2550–2560. doi: 10.1158/0008-5472.CAN-10-2828

Virmani, R., Kolodgie, F. D., Burke, A. P., Finn, A. V., Gold, H. K., Tulenko, T. N., et al. (2005). Atherosclerotic plaque progression and vulnerability to rupture: angiogenesis as a source of intraplaque hemorrhage. *Arterioscler. Thromb. Vasc. Biol.* 25, 2054–2061. doi: 10.1161/01.ATV.0000178991.71605.18

Vizán, P., Sanchez-Tena, S., Alcarraz-Vizan, G., Soler, M., Messeguer, R., Pujol, M. D., et al. (2009). Characterization of the metabolic changes underlying growth factor angiogenic activation: identification of new potential therapeutic targets. *Carcinogenesis* 30, 946–952. doi: 10.1093/carcin/bgp083

Wang, J. C., and Bennett, M. (2012). Aging and atherosclerosis: mechanisms, functional consequences, and potential therapeutics for cellular senescence. *Circ. Res.* 111, 245–259. doi: 10.1161/CIRCRESAHA.111.261388

Wang, K. C., Yeh, Y. T., Nguyen, P., Limqueco, E., Lopez, J., Thorossian, S., et al. (2016). Flow-dependent YAP/TAZ activities regulate endothelial phenotypes and atherosclerosis. *Proc. Natl. Acad. Sci. U.S.A.* 113, 11525–11530. doi: 10.1073/pnas.1613121113

Wang, L., Chen, Y., Li, X., Zhang, Y., Gulbins, E., and Zhang, Y. (2016a). Enhancement of endothelial permeability by free fatty acid through lysosomal cathepsin B-mediated Nlrp3 inflammasome activation. *Oncotarget* 7, 73229–73241. doi: 10.18632/oncotarget.12302

Wang, L., Luo, J. Y., Li, B., Tian, X. Y., Chen, L. J., Huang, Y., et al. (2016b). Integrin-YAP/TAZ-JNK cascade mediates atheroprotective effect of unidirectional shear flow. *Nature* 540, 579–582. doi: 10.1038/nature20602

Wei, X., Schneider, J. G., Shenouda, S. M., Lee, A., Towler, D. A., Chakravarthy, M. V., et al. (2011). *De novo* lipogenesis maintains vascular homeostasis through endothelial nitric-oxide synthase (eNOS) palmitoylation. *J. Biol. Chem.* 286, 2933–2945. doi: 10.1074/jbc.M110.193037

Wilhelm, K., Happel, K., Eelen, G., Schoors, S., Oellerich, M. F., Lim, R., et al. (2016). FOXO1 couples metabolic activity and growth state in the vascular endothelium. *Nature* 529, 216–220. doi: 10.1038/nature16498

Won, J. C., Park, J. Y., Kim, Y. M., Koh, E. H., Seol, S., Jeon, B. H., et al. (2010). Peroxisome proliferator-activated receptor-gamma coactivator 1-alpha overexpression prevents endothelial apoptosis by increasing ATP/ADP translocase activity. *Arterioscler. Thromb. Vasc. Biol.* 30, 290–297. doi: 10.1161/ATVBAHA.109.198721

Wong, B. W., Wang, X., Zecchin, A., Thienpont, B., Cornelissen, I., Kalucka, J., et al. (2017). The role of fatty acid beta-oxidation in lymphangiogenesis. *Nature* 542, 49–54. doi: 10.1038/nature21028

World Health Organization (2014). *Global Status Report on Noncommunicable Diseases 2014*. Geneva: World Health Organization.

Wu, D., Huang, R. T., Hamanaka, R. B., Krause, M., Oh, M. J., Kuo, C. H., et al. (2017). HIF-1alpha is required for disturbed flow-induced metabolic reprogramming in human and porcine vascular endothelium. *Elife* 6:e25217. doi: 10.7554/eLife.25217

Xiong, J., Kawagishi, H., Yan, Y., Liu, J., Wells, Q. S., Edmunds, L. R., et al. (2018). A metabolic basis for endothelial-to-mesenchymal transition. *Mol. Cell* 69, 689.e7–698.e7. doi: 10.1016/j.molcel.2018.01.010

Yu, P., Wilhelm, K., Dubrac, A., Tung, J. K., Alves, T. C., Fang, J. S., et al. (2017). FGF-dependent metabolic control of vascular development. *Nature* 545, 224–228. doi: 10.1038/nature22322

Zibrova, D., Vandermoere, F., Goransson, O., Peggie, M., Marino, K. V., Knierim, A., et al. (2017). GFAT1 phosphorylation by AMPK promotes VEGF-induced angiogenesis. *Biochem. J.* 474, 983–1001. doi: 10.1042/BCJ20160980

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REVIEW
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The Warburg Effect in Endothelial Cells and its Potential as an Anti-angiogenic Target in Cancer

*Gillian Fitzgerald†, Inés Soro-Arnaiz† and Katrien De Bock**

Laboratory of Exercise and Health, Department of Health Sciences and Technology, Swiss Federal Institute of Technology Zurich, Zurich, Switzerland

Endothelial cells (ECs) make up the lining of our blood vessels and they ensure optimal nutrient and oxygen delivery to the parenchymal tissue. In response to oxygen and/or nutrient deprivation, ECs become activated and sprout into hypo-vascularized tissues forming new vascular networks in a process termed angiogenesis. New sprouts are led by migratory tip cells and extended through the proliferation of trailing stalk cells. Activated ECs rewire their metabolism to cope with the increased energetic and biosynthetic demands associated with migration and proliferation. Moreover, metabolic signaling pathways interact and integrate with angiogenic signaling events. These metabolic adaptations play essential roles in determining EC fate and function, and are perturbed during pathological angiogenesis, as occurs in cancer. The angiogenic switch, or the growth of new blood vessels into an expanding tumor, increases tumor growth and malignancy. Limiting tumor angiogenesis has therefore long been a goal for anticancer therapy but the traditional growth factor targeted anti-angiogenic treatments have met with limited success. In recent years however, it has become increasingly recognized that focusing on altered tumor EC metabolism provides an attractive alternative anti-angiogenic strategy. In this review, we will describe the EC metabolic signature and how changes in EC metabolism affect EC fate during physiological sprouting, as well as in the cancer setting. Then, we will discuss the potential of targeting EC metabolism as a promising approach to develop new anti-cancer therapies.

Keywords: angiogenesis, endothelial, metabolism, cancer, vascular normalization

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Sapienza Università di Roma, Italy

***Correspondence:**
Katrien De Bock
katrien-debock@ethz.ch

†These authors have contributed equally to this work

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INTRODUCTION

The circulatory system is an ingenious and extended network of blood and lymphatic vessels that allows the transport of nutrients and oxygen from their site of uptake or production to peripheral tissues where they will be metabolized. At the same time, it removes any metabolic waste products away from these tissues. Vessels are lined by an impressive monolayer of endothelial cells (ECs), the surface of which can cover > 700 m^2 and has a weight of about 700g in an adult person (Wolinsky, 1980). Although in the adult organism ECs seldom proliferate and remain in a quiescent state for protracted periods, they retain the ability to rapidly initiate the formation of new vessels; a tightly coordinated process termed angiogenesis. The expansion of the vascular network via angiogenesis occurs in response to nutrient and oxygen deprivation; vascular expansion serves to accommodate these enhanced oxygen and nutrient requirements and to restore tissue metabolic homeostasis. Angiogenesis and metabolism are thus intimately linked.

This is particularly true in a cancer setting, where the nutrient and oxygen requirements of tumors exceeding a volume of 1 mm^3 surpass what can be provided through passive diffusion from the vessels of the surrounding host tissue. When this occurs, the tumor microenvironment starts releasing proangiogenic factors, such as the vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), ephrins, and angiopoietins, promoting the vascularization of the tumor and the restoration of oxygen and nutrient supply. This process, termed the angiogenic switch, is crucial for the growth and progression of the tumor. Anti-angiogenic therapy has therefore been put forward as an attractive therapeutic avenue for anti-cancer therapies (Folkman, 1971; Yuan et al., 1996). Therapeutically, anti-angiogenic treatment has been proposed to starve existing tumors of nutrients and oxygen preventing their continued growth. In recent years, the concept of angio-prevention has emerged as a prophylactic strategy to stop low grade undetected lesions from progressing by preemptively providing anti-angiogenic therapy to at risk patients (Albini et al., 2012). This preventative strategy complements the traditional therapeutic approach. Although several strategies to inhibit VEGF have been developed and approved for the treatment of cancer, they have shown only limited efficacy (Jayson et al., 2016; Fukumura et al., 2018). This is in part due to the upregulation of alternative pro-angiogenic growth factors within the tumor to overcome VEGF blockade (Bergers and Hanahan, 2008; Ellis and Hicklin, 2008; Carmeliet and Jain, 2011; Jayson et al., 2016; Fukumura et al., 2018). This has necessitated the development of novel treatment strategies that target not just the angiogenic growth factors but rather the endothelium itself. In recent years, it has become clear that ECs reprogram their metabolism during angiogenesis, and targeting endothelial metabolism provides a promising alternative therapeutic target in anti-cancer anti-angiogenic strategies (De Bock et al., 2013a; Cantelmo et al., 2017).

In this review, we will give a brief overview of angiogenic biology and the canonical signaling pathways involved in this process. For a more comprehensive overview of this topic, we refer the reader to the following reviews (Adams and Alitalo, 2007; Potente et al., 2011; Blanco and Gerhardt, 2013; Eelen et al., 2018). Although angiogenesis can occur via different mechanisms, endothelial metabolism has been exclusively investigated during the sprouting of new vessels out of existing ones (sprouting angiogenesis). We will therefore limit our review to sprouting angiogenesis, beginning with an overview of the Warburgian characteristics of ECs, and how they change their metabolism during angiogenesis. Then, we will highlight recent insights into the potential of targeting endothelial metabolism as a novel anti-angiogenic strategy for cancer therapy.

ANGIOGENESIS – THE CURRENT MODEL OF VESSEL SPROUTING

Vessel growth via sprouting angiogenesis is initiated via the secretion of angiogenic growth factors from the oxygen and nutrient deprived microenvironment, which triggers tip cell selection. Tip cells are characterized by a migratory (non-proliferative) phenotype with numerous and highly motile filopodia which explore the microenvironment and guide the nascent sprout toward the hypoxic/nutrient deprived area (Gerhardt et al., 2003). Importantly, the tip cell subsequently instructs the neighboring cells not to become tip cells. Instead, those cells then adopt a stalk cell fate, characterized by a proliferative (non-migratory) phenotype which provides a mechanism for sprout extension (Hellstrom et al., 2007; Potente et al., 2011). Moreover, stalk cells drive the formation of the nascent vascular lumen (Iruela-Arispe and Davis, 2009; Charpentier and Conlon, 2014; Betz et al., 2016). When two tip cells make filopodial contacts, the sprouts eventually anastomose, a new blood vessel is formed, and blood flow is initiated (Lenard et al., 2013; Betz et al., 2016). After the functional vascular network has been established it remodels in order to optimize tissue perfusion and oxygen/nutrient delivery (Korn and Augustin, 2015; Ricard and Simons, 2015). Ultimately, the secretion of angiogenic growth factors will cease and this, together with blood flow, will instruct ECs to return to quiescence. Those quiescent ECs, termed phalanx cells, secrete a basement membrane, recruit pericytes, and form tight junctions via the upregulation of VE-Cadherin expression (Mazzone et al., 2009).

Although many other angiogenic growth factors have been described and characterized, VEGF is a key regulator of sprouting angiogenesis. Following release by hypoxic and nutrient deprived cells, it binds to the VEGF receptor 2 (VEGFR2) that is expressed by ECs, and initiates a signaling cascade that promotes EC migration, proliferation, and survival. At the same time, VEGF induced cytoskeletal dynamics activate a transcriptional program by promoting the activation of the transcriptional co-activators YAP and TAZ (Kim J. et al., 2017; Wang et al., 2017; Neto et al., 2018). YAP/TAZ control cytoskeletal rearrangements for filopodia formation and junctional dynamics; their nuclear translocation promotes EC proliferation. VEGF signaling in the tip cell also results in the upregulation of delta like 4 (DLL4), which binds the Notch1 receptor of the neighboring stalk cells and prevents them from acquiring tip cell characteristics (Suchting et al., 2007; Benedito et al., 2009). Also, Notch signaling lowers VEGFR2 levels and enhances the expression of the VEGF trap VEGFR1, rendering the stalk cell less responsive to VEGF. Cell fates within the growing sprout are transient and ECs continuously overtake each other, alternating at the tip cell position (Bentley et al., 2009; Jakobsson et al., 2010; Arima et al., 2011). ECs stochastically change their fate during sprouting as a consequence of cellular motion during sprouting angiogenesis (Boas and Merks, 2015) and the cell at the tip is constantly replaced, even in absence of VEGF (Arima et al., 2011; Boas and Merks, 2015). Subsequently, VEGF-DLL4-Notch signaling ensures that the cell that ended up at the tip position adopts the tip cell phenotype (Arima et al., 2011).

While VEGF-DLL4-Notch is the main signaling hub involved in the control of vessel sprouting many other growth factor and metabolic signaling pathways interact with angiogenic signaling events (**Figure 1**). The NAD^+ dependent deacetylase SIRT1 nutrient sensor deacetylates and inactivates Notch to control

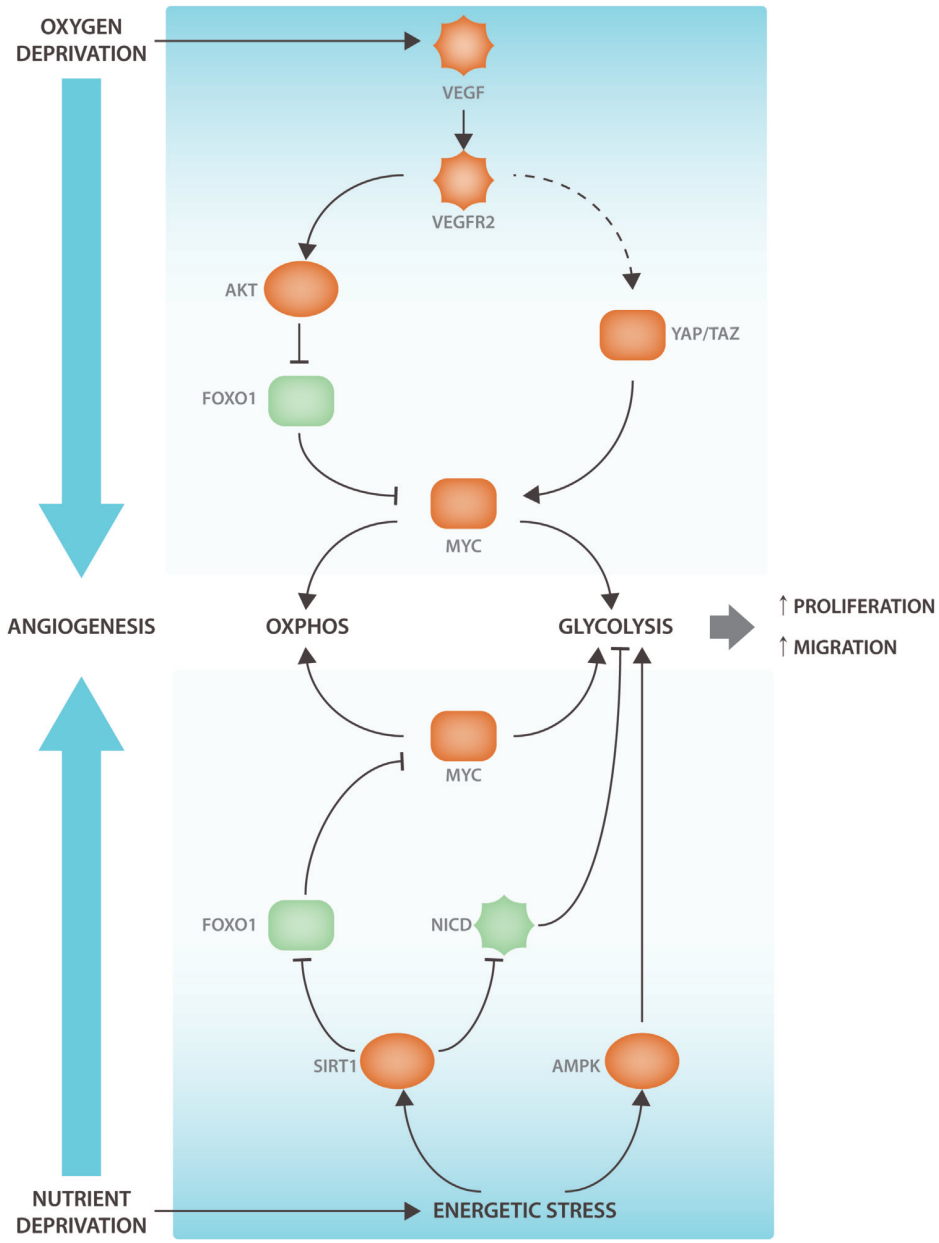

FIGURE 1 | Angiogenic signaling cascades interact with metabolic regulators. VEGF signaling interacts with the ECs' nutrient sensing apparatus to induce metabolic changes and promote angiogenesis. When oxygen levels decrease, the surrounding tissue will secrete VEGF that will bind to its receptor VEGFR2 present on the ECs. VEGF-induced AKT activation will lead to FOXO1 phosphorylation. When FOXO1 is phosphorylated, it will be excluded from the nucleus and therefore be unable to inhibit MYC transcriptional activity. Moreover, VEGF also leads (via cytoskeletal rearrangements) to YAP/TAZ activation, two transcription factors that have been shown to be involved in metabolic reprogramming via the transcription factor MYC. AMPK and SIRT1 are nutrient sensors that become activated under energetic stress conditions. SIRT1 modulates NICD and FOXO1 activity through deacetylation. AMPK activation enhances EC migration and proliferation by increasing glycolysis. Pro-angiogenic factors are indicated in red and anti-angiogenic factors are indicated in green.

sprouting (Guarani et al., 2011). SIRT1 also deacetylates the Forkhead box O 1 (FOXO1) transcription factor that controls many aspects of tissue growth, maintenance and metabolism and acts as a gatekeeper of EC quiescence by reducing vascular sprouting (Wilhelm et al., 2016). Endothelial FOXO1 is also inactivated by VEGFR2 in a PI3K/AKT dependent manner (Abid et al., 2004). FOXO1 acts through suppressing the expression of MYC, a crucial regulator of growth and metabolism in many cell types, including ECs (Dang, 2013; Wilhelm et al., 2016). MYC expression and activity is also controlled by YAP/TAZ (Kim J. et al., 2017), but it is not clear whether YAP and TAZ mediated control of proliferation and metabolism occurs exclusively via MYC. Intriguingly, it has been shown that YAP/TAZ can also promote EC proliferation by directly binding *cis* elements of

metabolic genes (Wang et al., 2017). Another sensor of EC nutrient depletion is AMPK, which promotes angiogenesis under hypoxic conditions (Dagher et al., 2001; Nagata et al., 2003). Thus, main drivers of cellular metabolism interact with VEGF-YAP/TAZ-Notch signaling at many levels to codetermine and modulate sprouting characteristics.

It has recently been shown that ECs change their metabolism during angiogenesis and increase their metabolic activity to meet the specific bioenergetic and anabolic demands that are required for increased migration and proliferation (De Bock et al., 2013a; Eelen et al., 2018). Interestingly, the metabolic switch in ECs not only accompanies the changes in cell phenotype but also plays a critical role in determining cellular behavior during angiogenesis (De Bock et al., 2013b; Schoors et al., 2015). In the following section, we will provide a review of how various metabolic pathways contribute to phenotypic changes during sprouting angiogenesis in healthy conditions as well as how these pathways can be targeted during pathological angiogenesis in cancer.

EC METABOLISM

Endothelial Cells Are Highly Glycolytic

In many cell types, mitochondria produce the majority of ATP via the oxidative phosphorylation (OXPHOS) of reducing equivalents which are generated in the tricarboxylic acid (TCA) cycle through the catabolism of nutrients. This process requires oxygen and most cells will only switch to glycolytic ATP production under hypoxic conditions. Although they are located next to the bloodstream, and therefore have access to the highest levels of oxygen, ECs predominantly produce ATP via aerobic glycolysis, also termed the Warburg effect. This means that almost all glucose is catabolized into lactate even with ample oxygen availability. Consequently, ECs generate more than 80% of their ATP via glycolysis (Krutzfeldt et al., 1990; Culic et al., 1997; De Bock et al., 2013b) and less than 1% of the pyruvate that is generated by glycolytic breakdown of glucose ends up in the TCA cycle (Krutzfeldt et al., 1990). In fact, blocking pyruvate conversion into lactate by inhibiting lactate dehydrogenase (LDHA), thereby allowing pyruvate entry into the mitochondria, impairs endothelial growth, indicating that recycling of NAD^+ by LDHA is required to keep EC glycolysis high (Parra-Bonilla et al., 2010). Glycolysis is critical for ECs, and its complete blockade by using 2-deoxy-glucose leads to decreased proliferation and migration and induces cell death (Delgado et al., 2010; Merchan et al., 2010; Schoors et al., 2014).

When compared to other cell types in the body, ECs have high glycolysis and exhibit similar glycolytic rates to many cancer cell lines (De Bock et al., 2013b). These high levels of glycolysis in ECs are maintained through control of several rate limiting steps such as the phosphorylation of glucose to glucose-6-phosphate by hexokinase 2 (HK2) and the conversion of fructose-6-phosphate to fructose-1,6-phosphate by phosphofructokinase 1 (PFK1). In ECs, the activity of PFK1 is controlled by phosphofructokinase-2/fructose-2,6-bisphosphatase 3 (PFKFB3), which produces fructose-2,6-bisphosphate ($F2,6P_2$), the main allosteric activator of PFK1 (Van Schaftingen et al., 1982).

Why Glycolysis?

Even though glycolytic ATP production occurs at a lower yield when compared to OXPHOS (2 versus 36 mole of ATP per mole of glucose), is less efficient and considered wasteful, several reasons might explain why ECs are highly glycolytic:

First, regulation of glycolytic flux occurs extremely fast (within seconds to minutes) while the response of OXPHOS to increased ATP requirement is at least 100 times slower (Pfeiffer et al., 2001). Glycolysis thus would allow ECs to rapidly adapt their metabolism to the increased energetic demands during proliferation and migration in response to VEGF stimulation and, therefore, to start sprouting immediately.

Second, glycolysis increases the rate of ATP production and can also provide precursors for biomass synthesis (Vander Heiden et al., 2009). This implies that more ATP can be produced during periods of migration where ATP requirements are peaking. Research from the cancer field has shown that production of ATP by glycolysis, rather than OXPHOS, supports cell migration (Yizhak et al., 2014). At the same time, metabolites are generated that can rapidly be shunted into biosynthetic pathways for EC proliferation (Vander Heiden, 2011). For instance, the hexosamine biosynthesis pathway (HBP) uses glutamine, acetyl-CoA and uridine to convert fructose-6-phosphate, a glycolytic intermediate, to glucosamine-6-phosphate and subsequently to uridine-5-diphosphate-*N*-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc is an important substrate for *O*- and *N*-glycosylation which determines the functionality of numerous proteins including VEGFR2 and Notch (Vaisman et al., 1990; Benedito et al., 2009). The HBP also controls the synthesis of hyaluronan, a critical component of the glycocalyx interface between the endothelium and the vascular lumen (Moretto et al., 2015). Via its dependence on the availability of several nutrients, the HBP potentially acts as a nutrient sensing mechanism that integrates nutrient availability with sprouting behavior. Inhibition of HBP reduces angiogenesis, but the underlying mechanisms still need to be defined (Merchan et al., 2010). Glucose can also leave the glycolytic pathway and enter the pentose phosphate pathway (PPP) to fuel the synthesis of ribose-5-phosphate, which is required for the biosynthesis of nucleotides (Pandolfi et al., 1995). The PPP consists of an oxidative (oxPPP) and non-oxidative branch (non-oxPPP), and inhibition of either of those branches impairs EC viability and migration (Vizan et al., 2009). The flux through the oxPPP is controlled by glucose-6-phosphate dehydrogenase, whose activity is partially controlled by VEGF (Pan et al., 2009). The oxPPP also produces NADPH from $NADP^+$ and thus couples nucleotide synthesis to cellular redox status. Glycolytic intermediates can also enter the serine biosynthesis pathway, which in ECs is required for proliferation and survival due to its role in the support of both nucleotide and heme synthesis (Vandekeere et al., 2018) (see below). Taken together, glycolysis will allow ECs to dynamically switch their metabolism when shuffling between the tip and stalk position during sprouting.

Third, because angiogenesis and the restoration of oxygen and nutrient delivery is crucial for survival of the tissue (or even the organism during embryo development) (Carmeliet et al., 1996; Ferrara et al., 1996), proper vascular remodeling

has the highest priority. Studies have shown that glycolysis gives cells a competitive advantage when compared to more oxidative cell types when they need to compete for the same glucose (Pfeiffer et al., 2001). Only high glycolytic flux will therefore allow ECs to invade the environment and to receive sufficient glucose for energy production, while the oxidative cells in the microenvironment can exploit alternative sources for ATP production. Glycolysis will also make ECs more resistant to hypoxia as they can use glycolysis anaerobically as long as glucose is available. An alternative way of glycolytic ATP production could be through the breakdown of glycogen during periods when the growing sprouts enter areas where glucose is scarce. Glycogen is an intracellular glucose store and its catabolism to pyruvate yields additional ATP since it does not require glucose uptake followed by hexokinase mediated phosphorylation. Glycogen breakdown might allow ECs to migrate and proliferate when glucose availability is compromised. Along this line, it has been shown that ECs can store glycogen (Amemiya, 1983; Vizan et al., 2009) and levels of GLUT1 are low at the migrating front of the developing retina (Kishimoto et al., 2016). However, whether glycogen breakdown occurs during sprouting and contributes to the metabolic 'fitness' of the tip cell, is not known.

Fourth, the preferential utilization of anaerobic ATP production would protect ECs from oxidative stress. By using anaerobic glycolysis, ECs reduce the production of reactive oxygen species (ROS) as a consequence of oxidative metabolism (De Bock et al., 2013a). Future research will be needed to reveal whether quiescent ECs that line the oxygen-rich bloodstream need additional metabolic adaptations to promote NAPDH (and glutathione) production to maintain redox balance.

And last, since providing oxygen and nutrients to the surrounding more oxidative cells is an important role of the vasculature, high glycolysis may allow maximal oxygen diffusion over the endothelial wall.

Are Mitochondria Important for ATP Production in ECs?

Endothelial cells rely mainly on glycolysis for ATP production when compared to other cells (De Bock et al., 2013b), and only produce a minor fraction of their ATP via the OXPHOS of reducing equivalents in the mitochondria (Krutzfeldt et al., 1990; Culic et al., 1997; De Bock et al., 2013b). The role of the mitochondrial derived ATP in EC metabolism during sprouting is still not completely understood and is influenced by many factors. For instance, while inhibiting the import of fatty acids (FAs) into the mitochondria does not affect mitochondrial ATP production under normal culturing conditions (Schoors et al., 2015), it does reduce oxygen consumption under conditions where ECs are quiescent and preloaded with oleic acid and rely more on lipid oxidation for ATP production (Kuo et al., 2017). This indicates that under specific conditions, nutrient availability affects the contribution of the mitochondria to ATP production and that ECs might exhibit a Pasteur effect. Moreover, interfering with mitochondrial metabolism does not only alter ATP production but also affects mitochondrial ROS production and cellular redox status, which can modulate EC function. Low mitochondrial ROS levels promote angiogenic signal transduction and migration upon angiogenic stimulation (Chua et al., 1998; Wright et al., 2008; Wang et al., 2011), while higher ROS levels can cause cell damage and death (Wellen and Thompson, 2010; Warren et al., 2014; Vandekeere et al., 2018).

Nonetheless, inhibition of mitochondrial ATP synthesis in ECs via inhibition of ATP synthase does not inhibit endothelial sprouting in a spheroid model (De Bock et al., 2013b). In fact, inhibiting OXPHOS activity might even promote EC migration and sprouting (De Bock et al., 2013b; Longchamp et al., 2018). This increase in migration was caused by an acute activation of the cellular energy sensor AMPK that resulted in a compensatory increase in glycolysis (Longchamp et al., 2018). The inhibition of mitochondrial ATP production might thus have been compensated for by enhanced glycolysis to drive migration. Indeed, inducing mitochondrial dysfunction in osteosarcoma cells enhances glycolysis to maintain NADH recycling, and this sufficed to drive faster migration (Gaude et al., 2018). On the other hand, increasing mitochondrial ATP production and oxygen consumption via supplementing pyruvate does not further promote sprouting nor does it rescue a PFKFB3 knockout driven sprouting defect suggesting that mitochondrial ATP production (from either glucose or FA oxidation) is dispensable during sprouting. Altogether, these data show that ECs tightly control overall energy balance. Although mitochondria do not reach filopodia and lamellipodia during migration, and mitochondrial ATP production occurs too far away from the actin cytoskeleton during sprouting, depleting ATP levels through OXPHOS inhibition results in a metabolic rewiring that promotes glycolysis even at distant sites in the cell and thus drives migration.

Tip Cells - Compartmentalized Glycolytic ATP Production Drives Migration

Glycolysis is particularly crucial for the migrating tip cell. Due to active cytoskeletal rearrangements (Pollard and Borisy, 2003) and the high activity of membrane channels (Schwab et al., 2012; Karlsson et al., 2013) during migration, ATP consumption in the tip cell is extremely high. To meet these increased energetic demands, tip cells upregulate glycolysis above the high baseline levels of glycolysis already found in non-sprouting ECs (**Figure 2**). Different angiogenic growth factors induce glycolytic activation indicating that this process is a crucial component of the angiogenic response. VEGF increases glycolysis by increasing PFKFB3 expression and FGF activates both HK2 as well as PFKFB3 (De Bock et al., 2013b; Yu et al., 2017). VEGF also upregulates GLUT1, the main endothelial glucose transporter (Yeh et al., 2008). The increase in glycolysis upon growth factor stimulation is required for sprouting since endothelial specific knockdown of PFKFB3 as well as HK2 impairs tip cell migration causing vascular defects *in vivo* (De Bock et al., 2013b; Xu et al., 2014; Yu et al., 2017). Thus, activation of glycolysis is required for vessel sprouting.

PFKFB3 and other glycolytic enzymes are mainly located in the perinuclear cytosol of contact inhibited cells but when ECs are sparsely seeded and start to migrate, these

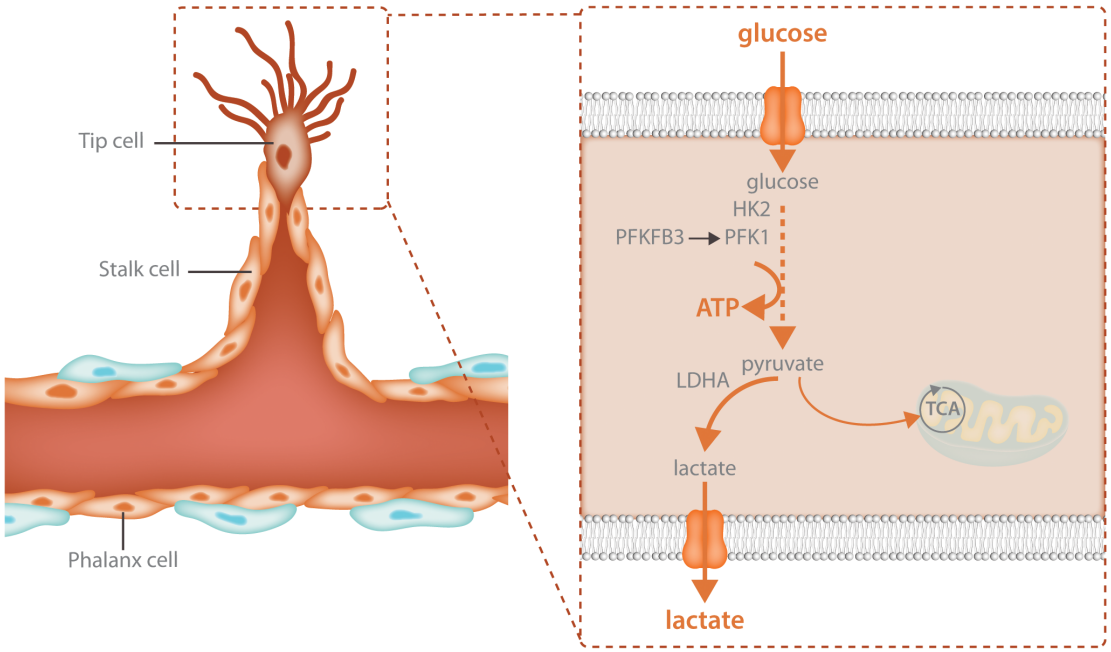

FIGURE 2 | Glycolysis fuels the migrating tip cell. Tip cells are dependent upon glycolytic ATP production and have even higher levels of glycolysis than other endothelial cells. Tip cells increase glycolysis through upregulation of the enzymes that control the rate limiting steps of the reaction: HK2 and PFKFB3. HK2 catalyzes the first reaction of glycolysis, the phosphorylation of glucose to glucose-6-phosphate while PFKFB3 produces fructose-2,6-bisphosphate, the main allosteric activator of PFK1. The majority of the pyruvate that is produced during glycolysis is further broken down into lactate while only 1% ends up in the TCA cycle. High glycolytic flux provides increased local ATP production for the energetically demanding cytoskeletal rearrangements involved in cellular migration.

enzymes partially relocate to the leading front to support the ATP-consuming process of cytoskeletal remodeling. Indeed, lamellipodia and filopodia contain a meshwork of polymerized actin where high levels of ATP are found. Knockdown of PFKFB3 reduced lamellipodial ATP levels, indicating that they are derived from glycolysis (De Bock et al., 2013b). In addition, PFKFB3 also immunoprecipitated with β-actin and was more abundant in the F-actin fraction, the filamentous form found in motile lamellipodia and filopodia of migrating ECs (De Bock et al., 2013b). The compartmentalization of glycolytic enzymes and their binding to actin has been documented in other cell types (Schmitt and An, 2017), and has several implications: migration speed and directionality are driven by local ATP production (van Horssen et al., 2009). In this regard, knockdown of PFKFB3 reduces migration speed as well as directionality. F-actin also provides docking sites for other glycolytic enzymes which stabilizes them, increases their activity and allows other enzymes to piggy-back (Real-Hohn et al., 2010; Araiza-Olivera et al., 2013). By lining up several glycolytic enzymes in a highly organized fashion using the actin as a scaffold, a so called 'metabolon' is formed. In these metabolons, metabolites are channeled, which indicates that the product of one enzyme is immediately transferred to the next enzyme, which enhances metabolic efficiency and further increases the flux through a specific pathway (Miura et al., 2013). The importance of glycolytic compartmentalization is underscored by the observation that flies which lack the ability to compartmentalize aldolase to the actin, results into the inability to fly, even when all enzymes are present (Wojtas et al., 1997).

The signals that control glycolytic enzyme localization in ECs are not known. In mammary epithelial cells, Rac/cdc42 dependent cytoskeletal rearrangements induced by PI3K signaling mobilize the glycolytic enzyme aldolase from the F-actin to control glycolysis (Hu et al., 2016). Along with cytoskeletal tethering, PFKFB3 and many other glycolytic enzymes can relocate to the nucleus. There, PFKFB3 produces $F2,6P_2$, which enhances cyclin-dependent kinase-mediated phosphorylation of $p27^{kip1}$ (a potent inhibitor of Cdk and G_1-to-S cell cycle phase transition) thereby promoting its proteasomal degradation. This results in increased proliferation, independent of increased glycolysis (Yalcin et al., 2009). Acetylation of PFKFB3 in HeLa cells leads to its cytoplasmic accumulation where it contributes to increasing glycolysis (Li et al., 2018). Interestingly, deacetylation of PFKFB3 seems to be regulated by SIRT1 (Li et al., 2018). Accordingly, HK2 localizes both in the cytoplasm as well as at the mitochondrial membrane, and its multifunctional role can be dependent or independent of its kinase activity (Pastorino and Hoek, 2008; Snaebjornsson and Schulze, 2018). Knockdown of pyruvate kinase M2 (PKM2) in ECs reduces spheroid sprouting (Boeckel et al., 2016), but it remains to be elucidated whether, in agreement with cancer cells (Yang et al., 2014), PKM2 can also be present in the nucleus under some conditions to control gene expression and proliferation independent of its pyruvate kinase activity. As many glycolytic enzymes have been shown to exert non-canonical functions,

which are dependent on their location, further studies on the exact localization and regulation of glycolytic enzymes will provide valuable insight into the compartmented organization of EC metabolism and how this affects sprouting.

Besides controlling tip cell migration, glycolysis also determines the ability of ECs to take the tip cell position. Mice that lack PFKFB3 or HK2 in ECs have a lower number of tip cells and the tip cells that are present have fewer and shorter filopodia (De Bock et al., 2013b; Yu et al., 2017). PFKFB3 overexpression promotes tip cell contribution even in cells that have been genetically instructed to exhibit a stalk cell phenotype via overexpressing the Notch intracellular domain (NICD), which leads to activation of the Notch transcriptional program. These data might have interesting implications that require further testing because acquiring the tip cell position by tip cell overtaking is considered to occur spontaneously (Arima et al., 2011; Boas and Merks, 2015). Therefore, increasing the potential for fast migration might increase the likelihood that a particular cell ends up at the tip position. In that case, tip cell competition is in fact a 'running' race for the tip that will be won by the fastest one, where speed is determined by the kinetics of ATP requiring processes such as actin cytoskeletal rearrangements and VE-Cadherin recycling (Cruys et al., 2016). Alternatively, since PFKFB3 positive ECs have more filopodia and lamellipodia compared to PFKFB3 knockout ECs, high glycolysis can promote the ability of ECs to execute the tip cell function once they have acquired the tip cell position.

Stalk Cells - When Mitochondria Contribute to Biomass Synthesis

Endothelial cells rely heavily on glycolytic ATP production as an energy source in not only the tip cell but in stalk cells as well (**Figure 3**). Reducing glycolysis in ECs leads to vascular defects by impairing tip cell function as well as stalk cell proliferation (Yu et al., 2017). While the role of mitochondria in the migrating tip cell requires more investigation, it has been shown that they critically contribute to EC metabolism in the stalk cell by acting as a biosynthetic hub for cellular proliferation. The TCA cycle is an important contributor to the generation of many metabolic intermediates for the *de novo* synthesis of nucleotides, proteins and lipids in many proliferating cell types (Pavlova and Thompson, 2016). Besides glucose, long chain FAs can produce acetyl-CoA upon beta-oxidation in the mitochondria. Transport of FAs into the mitochondria is controlled by carnitine palmitoyl transferase 1 alpha (CPT1A), the rate limiting enzyme of fat oxidation (FAO) (Eaton, 2002). In ECs, FA derived carbons are incorporated into many TCA cycle intermediates (Schoors et al., 2015) and loss of CPT1A causes endothelial sprouting defects (Schoors et al., 2015). This was due to reduced biomass synthesis, particularly reduced deoxyribonucleotide (dNTP) synthesis (Schoors et al., 2015) (**Figure 3**). FA derived acetyl-CoA was found to be the major carbon source for TCA cycle intermediates including citrate, α-ketoglutarate (αKG), glutamate, and importantly aspartate which is an essential carbon source for dNTP synthesis (Schoors et al., 2015). CPT1A knockdown in cultured ECs severely blunted the contribution of FA derived carbon to dNTPs and reduced totals levels of dNTPs (Schoors et al., 2015). The reduced dNTP synthesis resulted in decreased proliferation of ECs *in vitro* as well as reduced EC proliferation in the developing retinal vascular network, resulting in decreased sprout length as well as branching complexity *in vivo* (Schoors et al., 2015). However, CPT1A was not required for migration and did not change tip cell number nor the amount of filopodia (Schoors et al., 2015). The contribution of FA oxidation to non-lipid biomass seems to be a feature that is restricted to ECs as recent evidence indicates that in many other cell types, FAO does not provide carbon to non-lipid biomass (Hosios et al., 2016).

Along with a reliance on FAO for biomass production, ECs use the non-essential amino acid glutamine to sustain proliferation and macromolecular biosynthesis (Huang et al., 2017; Kim B. et al., 2017). Proliferating ECs consume high amounts of glutamine, more than other amino acids in the media (Krutzfeldt et al., 1990; Huang et al., 2017; Kim B. et al., 2017). Once inside the cell, glutamine is metabolized to glutamate by glutaminase (GLS). Glutamate is then converted to αKG by glutamate dehydrogenase (GDH) or transaminases and contributes to protein and nucleotide synthesis by entering the TCA cycle (**Figure 3**). αKG-derived oxalacetate may exit the TCA cycle and be converted to aspartate that will be further used for protein and/or nucleotide biosynthesis. Moreover, αKG could be metabolized to citrate via reductive carboxylation and contribute to FA synthesis. Glutamine depletion, or genetic inactivation of *GLS1*, blunts EC proliferation due to depletion of TCA metabolites and a subsequent decrease in macromolecular biosynthesis (Huang et al., 2017; Kim B. et al., 2017). However, it is unclear whether glutamine also contributes to ATP production in proliferative ECs. Kim B. et al. (2017) reported a 50% reduction in energy charge in glutamine depleted and siGLS1 cells, whereas Huang et al. (2017) showed that glutamine deprivation does not have an effect on ATP production in ECs. When glutamine is available, EC synthesize asparagine by converting glutamine-derived nitrogen (and aspartate) to asparagine via asparagine synthase (*ASNS*) to support cell growth, mTORC activation and protein synthesis as well as to reduce endoplasmic reticulum (ER) stress (Huang et al., 2017) (**Figure 3**). Accordingly, increased ER stress induced by glutamine depletion was alleviated when αKG and asparagine were provided (Huang et al., 2017). Moreover, reduced proliferation in glutamine-deprived ECs could be restored by αKG and asparagine supplementation (Huang et al., 2017), indicating that both carbon and nitrogen sources are important for EC proliferation. In addition, glutamine-deprived ECs activate macropinocytosis in order to take up asparagine (and other nutrients) from the extracellular milieu (Kim B. et al., 2017). However, whether macropinocytosis also ensures endothelial asparagine uptake *in vivo* is not known and further studies using genetic inactivation of both pathways are needed to validate the *in vitro* data.

The role of other amino acids in EC biology needs to be further characterized. In this regard, recent data show that VEGF increases the expression of the glycine transporter 1 and enhances intracellular glycine availability (Guo et al., 2017).

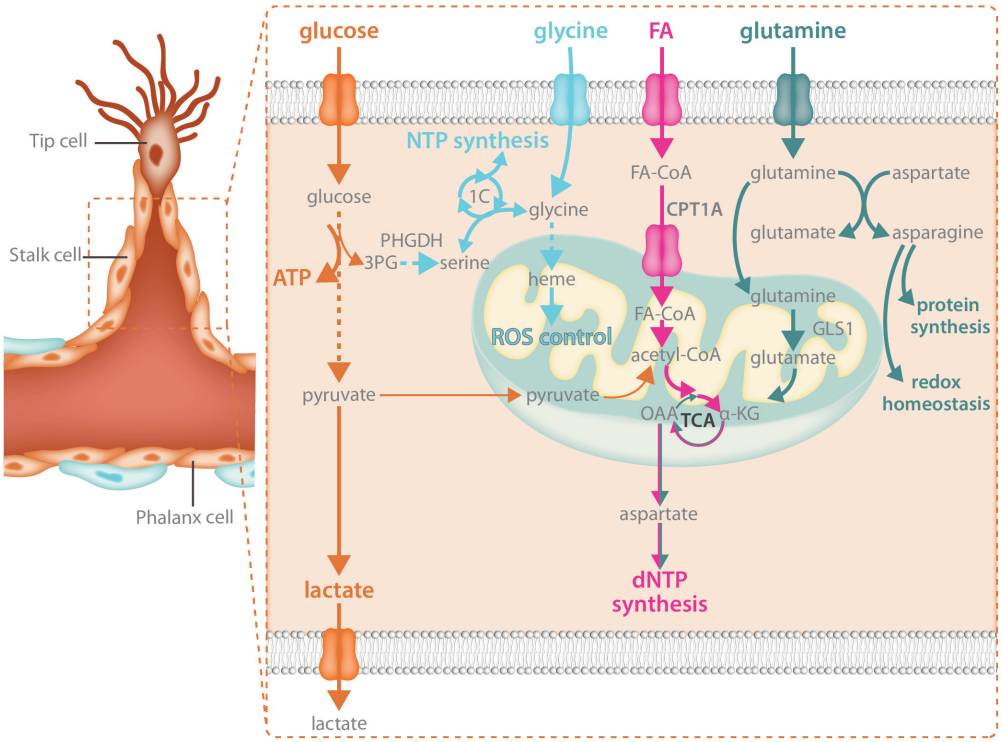

FIGURE 3 | Stalk cells use fatty acid oxidation and amino acid metabolism for biomass synthesis. Like all endothelial cells, stalk cells rely heavily on glycolysis for ATP production. Fatty acid oxidation and amino acid metabolism are required to produce the macromolecules necessary for stalk cell proliferation. Carbon derived from fatty acid oxidation and glutamine catabolism contribute to nucleotide synthesis while glutamine and asparagine catabolism contribute to both protein synthesis and maintenance of redox homeostasis. The intracellular serine synthesis pathway links glucose metabolism with amino acid metabolism in stalk cells for the production of NTPs as well as heme synthesis, which maintains mitochondrial function and controls ROS production.

This promotes tube formation *in vitro* and perfusion of the ischemic hindlimb *in vivo* by improving mitochondrial coupling, reducing ROS, and promoting NO synthesis. At the same time, ECs are also dependent on intracellular glycine synthesis via the serine biosynthesis pathway. Indeed, VEGF increases the expression of enzymes involved in serine biosynthesis, and intracellular glycine levels cannot be maintained in the absence of phosphoglycerate dehydrogenase (PHGDH), a crucial enzyme within the serine biosynthesis pathway. Interestingly, knockdown of PHGDH has recently been reported to critically inhibit angiogenesis *in vitro* and *in vivo* (Vandekeere et al., 2018). *In vivo*, endothelial specific PHGDH knockout mice exhibit decreased EC proliferation as well as increased vessel regression due to lower EC survival and pups die around P9 while *in vitro*, PHGDH knockdown in ECs impairs vessel sprouting from a spheroid due to decreased proliferation. These defects were not only dependent on decreased nucleotide synthesis leading to decreased proliferation but were also – in contrast to many other cell types – dependent on reduced heme synthesis (**Figure 3**). Since heme is crucial to maintain the function of the electron transport chain, impaired heme synthesis increased oxidative stress thereby reducing EC survival (Vandekeere et al., 2018). Amino acids might also control EC tip cell behavior through yet undefined mechanisms. GLS1 inhibition affects EC migration, reduces the number of tip cell in the developing retina *in vivo* and impairs the ability to obtain the tip cell position in competition assays, indicating that amino acid metabolism also contributes to tip cell behavior (Huang et al., 2017). In support of this, microarray data from retinal tip cells showed enrichment of GLS2, though the role of GLS2 in sprouting still needs to be investigated (Strasser et al., 2010).

Phalanx

As mentioned before, when a new blood vessel is fully formed, ECs stop migrating and proliferating, and become quiescent. These phalanx cells form a tight monolayer of cobblestone-like cells which resembles the phalanx formation of ancient Greek soldiers (Mazzone et al., 2009). Quiescent phalanx cells are characterized by an exit from the cell cycle, entering a state of reversible cell cycle arrest in G0/G1, a condition in which they can stay for years. Although termed 'quiescent,' phalanx cells need to actively secrete a glycocalyx layer to ensure optimal perfusion, protect themselves from the harmful oxygen rich environment by maintaining redox status, provide optimal barrier function, and maintain vasoregulation (Pries et al., 2000; Kops et al., 2002; Potente et al., 2011). In addition, while it is plausible that they need to adapt their own metabolism to this multifaceted role, at the same time, they need to provide optimal oxygen and nutrient availability to the microenvironment. However, little is known about how ECs change their metabolism during transition

to the quiescent state. Moreover, how this quiescent state is metabolically maintained, and how metabolic interaction with the microenvironment that the blood vessel serves is controlled, is not known.

One of the factors that induces quiescence is shear stress, induced by the force that laminar flow exerts on ECs (Lee et al., 2012). Krüpple-like factor 2 (KLF2) is a transcription factor that is induced by shear stress and that orchestrates a network of genes that control EC quiescence (Doddaballapur et al., 2015). Shear stress reduces the expression of the glycolytic enzymes HK2, PFK1 as well as PFKFB3 through the direct binding of KLF2 to its gene promoter (Doddaballapur et al., 2015). Overexpression of PFKFB3 partially blunts the KLF2 mediated reduction in sprouting, indicating that the ability of KLF2 to induce quiescence is co-determined by its ability to repress glycolysis. Knockdown of PFKFB3 in ECs is indeed sufficient to induce a quiescent phenotype (Schoors et al., 2014). Shear stress also reduces oxygen consumption *in vitro* but this is highly dependent on the oxygen tension in which cells are maintained (Jones et al., 2008), so it is not clear yet whether quiescent ECs also respire less *in vivo*. Opposite to this, disturbed flow activates glycolysis and downregulates mitochondrial respiration, and targeting this glycolytic switch reduces inflammation (Wu et al., 2017). Besides KLF2, quiescent ECs show high levels of active FOXO1, which couples reduced EC proliferation with reduced glycolysis as well as mitochondrial activity. Mechanistically, FOXO1 decelerates EC metabolism and reduces metabolic rate by repressing MYC signaling (see above) (Wilhelm et al., 2016). As mentioned above, FOXO1 is inhibited by PI3K/AKT mediated phosphorylation which leads to its nuclear exclusion. FOXO1 is also inhibited following SIRT1 mediated deacetylation (Abid et al., 2004; Wilhelm et al., 2016). The endothelium is extremely sensitive to alterations in FOXO1 levels. During development, FOXO1 deficiency leads to EC hyper-proliferation, hyperplasia and vessel enlargement, while FOXO1 overexpression results in defective blood vessel development and hypobranching (Wilhelm et al., 2016). Moreover, in phalanx ECs intracrine VEGF signaling controls viability by preventing FOXO1 overactivation and subsequent suppression of glycolysis, mitochondrial respiration and FA synthesis (Domigan et al., 2015).

Endothelial cells rewire their metabolism by decreasing glycolysis and mitochondrial activity when becoming quiescent (Doddaballapur et al., 2015; Patella et al., 2015; Wilhelm et al., 2016) while at the same time increasing FAO (Patella et al., 2015). When fully assembled into a three dimensional network, ECs use FAO to replenish TCA cycle intermediates to sustain ATP production (Patella et al., 2015). Inhibition of CPT1A lowered ATP levels and glucose consumption which could almost completely be restored by forcing glucose entry into the TCA cycle. Interestingly, inhibition of FAO increased EC permeability *in vitro* and vessel leakiness *in vivo*, and this was dependent on the contribution of FAO to OXPHOS, as inhibition of oligomycin had similar effects. However, the reduction in ATP levels was only around 10%, indicating that other mechanisms might potentially contribute to the ability of FAO to maintain barrier function. In this regard, the production of FAO derived NADPH, which is key to counteract oxidative stress in tumor cells (Pike et al., 2011), might be an additional mechanism through which FAO maintains vascular health. In addition, recent reports have shown that ECs also have the capacity to store lipids in lipid droplets (Kuo et al., 2017). These lipids can be used for the production of ATP via FAO or potentially NADPH. It is not yet clear to what extent the formation of lipid droplets in ECs is relevant to their energy homeostasis, although a role in the regulation of energetic substrate provision to parenchymal tissue has been proposed (Dagher et al., 2001; Ibrahim and Arany, 2017; Kuo et al., 2017). Recent *in vivo* visualization of FA flux over the endothelium however failed to show lipid accumulation inside ECs (He et al., 2018), indicating that more research is needed to identify how quiescent ECs metabolically interact with their microenvironment.

Quiescent ECs expressing Notch1 and NICD (indicating active Notch1 signaling) have been observed at least in heart ECs (Jabs et al., 2018). In addition, activation of Notch signaling is one of the mechanisms through which ECs become contact inhibited when grown until confluence (Noseda et al., 2004; Rostama et al., 2015). Chronic treatment with anti-DLL4 antibodies induces vascular neoplasms due to pathological activation of ECs in mice (Yan et al., 2010) and leads to congestive heart failure in humans in phase I clinical studies (Smith et al., 2014; Chiorean et al., 2015). These data suggest that Notch has an important role in the maintenance of vascular quiescence. Whether it does so via altering EC metabolism still needs to be explored, however, contact inhibited cells have lower glycolysis and NICD overexpression in ECs reduces PFKFB3 and glycolysis (Schoors et al., 2014). Moreover, Notch engages in the metabolic crosstalk between the endothelium and the periphery by controlling vascular lipolysis and transendothelial transport of FAs into the heart by transcriptionally controlling key genes in these processes (Jabs et al., 2018). In addition, global inhibition of Notch improves insulin sensitivity, but it is not known whether this effect is (co-)mediated by the vasculature (Pajvani et al., 2011).

Nutrient Depletion and Angiogenesis

Although mounting evidence indicates that sprouting critically depends on glycolysis, FAO, and glutaminolysis, it is paradoxical that angiogenesis is in fact driven by tissue nutrient deprivation. Nascent sprouts must be instructed to grow, divide, and migrate by the nutrient limited conditions of the host tissue. Recent work has shed light on how nutrient deprivation can initiate angiogenesis. The NAD^+ dependent deacetylase SIRT1 is activated by increases in NAD^+ levels during nutrient deprivation or cellular energy shortage. Upon nutrient restriction, deacetylation of NICD by SIRT1 lowers NICD stability and desensitizes ECs to Notch activation by dampening the Notch response. Accordingly, loss of EC SIRT1 imposes a non-sprouting, stalk cell phenotype (Guarani et al., 2011). In addition to NICD, SIRT1 also deacetylates FOXO1, thereby limiting its antiangiogenic activity (see below) (Wilhelm et al., 2016). The fact that nutrient restriction can directly alter angiogenic behavior of ECs is underscored by the observation that dietary restriction by reducing caloric intake promotes angiogenesis in the ischemic hindlimb (Kondo et al., 2009).

In fact, removing the sulfur amino acids (SAA) methionine and cysteine from the diet phenocopies many aspects of dietary restriction (Orentreich et al., 1993). Depleting SAA alone increases vascular density in skeletal muscle in a VEGF dependent manner and improves neoangiogenesis after femoral artery ligation (Longchamp et al., 2018). SAA restriction of cultured ECs increased angiogenic capacity in a VEGF and SIRT1 dependent manner. Mechanistically, this was due to an increase in the production of hydrogen sulfide (H_2S) by endothelial cystathionine-gamma-lyase (CGL) during SAA restriction. H_2S inhibits complex I and IV of the electron transport chain thereby reducing OXPHOS dependent oxygen consumption. This evoked an AMPK-dependent compensatory increase in glycolytic ATP production as well as increased flux through the PPP (Longchamp et al., 2018) each of which are required for angiogenesis (Vizan et al., 2009; De Bock et al., 2013b). These data shed light on how the balance between the absence of specific nutrients and the presence of others can drive angiogenesis.

TUMOR ECS - TARGETING ENDOTHELIAL METABOLISM FOR ANTI-ANGIOGENESIS OR VESSEL NORMALIZATION

Angiogenesis is crucial to support tumor growth and malignancy (Folkman, 1971; Hanahan and Folkman, 1996). Indeed, rapidly dividing cancer cells have an increased requirement for nutrients and oxygen to support biomass synthesis and ATP production, and at the same time their own rapid growth causes hypoxia. This leads to an uncontrolled and relentless production of pro-angiogenic factors which induces hyperactive and abnormal vessel growth. Tumor vessels are abnormal in both structure and function, being characterized as tortuous, morphologically heterogeneous, and disorganized. Tumor ECs (TECs) are poorly interconnected due to lower levels of the junctional molecule VE-Cadherin, have lost polarity, are poorly covered with pericytes and often leave gaps which reduces barrier function and allows tumor cells to escape and metastasize to distant organs (Jain, 2005; Carmeliet and Jain, 2011; Cantelmo et al., 2016) (**Figure 4A**). In addition, these TECs are irregular and no longer form a tight monolayer but become stacked and protrude into the vessels lumen. These abnormalities lead to perturbed perfusion of the tumor and worsening of the hypoxic, acidic and nutrient deprived conditions within the tumor microenvironment. This results in the secretion of even more angiogenic growth factors thereby establishing a loop of non-productive angiogenesis further promoting vessel abnormalization and tumor malignancy. Because the abnormality of the tumor blood vessels contributes to the severity of tumor malignancy, blood vessel normalization has become a promising strategy for anti-angiogenic cancer therapy. The initially proposed concept of anti-angiogenic treatment, which was intended to induce vessel pruning and starve the tumor to death, has met with little success due to development of resistance as well as an intensification of the hypoxic/acidic microenvironment which can promote metastatic spreading (Ebos et al., 2009; Paez-Ribes et al., 2009). In contrast, promoting vascular normalization would improve tumor perfusion, reduce hypoxia, prevent invasion/metastasis, and increase delivery and efficacy of chemotherapy (Jain, 2001; Carmeliet and Jain, 2011; Goel et al., 2012).

Over the last few years, it has become clear that TECs have an altered metabolic profile (Carmeliet and Jain, 2011; Cantelmo et al., 2016). Indeed, TECs show an even higher reliance on glycolysis (**Figure 4A**) when compared to normal proliferating ECs and have an increased expression of almost all glycolytic enzymes, including GLUT1, at the transcriptional level as well as increased abundance of glycolytic metabolites (Cantelmo et al., 2016; Jayaraman et al., 2018). Lowering glycolysis by low dose pharmacological PFKFB3 inhibition using 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) or endothelial PFKFB3 haplodeficiency did not affect tumor growth but normalized tumor vessels leading to lower cancer cell invasion, intravasation and metastasis (Cantelmo et al., 2016). Moreover, these tumors had more mature vessels and showed enhanced vessel perfusion which improved tumor oxygenation. This lead to improved delivery of chemotherapy into the tumor and increased chemotherapeutic efficacy due to higher oxygen availability (Cantelmo et al., 2016) (**Figure 4C**). Mechanistically, PFKFB3 inhibition reduced the endocytosis of VE-Cadherin, thereby tightening the vascular barrier and rendered pericytes more quiescent and adhesive. The effect of PFKFB3 inhibition on the tumor vasculature is dependent though on the degree of inhibition. Tumors implanted in mice with complete PFKFB3 deletion grow more slowly due to reduced tumor perfusion (Xu et al., 2014). Similarly, high doses of the PFKFB3 inhibitor 3PO lead to tumor vessel disintegration. The increase in vascular leakiness that accompanies tumor vessel disintegration promoted tumor cell metastasis (Conradi et al., 2017) (**Figure 4B**). It seems that the balance between vessel pruning and vessel normalization depends on how big the brake on endothelial metabolism is. Indeed, under the metabolically stressful and hypoxic conditions of the microenvironment, complete high dose PFKFB3 inhibition induced TEC apoptosis (Cantelmo et al., 2016). PFKFB3 knockdown also induced apoptosis under hypoxic conditions *in vitro* (Xu et al., 2014). Moreover, ECs infected with Kaposi's sarcoma associated herpesvirus which leads to endothelial tumor formation, are more sensitive to glycolytic inhibition associated apoptosis (Delgado et al., 2010). Taken together, targeting endothelial glycolysis can offer an attractive therapeutic opportunity, but caution is warranted since its effects are highly dose-dependent. This notion is particularly relevant in the cancer setting, where clinical trials for glycolytic inhibitors for anti-cancer treatment are currently ongoing, and where effects on the vasculature might co-determine long-term treatment outcome for the patient.

It is not clear yet which factors control the activation of glycolysis in the tumor. Although it has previously been shown that VEGF secreted from hypoxic glioma tumor cells can increase GLUT1 expression in ECs (Yeh et al., 2008), several other factors that are present in the tumor microenvironment such as cytokines, hypoxia and estrogen (Yizhak et al., 2014;

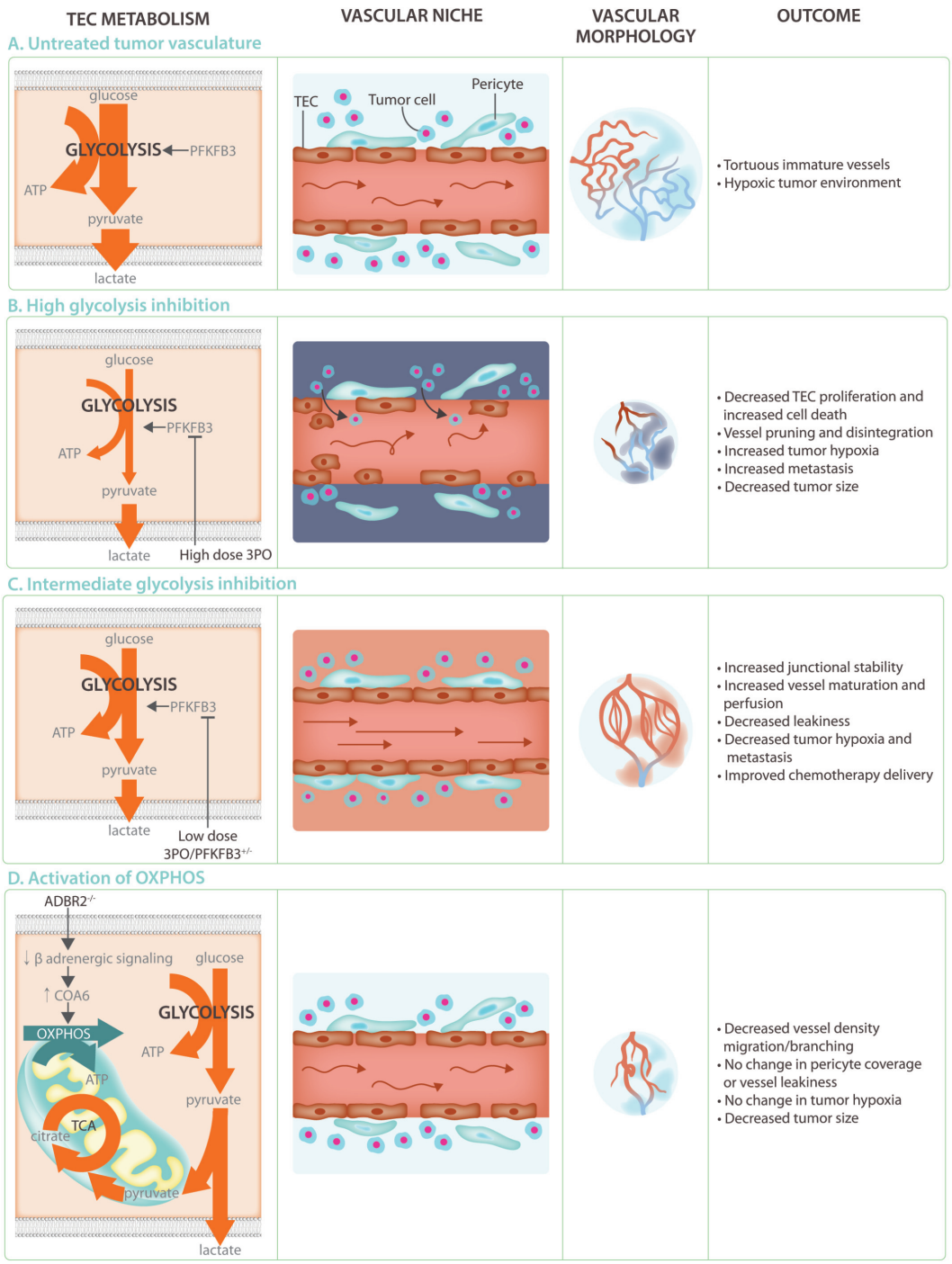

FIGURE 4 | Targeting TEC metabolism as anti-cancer treatment. **(A)** The hypoxic and acidic tumor microenvironment leads to hyperactivation of the tumor endothelium. This leads to TECs that are hyperglycolytic in comparison to normal endothelial cells, weakly connected due to decreased VE-Cadherin, and poorly covered by pericytes. These abnormal characteristics lead to poor perfusion and a worsening of the hypoxic and acidic tumor microenvironment establishing a loop of non-productive angiogenesis creating tortuous and immature tumor vasculature. **(B)** Targeting the hyperglycolytic TEC metabolism by high dose 3PO treatment decreases TEC proliferation and increases endothelial cell death. While this leads to increased vessel pruning, disintegration, and decreased tumor size, it also increases tumor hypoxia and metastasis. **(C)** Targeting TEC glycolysis by using low dose 3PO treatment, or in PFKFB3+/– mice, induces an intermediate brake on TEC metabolism leading to vessel normalization. Increased junctional stability due to decreased endocytosis of the junctional molecule VE-Cadherin allows for decreased vascular leakiness and increased vessel maturation. Normalization of the tumor vasculature improves tumor perfusion, hypoxia, and chemotherapy delivery while reducing metastasis. **(D)** While TECs rely mainly on glycolysis for ATP production, increasing OXPHOS through decreasing β-adrenergic signaling decreases migration and proliferation thereby preventing the angiogenic switch. This does not improve vessel maturation nor influence tumor hypoxia but reduces tumor growth.

Cantelmo et al., 2016; Trenti et al., 2017) can also increase EC PFKFB3 expression. Interestingly, the activated glycolytic transcriptional pattern and increased glycolytic flux can be maintained upon culturing (Cantelmo et al., 2016). It is therefore unlikely that enhanced glycolysis is solely caused by acute environmental conditions within the tumor microenvironment but that other mechanisms including epigenetic modifications (potentially induced by the tumor microenvironment) co-determine TEC glycolysis. Also, recent single cell data obtained from TECs isolated from tumors treated with anti-DLL4 (which inhibits Notch signaling and results in an increased, non-functional vasculature) and/or VEGF inhibition (which reduces tumor vessel density) showed that glycolytic genes were amongst the most activated ones in tip cell-like TECs upon both anti-angiogenic treatments (Zhao et al., 2018). This is remarkable, since reducing VEGF signaling would favor reduced glycolysis. Nonetheless, given the concomitant increase in the expression of hypoxia genes induced by both treatments, it is possible that hypoxia contributes to metabolic regulation of ECs in the tumor. As it is known that endothelial hypoxia signaling mediates the tumor vascular phenotype (Branco-Price et al., 2012), it will be exciting to explore the *in vivo* behavior of highly glycolytic, tip cell-like TECs and how they contribute to anti-angiogenic resistance.

Besides glycolysis, many other metabolic pathways such as the PPP, and the serine biosynthesis pathway are transcriptionally deregulated in TECS (Cantelmo et al., 2016). In addition, culturing ECs in tumor cell derived conditioned medium revealed significant changes in their metabolite profile that were dependent on the type of cancer cell (Jayaraman et al., 2018). Metabolite pathway analysis showed activated glycolysis and purine metabolism as well as FAO which was underscored by a pronounced increase in the levels of acetyl carnitine. The hyperproliferative nature of TECs might require active mitochondria for biomass synthesis and it cannot be excluded that in a competitive cancer setting, where glycolysis is already maximized, mitochondrial ATP synthesis is required for angiogenesis. Indeed, it has been shown that treatment of proliferating ECs with Embelin, a weak mitochondrial uncoupler, causes a reduction in OXPHOS which leads to reduced tumor growth and decreased microvessel density in murine tumor models (Coutelle et al., 2014). Conversely, a recent report indicates that inducing a shift to oxidative metabolism through inhibition of adrenergic signaling in ECs, can prevent the angiogenic switch in a mouse model of prostate cancer leading to decreased tumor growth (Zahalka et al., 2017). Indeed, EC specific knockout of the β_2-adrenergic receptor (ADBR2) increased the expression of cytochrome C oxidase assembly factor 6 (COA6), leading to an increase in OXPHOS activity. This was supported by increased glucose uptake and an increased contribution of glucose and glutamine oxidation to the TCA cycle without decreasing intracellular lactate. Interestingly, this increase in OXPHOS lead to decreased EC migration and proliferation, despite increased ATP levels (**Figure 4D**). This data indicates that increasing OXPHOS in TECs may directly alter EC migratory and proliferative capacity independent of levels of glycolysis (Zahalka et al., 2017). While the differences between these reports remain to be reconciled, they open up the possibility of pursuing non-glycolytic targets of TEC metabolism as cancer therapies.

TECs are part of a complex tumor microenvironment and are surrounded by not only the malignant cancer cells but also tumor associated macrophages (TAMs), fibroblasts and other stromal cells. The specific context of the tumor microenvironment imposes great metabolic challenges: the uncontrolled and rapid proliferation of cancer cells rapidly creates a hypoxic environment which is exacerbated by the abnormal characteristics of the tumor vasculature. This hypoxic response enhances glycolytic flux in tumor cells leading to a highly acidic microenvironment caused by the production of high levels of lactate (Cairns et al., 2011; Vander Heiden, 2011; Harjes et al., 2012). Lactate can be taken up by TECs through monocarboxylate transporter 1 (MCT1) which promotes angiogenesis. This occurs through increased VEGFR2 levels following the stabilization of hypoxia inducible factor 1 (HIF1) in an αKG and ROS dependent fashion rendering them more responsive to the pro-angiogenic action of VEGF (Vegran et al., 2011; Sonveaux et al., 2012). Incubation of ECs with conditioned medium from glioblastoma tumor cells increases MCT1 expression (Miranda-Goncalves et al., 2017). Moreover, lactate increases PI3K/AKT signaling downstream of angiogenic receptor activation due to increased production of pro-angiogenic factors (Ruan and Kazlauskas, 2013). Increased lactate levels in the hypoxic tumor will thus further tip the balance in favor of vessel abnormalization. *In vivo*, inhibiting lactate transport through MCT1 reduces tumor angiogenesis (Sonveaux et al., 2012). Also, upon exposure to conditioned medium from cancer cells, ECs increase expression of GLUT1 and metabolically prepare for increased angiogenic activity (Yeh et al., 2008). High succinate concentrations in the tumor microenvironment also promote glucose uptake by TECs but it is not clear whether this is via metabolic effects, HIF stabilization or via activation of the succinate receptor GPR91 (Garrigue et al., 2017).

Nutrient limitation in the tumor microenvironment provides an additional metabolic challenge in which different cell types need to compete for nutrients to support biomass generation, bioenergetic needs, as well as effector functions (Lyssiotis and Kimmelman, 2017). For instance, TAMs compete with TECs for the limited glucose in the tumor microenvironment, and stimulating glucose metabolism in TAMs induces vessel normalization (Wenes et al., 2016). The hyperglycolytic TAMs lower glucose availability for TECs so that the latter are subsequently forced toward quiescence and a more normalized phenotype. These glucose starved TECs have tighter VE-Cadherin positive junctions which, like 3PO treatment, increased perfusion, reduced tumor hypoxia and prevented metastasis. Although the metabolic crosstalk and competition between TECs and other cells in the tumor microenvironment remain largely unexplored, due to the unique and extreme conditions within tumor microenvironment, targeting this crosstalk offers windows for therapeutic opportunities. The metabolic cross talk between these cell types is therefore an intriguing topic for exploration.

FUTURE PERSPECTIVES

The role of EC metabolism in vessel sprouting has received considerable attention over the last few years. While our knowledge on how metabolism and angiogenesis interact is growing, only a few pathways have been characterized to date. Undoubtedly, extending our insight into the role of other pathways will offer tremendous insight into basic mechanisms of sprouting and non-sprouting angiogenesis. Furthermore, since metabolism drives angiogenesis, understanding the metabolic differences between healthy and diseased ECs might offer novel treatment opportunities for many diseases such as cancer but also for regenerative purposes. In addition, EC metabolism research has exclusively been performed under *in vitro* conditions and/or using preclinical mouse models. It still remains to be confirmed whether ECs have similar metabolic characteristics in humans and whether those can be exploited for therapy. Another exciting outstanding question is whether ECs alter their metabolism dependent upon the microenvironment in which they reside and the nutrients they have available. This will provide further insight into how they interact with their microenvironment. In this regard, the development of genetic tools that allow tissue restricted endothelial gene regulation will become crucial to overcome limitations of currently available models.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Abid, M. R., Guo, S., Minami, T., Spokes, K. C., Ueki, K., Skurk, C., et al. (2004). Vascular endothelial growth factor activates PI3K/Akt/forkhead signaling in endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 24, 294–300. doi: 10.1161/01.ATV.0000110502.10593.06

Adams, R. H., and Alitalo, K. (2007). Molecular regulation of angiogenesis and lymphangiogenesis. *Nat. Rev. Mol. Cell Biol.* 8, 464–478. doi: 10.1038/nrm2183

Albini, A., Tosetti, F., Li, V. W., Noonan, D. M., and Li, W. W. (2012). Cancer prevention by targeting angiogenesis. *Nat. Rev. Clin. Oncol.* 9, 498–509. doi: 10.1038/nrclinonc.2012.120

Amemiya, T. (1983). Glycogen metabolism in the capillary endothelium. Electron histochemical study of glycogen synthetase and phosphorylase in the pecten capillary of the chick. *Acta Histochem.* 73, 93–96. doi: 10.1016/S0065-1281(83)80080-4

Araiza-Olivera, D., Chiquete-Felix, N., Rosas-Lemus, M., Sampedro, J. G., Pena, A., Mujica, A., et al. (2013). A glycolytic metabolon in *Saccharomyces cerevisiae* is stabilized by F-actin. *FEBS J.* 280, 3887–3905. doi: 10.1111/febs.12387

Arima, S., Nishiyama, K., Ko, T., Arima, Y., Hakozaki, Y., Sugihara, K., et al. (2011). Angiogenic morphogenesis driven by dynamic and heterogeneous collective endothelial cell movement. *Development* 138, 4763–4776. doi: 10.1242/dev.068023

Benedito, R., Roca, C., Sorensen, I., Adams, S., Gossler, A., Fruttiger, M., et al. (2009). The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell* 137, 1124–1135. doi: 10.1016/j.cell.2009.03.025

Bentley, K., Mariggi, G., Gerhardt, H., and Bates, P. A. (2009). Tipping the balance: robustness of tip cell selection, migration and fusion in angiogenesis. *PLoS Comput. Biol.* 5:e1000549. doi: 10.1371/journal.pcbi.1000549

Bergers, G., and Hanahan, D. (2008). Modes of resistance to anti-angiogenic therapy. *Nat. Rev. Cancer* 8, 592–603. doi: 10.1038/nrc2442

Betz, C., Lenard, A., Belting, H. G., and Affolter, M. (2016). Cell behaviors and dynamics during angiogenesis. *Development* 143, 2249–2260. doi: 10.1242/dev.135616

Blanco, R., and Gerhardt, H. (2013). VEGF and Notch in tip and stalk cell selection. *Cold Spring Harb. Perspect. Med.* 3:a006569. doi: 10.1101/cshperspect.a006569

Boas, S. E., and Merks, R. M. (2015). Tip cell overtaking occurs as a side effect of sprouting in computational models of angiogenesis. *BMC Syst. Biol.* 9:86. doi: 10.1186/s12918-015-0230-7

Boeckel, J. N., Derlet, A., Glaser, S. F., Luczak, A., Lucas, T., Heumuller, A. W., et al. (2016). JMJD8 regulates angiogenic sprouting and cellular metabolism by interacting with pyruvate kinase M2 in endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 36, 1425–1433. doi: 10.1161/ATVBAHA.116.307695

Branco-Price, C., Zhang, N., Schnelle, M., Evans, C., Katschinski, D. M., Liao, D., et al. (2012). Endothelial cell HIF-1alpha and HIF-2alpha differentially regulate metastatic success. *Cancer Cell* 21, 52–65. doi: 10.1016/j.ccr.2011.11.017

Cairns, R. A., Harris, I. S., and Mak, T. W. (2011). Regulation of cancer cell metabolism. *Nat. Rev. Cancer* 11, 85–95. doi: 10.1038/nrc2981

Cantelmo, A. R., Conradi, L. C., Brajic, A., Goveia, J., Kalucka, J., Pircher, A., et al. (2016). Inhibition of the glycolytic activator PFKFB3 in endothelium induces tumor vessel normalization, impairs metastasis, and improves chemotherapy. *Cancer Cell* 30, 968–985. doi: 10.1016/j.ccell.2016.10.006

Cantelmo, A. R., Pircher, A., Kalucka, J., and Carmeliet, P. (2017). Vessel pruning or healing: endothelial metabolism as a novel target? *Expert Opin. Ther. Targets* 21, 239–247. doi: 10.1080/14728222.2017.1282465

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., et al. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380, 435–439. doi: 10.1038/380435a0

Carmeliet, P., and Jain, R. K. (2011). Molecular mechanisms and clinical applications of angiogenesis. *Nature* 473, 298–307. doi: 10.1038/nature10144

Charpentier, M. S., and Conlon, F. L. (2014). Cellular and molecular mechanisms underlying blood vessel lumen formation. *Bioessays* 36, 251–259. doi: 10.1002/bies.201300133

Chiorean, E. G., LoRusso, P., Strother, R. M., Diamond, J. R., Younger, A., Messersmith, W. A., et al. (2015). A phase I first-in-human study of enoticumab (REGN421), a fully human delta-like ligand 4 (Dll4) monoclonal antibody in patients with advanced solid tumors. *Clin. Cancer Res.* 21, 2695–2703. doi: 10.1158/1078-0432.CCR-14-2797

Chua, C. C., Hamdy, R. C., and Chua, B. H. (1998). Upregulation of vascular endothelial growth factor by H2O2 in rat heart endothelial

cells. *Free Radic. Biol. Med.* 25, 891–897. doi: 10.1016/S0891-5849(98)00115-4

Conradi, L. C., Brajic, A., Cantelmo, A. R., Bouché, A., Kalucka, J., Pircher, A., et al. (2017). Tumor vessel disintegration by maximum tolerable PFKFB3 blockade. *Angiogenesis* 20, 599–613. doi: 10.1007/s10456-017-9573-6

Coutelle, O., Hornig-Do, H. T., Witt, A., Andree, M., Schiffmann, L. M., Piekarek, M., et al. (2014). Embelin inhibits endothelial mitochondrial respiration and impairs neoangiogenesis during tumor growth and wound healing. *EMBO Mol. Med.* 6, 624–639. doi: 10.1002/emmm.201303016

Cruys, B., Wong, B. W., Kuchnio, A., Verdegem, D., Cantelmo, A. R., Conradi, L. C., et al. (2016). Glycolytic regulation of cell rearrangement in angiogenesis. *Nat. Commun.* 7:12240. doi: 10.1038/ncomms12240

Culic, O., Gruwel, M. L., and Schrader, J. (1997). Energy turnover of vascular endothelial cells. *Am. J. Physiol.* 273, C205–C213. doi: 10.1152/ajpcell.1997.273.1.C205

Dagher, Z., Ruderman, N., Tornheim, K., and Ido, Y. (2001). Acute regulation of fatty acid oxidation and amp-activated protein kinase in human umbilical vein endothelial cells. *Circ. Res.* 88, 1276–1282. doi: 10.1161/hh1201.092998

Dang, C. V. (2013). MYC, metabolism, cell growth, and tumorigenesis. *Cold Spring Harb. Perspect. Med.* 3:a014217. doi: 10.1101/cshperspect.a014217

De Bock, K., Georgiadou, M., and Carmeliet, P. (2013a). Role of endothelial cell metabolism in vessel sprouting. *Cell Metab.* 18, 634–647. doi: 10.1016/j.cmet.2013.08.001

De Bock, K., Georgiadou, M., Schoors, S., Kuchnio, A., Wong, B. W., Cantelmo, A. R., et al. (2013b). Role of PFKFB3-driven glycolysis in vessel sprouting. *Cell* 154, 651–663. doi: 10.1016/j.cell.2013.06.037

Delgado, T., Carroll, P. A., Punjabi, A. S., Margineantu, D., Hockenbery, D. M., and Lagunoff, M. (2010). Induction of the Warburg effect by Kaposi's sarcoma herpesvirus is required for the maintenance of latently infected endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 107, 10696–10701. doi: 10.1073/pnas.1004882107

Doddaballapur, A., Michalik, K. M., Manavski, Y., Lucas, T., Houtkooper, R. H., You, X., et al. (2015). Laminar shear stress inhibits endothelial cell metabolism via KLF2-mediated repression of PFKFB3. *Arterioscler. Thromb. Vasc. Biol.* 35, 137–145. doi: 10.1161/ATVBAHA.114.304277

Domigan, C. K., Warren, C. M., Antanesian, V., Happel, K., Ziyad, S., Lee, S., et al. (2015). Autocrine VEGF maintains endothelial survival through regulation of metabolism and autophagy. *J. Cell Sci.* 128, 2236–2248. doi: 10.1242/jcs.163774

Eaton, S. (2002). Control of mitochondrial beta-oxidation flux. *Prog. Lipid Res.* 41, 197–239. doi: 10.1016/S0163-7827(01)00024-8

Ebos, J. M., Lee, C. R., Cruz-Munoz, W., Bjarnason, G. A., Christensen, J. G., and Kerbel, R. S. (2009). Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell* 15, 232–239. doi: 10.1016/j.ccr.2009.01.021

Eelen, G., de Zeeuw, P., Treps, L., Harjes, U., Wong, B. W., and Carmeliet, P. (2018). Endothelial cell metabolism. *Physiol. Rev.* 98, 3–58. doi: 10.1152/physrev.00001.2017

Ellis, L. M., and Hicklin, D. J. (2008). VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nat. Rev. Cancer* 8, 579–591. doi: 10.1038/nrc2403

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., et al. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380, 439–442. doi: 10.1038/380439a0

Folkman, J. (1971). Tumor angiogenesis: therapeutic implications. *N. Engl. J. Med.* 285, 1182–1186. doi: 10.1056/NEJM197111182852108

Fukumura, D., Kloepper, J., Amoozgar, Z., Duda, D. G., and Jain, R. K. (2018). Enhancing cancer immunotherapy using antiangiogenics: opportunities and challenges. *Nat. Rev. Clin. Oncol.* 15, 325–340. doi: 10.1038/nrclinonc.2018.29

Garrigue, P., Bodin-Hullin, A., Balasse, L., Fernandez, S., Essamet, W., Dignat-George, F., et al. (2017). The evolving role of succinate in tumor metabolism: an (18)F-FDG-based study. *J. Nucl. Med.* 58, 1749–1755. doi: 10.2967/jnumed.117.192674

Gaude, E., Schmidt, C., Gammage, P. A., Dugourd, A., Blacker, T., Chew, S. P., et al. (2018). NADH shuttling couples cytosolic reductive carboxylation of glutamine with glycolysis in cells with mitochondrial dysfunction. *Mol. Cell.* 69, 581.e–593.e. doi: 10.1016/j.molcel.2018.01.034

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., et al. (2003). VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J. Cell Biol.* 161, 1163–1177. doi: 10.1083/jcb.200302047

Goel, S., Wong, A. H., and Jain, R. K. (2012). Vascular normalization as a therapeutic strategy for malignant and nonmalignant disease. *Cold Spring Harb. Perspect. Med.* 2:a006486. doi: 10.1101/cshperspect.a006486

Guarani, V., Deflorian, G., Franco, C. A., Kruger, M., Phng, L. K., Bentley, K., et al. (2011). Acetylation-dependent regulation of endothelial Notch signalling by the SIRT1 deacetylase. *Nature* 473, 234–238. doi: 10.1038/nature09917

Guo, D., Murdoch, C. E., Xu, H., Shi, H., Duan, D. D., Ahmed, A., et al. (2017). Vascular endothelial growth factor signaling requires glycine to promote angiogenesis. *Sci. Rep.* 7:14749. doi: 10.1038/s41598-017-15246-3

Hanahan, D., and Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86, 353–364. doi: 10.1016/S0092-8674(00)80108-7

Harjes, U., Bensaad, K., and Harris, A. L. (2012). Endothelial cell metabolism and implications for cancer therapy. *Br. J. Cancer* 107, 1207–1212. doi: 10.1038/bjc.2012.398

He, C., Weston, T. A., Jung, R. S., Heizer, P., Larsson, M., Hu, X., et al. (2018). NanoSIMS analysis of intravascular lipolysis and lipid movement across capillaries and into cardiomyocytes. *Cell Metab.* 27, 1055–1066.e3. doi: 10.1016/j.cmet.2018.03.017

Hellstrom, M., Phng, L. K., Hofmann, J. J., Wallgard, E., Coultas, L., Lindblom, P., et al. (2007). Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature* 445, 776–780. doi: 10.1038/nature05571

Hosios, A. M., Hecht, V. C., Danai, L. V., Johnson, M. O., Rathmell, J. C., Steinhauser, M. L., et al. (2016). Amino acids rather than glucose account for the majority of cell mass in proliferating mammalian cells. *Dev. Cell* 36, 540–549. doi: 10.1016/j.devcel.2016.02.012

Hu, H., Juvekar, A., Lyssiotis, C. A., Lien, E. C., Albeck, J. G., Oh, D., et al. (2016). Phosphoinositide 3-Kinase regulates glycolysis through mobilization of aldolase from the actin cytoskeleton. *Cell* 164, 433–446. doi: 10.1016/j.cell.2015.12.042

Huang, H., Vandekeere, S., Kalucka, J., Bierhansl, L., Zecchin, A., Brüning, U., et al. (2017). Role of glutamine and interlinked asparagine metabolism in vessel formation. *EMBO J.* 36, 2334–2352. doi: 10.15252/embj.201695518

Ibrahim, A., and Arany, Z. (2017). Does endothelium buffer fat? *Circ. Res.* 120, 1219–1221. doi: 10.1161/CIRCRESAHA.117.310865

Iruela-Arispe, M. L., and Davis, G. E. (2009). Cellular and molecular mechanisms of vascular lumen formation. *Dev. Cell* 16, 222–231. doi: 10.1016/j.devcel.2009.01.013

Jabs, M., Rose, A. J., Lehmann, L. H., Taylor, J., Moll, I., Sijmonsma, T. P., et al. (2018). Inhibition of endothelial notch signaling impairs fatty acid transport and leads to metabolic and vascular remodeling of the adult heart. *Circulation* 137, 2592–2608. doi: 10.1161/CIRCULATIONAHA.117.029733

Jain, R. K. (2001). Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. *Nat. Med.* 7, 987–989. doi: 10.1038/nm0901-987

Jain, R. K. (2005). Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 307, 58–62. doi: 10.1126/science.1104819

Jakobsson, L., Franco, C. A., Bentley, K., Collins, R. T., Ponsioen, B., Aspalter, I. M., et al. (2010). Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. *Nat. Cell Biol.* 12, 943–953. doi: 10.1038/ncb2103

Jayaraman, A., Kumar, P., Marin, S., de Atauri, P., Mateo, F. M., Thomson, T., et al. (2018). Untargeted metabolomics reveals distinct metabolic reprogramming in endothelial cells co-cultured with CSC and non-CSC prostate cancer cell subpopulations. *PLoS One* 13:e0192175. doi: 10.1371/journal.pone.0192175

Jayson, G. C., Kerbel, R., Ellis, L. M., and Harris, A. L. (2016). Antiangiogenic therapy in oncology: current status and future directions. *Lancet* 388, 518–529. doi: 10.1016/S0140-6736(15)01088-0

Jones, C. I. III, Han, Z., Presley, T., Varadharaj, S., Zweier, J. L., Ilangovan, G., et al. (2008). Endothelial cell respiration is affected by the oxygen tension during shear exposure: role of mitochondrial peroxynitrite. *Am. J. Physiol. Cell Physiol.* 295, C180–C191. doi: 10.1152/ajpcell.00549.2007

Karlsson, T., Bolshakova, A., Magalhaes, M. A., Loitto, V. M., and Magnusson, K. E. (2013). Fluxes of water through aquaporin 9 weaken membrane-cytoskeleton

anchorage and promote formation of membrane protrusions. *PLoS One* 8:e59901. doi: 10.1371/journal.pone.0059901

Kim, B., Li, J., Jang, C., and Arany, Z. (2017). Glutamine fuels proliferation but not migration of endothelial cells. *EMBO J.* 36, 2321–2333. doi: 10.15252/embj.201796436

Kim, J., Kim, Y. H., Kim, J., Park, D. Y., Bae, H., Lee, D. H., et al. (2017). YAP/TAZ regulates sprouting angiogenesis and vascular barrier maturation. *J. Clin. Invest.* 127, 3441–3461. doi: 10.1172/JCI93825

Kishimoto, A., Takahashi-Iwanaga, H., Watanabe, M. M., and Iwanaga, T. (2016). Differential expression of endothelial nutrient transporters (MCT1 and GLUT1) in the developing eyes of mice. *Exp. Eye Res.* 153, 170–177. doi: 10.1016/j.exer.2016.10.019

Kondo, M., Shibata, R., Miura, R., Shimano, M., Kondo, K., Li, P., et al. (2009). Caloric restriction stimulates revascularization in response to ischemia via adiponectin-mediated activation of endothelial nitric-oxide synthase. *J. Biol. Chem.* 284, 1718–1724. doi: 10.1074/jbc.M805301200

Kops, G. J., Dansen, T. B., Polderman, P. E., Saarloos, I., Wirtz, K. W., Coffer, P. J., et al. (2002). Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature* 419, 316–321. doi: 10.1038/nature01036

Korn, C., and Augustin, H. G. (2015). Mechanisms of vessel pruning and regression. *Dev. Cell* 34, 5–17. doi: 10.1016/j.devcel.2015.06.004

Krutzfeldt, A., Spahr, R., Mertens, S., Siegmund, B., and Piper, H. M. (1990). Metabolism of exogenous substrates by coronary endothelial cells in culture. *J. Mol. Cell Cardiol.* 22, 1393–1404. doi: 10.1016/0022-2828(90)90984-A

Kuo, A., Lee, M. Y., and Sessa, W. C. (2017). Lipid droplet biogenesis and function in the endothelium. *Circ. Res.* 120, 1289–1297. doi: 10.1161/CIRCRESAHA.116.310498

Lee, D. Y., Lee, C. I., Lin, T. E., Lim, S. H., Zhou, J., Tseng, Y. C., et al. (2012). Role of histone deacetylases in transcription factor regulation and cell cycle modulation in endothelial cells in response to disturbed flow. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1967–1972. doi: 10.1073/pnas.1121214109

Lenard, A., Ellertsdottir, E., Herwig, L., Krudewig, A., Sauteur, L., Belting, H. G., et al. (2013). In vivo analysis reveals a highly stereotypic morphogenetic pathway of vascular anastomosis. *Dev. Cell* 25, 492–506. doi: 10.1016/j.devcel.2013.05.010

Li, F. L., Liu, J. P., Bao, R. X., Yan, G., Feng, X., Xu, Y. P., et al. (2018). Acetylation accumulates PFKFB3 in cytoplasm to promote glycolysis and protects cells from cisplatin-induced apoptosis. *Nat. Commun.* 9:508. doi: 10.1038/s41467-018-02950-5

Longchamp, A., Mirabella, T., Arduini, A., MacArthur, M. R., Das, A., Trevino-Villarreal, J. H., et al. (2018). Amino acid restriction triggers angiogenesis via GCN2/ATF4 regulation of VEGF and H2S production. *Cell* 173, 117–129.e14. doi: 10.1016/j.cell.2018.03.001

Lyssiotis, C. A., and Kimmelman, A. C. (2017). Metabolic interactions in the tumor microenvironment. *Trends Cell Biol.* 27, 863–875. doi: 10.1016/j.tcb.2017.06.003

Mazzone, M., Dettori, D., de Oliveira, R. L., Loges, S., Schmidt, T., Jonckx, B., et al. (2009). Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. *Cell* 136, 839–851. doi: 10.1016/j.cell.2009.01.020

Merchan, J. R., Kovacs, K., Railsback, J. W., Kurtoglu, M., Jing, Y., Pina, Y., et al. (2010). Antiangiogenic activity of 2-deoxy-D-glucose. *PLoS One* 5:e13699. doi: 10.1371/journal.pone.0013699

Miranda-Goncalves, V., Bezerra, F., Costa-Almeida, R., Freitas-Cunha, M., Soares, R., Martinho, O., et al. (2017). Monocarboxylate transporter 1 is a key player in glioma-endothelial cell crosstalk. *Mol. Carcinog.* 56, 2630–2642. doi: 10.1002/mc.22707

Miura, N., Shinohara, M., Tatsukami, Y., Sato, Y., Morisaka, H., Kuroda, K., et al. (2013). Spatial reorganization of *Saccharomyces cerevisiae* enolase to alter carbon metabolism under hypoxia. *Eukaryot. Cell* 12, 1106–1119. doi: 10.1128/EC.00093-13

Moretto, P., Karousou, E., Viola, M., Caon, I., D'Angelo, M. L., De Luca, G., et al. (2015). Regulation of hyaluronan synthesis in vascular diseases and diabetes. *J. Diabetes Res.* 2015:167283. doi: 10.1155/2015/167283

Nagata, D., Mogi, M., and Walsh, K. (2003). AMP-activated protein kinase (AMPK) signaling in endothelial cells is essential for angiogenesis in response to hypoxic stress. *J. Biol. Chem.* 278, 31000–31006. doi: 10.1074/jbc.M300643200

Neto, F., Klaus-Bergmann, A., Ong, Y. T., Alt, S., Vion, A. C., Szymborska, A., et al. (2018). YAP and TAZ regulate adherens junction dynamics and endothelial cell distribution during vascular development. *eLife* 7:e31037. doi: 10.7554/eLife.31037

Noseda, M., Chang, L., McLean, G., Grim, J. E., Clurman, B. E., Smith, L. L., et al. (2004). Notch activation induces endothelial cell cycle arrest and participates in contact inhibition: role of p21Cip1 repression. *Mol. Cell. Biol.* 24, 8813–8822. doi: 10.1128/MCB.24.20.8813-8822.2004

Orentreich, N., Matias, J. R., DeFelice, A., and Zimmerman, J. A. (1993). Low methionine ingestion by rats extends life span. *J. Nutr.* 123, 269–274.

Paez-Ribes, M., Allen, E., Hudock, J., Takeda, T., Okuyama, H., Vinals, F., et al. (2009). Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 15, 220–231. doi: 10.1016/j.ccr.2009.01.027

Pajvani, U. B., Shawber, C. J., Samuel, V. T., Birkenfeld, A. L., Shulman, G. I., Kitajewski, J., et al. (2011). Inhibition of Notch signaling ameliorates insulin resistance in a FoxO1-dependent manner. *Nat. Med.* 17, 961–967. doi: 10.1038/nm.2378

Pan, S., World, C. J., Kovacs, C. J., and Berk, B. C. (2009). Glucose 6-phosphate dehydrogenase is regulated through c-Src-mediated tyrosine phosphorylation in endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 29, 895–901. doi: 10.1161/ATVBAHA.109.184812

Pandolfi, P. P., Sonati, F., Rivi, R., Mason, P., Grosveld, F., and Luzzatto, L. (1995). Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress. *EMBO J.* 14, 5209–5215.

Parra-Bonilla, G., Alvarez, D. F., Al-Mehdi, A. B., Alexeyev, M., and Stevens, T. (2010). Critical role for lactate dehydrogenase A in aerobic glycolysis that sustains pulmonary microvascular endothelial cell proliferation. *Am. J. Physiol. Lung. Cell Mol. Physiol.* 299, L513–L522. doi: 10.1152/ajplung.00274.2009

Pastorino, J. G., and Hoek, J. B. (2008). Regulation of hexokinase binding to VDAC. *J. Bioenerg. Biomembr.* 40, 171–182. doi: 10.1007/s10863-008-9148-8

Patella, F., Schug, Z. T., Persi, E., Neilson, L. J., Erami, Z., Avanzato, D., et al. (2015). Proteomics-based metabolic modeling reveals that fatty acid oxidation (FAO) controls endothelial cell (EC) permeability. *Mol. Cell. Proteom.* 14, 621–634. doi: 10.1074/mcp.M114.045575

Pavlova, N. N., and Thompson, C. B. (2016). The emerging hallmarks of cancer metabolism. *Cell Metab.* 23, 27–47. doi: 10.1016/j.cmet.2015.12.006

Pfeiffer, T., Schuster, S., and Bonhoeffer, S. (2001). Cooperation and competition in the evolution of ATP-producing pathways. *Science* 292, 504–507. doi: 10.1126/science.1058079

Pike, L. S., Smift, A. L., Croteau, N. J., Ferrick, D. A., and Wu, M. (2011). Inhibition of fatty acid oxidation by etomoxir impairs NADPH production and increases reactive oxygen species resulting in ATP depletion and cell death in human glioblastoma cells. *Biochim. Biophys. Acta* 1807, 726–734. doi: 10.1016/j.bbabio.2010.10.022

Pollard, T. D., and Borisy, G. G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. *Cell* 112, 453–465. doi: 10.1016/S0092-8674(03)00120-X

Potente, M., Gerhardt, H., and Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. *Cell* 146, 873–887. doi: 10.1016/j.cell.2011.08.039

Pries, A. R., Secomb, T. W., and Gaehtgens, P. (2000). The endothelial surface layer. *Pflugers. Arch.* 440, 653–666. doi: 10.1007/s004240000307

Real-Hohn, A., Zancan, P., Da Silva, D., Martins, E. R., Salgado, L. T., Mermelstein, C. S., et al. (2010). Filamentous actin and its associated binding proteins are the stimulatory site for 6-phosphofructo-1-kinase association within the membrane of human erythrocytes. *Biochimie* 92, 538–544. doi: 10.1016/j.biochi.2010.01.023

Ricard, N., and Simons, M. (2015). When it is better to regress: dynamics of vascular pruning. *PLoS Biol.* 13:e1002148. doi: 10.1371/journal.pbio.\break1002148

Rostama, B., Turner, J. E., Seavey, G. T., Norton, C. R., Gridley, T., Vary, C. P., et al. (2015). DLL4/Notch1 and BMP9 interdependent signaling induces human endothelial cell quiescence via P27KIP1 and Thrombospondin-1.

Arterioscler. Thromb. Vasc. Biol. 35, 2626–2637. doi: 10.1161/ATVBAHA.115.306541

Ruan, G. X., and Kazlauskas, A. (2013). Lactate engages receptor tyrosine kinases Axl, Tie2, and vascular endothelial growth factor receptor 2 to activate phosphoinositide 3-kinase/Akt and promote angiogenesis. *J. Biol. Chem.* 288, 21161–21172. doi: 10.1074/jbc.M113.474619

Schmitt, D. L., and An, S. (2017). Spatial organization of metabolic enzyme complexes in cells. *Biochemistry* 56, 3184–3196. doi: 10.1021/acs.biochem.7b00249

Schoors, S., Bruning, U., Missiaen, R., Queiroz, K. C., Borgers, G., Elia, I., et al. (2015). Fatty acid carbon is essential for dNTP synthesis in endothelial cells. *Nature* 520, 192–197. doi: 10.1038/nature14362

Schoors, S., De Bock, K., Cantelmo Anna, R., Georgiadou, M., Ghesquière, B., Cauwenberghs, S., et al. (2014). Partial and transient reduction of glycolysis by PFKFB3 blockade reduces pathological angiogenesis. *Cell Metab.* 19, 37–48. doi: 10.1016/j.cmet.2013.11.008

Schwab, A., Fabian, A., Hanley, P. J., and Stock, C. (2012). Role of ion channels and transporters in cell migration. *Physiol. Rev.* 92, 1865–1913. doi: 10.1152/physrev.00018.2011

Smith, D. C., Eisenberg, P. D., Manikhas, G., Chugh, R., Gubens, M. A., Stagg, R. J., et al. (2014). A phase I dose escalation and expansion study of the anticancer stem cell agent demcizumab (anti-DLL4) in patients with previously treated solid tumors. *Clin. Cancer Res.* 20, 6295–6303. doi: 10.1158/1078-0432.CCR-14-1373

Snaebjornsson, M. T., and Schulze, A. (2018). Non-canonical functions of enzymes facilitate cross-talk between cell metabolic and regulatory pathways. *Exp. Mol. Med.* 50:34. doi: 10.1038/s12276-018-0065-6

Sonveaux, P., Copetti, T., de Saedeleer, C. J., Végran, F., Verrax, J., Kennedy, K. M., et al. (2012). Targeting the lactate transporter MCT1 in endothelial cells inhibits lactate-induced HIF-1 activation and tumor angiogenesis. *PLoS One* 7:e33418. doi: 10.1371/journal.pone.0033418

Strasser, G. A., Kaminker, J. S., and Tessier-Lavigne, M. (2010). Microarray analysis of retinal endothelial tip cells identifies CXCR4 as a mediator of tip cell morphology and branching. *Blood* 115, 5102–5110. doi: 10.1182/blood-2009-07-230284

Suchting, S., Freitas, C., le Noble, F., Benedito, R., Breant, C., Duarte, A., et al. (2007). The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3225–3230. doi: 10.1073/pnas.0611177104

Trenti, A., Tedesco, S., Boscaro, C., Ferri, N., Cignarella, A., Trevisi, L., et al. (2017). The glycolytic enzyme PFKFB3 is involved in estrogen-mediated angiogenesis via GPER1. *J. Pharmacol. Exp. Ther.* 361, 398–407. doi: 10.1124/jpet.116.238212

Vaisman, N., Gospodarowicz, D., and Neufeld, G. (1990). Characterization of the receptors for vascular endothelial growth factor. *J. Biol. Chem.* 265, 19461–19466.

van Horssen, R., Janssen, E., Peters, W., van de Pasch, L., Lindert, M. M., van Dommelen, M. M., et al. (2009). Modulation of cell motility by spatial repositioning of enzymatic ATP/ADP exchange capacity. *J. Biol. Chem.* 284, 1620–1627. doi: 10.1074/jbc.M806974200

Van Schaftingen, E., Lederer, B., Bartrons, R., and Hers, H. G. (1982). A kinetic study of pyrophosphate: fructose-6-phosphate phosphotransferase from potato tubers. Application to a microassay of fructose 2,6-bisphosphate. *Eur. J. Biochem.* 129, 191–195. doi: 10.1111/j.1432-1033.1982.tb07039.x

Vandekeere, S., Dubois, C., Kalucka, J., Sullivan, M. R., García-Caballero, M., Goveia, J., et al. (2018). Serine synthesis via PHGDH is essential for heme production in endothelial cells. *Cell Metab.* doi: 10.1016/j.cmet.2018.06.009 [Epub ahead of print].

Vander Heiden, M. G. (2011). Targeting cancer metabolism: a therapeutic window opens. *Nat. Rev. Drug Discov.* 10, 671–684. doi: 10.1038/nrd3504

Vander Heiden, M. G., Cantley, L. C., and Thompson, C. B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 324, 1029–1033. doi: 10.1126/science.1160809

Vegran, F., Boidot, R., Michiels, C., Sonveaux, P., and Feron, O. (2011). Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF-kappaB/IL-8 pathway that drives tumor angiogenesis. *Cancer Res.* 71, 2550–2560. doi: 10.1158/0008-5472.CAN-10-2828

Vizan, P., Sanchez-Tena, S., Alcarraz-Vizan, G., Soler, M., Messeguer, R., Pujol, M. D., et al. (2009). Characterization of the metabolic changes underlying growth factor angiogenic activation: identification of new potential therapeutic targets. *Carcinogenesis* 30, 946–952. doi: 10.1093/carcin/bgp083

Wang, X., Freire Valls, A., Schermann, G., Shen, Y., Moya, I. M., Castro, L., et al. (2017). YAP/TAZ orchestrate VEGF signaling during developmental angiogenesis. *Dev. Cell* 42, 462–478e467. doi: 10.1016/j.devcel.2017.08.002

Wang, Y., Zang, Q. S., Liu, Z., Wu, Q., Maass, D., Dulan, G., et al. (2011). Regulation of VEGF-induced endothelial cell migration by mitochondrial reactive oxygen species. *Am. J. Physiol. Cell Physiol.* 301, C695–C704. doi: 10.1152/ajpcell.00322.2010

Warren, C. M., Ziyad, S., Briot, A., Der, A., and Iruela-Arispe, M. L. (2014). A ligand-independent VEGFR2 signaling pathway limits angiogenic responses in diabetes. *Sci. Signal.* 7:ra1. doi: 10.1126/scisignal.2004235

Wellen, K. E., and Thompson, C. B. (2010). Cellular metabolic stress: considering how cells respond to nutrient excess. *Mol. Cell.* 40, 323–332. doi: 10.1016/j.molcel.2010.10.004

Wenes, M., Shang, M., Di Matteo, M., Goveia, J., Martín-Pérez, R., Serneels, J., et al. (2016). Macrophage metabolism controls tumor blood vessel morphogenesis and metastasis. *Cell Metab.* 24, 701–715. doi: 10.1016/j.cmet.2016.09.008

Wilhelm, K., Happel, K., Eelen, G., Schoors, S., Oellerich, M. F., Lim, R., et al. (2016). FOXO1 couples metabolic activity and growth state in the vascular endothelium. *Nature* 529, 216–220. doi: 10.1038/nature16498

Wojtas, K., Slepecky, N., von Kalm, L., and Sullivan, D. (1997). Flight muscle function in Drosophila requires colocalization of glycolytic enzymes. *Mol. Biol. Cell* 8, 1665–1675. doi: 10.1091/mbc.8.9.1665

Wolinsky, H. (1980). A proposal linking clearance of circulating lipoproteins to tissue metabolic activity as a basis for understanding atherogenesis. *Circ. Res.* 47, 301–311. doi: 10.1161/01.RES.47.3.301

Wright, G. L., Maroulakou, I. G., Eldridge, J., Liby, T. L., Sridharan, V., Tsichlis, P. N., et al. (2008). VEGF stimulation of mitochondrial biogenesis: requirement of AKT3 kinase. *FASEB J.* 22, 3264–3275. doi: 10.1096/fj.08-106468

Wu, D., Huang, R. T., Hamanaka, R. B., Krause, M., Oh, M. J., Kuo, C. H., et al. (2017). HIF-1alpha is required for disturbed flow-induced metabolic reprogramming in human and porcine vascular endothelium. *eLife* 6:e25217. doi: 10.7554/eLife.25217

Xu, Y., An, X., Guo, X., Habtetsion, T. G., Wang, Y., Xu, X., et al. (2014). Endothelial PFKFB3 plays a critical role in angiogenesis. *Arterioscler. Thromb. Vasc. Biol.* 34, 1231–1239. doi: 10.1161/ATVBAHA.113.303041

Yalcin, A., Clem, B. F., Simmons, A., Lane, A., Nelson, K., Clem, A. L., et al. (2009). Nuclear targeting of 6-phosphofructo-2-kinase (PFKFB3) increases proliferation via cyclin-dependent kinases. *J. Biol. Chem.* 284, 24223–24232. doi: 10.1074/jbc.M109.016816

Yan, M., Callahan, C. A., Beyer, J. C., Allamneni, K. P., Zhang, G., Ridgway, J. B., et al. (2010). Chronic DLL4 blockade induces vascular neoplasms. *Nature* 463, E6–E7. doi: 10.1038/nature08751

Yang, W., Xia, Y., Hawke, D., Li, X., Liang, J., Xing, D., et al. (2014). PKM2 phosphorylates histone H3 and promotes gene transcription and tumorigenesis. *Cell* 150, 685–696. doi: 10.1016/j.cell.2012.07.018

Yeh, W. L., Lin, C. J., and Fu, W. M. (2008). Enhancement of glucose transporter expression of brain endothelial cells by vascular endothelial growth factor derived from glioma exposed to hypoxia. *Mol. Pharmacol.* 73, 170–177. doi: 10.1124/mol.107.038851

Yizhak, K., Le Devedec, S. E., Rogkoti, V. M., Baenke, F., de Boer, V. C., Frezza, C., et al. (2014). A computational study of the Warburg effect identifies metabolic targets inhibiting cancer migration. *Mol. Syst. Biol.* 10:744. doi: 10.15252/msb.20134993

Yu, P., Wilhelm, K., Dubrac, A., Tung, J. K., Alves, T. C., Fang, J. S., et al. (2017). FGF-dependent metabolic control of vascular development. *Nature* 545, 224–228. doi: 10.1038/nature22322

Yuan, F., Chen, Y., Dellian, M., Safabakhsh, N., Ferrara, N., and Jain, R. K. (1996). Time-dependent vascular regression and permeability changes in established human tumor xenografts induced by an anti-vascular endothelial growth

factor/vascular permeability factor antibody. *Proc. Natl. Acad. Sci. U.S.A.* 93, 14765–14770. doi: 10.1073/pnas.93.25.14765

Zahalka, A. H., Arnal-Estape, A., Maryanovich, M., Nakahara, F., Cruz, C. D., Finley, L. W. S., et al. (2017). Adrenergic nerves activate an angio-metabolic switch in prostate cancer. *Science* 358, 321–326. doi: 10.1126/science.aah5072

Zhao, Q., Eichten, A., Parveen, A., Adler, C., Huang, Y., Wang, W., et al. (2018). Single-cell transcriptome analyses reveal endothelial cell heterogeneity in tumors and changes following antiangiogenic treatment. *Cancer Res.* 78, 2370–2382. doi: 10.1158/0008-5472.CAN-17-2728

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MINI REVIEW
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mTOR Regulation of Glycolytic Metabolism in T Cells

*Robert J. Salmond**

Leeds Institute of Cancer and Pathology, St James's University Hospital, University of Leeds, Leeds, United Kingdom

T cell activation, differentiation and effector function is intrinsically linked to the regulation of metabolic pathways. Evidence has shown that inflammatory T cell responses are dependent upon the adoption of aerobic glycolytic metabolism. Furthermore, activation and regulation of the mechanistic target of rapamycin signaling pathway serves a key determinant of T cell metabolism, with subsequent effects on T cell effector responses. In this mini-review, we discuss the mechanisms underpinning the function of the Warburg effect in T cell responses and the role of mTOR in these processes.

Keywords: T cell, aerobic glycolysis, mTOR, metabolism, immune responses

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Andras Perl,
Upstate Medical University,
United States
Gabriela Andrejeva,
Vanderbilt University Medical Center,
United States

***Correspondence:**
Robert J. Salmond
r.j.salmond@leeds.ac.uk

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INTRODUCTION

T cells serve as cellular effectors and orchestrators of adaptive immune responses during infection and cancer. In the past decade, a wealth of data has determined that T cell activation, clonal expansion, effector differentiation, and function is closely linked to and dependent upon the regulation of basic cellular metabolic processes. It has become clear that in effector T cells, the pyruvate produced by glycolysis is preferentially fermented to lactate even in the presence of oxygen; a classic example of the Warburg effect in non-transformed cells. In this review, we discuss how the engagement of aerobic glycolysis influences T cell activation and describe the role of the mechanistic target of rapamycin (mTOR) pathway in these processes.

METABOLIC REPROGRAMMING DURING T CELL ACTIVATION

Prior to encountering antigen, T cells are quiescent and lack effector function. These naïve T cells uptake low levels of glucose and amino acids, and rely on mitochondrial oxidative phosphorylation (OXPHOS) to maintain cellular ATP levels [reviewed in Geltink et al. (2018)]. Naïve T cells may survive for years circulating through the blood and lymph, only rarely undergoing cell division. Upon encounter with peptide antigen-major histocompatibility complexes (MHC) presented by antigen-presenting cells, the differentiation of naïve $CD4^+$ T cells to a plethora of specialized helper T cell (Th) subsets enables the immune system to respond appropriately to a huge variety of pathogens, from extracellular parasitic worms to intracellular viruses and bacteria. In this regard, $CD4^+$ Th cells modulate the activity and function of innate and adaptive immune cells by secreting cytokines. $CD4^+$ Th1 cells promote cell-mediated immunity by secreting interleukin (IL)-2, interferon (IFN)-γ and tumor necrosis factor (TNF) whereas Th2 cells promote humoral immunity through the production of IL-4, IL-5 and IL-13 (Asnagli and Murphy, 2001). Th17 cells produce high levels of IL-17 and are important for maintenance of homeostasis and protection from pathogens at barrier sites, such as the intestine (Stockinger and Omenetti, 2017). By contrast, regulatory $CD4^+$ T cells (Treg), characterized by expression of the transcription factor forkhead box P3 (FOXP3), have a key role in limiting inflammation and preventing autoimmunity by suppressing the activity of other immune cell types (Sakaguchi et al., 2010). Cytotoxic $CD8^+$ T cells have the capacity to target and kill infected and transformed cells, and produce inflammatory

cytokines such as IFNγ (Halle et al., 2017). Upon resolution of an immune response, a number of memory T cell populations capable of responding rapidly to a second antigenic encounter are retained, facilitating life-long protection from re-infection.

The processes of T cell activation are bioenergetically expensive; for example, it has been estimated that, during infection, virus-specific $CD8^+$ T cells undergo rapid proliferation with a population doubling time of only ∼8 h (De Boer et al., 2003). Therefore, a key question in immunology is: how do T cells fuel the processes of activation, proliferation and differentiation? Whereas cytokines such as IL-7 maintain low level glycolytic metabolism in naïve T cells (Jacobs et al., 2010), triggering of the T cell antigen receptor (TCR) by cognate peptide antigen-MHC presented on the surface of antigen-presenting cells, results in the upregulation of anabolic biosynthetic pathways in order to facilitate T cell activation. The integration of TCR, CD28 co-stimulation and cytokine receptor signals determines T cell metabolism and subsequently impacts upon differentiation, and effector function (Fox et al., 2005; Cornish et al., 2006; Jacobs et al., 2010; Michalek et al., 2011; Shi et al., 2011; Finlay et al., 2012; Gubser et al., 2013; Ray et al., 2015; Richer et al., 2015; Tan et al., 2017; Geltink et al., 2018). The regulation of aerobic glycolysis is central to these fate decisions.

AEROBIC GLYCOLYSIS DRIVES EFFECTOR T CELL DIFFERENTIATION

An important role for glucose uptake, and glycolysis in T cell function was suggested four decades ago by the demonstration that the glycolysis inhibitor 2-deoxyglucose (2-DG) impaired T cell cytotoxic capacity (MacDonald, 1977; MacDonald and Cerottini, 1979). Furthermore, studies indicated that 2-DG treatment selectively reduced the expression of key effector molecules, including IFNγ and granzymes, and cell cycle proteins in both mouse (Cham and Gajewski, 2005; Cham et al., 2008) and human (Renner et al., 2015) $CD8^+$ T cells. At low doses that do not impact upon TCR-induced proliferation, 2-DG also inhibits $CD4^+$ Th2 (Yang et al., 2016) and Th17 (Shi et al., 2011) cell differentiation, but promotes Treg differentiation (Shi et al., 2011). Together, these studies indicate that the regulation of glycolytic flux plays a central role in cell fate decisions, and T cell differentiation. In recent years, mass-spectrometry based proteomic analyses have further informed our understanding of the extent to which the regulation of metabolic pathways is prioritized by T cells. Thus, studies from the Cantrell lab have shown that 41 glycolytic proteins represent 7% of the total protein molecules in effector cytotoxic $CD8^+$ T cells (Hukelmann et al., 2016).

Upon TCR triggering, expression of plasma membrane glucose transporters is enhanced as part of the general process of metabolic reprogramming. T cell-specific knockout of the glucose transporter SLC2A1/GLUT1 substantially inhibited the activation of mouse $CD4^+$ T cells (Macintyre et al., 2014). Whilst the homeostasis and survival of naïve T cells was unaffected by the absence of GLUT1, TCR-induced $CD4^+$ T cell growth, and proliferation were profoundly impaired. Furthermore, differentiation of *Slc2a1*$^{-/-}$ T cells to effector Th1, Th2 and Th17, but not Treg, lineages was blocked (Macintyre et al., 2014), consistent with the known effects on T cell differentiation of inhibiting glycolytic flux with 2-DG. As anticipated, T cell activation defects in TCR-stimulated *Slc2a1*$^{-/-}$ T cells were associated with reduced rates of glucose uptake, glycolysis and lactate production (Macintyre et al., 2014). The lack of a catastrophic impact of GLUT1-deficiency on glycolytic flux, and cell survival in naïve T cells is likely to be a consequence of expression of additional glucose transporters, including GLUT3, by T cells (Macintyre et al., 2014; Hukelmann et al., 2016). The importance of glucose uptake in T cell responses *in vivo* has been further highlighted by recent studies indicating that T cells and cancer cells directly compete for nutrients in the tumor microenvironment (Chang et al., 2015; Ho et al., 2015; Siska et al., 2017). Thus, highly glycolytic tumor variants suppress the activity of anti-tumor T cells, at least in part, by reducing the bioavailability of glucose.

In addition to upregulating glycolytic metabolism, activated T cells also increase uptake and hydrolysis of amino acids such as glutamine, and modulate mitochondrial, and lipid metabolism [reviewed in Geltink et al. (2018)]. Distinct T cell populations differ in their utilization, and dependence upon these metabolic programs. Effector $CD8^+$ T cells, and Th1, Th2 and Th17 $CD4^+$ T cells are highly glycolytic, whereas Tregs are dependent upon fatty acid oxidation (FAO) (Michalek et al., 2011; Shi et al., 2011 Berod et al., 2014) (**Figure 1**). Based on the use of chemical inhibitors, FAO has also been suggested to be important for the development of memory T cells (reviewed in (Lochner et al., 2015)); although recent evidence using genetic mouse models suggest that the requirement for FAO is not absolute (Pan et al., 2017; Raud et al., 2018). The use of electron microscopy has determined that memory T cells have altered mitochondrial morphology with fused cristae, that appears to favor OXPHOS and FAO (Buck et al., 2016). Furthermore, a recent study identified a crucial role for CD28 co-stimulatory signals during initial T cell activation to 'prime' mitochondria with elevated spare respiratory capacity, that is necessary for the rapid recall responses of memory T cells (Klein Geltink et al., 2017). The ability of quiescent memory T cells to re-acquire effector function rapidly upon TCR triggering is also dependent upon immediate re-engagement of glycolysis (Gubser et al., 2013; Klein Geltink et al., 2017). Therefore, in general terms, a highly glycolytic metabolism is associated with T cell effector responses, whereas low level glycolysis and lipid metabolism is associated with memory and regulatory T cell responses.

MECHANISMS UNDERPINNING THE ROLE OF AEROBIC GLYCOLYSIS IN T CELL FUNCTION

The function of the Warburg effect in activated effector T cells is incompletely understood. Aerobic glycolysis is an inefficient means of energy production, producing only 2 molecules of ATP as compared to between 30 and 36 produced by OXPHOS. Furthermore, experiments using the ATP synthase inhibitor

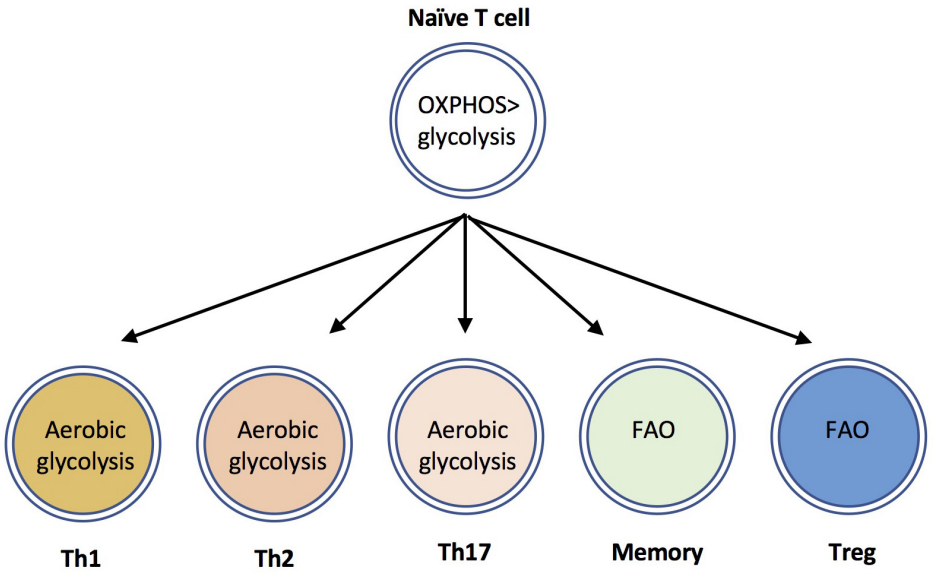

FIGURE 1 | $CD4^+$ T cell subsets use distinct metabolic programs. Naïve T cells uptake low levels of glucose and primarily utilize mitochondrial oxidative phosphorylation to maintain homeostasis. The differentiation and effector functions of inflammatory Th1, Th2, and Th17 cells relies on engagement of aerobic glycolysis. By contrast, memory T cells and Tregs are dependent upon fatty acid oxidation (FAO) pathways.

oligomycin demonstrated that mitochondrial ATP production via OXPHOS is required for initial stages of T cell activation and proliferation (Chang et al., 2013). By contrast, data indicate that expression of the rate-limiting glycolytic enzyme hexokinase 2 (HK2) is actually dispensable for early stages of T cell activation (Tan et al., 2017). It has been suggested that a key advantage of the Warburg effect for cancer cells, and presumably for all proliferating cells, is that it allows the metabolic flexibility required to build biomass (Vander Heiden et al., 2009). Thus, a key function of a switch to aerobic glycolysis might be to enable T cells to use glucose for the generation of biosynthetic precursors for amino acids and nucleic acids, critical for rapid growth, and population expansion, via the pentose phosphate pathway (PPP) (reviewed in (Lunt and Vander Heiden, 2011)). Carbon tracing experiments indicate that, in activated T cells, up to 85% of glucose is excreted as lactate (Fox et al., 2005), indicating that only a minor proportion of glucose-derived carbon is used to fuel biosynthetic pathways. Rather, the NADPH generated by the PPP is rate-limiting in the production of amino acids, nucleic acids, and fatty acids in T cells, and it is likely that aerobic glycolysis allows a faster flux through this pathway as compared to mitochondrial respiration (Vander Heiden et al., 2009). Indeed, blockade of lactate excretion using pharmacological inhibitors of the monocarboxylate transporter MCT1 inhibits T cell proliferation (Murray et al., 2005). Furthermore, a number of recent studies have provided evidence that elevated glucose uptake, and engagement of aerobic glycolysis modulates T cell effector responses through additional mechanisms.

Chang et al. (2013) demonstrated an important role for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in regulating effector T cell function via a post-transcriptional mechanism. These researchers showed that, in addition to functioning as a glycolytic enzyme, GAPDH binds to the 3′-untranslated region (UTR) of IFNγ mRNA and prevents efficient translation (Chang et al., 2013). By engaging glycolysis, effector T cells sequester GAPDH away from IFNγ mRNA and thereby enhance cytokine production. Further mechanistic insight into the role of glycolysis in inflammatory cytokine production comes from studies of lactate dehydrogenase A (LDHA) function in T cells. LDHA expression is enhanced in activated T cells and is required to support aerobic glycolysis (Peng et al., 2016). Furthermore, IFNγ production was reduced in LDHA-deficient $CD4^+$ Th1 cells as compared to control cells, consistent with the known role of glycolysis in T cell effector function. This effect was independent of the *Ifng* 3′-UTR, indicating a distinct effect on cytokine production from that mediated by GAPDH. In the absence of LDHA, histone 3 acetylation at lysine 9 (H3K9Ac) and lysine 27 (H3K27Ac) within the *Ifng* promoter region was substantially decreased (Peng et al., 2016). This glycolysis-dependent epigenetic regulation of IFNγ expression via histone acetylation was mediated by LDHA-dependent maintenance of high levels of acetyl-CoA in effector Th1 cells (Peng et al., 2016).

Studies from the Kaech laboratory revealed a further role for glycolytic flux in T cell activation. Thus, production of the glycolytic metabolite phosphoenolpyruvate (PEP) via enolase promotes prolonged Ca^{2+} responses and activation of the transcription factor nuclear factor of activated T cells (NFAT) (Ho et al., 2015). Nuclear translocation and the transcriptional activity of NFAT regulates the expression of key effector molecules such as IL-2, IFNγ and CD40L in T cells (Hogan, 2017). PEP binds and inhibits the activity of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), preventing transfer of Ca^{2+} from the cytosol to the SR and prolonging NFAT activation (Ho et al., 2015). Importantly, defects in

Ca^{2+}/NFAT signaling and T cell activation under conditions of low glucose could be partially corrected by restoration of PEP levels following enforced expression of the gluconeogenesis enzyme PEP carboxykinase 1 (PEPCK1) (Ho et al., 2015). Furthermore, PEPCK1-overexpressing $CD4^+$ (Ho et al., 2015) and $CD8^+$ (Ma et al., 2018) T cells had elevated anti-tumor responses as compared to control cells, indicating that PEP production serves as a metabolic checkpoint *in vivo*. In NFATc1-deficient T cells, transcript levels of glycolytic proteins such as GLUT1, GLUT3 and HK2 were substantially reduced with a concomitant impairment of glycolytic flux, an effect that could be rescued partially by IL-2 (Klein-Hessling et al., 2017). Therefore NFATc1 regulates T cell activation and upregulation of the glycolytic pathway, which in turn acts in a positive-feedback loop to prolong NFATc1 signaling via PEP.

A novel role for the glycolytic enzyme enolase-1 (Eno1) in inducible Treg function was recently described by Materese and colleagues. These researchers showed that inhibition of glycolysis using 2-DG limited *FOXP3* gene splicing and expression in human Tregs (De Rosa et al., 2015). In Tregs treated with 2-DG, a substantially increased proportion of Eno1 was recruited to the *FOXP3* promoter and regulatory elements, whilst shRNA knockdown of Eno1 expression restored FOXP3 expression (De Rosa et al., 2015). These data suggest that nuclear Eno1 regulates FOXP3 splicing and that engagement of the glycolytic function of Eno1 interferes with this nuclear role, thereby stabilizing the Treg phenotype and function.

A further key role for glucose in T cell activation is to fuel protein O-GlcNacylation. In this pathway, glucose is diverted from the glycolytic pathway (at the level of fructose-6-phosphate) into the hexosamine biosynthetic pathway, which ultimately provides the donor substrate for O-GlcNacylation (Yang and Qian, 2017). TCR triggering results in a substantial increase in the pool of intracellular UDP-GlcNac, resulting in post-translational modification of Ser / Thr residues, and modifying the activity or stability of key proteins, including c-Myc (Swamy et al., 2016). Experiments investigating the impact of T cell-specific deletion of O-GlcNAc transferase (OGT) demonstrated a requirement for this pathway in T cell development in the thymus as well as the clonal expansion of mature T cells (Swamy et al., 2016). Supplementation of *in vitro* T cell cultures with GlcNAc favors Treg differentiation, at the expense of inflammatory Th17 cells, by promoting IL-2R signaling (Araujo et al., 2017). Thus, it is possible that aerobic glycolysis might impinge on Treg differentiation by limiting the supply of metabolites to the hexosamine and O-GlcNAc biosynthetic pathways.

In summary, it is clear that engagement of aerobic glycolysis impacts on T cell function through a number of distinct mechanisms: (i) glycolysis provides a source of ATP and enables the production of biosynthetic precursors to enable proliferation and cell growth; (ii) engagement of the glycolytic pathway and enzymes such as Eno1 and GAPDH diverts their function away from non-glycolytic functions that impinge on T cell gene expression; (iii) glycolytic metabolites such as PEP have additional signaling functions in T cells; (iv) the engagement of glycolysis interacts in a complex network with additional metabolic pathways such as the hexosamine pathway and glutaminolysis to regulate T cell behavior. Further investigation into the function of the Warburg effect in T cells will, no doubt, add to this list of mechanisms in the coming years.

mTOR REGULATES T CELL DIFFERENTIATION

The signaling pathways that regulate T cell metabolic reprogramming have been the subject of intense research in the past decade. mTOR is an evolutionarily conserved ser/thr kinase that, in T cells, integrates nutrient sensing and antigen-receptor signaling (reviewed in (Salmond and Zamoyska, 2010, 2011; Powell et al., 2012)). mTOR forms two main signaling complexes, mTORC1 and mTORC2, that differ in their sensitivity to the macrolide inhibitor rapamycin. mTORC1 is composed of mTOR in complex with the adapter protein raptor, mammalian lethal with SEC13 protein 8 (MLST8) and proline-rich Akt substrate (PRAS) 1, an endogenous regulator DEPTOR, and is sensitive to rapamycin. By contrast, mTORC2, composed of mTOR, rictor, GβL, and mammalian stress-activated protein kinase interacting protein 1 (mSIN1), is insensitive to acute inhibition by rapamycin. The pathways that regulate mTOR activation in T cells are summarized in **Figure 2**. In brief, mTORC1 activity is regulated by intracellular amino acids via the nutrient sensing Rag GTPases (Wolfson and Sabatini, 2017). Upon TCR stimulation, T cells upregulate the expression of plasma membrane transporters that enable the uptake of amino acids such as leucine and glutamine from the extracellular environment, that in turn sustain mTORC1 activation. Knockout mouse studies have shown that upregulation of the System L amino acid transporter SLC7A5 (Sinclair et al., 2013) and glutamine-transporter SLC1A5 (Nakaya et al., 2014) are both essential for mTORC1 activity in T cells. Glucose levels also regulate mTORC1 by influencing the activity of the negative regulator AMP kinase (AMPK) (Rolf et al., 2013). Furthermore, recent work has shown that, following TCR signaling, the kinase activity of mTORC1 is activated via the upstream kinase PDK1, in a PI3K/Akt-independent manner (Finlay et al., 2012). In addition, co-stimulation through CD28 and signaling mediated via cytokines such as IL-2 and IL-15 contribute to the magnitude of mTOR activation in T cells (Cornish et al., 2006; Ray et al., 2015). Key downstream targets / effectors of mTORC1 include the translational regulators 4E-binding proteins (4E-BPs) and ribosomal protein S6 kinases (S6Ks). The mechanism by which mTORC2 is activated is less well understood but likely involves PI3K/Akt activity (Zinzalla et al., 2011; Yang et al., 2015). mTORC2 targets include Akt and serum and glucocorticoid-induced protein kinase (SGK).

Whilst the anti-proliferative and immunosuppressive properties of rapamycin have been known for decades, seminal studies published in 2009 determined that mTOR activity also influences T cell effector-memory cell fate decisions *in vivo* (Araki et al., 2009). Thus, rapamycin treatment enhanced the quantity and quality of virus-specific $CD8^+$ memory T cells in mice. Knockdown of mTORC1 targets S6K1 and 4E-BPs also impacted upon T cell memory differentiation (Araki

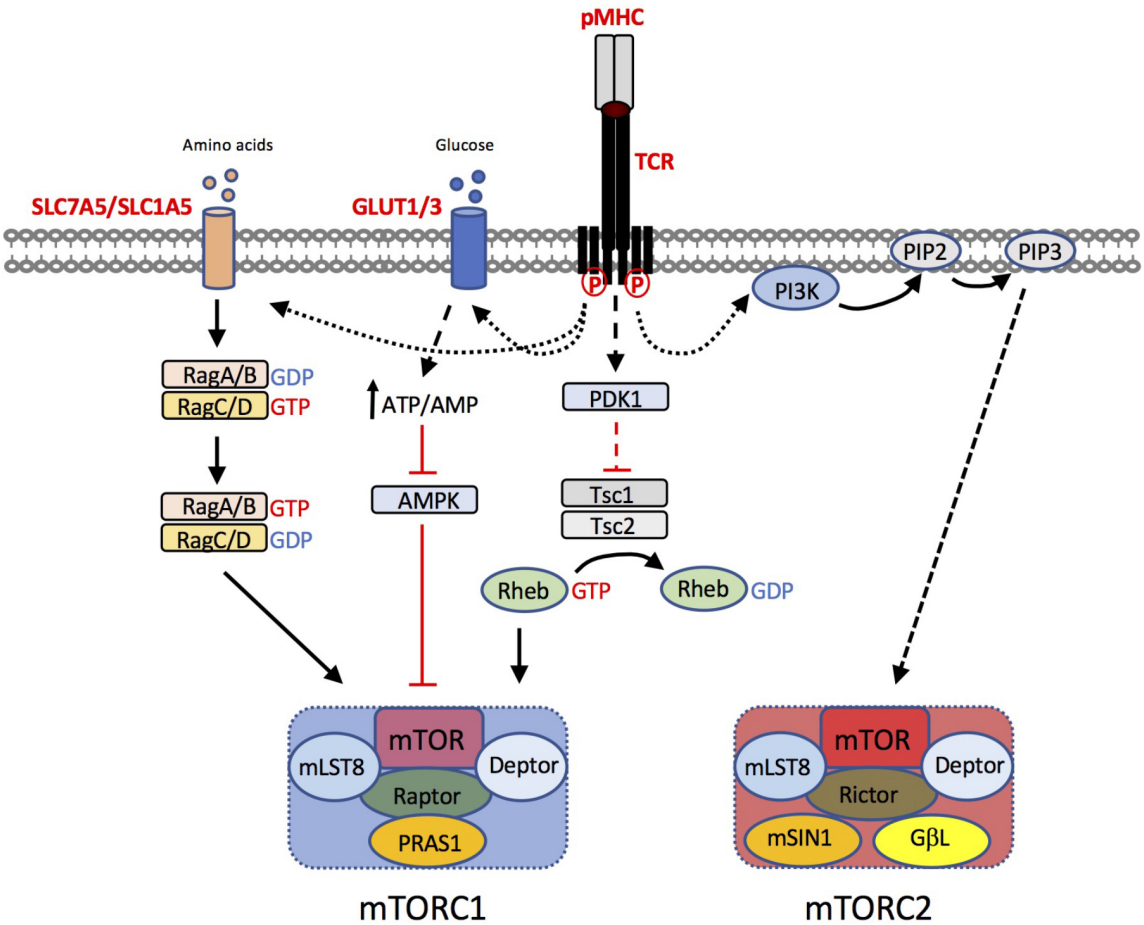

FIGURE 2 | T cell pathways to mTORC1 and mTORC2 activation. T cell receptor (TCR) triggering by peptide (p)MHC complexes results in upregulation of amino acid transporters such as SLC7A5 and SLC1A5. Leucine and glutamine are critical amino acids for the activation of the Rag GTPases, that in turn regulate mTORC1 activation at the lysosome. TCR induced upregulation of glucose transporters GLUT1 and GLUT3 enhances glucose uptake. Increases in the intracellular ATP:AMP ratio as a consequence of increased glucose availability, suppresses the activity of AMP kinase (AMPK), preventing its inhibitory effect on mTORC1 activity. Furthermore, TCR-induced phosphoinositide-dependent kinase (PDK)1, is critical for mTOR activation. PDK1 dependent signals prevent the GTPase activating protein (GAP) activity of the Tsc1/Tsc2 complex. Loss of Tsc GAP activity enables Rheb-GTP to activate mTORC1 through an incompletely understood mechanism. The mechanism of mTORC2 activation is less well understood but is likely to be downstream of PI3K/PIP3-dependent pathways.

et al., 2009). Consistent with these findings, activation of the AMPK1 pathway via metformin or via glucose-deprivation restrains mTOR activity (Pearce et al., 2009; Rolf et al., 2013) and enhances T cell memory. Furthermore, experiments have shown that IL-2 drives high levels of mTOR activation and effector $CD8^+$ T cell differentiation, whereas IL-15 drives lower levels of mTOR activation and favors memory cell formation (Cornish et al., 2006; Pipkin et al., 2010; Ray et al., 2015; Richer et al., 2015). Recent studies examining daughter cells from the first cell division following TCR stimulation indicate that mTORC1 activity is asymmetrically inherited (Pollizzi et al., 2016). Importantly, the asymmetric inheritance of mTORC1 influences T cell metabolic capacity and cell fate. Thus, daughter cells with high mTORC1 activity had elevated glycolytic flux and generated T cell populations with enhanced effector capacity, whereas cells with lower mTORC1 activity generated long-lived memory cells (Pollizzi et al., 2016).

Studies using mice with T cell-specific deficiencies in mTOR or with selective ablation or hyperactivation of mTORC1 or mTORC2 signaling pathways have defined an important role for both of these signaling complexes in T cell activation, differentiation and effector function (Delgoffe et al., 2009, 2011; Lee et al., 2010; Yang et al., 2011; Zeng et al., 2013, 2016; Pollizzi et al., 2015). $CD4^+$ T cells completely lacking mTOR fail to differentiate into Th1, Th2 or Th17 lineages and instead differentiate preferentially into $FOXP3^+$ regulatory T cells (Delgoffe et al., 2009). Nonetheless, whilst mTORC1 signals negatively regulate *de novo* Treg differentiation, the suppressive function of fully differentiated Treg also requires mTORC1 activity (Zeng et al., 2013; Gerriets et al., 2016; Chapman et al., 2018; Sun et al., 2018). T cells deficient in the upstream activator of mTORC1, Rheb, are defective in Th1 and Th17 differentiation (Delgoffe et al., 2011) whilst raptor deficiency also impinges upon Th2 differentiation (Yang et al., 2013).

By contrast, deletion of tuberous sclerosis 1 (Tsc1) or Tsc2 results in hyperactive mTORC1 and a subsequent loss of naïve T cell quiescence, indicating that restraining mTOR activity is important for the maintenance of immune homeostasis (Yang et al., 2011; Pollizzi et al., 2015). Rictor deficiency (i.e., loss of mTORC2) has a milder effect on Th1 cell activation *in vivo* as compared to loss of mTORC1 function (Yang et al., 2013) but compromises $CD4^+$ Th2 differentiation (Lee et al., 2010). Similarly, Rheb/mTORC1-dependent signals are also required for $CD8^+$ T cell differentiation whilst rictor/mTORC2-dependent signals regulate $CD8^+$ T cell memory (Pollizzi et al., 2015; Zhang et al., 2016).

Thus, studies of knockout mouse models have shed significant insight into the multifarious roles of mTOR in T cell differentiation and effector function. In this regard, mTORC1 has dual and apparently opposing roles in Treg biology; on the one hand, elevated mTORC1 favors the differentiation of effector T cells at the expense of Tregs, whilst on the other, mTOR expression in Tregs is essential to prevent autoimmunity. Furthermore, these studies have suggested distinct roles for the mTORC1 and mTORC2 complexes in T cells; for example, in Th1 vs. Th2 differentiation. Importantly, evidence indicates that the regulation of T cell metabolism by mTOR complexes is central to these complex phenotypes.

REGULATION OF T CELL METABOLISM BY mTOR

In T cells, mTORC1 signaling serves to promote aerobic glycolysis and as a consequence impacts upon T cell differentiation and effector function. Rapamycin treatment substantially impairs the initial TCR-induced upregulation of glucose transporters, glucose uptake and glycolytic enzymes in both $CD4^+$ and $CD8^+$ T cells (Shi et al., 2011; Finlay, 2012). Similarly, genetic ablation of Rheb (Pollizzi et al., 2015) or raptor (Yang et al., 2013) impairs the upregulation of aerobic glycolysis in TCR-stimulated T cells, whilst hyperactivation of mTORC1 in Tsc1 or Tsc2-deficient T cells is associated with enhanced glycolytic metabolism. Furthermore, mTORC1 activity is required to sustain high levels of aerobic glycolysis in effector T cells (Finlay, 2012; Hukelmann et al., 2016). In this regard, rapamycin treatment caused an approximate 50% reduction in levels of GLUT1 and GLUT3 in IL-2 maintained effector CTLs and a proportional decrease in glucose uptake and lactate production (Hukelmann et al., 2016). By contrast, inhibition of mTORC2 activity actually increases the metabolic capacity of $CD8^+$ T cells. Thus, Rictor-deficient $CD8^+$ T cells have elevated glycolytic flux, spare respiratory capacity (SRC) and FAO (Pollizzi et al., 2015; Zhang et al., 2016). The mechanism by which deletion of mTORC2 results in increased metabolic fitness has not been fully elucidated but may involve stabilization of nuclear Foxo1 transcription factor (Zhang et al., 2016). Thus, knockdown of Foxo1 reverses the impact of Rictor-deficiency on T cell memory formation whilst expression of a constitutively active Foxo1 in $CD8^+$ T cells results in elevated SRC and FAO (Zhang et al., 2016).

The molecular mechanisms by which mTORC1 signals regulate glycolytic pathways in T cells are also incompletely understood. Studies have identified transcription factors including Myc (Wang et al., 2011) and hypoxia inducible factor 1 alpha (HIF-1α) (Shi et al., 2011; Finlay et al., 2012) as key drivers of metabolic reprogramming in T cells. Myc-deficient T cells are defective in TCR-induced upregulation of glucose transporters and glycolytic enzymes and have substantially reduced glycolytic flux (Wang et al., 2011). HIF-1α is upregulated strongly in Th17 cells (Shi et al., 2011) and effector $CD8^+$ T cells (Finlay et al., 2012) and, similar to Myc, is important for the upregulation of aerobic glycolysis in these T cell subsets. Importantly, rapamycin impairs the TCR-induced expression of both Myc and HIF-1α (Shi et al., 2011; Wang et al., 2011; Finlay et al., 2012; Pollizzi et al., 2016) indicating that mTOR serves to regulate aerobic glycolysis, at least in part, through regulation of Myc and HIF-1α expression and their subsequent downstream transcriptional programs. mTORC1 has been reported to regulate Myc expression via post-transcriptional mechanisms as levels of Myc protein, but not mRNA, were reduced in Raptor-deficient T cells as compared to controls (Yang et al., 2013).

In addition to the role of mTOR in promoting glycolytic metabolism in effector T cells, recent evidence has shown that mTOR also has a vital role in the regulation of mitochondrial metabolism. Gene expression and pathway analysis identified the regulation of both glycolysis and OXPHOS as being significantly impacted by hyperactive mTOR activity in $Tsc1^{-/-}$ T cells (Shrestha et al., 2014). Furthermore, mTOR catalytic site inhibitors reduced expression of mitochondrial and OXPHOS genes in activated Tregs (Chapman et al., 2018). In both cases, mTOR activity was required for the transcriptional programs driving OXPHOS in T cells, highlighting the dual role for this kinase in regulating mitochondrial and glycolytic metabolism.

ACTIVATED mTOR LEADS TO ALTERED T CELL METABOLISM IN AUTOIMMUNITY

The use of rapamycin/sirolimus as an immunosuppressive agent in the clinic was approved by the US FDA in 1999. Rapamycin and its derivatives have been used extensively in transplantation to limit organ rejection, however, recent evidence has shown that these compounds may have broader applicability in the treatment of cancers and autoimmunity, as well as in vaccine design (reviewed in Perl, 2015, 2016). Importantly, the clinical benefits of mTOR blockade in inflammatory diseases has been linked to the modulation of T cell metabolism. For example, inflammatory T cells from systemic lupus erythematosus (SLE) patients have substantially elevated glycolytic and mitochondrial metabolism (Yin et al., 2015) and mTOR activity (Kato and Perl, 2014), as compared to healthy controls. Importantly, in both mouse models and in human patients, T cell metabolism and inflammatory cytokine production could be normalized by reducing mTOR activity through metformin or rapamycin treatment (Kato and Perl, 2014; Yin et al., 2015). Furthermore, and consistent with an important role for mTOR-driven

inflammatory T cells in the pathogenesis of lupus, a recent phase 1/2 trial reported that rapamycin had a beneficial impact on clinical disease scores in a cohort of 43 SLE patients (Lai et al., 2018). Improved disease outcomes were associated with decreased inflammatory T cell activity and increased Treg numbers (Lai et al., 2018), consistent with the known role of mTOR in regulating T cell metabolism and effector responses.

CONCLUDING REMARKS

Our understanding of the close links between the regulation of aerobic glycolysis and T cell function has been transformed in the past decade. Furthermore, mTOR signals have emerged as a key driver of these processes. As our understanding of the molecular details and signaling pathways leading to metabolic reprogramming increases, then the opportunity to translate these findings into the clinic should emerge. In this regard, evidence for distinct roles for mTORC1 and mTORC2 in modulating T cell metabolism and activation gives scope for more precise manipulation of these pathways in the future.

AUTHOR CONTRIBUTIONS

RS wrote the manuscript and generated the figures.

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REFERENCES

Araki, K., Turner, A. P., Shaffer, V. O., Gangappa, S., Keller, S. A., Bachmann, M. F., et al. (2009). mTOR regulates memory CD8 T-cell differentiation. *Nature* 460, 108–112. doi: 10.1038/nature08155

Araujo, L., Khim, P., Mkhikian, H., Mortales, C. L., and Demetriou, M. (2017). Glycolysis and glutaminolysis cooperatively control T cell function by limiting metabolite supply to N-glycosylation. *eLife* 6:e21330. doi: 10.7554/eLife.21330

Asnagli, H., and Murphy, K. M. (2001). Stability and commitment in T helper cell development. *Curr. Opin. Immunol.* 13, 242–247. doi: 10.1016/S0952-7915(00)00210-7

Berod, L., Friedrich, C., Nandan, A., Freitag, J., Hagemann, S., Harmrolfs, K., et al. (2014). De novo fatty acid synthesis controls the fate between regulatory T and T helper 17 cells. *Nat. Med.* 20, 1327–1333. doi: 10.1038/nm.3704

Buck, M. D., O'Sullivan, D., Klein, Geltink RI, Curtis, J. D., Chang, C. H., Sanin, D. E., et al. (2016). Mitochondrial dynamics controls T cell fate through metabolic programming. *Cell* 166, 63–76. doi: 10.1016/j.cell.2016.05.035

Cham, C. M., Driessens, G., O'Keefe, J. P., and Gajewski, T. F. (2008). Glucose deprivation inhibits multiple key gene expression events and effector functions in CD8+ T cells. *Eur. J. Immunol.* 38, 2438–2450. doi: 10.1002/eji.200838289

Cham, C. M., and Gajewski, T. F. (2005). Glucose availability regulates IFN-gamma production and p70S6 kinase activation in CD8+ effector T cells. *J. Immunol.* 174, 4670–4677. doi: 10.4049/jimmunol.174.8.4670

Chang, C. H., Curtis, J. D., Maggi, L. B. Jr, Faubert, B., Villarino, A. V., O'Sullivan, D., et al. (2013). Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* 153, 1239–1251. doi: 10.1016/j.cell.2013.05.016

Chang, C. H., Qiu, J., O'Sullivan, D., Buck, M. D., Noguchi, T., Curtis, J. D., et al. (2015). Metabolic competition in the tumor microenvironment is a driver of cancer progression. *Cell* 162, 1229–1241. doi: 10.1016/j.cell.2015.08.016

Chapman, N. M., Zeng, H., Nguyen, T. M., Wang, Y., Vogel, P., Dhungana, Y., et al. (2018). mTOR coordinates transcriptional programs and mitochondrial metabolism of activated Treg subsets to protect tissue homeostasis. *Nat. Commun.* 9:2095. doi: 10.1038/s41467-018-04392-5

Cornish, G. H., Sinclair, L. V., and Cantrell, D. A. (2006). Differential regulation of T-cell growth by IL-2 and IL-15. *Blood* 108, 600–608. doi: 10.1182/blood-2005-12-4827

De, Rosa V, Galgani, M., Porcellini, A., Colamatteo, A., Santopaolo, M., Zuchegna, C., et al. (2015). Glycolysis controls the induction of human regulatory T cells by modulating the expression of FOXP3 exon 2 splicing variants. *Nat. Immunol.* 16, 1174–1184. doi: 10.1038/ni.3269

De Boer, R. J., Homann, D., and Perelson, A. S. (2003). Different dynamics of CD4+ and CD8+ T cell responses during and after acute lymphocytic choriomeningitis virus infection. *J. Immunol.* 171, 3928–3935. doi: 10.4049/jimmunol.171.8.3928

Delgoffe, G. M., Kole, T. P., Zheng, Y., Zarek, P. E., Matthews, K. L., Xiao, B., et al. (2009). The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity* 30, 832–844. doi: 10.1016/j.immuni.2009.04.014

Delgoffe, G. M., Pollizzi, K. N., Waickman, A. T., Heikamp, E., Meyers, D. J., Horton, M. R., et al. (2011). The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. *Nat. Immunol.* 12, 295–303. doi: 10.1038/ni.2005

Finlay, D. K. (2012). Regulation of glucose metabolism in T cells: new insight into the role of Phosphoinositide 3-kinases. *Front. Immunol.* 3:247. doi: 10.3389/fimmu.2012.00247

Finlay, D. K., Rosenzweig, E., Sinclair, L. V., Feijoo-Carnero, C., Hukelmann, J. L., Rolf, J., et al. (2012). PDK1 regulation of mTOR and hypoxia-inducible factor 1 integrate metabolism and migration of CD8+ T cells. *J. Exp. Med.* 209, 2441–2453. doi: 10.1084/jem.20112607

Fox, C. J., Hammerman, P. S., and Thompson, C. B. (2005). Fuel feeds function: energy metabolism and the T-cell response. *Nat. Rev. Immunol.* 5, 844–852. doi: 10.1038/nri1710

Geltink, R. I. K., Kyle, R. L., and Pearce, E. L. (2018). Unraveling the complex interplay between T cell metabolism and function. *Annu. Rev. Immunol.* 36, 461–488. doi: 10.1146/annurev-immunol-042617-053019

Gerriets, V. A., Kishton, R. J., Johnson, M. O., Cohen, S., Siska, P. J., Nichols, A. G., et al. (2016). Foxp3 and Toll-like receptor signaling balance Treg cell anabolic metabolism for suppression. *Nat. Immunol.* 17, 1459–1466. doi: 10.1038/ni.3577

Gubser, P. M., Bantug, G. R., Razik, L., Fischer, M., Dimeloe, S., Hoenger, G., et al. (2013). Rapid effector function of memory CD8+ T cells requires an immediate-early glycolytic switch. *Nat. Immunol.* 14, 1064–1072. doi: 10.1038/ni.2687

Halle, S., Halle, O., and Forster, R. (2017). Mechanisms and dynamics of T cell-mediated cytotoxicity *In Vivo*. *Trends Immunol.* 38, 432–443. doi: 10.1016/j.it.2017.04.002

Ho, P. C., Bihuniak, J. D., Macintyre, A. N., Staron, M., Liu, X., Amezquita, R., et al. (2015). Phosphoenolpyruvate is a metabolic checkpoint of anti-tumor T cell responses. *Cell* 162, 1217–1228. doi: 10.1016/j.cell.2015.08.012

Hogan, P. G. (2017). Calcium-NFAT transcriptional signalling in T cell activation and T cell exhaustion. *Cell Calcium* 63, 66–69. doi: 10.1016/j.ceca.2017.01.014

Hukelmann, J. L., Anderson, K. E., Sinclair, L. V., Grzes, K. M., Murillo, A. B., Hawkins, P. T., et al. (2016). The cytotoxic T cell proteome and its shaping by the kinase mTOR. *Nat. Immunol.* 17, 104–112. doi: 10.1038/ni.3314

Jacobs, S. R., Michalek, R. D., and Rathmell, J. C. (2010). IL-7 is essential for homeostatic control of T cell metabolism in vivo. *J. Immunol.* 184, 3461–3469. doi: 10.4049/jimmunol.0902593

Kato, H., and Perl, A. (2014). Mechanistic target of rapamycin complex 1 expands Th17 and IL-4+ CD4-CD8- double-negative T cells and contracts regulatory T cells in systemic lupus erythematosus. *J. Immunol.* 192, 4134–4144. doi: 10.4049/jimmunol.1301859

Klein Geltink, R. I., O'Sullivan, D., Corrado, M., Bremser, A., Buck, M. D., Buescher, J. M., et al. (2017). Mitochondrial priming by CD28. *Cell* 171, 385.e11–397.e11. doi: 10.1016/j.cell.2017.08.018

Klein-Hessling, S., Muhammad, K., Klein, M., Pusch, T., Rudolf, R., Flöter, J., et al. (2017). NFATc1 controls the cytotoxicity of CD8(+) T cells. *Nat. Commun.* 8:511. doi: 10.1038/s41467-017-00612-6

Lai, Z. W., Kelly, R., Winans, T., Marchena, I., Shadakshari, A., Yu, J., et al. (2018). Sirolimus in patients with clinically active systemic lupus erythematosus resistant to, or intolerant of, conventional medications: a single-arm, open-label, phase 1/2 trial. *Lancet* 391, 1186–1196. doi: 10.1016/S0140-6736(18)30485-9

Lee, K., Gudapati, P., Dragovic, S., Spencer, C., Joyce, S., Killeen, N., et al. (2010). Mammalian target of rapamycin protein complex 2 regulates differentiation of Th1 and Th2 cell subsets via distinct signaling pathways. *Immunity* 32, 743–753. doi: 10.1016/j.immuni.2010.06.002

Lochner, M., Berod, L., and Sparwasser, T. (2015). Fatty acid metabolism in the regulation of T cell function. *Trends Immunol.* 36, 81–91. doi: 10.1016/j.it.2014.12.005

Lunt, S. Y., and Vander Heiden, M. G. (2011). Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu. Rev. Cell Dev. Biol.* 27, 441–464. doi: 10.1146/annurev-cellbio-092910-154237

Ma, R., Ji, T., Zhang, H., Dong, W., Chen, X., Xu, P., et al. (2018). A Pck1-directed glycogen metabolic program regulates formation and maintenance of memory CD8(+) T cells. *Nat. Cell Biol.* 20, 21–27. doi: 10.1038/s41556-017-0002-2

MacDonald, H. R. (1977). Energy metabolism and T-cell-mediated cytolysis. II. selective inhibition of cytolysis by 2-deoxy-D-glucose. *J. Exp. Med.* 146, 710–719. doi: 10.1084/jem.146.3.710

MacDonald, H. R., and Cerottini, J. C. (1979). Inhibition of T cell-mediated cytolysis by 2-deoxy-D-glucose:dissociation of the inhibitory effect from glycoprotein synthesis. *Eur. J. Immunol.* 9, 466–470. doi: 10.1002/eji.1830090610

Macintyre, A. N., Gerriets, V. A., Nichols, A. G., Michalek, R. D., Rudolph, M. C., Deoliveira, D., et al. (2014). The glucose transporter Glut1 is selectively essential for CD4 T cell activation and effector function. *Cell Metab.* 20, 61–72. doi: 10.1016/j.cmet.2014.05.004

Michalek, R. D., Gerriets, V. A., Jacobs, S. R., Macintyre, A. N., MacIver, N. J., Mason, E. F., et al. (2011). Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. *J. Immunol.* 186, 3299–3303. doi: 10.4049/jimmunol.1003613

Murray, C. M., Hutchinson, R., Bantick, J. R., Belfield, G. P., Benjamin, A. D., Brazma, D., et al. (2005). Monocarboxylate transporter MCT1 is a target for immunosuppression. *Nat. Chem. Biol.* 1, 371–376. doi: 10.1038/nchembio744

Nakaya, M., Xiao, Y., Zhou, X., Chang, J. H., Chang, M., Cheng, X., et al. (2014). Inflammatory T cell responses rely on amino acid transporter ASCT2 facilitation of glutamine uptake and mTORC1 kinase activation. *Immunity* 40, 692–705. doi: 10.1016/j.immuni.2014.04.007

Pan, Y., Tian, T., Park, C. O., Lofftus, S. Y., Mei, S., Liu, X., et al. (2017). Survival of tissue-resident memory T cells requires exogenous lipid uptake and metabolism. *Nature* 543, 252–256. doi: 10.1038/nature21379

Pearce, E. L., Walsh, M. C., Cejas, P. J., Harms, G. M., Shen, H., Wang, L. S., et al. (2009). Enhancing CD8 T-cell memory by modulating fatty acid metabolism. *Nature* 460, 103–107. doi: 10.1038/nature08097

Peng, M., Yin, N., Chhangawala, S., Xu, K., Leslie, C. S., and Li, M. O. (2016). Aerobic glycolysis promotes T helper 1 cell differentiation through an epigenetic mechanism. *Science* 354, 481–484. doi: 10.1126/science.aaf6284

Perl, A. (2015). mTOR activation is a biomarker and a central pathway to autoimmune disorders, cancer, obesity, and aging. *Ann. N. Y. Acad. Sci.* 1346, 33–44. doi: 10.1111/nyas.12756

Perl, A. (2016). Activation of mTOR (mechanistic target of rapamycin) in rheumatic diseases. *Nat. Rev. Rheumatol.* 12, 169–182. doi: 10.1038/nrrheum.2015.172

Pipkin, M. E., Sacks, J. A., Cruz-Guilloty, F., Lichtenheld, M. G., Bevan, M. J., and Rao, A. (2010). Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity* 32, 79–90. doi: 10.1016/j.immuni.2009.11.012

Pollizzi, K. N., Patel, C. H., Sun, I. H., Oh, M. H., Waickman, A. T., Wen, J., et al. (2015). mTORC1 and mTORC2 selectively regulate CD8(+) T cell differentiation. *J. Clin. Invest.* 125, 2090–2108. doi: 10.1172/JCI77746

Pollizzi, K. N., Sun, I. H., Patel, C. H., Lo, Y. C., Oh, M. H., Waickman, A. T., et al. (2016). Asymmetric inheritance of mTORC1 kinase activity during division dictates CD8(+) T cell differentiation. *Nat. Immunol.* 17, 704–711. doi: 10.1038/ni.3438

Powell, J. D., Pollizzi, K. N., Heikamp, E. B., and Horton, M. R. (2012). Regulation of immune responses by mTOR. *Annu. Rev. Immunol.* 30, 39–68. doi: 10.1146/annurev-immunol-020711-075024

Raud, B., McGuire, P. J., Jones, R. G., Sparwasser, T., and Berod, L. (2018). Fatty acid metabolism in CD8(+) T cell memory: challenging current concepts. *Immunol. Rev.* 283, 213–231. doi: 10.1111/imr.12655

Ray, J. P., Staron, M. M., Shyer, J. A., Ho, P. C., Marshall, H. D., Gray, S. M., et al. (2015). The Interleukin-2-mTORc1 kinase axis defines the signaling, differentiation, and metabolism of T Helper 1 and follicular B helper T cells. *Immunity* 43, 690–702. doi: 10.1016/j.immuni.2015.08.017

Renner, K., Geiselhöringer, A. L., Fante, M., Bruss, C., Färber, S., Schönhammer, G., et al. (2015). Metabolic plasticity of human T cells: preserved cytokine production under glucose deprivation or mitochondrial restriction, but 2-deoxy-glucose affects effector functions. *Eur. J. Immunol.* 45, 2504–2516. doi: 10.1002/eji.201545473

Richer, M. J., Pewe, L. L., Hancox, L. S., Hartwig, S. M., Varga, S. M., and Harty, J. T. (2015). Inflammatory IL-15 is required for optimal memory T cell responses. *J. Clin. Invest.* 125, 3477–3490. doi: 10.1172/JCI81261

Rolf, J., Zarrouk, M., Finlay, D. K., Foretz, M., Viollet, B., and Cantrell, D. A. (2013). AMPKalpha1: a glucose sensor that controls CD8 T-cell memory. *Eur. J. Immunol.* 43, 889–896. doi: 10.1002/eji.201243008

Sakaguchi, S., Miyara, M., Costantino, C. M., and Hafler, D. A. (2010). FOXP3+ regulatory T cells in the human immune system. *Nat. Rev. Immunol.* 10, 490–500. doi: 10.1038/nri2785

Salmond, R. J., and Zamoyska, R. (2010). How does the mammalian target of rapamycin (mTOR) influence CD8 T cell differentiation? *Cell Cycle* 9, 2952–2957. doi: 10.4161/cc.9.15.12358

Salmond, R. J., and Zamoyska, R. (2011). The influence of mTOR on T helper cell differentiation and dendritic cell function. *Eur. J. Immunol.* 41, 2137–2141. doi: 10.1002/eji.201141523

Shi, L. Z., Wang, R., Huang, G., Vogel, P., Neale, G., Green, D. R., et al. (2011). HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J. Exp. Med.* 208, 1367–1376. doi: 10.1084/jem.20110278

Shrestha, S., Yang, K., Wei, J., Karmaus, P. W., Neale, G., and Chi, H. (2014). Tsc1 promotes the differentiation of memory CD8+ T cells via orchestrating the transcriptional and metabolic programs. *Proc. Natl. Acad. Sci. U.S.A.* 111, 14858–14863. doi: 10.1073/pnas.1404264111

Sinclair, L. V., Rolf, J., Emslie, E., Shi, Y. B., Taylor, P. M., and Cantrell, D. A. (2013). Control of amino-acid transport by antigen receptors coordinates the metabolic reprogramming essential for T cell differentiation. *Nat. Immunol.* 14, 500–508. doi: 10.1038/ni.2556

Siska, P. J., Beckermann, K. E., Mason, F. M., Andrejeva, G., Greenplate, A. R., Sendor, A. B., et al. (2017). Mitochondrial dysregulation and glycolytic insufficiency functionally impair CD8 T cells infiltrating human renal cell carcinoma. *JCI Insight* 2:93411. doi: 10.1172/jci.insight.93411

Stockinger, B., and Omenetti, S. (2017). The dichotomous nature of T helper 17 cells. *Nat. Rev. Immunol.* 17, 535–544. doi: 10.1038/nri.2017.50

Sun, I. H., Oh, M. H., Zhao, L., Patel, C. H., Arwood, M. L., Xu, W., et al. (2018). mTOR complex 1 signaling regulates the generation and function of central and effector foxp3(+) regulatory T cells. *J. Immunol.* 201, 481–492. doi: 10.4049/jimmunol.1701477

Swamy, M., Pathak, S., Grzes, K. M., Damerow, S., Sinclair, L. V., van, Aalten DM, et al. (2016). Glucose and glutamine fuel protein O-GlcNAcylation to

control T cell self-renewal and malignancy. *Nat. Immunol.* 17, 712–720. doi: 10.1038/ni.3439

Tan, H., Yang, K., Li, Y., Shaw, T. I., Wang, Y., Blanco, D. B., et al. (2017). Integrative proteomics and phosphoproteomics profiling reveals dynamic signaling networks and bioenergetics pathways underlying T cell activation. *Immunity* 46, 488–503. doi: 10.1016/j.immuni.2017.02.010

Vander Heiden, M. G., Cantley, L. C., and Thompson, C. B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 324, 1029–1033. doi: 10.1126/science.1160809

Wang, R., Dillon, C. P., Shi, L. Z., Milasta, S., Carter, R., Finkelstein, D., et al. (2011). The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. *Immunity* 35, 871–882. doi: 10.1016/j.immuni.2011.09.021

Wolfson, R. L., and Sabatini, D. M. (2017). The dawn of the age of amino acid sensors for the mTORC1 pathway. *Cell Metab.* 26, 301–309. doi: 10.1016/j.cmet.2017.07.001

Yang, G., Murashige, D. S., Humphrey, S. J., and James, D. E. (2015). A positive feedback loop between Akt and mTORC2 via SIN1 phosphorylation. *Cell Rep.* 12, 937–943. doi: 10.1016/j.celrep.2015.07.016

Yang, J. Q., Kalim, K. W., Li, Y., Zhang, S., Hinge, A., Filippi, M. D., et al. (2016). RhoA orchestrates glycolysis for TH2 cell differentiation and allergic airway inflammation. *J. Allergy Clin. Immunol.* 137, 231.e4–245.e4. doi: 10.1016/j.jaci.2015.05.004

Yang, K., Neale, G., Green, D. R., He, W., and Chi, H. (2011). The tumor suppressor Tsc1 enforces quiescence of naive T cells to promote immune homeostasis and function. *Nat. Immunol.* 12, 888–897. doi: 10.1038/ni.2068

Yang, K., Shrestha, S., Zeng, H., Karmaus, P. W., Neale, G., Vogel, P., et al. (2013). T cell exit from quiescence and differentiation into Th2 cells depend on Raptor-mTORC1-mediated metabolic reprogramming. *Immunity* 39, 1043–1056. doi: 10.1016/j.immuni.2013.09.015

Yang, X., and Qian, K. (2017). Protein O-GlcNAcylation: emerging mechanisms and functions. *Nat. Rev. Mol. Cell Biol.* 18, 452–465. doi: 10.1038/nrm.2017.22

Yin, Y., Choi, S. C., Xu, Z., Perry, D. J., Seay, H., Croker, B. P., et al. (2015). Normalization of CD4+ T cell metabolism reverses lupus. *Sci. Transl. Med.* 7:274ra218. doi: 10.1126/scitranslmed.aaa0835

Zeng, H., Cohen, S., Guy, C., Shrestha, S., Neale, G., Brown, S. A., et al. (2016). mTORC1 and mTORC2 kinase signaling and glucose metabolism drive follicular helper T cell differentiation. *Immunity* 45, 540–554. doi: 10.1016/j.immuni.2016.08.017

Zeng, H., Yang, K., Cloer, C., Neale, G., Vogel, P., and Chi, H. (2013). mTORC1 couples immune signals and metabolic programming to establish T(reg)-cell function. *Nature* 499, 485–490. doi: 10.1038/nature12297

Zhang, L., Tschumi, B. O., Lopez-Mejia, I. C., Oberle, S. G., Meyer, M., Samson, G., et al. (2016). Mammalian target of rapamycin complex 2 controls CD8 T cell memory differentiation in a foxo1-dependent manner. *Cell Rep.* 14, 1206–1217. doi: 10.1016/j.celrep.2015.12.095

Zinzalla, V., Stracka, D., Oppliger, W., and Hall, M. N. (2011). Activation of mTORC2 by association with the ribosome. *Cell* 144, 757–768. doi: 10.1016/j.cell.2011.02.014

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REVIEW
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Emerging Roles of Cellular Metabolism in Regulating Dendritic Cell Subsets and Function

*Xingrong Du, Nicole M. Chapman and Hongbo Chi**

Department of Immunology, St. Jude Children's Research Hospital, Memphis, TN, United States

Dendritic cells (DCs) are the bridge between innate and T cell-dependent adaptive immunity and are promising therapeutic targets for cancer and immune-mediated disorders. Upon stimulation by pathogen or danger-sensing receptors, DCs become activated and poised to induce T cell priming. Recent studies have identified critical roles of metabolic pathways, including glycolysis, oxidative phosphorylation, and fatty acid metabolism, in orchestrating DC function. In this review, we discuss the shared and distinct metabolic programs shaping the functional specification of different DC subsets, including conventional DCs, bone marrow-derived DCs, and plasmacytoid DCs. We also briefly discuss the signaling networks that tune metabolic programs in DC subsets.

Keywords: dendritic cell, metabolism, glycolysis, oxidative phosphorylation, fatty acid

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Van Andel Research Institute (VARI), United States

***Correspondence:**
Hongbo Chi
hongbo.chi@stjude.org

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INTRODUCTION

Dendritic cells (DCs) are an innate immune cell population with the capacity to process and present antigenic peptides on major histocompatibility complex (MHC) molecules to antigen-specific T cells. Thus, the hallmark function of DCs is to induce T cell-mediated immunity to foreign antigens and tolerance to self-antigens (Banchereau et al., 2000; Merad et al., 2013). DCs are also essential regulators of other immunological processes, including introduction of innate inflammatory responses to pathogens (Clark et al., 2000; Bedoui and Greyer, 2014). DCs must receive activation signals to efficiently promote T cell activation. DCs are activated by pathogens bearing pathogen-associated molecular patterns (PAMPs), which stimulate pattern recognition receptors, such as toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors, and members of the C-type lectin family (Hemmi and Akira, 2005; Watts et al., 2010; Hammer and Ma, 2013; Krishnaswamy et al., 2013). Besides PAMPs, DCs are also activated by inflammatory cytokines and ligation of selective cell surface receptors, including CD40. These activation signals promote upregulation of the expression of co-stimulatory molecules, increased production of cytokines and chemokines, and augmented antigen processing capacity. Thus, activated DCs have enhanced immunogenic ability to prime T cells.

DCs are a heterogeneous population that are divided into three major subsets, including conventional DCs (cDCs), plasmacytoid DCs (pDCs), and monocyte-derived DCs (moDCs; Merad et al., 2013; Pearce and Everts, 2015; Murphy, 2016; Segura, 2016). cDCs are further divided into cDC1 or cDC2 cells based on the expression of several surface molecules and transcription factors (Guilliams et al., 2014, 2016; Ginhoux et al., 2016). Whereas cDC and pDC directly arise from bone marrow-derived precursors in response to FLT3-FLT3L interactions, monocytes differentiate into moDCs within inflamed tissues or under steady state in selective tissues, such as the dermis, the intestinal lamina propria, and the lung. In addition to developmental

differences, DC subsets have discrete functions, with cDCs serving as potent inducers of T cell-dependent adaptive immunity through direct antigen presentation and co-stimulation and pDCs producing type I interferons (IFNs) following viral infections (Merad et al., 2013; Anderson et al., 2017). In addition to these DC subsets, studies using bone marrow-derived DCs (BMDCs), which can be generated *in vitro* from GM-CSF (GM-CSF-BMDCs) or FLT3L (FLT3L-BMDCs), have been essential for advancing our understanding of DC biology. It is important to note that BMDCs are heterogeneous, but certain subpopulations have reported similarities between moDCs, pDCs, and cDCs (Inaba et al., 1992; Lutz et al., 1999; Naik et al., 2005; Helft et al., 2015; Pearce and Everts, 2015).

Metabolism is the process by which cells acquire and process nutrients to fulfill energy and biosynthetic requirements for biological functions (O'Neill et al., 2016). Recently, cellular metabolism has emerged as an essential regulator of DC development and functional responses (Pearce and Everts, 2015; O'Neill and Pearce, 2016). In this review, we summarize and discuss recent progress in DC metabolic studies, focusing on the metabolic regulation of the function of different DC subsets.

GLUCOSE AND MITOCHONDRIAL METABOLISM IN THE FUNCTIONAL REGULATION OF GM-CSF-BMDCs AND cDCs

Compared to resting DCs, activated DCs have increased bioenergetic and biosynthetic demands that are required for protein and membrane synthesis to promote DC maturation. These requirements are met by rapid rewiring of glucose metabolism. Glucose uptake increases upon TLR stimulation, where it can be shuttled into the glycolytic pathway and serve as a precursor for adenosine triphosphate (ATP) production generated by conversion of pyruvate into lactate, even in the presence of oxygen. These are classical features of aerobic glycolysis or Warburg metabolism (Lunt and Vander Heiden, 2011; Liberti and Locasale, 2016).

Both GM-CSF-BMDCs and cDCs rapidly increase their glycolytic rate within minutes after TLR stimulation, which is maintained for several hours and returns to prestimulation levels when inducible nitric oxide synthase (iNOS) is not expressed (Everts and Pearce, 2014; Everts et al., 2014). Moreover, glycolysis is important for the maturation and function of both cDCs and GM-CSF-BMDCs. Treatment with 2-deoxyglucose [2-DG, a hexokinase (HK) inhibitor that dampens glycolysis] impairs the expression of co-stimulatory markers and production of IL-12 by both GM-CSF-BMDCs and cDCs, as well as their function to prime T cells (Everts et al., 2014). Glucose can also promote GM-CSF-BMDC and cDC migration toward CCL21, which is suppressed by 2-DG treatment (Guak et al., 2018). Further, glycolysis is also required to maintain the elongated cell shape of GM-CSF-BMDCs and promote CCR7 oligomerization that directs motility and migration to draining lymph nodes (Guak et al., 2018). In addition to extracellular glucose, an elegant study has shown that intracellular glycogen can be used as the nutrient source to fuel the metabolic requirements of GM-CSF-BMDCs by supporting glycolysis after TLR-induced activation (Thwe et al., 2017). Disruption of glycogen metabolism, by the glycogen phosphorylase inhibitor CP91149, significantly impairs DC maturation and function, especially at the earliest stage of GM-CSF-BMDC activation. It would be interesting to determine whether cDCs can also use glycogen as a nutrient source during activation. In contrast to the roles of glycolysis in promoting DC activation and pro-inflammatory function abovementioned, another independent study showed that glucose represses the pro-inflammatory functions of GM-CSF-BMDCs, and inhibits the induction of CD8 T cell proliferation and IFNγ production (Lawless et al., 2017). The differences between this study and other studies may be due to the discrete experimental systems. The latter one uses glucose deprivation and galactose treatment to block glycolysis, whereas the earlier ones use 2-DG treatment. The results from different systems (glucose deprivation versus inhibitor treatment) also strongly suggest the need for genetic models to study glycolysis in the functional regulation of DCs. Collectively, these studies demonstrate that glycolysis is an essential regulator of the pro-inflammatory functions of GM-CSF-BMDCs and cDCs.

While initial studies suggested that GM-CSF-BMDCs undergo a "metabolic shift" where oxidative phosphorylation (OXPHOS) is favored over glycolysis under resting conditions and *vice versa* after activation (Pearce and Everts, 2015), a more nuanced view of metabolic reprogramming has recently emerged. Indeed, GM-CSF-BMDCs are also glycolytic at rest, and this increases upon activation. Further, a recent study has investigated the metabolic profile of cDCs directly *ex vivo*. cDC1 retain higher levels of both glycolysis and mitochondrial metabolism than cDC2, and inhibition of either glycolysis or mitochondrial function impedes cDC1-dependent priming of CD8 T cells (Du et al., 2018). Thus, mitochondrial and glycolytic metabolism are also important for the functional responses of cDCs under resting conditions.

What regulates the balance between glycolysis and OXPHOS during DC activation? GM-CSF-BMDCs and cDCs have different ways to regulate these pathways. GM-CSF-BMDCs upregulate the expression of iNOS following TLR stimulation (Everts et al., 2012). iNOS generates nitric oxide (NO) by combining an oxygen radical with a nitrogen atom derived from arginine (Förstermann and Sessa, 2012). The production of NO, in turn, inhibits the mitochondrial electron transport chain and therefore OXPHOS (Cleeter et al., 1994). Suppression of OXPHOS by NO enforces glycolysis in DCs, which serves as the major source for intracellular ATP (Everts et al., 2012). The upregulation of hypoxia-inducible factor 1-alpha (HIF1α) contributes to the induction of iNOS expression in GM-CSF-BMDCs after LPS stimulation (Lawless et al., 2017). Moreover, repression of HIF1α expression impairs glucose metabolism in GM-CSF-BMDCs and consequently reduces DC maturation and their functional capacity to stimulate allogeneic T cells (Jantsch et al., 2008). By contrast, glycolytic reprogramming is mediated by HIF1α in an iNOS-independent way in cDCs. The expression of HIF1α increases in cDCs isolated from mice treated with poly(I:C), a

TLR3 agonist (Pantel et al., 2014). This upregulation is mediated by type I IFN signaling via IFNAR (IFNα receptor; **Figure 1**), suggesting that poly(I:C) could act directly or indirectly on cDCs to promote glycolysis. Furthermore, upregulation of HIF1α is essential for sustaining the glycolytic program and suppressing OXPHOS in cDCs. However, recent studies in macrophages suggest that mitochondrial metabolism itself can reinforce glycolysis through regulating the expression or function of HIF1α (O'Neill and Pearce, 2016). In summary, HIF1α is involved in maintaining the balance between glycolysis and OXPHOS in both GM-CSF-BMDCs and cDCs, but this occurs through respective iNOS-dependent or -independent mechanisms (**Table 1**). It would also be interesting to determine if iNOS signaling in GM-CSF-BMDCs and cDCs differs due to distinct regulation of arginine metabolism.

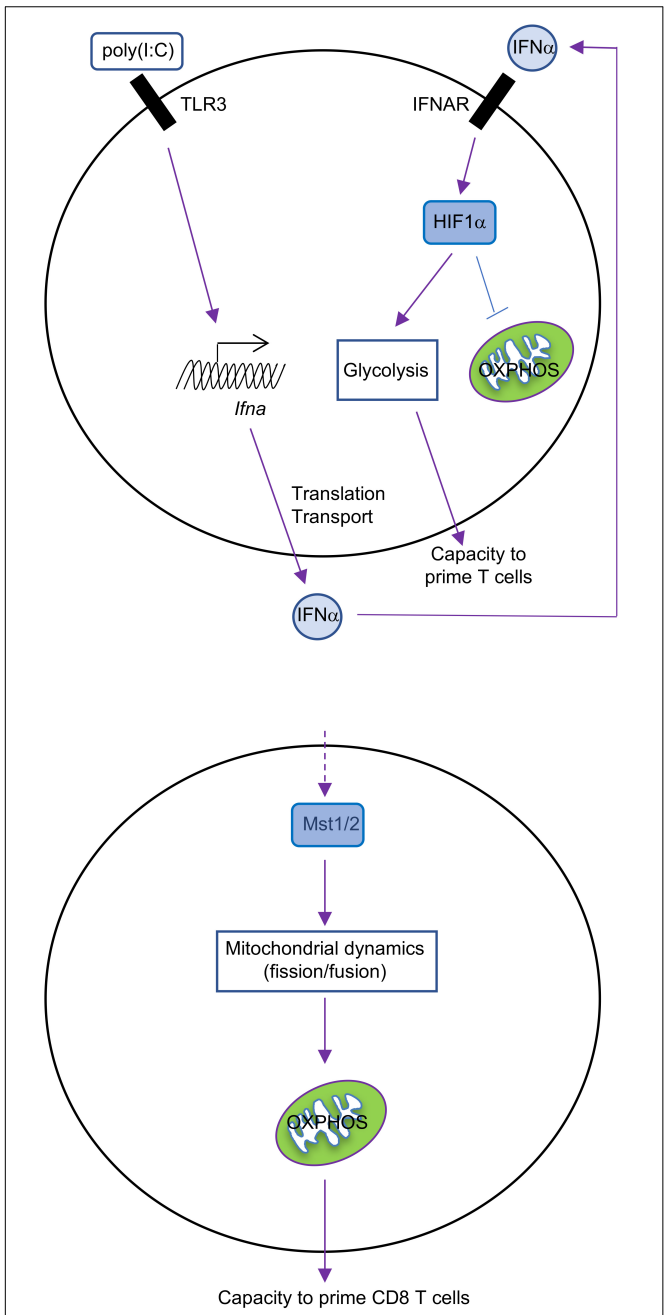

FIGURE 1 | Metabolic regulation in cDCs. IFNα-HIF1α signaling regulates glycolysis and OXPHOS in activated cDCs after long-term TLR ligation (over 14 h). TLR3 agonist [poly(I:C)]-treated cDCs upregulate expression of IFNα and HIF1α, and HIF1α then mediates the metabolic reprogramming from OXPHOS to glycolysis, which is required for cDC function (top). Mst1/2 signaling promotes cDC1 OXPHOS and function to prime CD8 T cells through orchestrating mitochondrial dynamics and function under steady state (bottom).

FATTY ACID AND LIPID METABOLISM IN THE FUNCTIONAL REGULATION OF GM-CSF-BMDCs, cDCs, AND pDCs

As mentioned above, activated DCs also have increased biosynthetic demands that are required for protein and membrane synthesis. Intermediates produced from glycolysis and mitochondrial metabolism are also important biosynthesis intermediates. Among these intermediates, acetyl-CoA is an important precursor used to generate fatty acids through the enzymes acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN; Lunt and Vander Heiden, 2011; Ganeshan and Chawla, 2014). Upon DC activation, citrate is produced from glucose-derived pyruvate and transported to the cytoplasm by Slc25a1 and converted to acetyl-CoA by ATP citrate lyase (Everts et al., 2014). LPS-induced activation is reduced in GM-CSF-BMDCs with Slc25a1 deficiency or treated with C75 (FASN inhibitor) or TOFA (ACC1 inhibitor). Thus, mitochondrial-derived acetyl-CoA serves as the nutrient source for *de novo* fatty acid synthesis that is essential for DC activation.

DC activation is associated with increased capacity for antigen processing and presentation and synthesis of proteins that are expressed on the cell surface (e.g., co-stimulatory molecules) or secreted (e.g., cytokines and chemokines). These processes are regulated by the fatty acid synthesis-dependent expansion of the endoplasmic reticulum (ER) and Golgi networks in GM-CSF-BMDCs (Everts et al., 2014). Consistent with these observations, blocking fatty acid synthesis by TOFA also impairs cDC immunogenicity, including reduced expression of TNFα, IL-6, and TLRs; these defects also lead to functional impairments for activating antigen-restricted CD4 T cells or NK cells (Ibrahim et al., 2012). However, different observations have been reported by others. One group has reported that TOFA treatment of GM-CSF-BMDCs enhances cytokine and chemokine production by DCs and increases DC-dependent activation of CD4 and CD8 T cells. Furthermore, treatment of mice with C75 results in increased capacity of splenic cDCs to capture antigen *in vivo* (Rehman et al., 2013). A third independent study suggests that fatty acid synthesis is dispensable for the activation and function of DCs. Indeed, ACC1-deficient iCD103 DCs (a BMDC subset that resembles and functions like $CD103^+$ cDCs) or GM-CSF-BMDCs have normal functional capacity and activation, as revealed by normal expression of

TABLE 1 | Changes in metabolism of different DC types during activation.

DC types	cDCs	pDCs	GM-CSF-BMDCs
Important metabolic profile changes	Glycolysis is increased rapidly, fatty acid synthesis is increased, OXPHOS is decreased	OXPHOS is increased, FAO is increased	Glycolysis is increased rapidly, fatty acid synthesis is increased, OXPHOS is decreased
Upstream stimulators	LPS, poly(I:C), type I IFNs	CpG-A and type I IFNs	LPS, R-848, CpG-B, poly(I:C), Pam_3CSK_4 and Pam_2CSK_4
Downstream signaling pathways	Akt, TBK1-IKKϵ, HKII HIF1α	PPARα	Akt, TBK1-IKKϵ, HKII, AMPK, IL-10, mTORC1, HIF1α, iNOS, NO

CD86 and MHC II, secretion of IL-12/23p40 and TNFα, and T cell priming ability following a bacterial infection (Stüve et al., 2018). Thus, ACC1-mediated *de novo* fatty acid synthesis is dispensable for the function of iCD103 cDCs and GM-CSF-BMDCs. The precise reasons for these inconsistencies are not clear, but could be explained by different requirements of fatty acid synthesis in DC types, the discrete effects between inhibitors and genetic models in suppressing fatty acid synthesis, and the unique treatment methods with inhibitors. In summary, the role of fatty acid synthesis in DC function still remains unclear.

It is also possible that the accumulation of total lipids, but not the synthesis of fatty acids *per se*, dictates DC function. In support of this view, activation-induced fatty acid synthesis results in increased lipid storage in lipid droplets in GM-CSF-BMDCs and cDCs (Maroof et al., 2005). Several studies have also demonstrated that the levels of intracellular lipids are linked to DC function. Pharmacological inhibition of lipid bodies using xanthohumol [an acyl-CoA: diacylglycerol acyltransferase (DGAT) inhibitor] inhibits the cross-presentation function of GM-CSF-BMDCs, a process important for activating CD8 T cells (Bougnères et al., 2009). Additionally, liver-derived cDCs that contain high concentrations of intracellular lipids are more potent activators of pro-inflammatory T cell, NK cell, and NKT cell responses, whereas liver-derived cDCs are more potent inducers of regulatory T cell-mediated tolerance if they express low levels of intracellular lipids (Ibrahim et al., 2012). By contrast, the proliferation of allogeneic T cells is reduced when T cells are primed using DCs containing high concentrations of intracellular lipids (Herber et al., 2010; Cao et al., 2014; Cubillos-Ruiz et al., 2015). Consistent with these results, splenic DCs in tumor-bearing mice have higher amounts of triglycerides than DCs from tumor-free mice, and lipid accumulation in DCs dampens their ability to process and present antigens and activate allogeneic T cells (Herber et al., 2010; Cao et al., 2014). This accumulation of lipids in cDCs from tumor-bearing mice is due to enhanced uptake of extracellular fatty acids mediated by the upregulation of Msr1, a scavenger receptor that facilitates the transport of lipids into the cell (Herber et al., 2010). The ER stress response that is triggered by suppression of fatty acid synthesis may regulate the accumulation of intracellular lipids, since limiting ER stress by deletion of Xbp1, an ER stress response factor (Oakes and Papa, 2015), decreases tumor-derived lipid accumulation in cDCs and enhances their ability to prime CD8 T cells (Cubillos-Ruiz et al., 2015). Whether different types of lipids (e.g., short-chain versus long-chain) exert unique effects on the functions in DC subsets remains to be explored, but could help determine why lipid metabolism can exert such a wide range of and sometimes divergent effects on DC function.

Aside from storage in lipid droplets, fatty acids can also be transported into the mitochondria and oxidized into acetyl-CoA in a process termed fatty acid oxidation (FAO). The transport of fatty acids into the mitochondria is mediated by Cpt1a (Houten et al., 2016). DCs can also take up extracellular free fatty acids, such as palmitic acid and oleic acid, which can augment the secretion of IL-23 and IL-1β by GM-CSF-BMDCs after LPS stimulation (Stelzner et al., 2016). While our understanding of metabolic reprogramming in pDCs is more limited, recent studies demonstrated that pDCs have a delayed increase in glycolytic flux and OXPHOS, occurring at approximately 24 h after TLR9 stimulation (Wu et al., 2016).

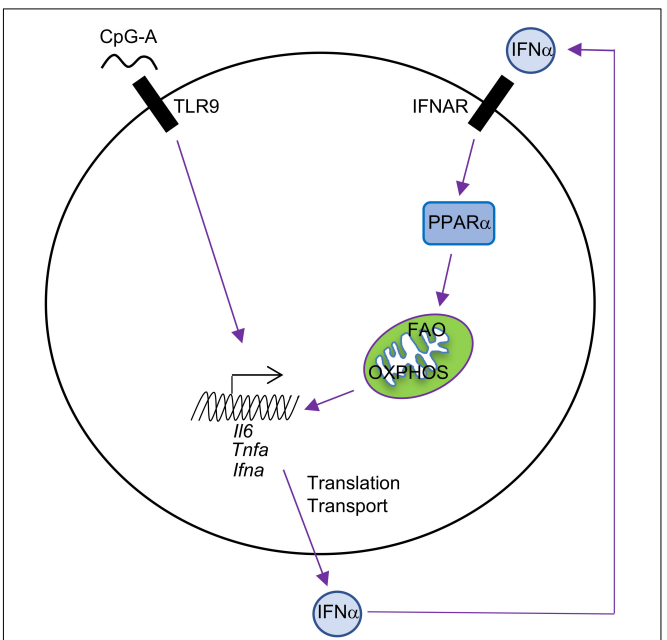

FIGURE 2 | Autocrine type I IFN signaling in the metabolic regulation of pDCs. pDC activation by CpG-A leads to IFNα production, and IFNα then induces cellular metabolic changes, including increased FAO and OXPHOS, through upregulation of PPARα in an autocrine manner. FAO and OXPHOS can further boost pDC activation, including production of IFNα, TNFα, and IL-6.

Interestingly, the increase in mitochondrial metabolism is due to enhanced FAO of *de novo* synthesized fatty acids. Two independent studies reported that pharmaceutical suppression of fatty acid synthesis (using C75 and TOFA) or blocking the function of Cpt1a (using the drug etomoxir or short-hairpin RNA) decreases TLR-induced production of IFNα, TNFα, and IL-6 by pDCs (Wu et al., 2016; Qiu et al., 2017). Qiu et al. (2017) also found that inhibition of glycolysis suppresses pDC activation, suggesting that glucose may serve as the precursor for *de novo* fatty acid synthesis. T cell priming by pDCs may be influenced by mitochondrial metabolism, as suppression of FAO can limit the expression of CD86 (a co-stimulatory receptor ligand) on pDCs (Wu et al., 2016). Mitochondrial-derived reactive oxygen species (ROS) are also increased upon TLR3 stimulation in pDC, which allows them to activate CD8 T cell responses via cross-presentation (Oberkampf et al., 2018). The combined defects in cytokine production (e.g., IFNα) and T cell-mediated immunity likely explain why etomoxir-treated mice have higher viral burdens in an LCMV infection model. These studies establish that the coordinated actions of mitochondrial and fatty acid metabolism are crucial for pDC function.

Mechanistically, autocrine or paracrine type I IFNs likely regulate this futile cycle of fatty acid synthesis/FAO in pDCs (Wu et al., 2016), an idea that is supported by several key experiments. First, there are two phases of type I IFN production by pDCs following TLR9 stimulation with CpG-A. The early phase is between 0 and 6 h after stimulation, during which only a small amount of cytokine is produced. The late phase is between 12 and 24 h, during which the majority of IFNα is produced after pDC activation. This two-phase regulation of type I IFN production is consistent with auto-induction of IFNα. Second, IFNAR deficiency inhibits IFNα production and FAO upregulation in response to TLR9 or imiquimod (a drug that induces type I IFN production) stimulation in pDCs. Third, IFNα itself induces upregulation of FAO, which is revealed by increased basal oxygen consumption rate (OCR), spare respiratory capacity (SRC, a parameter showing the difference between the maximal and basal mitochondrial OCR), and sensitivity of SRC to etomoxir treatment. This IFNα-inducing effect is abolished in IFNAR-deficient pDCs. PPARα is identified as the downstream target of type I IFNs in pDCs, and the PPARα antagonist GW6471 inhibits TLR9-induced IFNα production and OXPHOS in pDCs. Consistent with this observation, the PPARα agonist gemfibrozil increases basal OCR in pDCs. Thus, TLR9 stimulation enforces a feedforward loop whereby autocrine or paracrine type I IFN signaling upregulates the PPARα-dependent induction of FAO and mitochondrial oxidative metabolism (**Figure 2**). Of note, this is different from

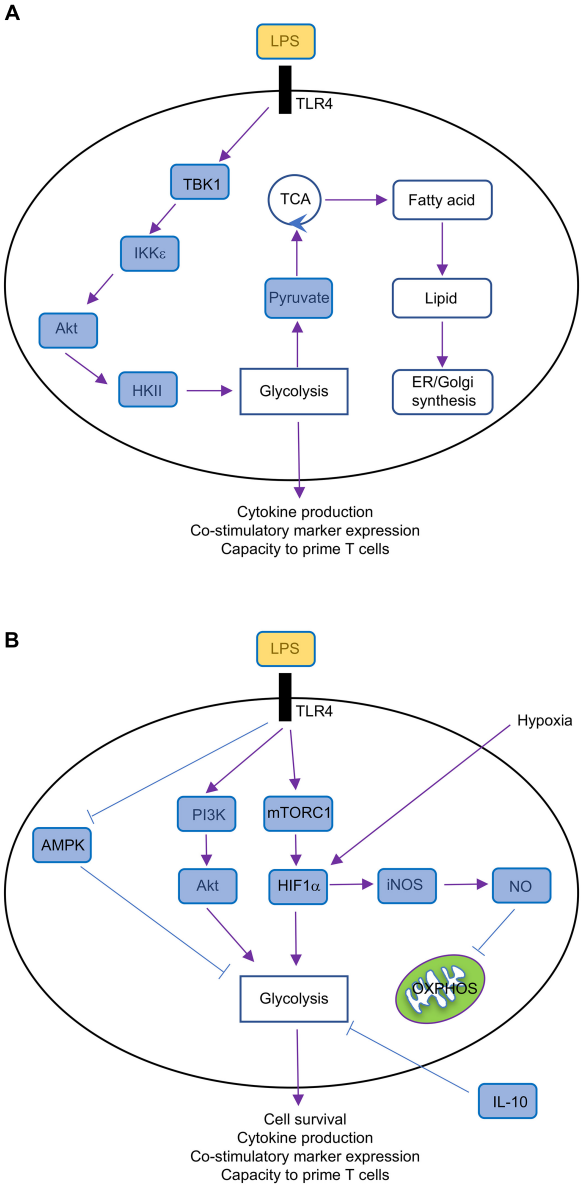

FIGURE 3 | Metabolic regulation in GM-CSF-BMDCs. **(A)** Short-term (minutes to several hours after TLR ligation) metabolic regulation in GM-CSF-BMDCs, which undergo rapid metabolic reprogramming controlled by TBK1-IKKε-Akt-HKII signaling. This signaling pathway promotes glycolysis and subsequently fuels other metabolic activities, such as fatty acid synthesis and lipid production, thereby supporting DC activation. **(B)** Long-term (over 14 h after TLR ligation) metabolic regulation in GM-CSF-BMDCs. TLR ligation triggers multiple signaling pathways, including PI3K-Akt, mTORC1, HIF1α, AMPK, and iNOS. TLR-triggered PI3K-Akt, mTORC1-HIF1α, and AMPK pathways promote glycolysis, which is antagonized by IL-10, whereas iNOS-NO suppresses OXPHOS.

BOX 1 | Important and unsolved questions in the field of metabolic regulation of DCs.

1. Does the function or differentiation of different DC subsets rely on distinct metabolic programs?
2. What are the precise roles of different metabolites in orchestrating the function or differentiation of DC subsets?
3. Is there any cross-talk between metabolites and other signaling pathways, such as epigenetic regulation, for specifying the function or differentiation of DC subsets?
4. Could reprogramming of DC metabolism be used as an efficient method for DC-based immunotherapy?

type I IFN/HIF1α-mediated inhibition of OXPHOS in cDCs, and the unique signaling pathways induced downstream of type I IFNs in DC subsets may account for the differences (**Table 1**).

EVOLUTIONARILY CONSERVED AND IMPORTANT SIGNALING PATHWAYS IN ORCHESTRATION OF DC METABOLISM

The phosphatidylinositol-3-OH kinase (PI3K)-Akt is a key regulator of metabolic reprogramming (Saxton and Sabatini, 2017). Indeed, LPS stimulation through TLR4 promotes the rapid upregulation of glycolysis in GM-CSF-BMDCs and cDCs by activating Akt, which phosphorylates the glycolytic enzyme HKII, thereby anchoring HKII to the mitochondrial membrane where its activity is enhanced (**Figure 3A**). Interestingly, glycolytic reprogramming by Akt occurs via discrete mechanisms that are regulated across time. The activation of PI3K promotes the PDK1 and mechanistic target of rapamycin complex 2 (mTORC2)-dependent phosphorylation of Akt (Chi, 2012), but Akt is also phosphorylated by TBK1 or IKKε (Xie et al., 2011). The early upregulation of TLR-induced glycolytic reprogramming requires Akt, which is activated by TBK1-IKKε but not PI3K or mTOR (Everts et al., 2014). In contrast, PI3K activity is required for the activation of Akt at later time points, which is necessary to sustain glycolysis (Krawczyk et al., 2010).

mTORC1 is also a key regulator of metabolic reprogramming. mTORC1 induces HIF1α expression (Land and Tee, 2007), which as discussed above, promotes glycolysis in DCs. The mTOR-dependent upregulation of HIF1α is also critical to upregulate iNOS expression in GM-CSF-BMDCs (Lawless et al., 2017), which inhibits OXPHOS as discussed above. mTORC1 also promotes glycolysis by regulating Myc expression, as we have found that tuberous sclerosis 1 (Tsc1), a negative regulator of mTOR (Chi, 2012), suppresses glycolytic gene expression and glycolysis in FLT3L-BMDCs through inhibiting Myc expression (Wang et al., 2013). The duration of glycolytic remodeling is blunted upon treatment with IL-10 or activation of adenosine monophosphate (AMP)—activated protein (AMPK; **Figure 3B**), the latter of which is known to suppress mTORC1 activation in other cellular systems (Chi, 2012). mTORC1 also induces mitochondrial metabolism and fatty acid synthesis, suggesting that it may be a central regulator of DC responses. Indeed, we recently demonstrated that mTOR-deficient cDC1 have reduced ability to prime CD8 T cell proliferation *in vitro* (Du et al., 2018). Of note, mTORC1 antagonizes FAO in other systems (Um et al., 2004; Soliman, 2011), suggesting that IFNAR signaling may modulate mTORC1 activation to enforce FAO in TLR9-stimulated pDCs.

The Hippo pathway is another evolutionarily conserved pathway that has recently been implicated in controlling metabolic reprogramming. The canonical Hippo pathway is induced by Mst1 and Mst2 (Mst1/2), the serine/threonine kinases whose activity are necessary to limit organ size and suppress tumorigenesis (Meng et al., 2016). We recently uncovered a novel function for Mst1/2 in cDC metabolism and function (Du et al., 2018). Upon ablation of Mst1/2, we found that cDC1-induced activation of CD8 T cells is reduced, while cDC2 retain their ability to induce CD4 T cell proliferation, likely owing to increased activity of Mst1 in cDC1 than cDC2. These defects lead to impaired anti-tumor and anti-bacterial immunity *in vivo,* further demonstrating that the function of CD8 T cells is diminished. We found that Mst1/2-deficient cDC1 accumulate enlarged mitochondria that have disorganized cristae, which are necessary to support OXPHOS (Cogliati et al., 2013; Buck et al., 2016). Consequently, Mst1/2-deficient cDC1 have reductions in OXPHOS (**Figure 1**). However, they also have reductions in glycolysis, further suggesting that there may be cooperation between these pathways for regulating cDC1 function. Of note, these alterations do not appear to be linked to reductions of mTORC1 activation, as its activity is not reduced in Mst1/2-deficient cDC1 compared with controls. Future studies are still required to uncover how Mst1/2 activity is regulated to mediate cDC1 function.

CONCLUSION AND FUTURE PERSPECTIVES

Emerging evidence provides new insight into the metabolic regulation in DCs. However, we are just starting to understand DC metabolism, and many interesting questions remain to be answered (**Box 1**). Insight into the metabolic regulation of DC subsets and functions could have a significant impact on our understanding of DC biology and immune regulation. Further, it could manifest in legitimate opportunities for treating autoimmune diseases and tumors, through DC-based immunotherapies or by tuning endogenous T cell responses.

AUTHOR CONTRIBUTIONS

XD wrote the manuscript and organized the review. NC wrote part of the manuscript. HC wrote and edited the manuscript and provided overall instructions.

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REFERENCES

Anderson, D. A., Murphy, K. M., and Briseño, C. G. (2017). Development, diversity, and function of dendritic cells in mouse and human. *Cold Spring Harb. Perspect. Biol.* 10: a028613. doi: 10.1101/cshperspect.a028613

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., et al. (2000). Immunobiology of dendritic cells. *Annu. Rev. Immunol.* 18, 767–811. doi: 10.1146/annurev.immunol.18.1.767

Bedoui, S., and Greyer, M. (2014). The role of dendritic cells in immunity against primary herpes simplex virus infections. *Front. Microbiol.* 5:533. doi: 10.3389/fmicb.2014.00533

Bougnères, L., Helft, J., Tiwari, S., Vargas, P., Chang, B. H. J., Chan, L., et al. (2009). A role for lipid bodies in the cross-presentation of phagocytosed antigens by MHC class I in dendritic cells. *Immunity* 31, 232–244. doi: 10.1016/j.immuni.2009.06.022

Buck, M. D. D., O'Sullivan, D., Klein Geltink, R. I. I., Curtis, J. D. D., Chang, C. H., Sanin, D. E. E., et al. (2016). Mitochondrial dynamics controls T cell fate through metabolic programming. *Cell* 166, 63–76. doi: 10.1016/j.cell.2016.05.035

Cao, W., Ramakrishnan, R., Tuyrin, V. A., Veglia, F., Condamine, T., Amoscato, A., et al. (2014). Oxidized lipids block antigen cross-presentation by dendritic cells in cancer. *J. Immunol.* 192, 2920–2931. doi: 10.4049/jimmunol.1302801

Chi, H. (2012). Regulation and function of mTOR signalling in T cell fate decisions. *Nat. Rev. Immunol.* 12, 325–338. doi: 10.1038/nri3198

Clark, G. J., Angel, N., Kato, M., López, J. A., MacDonald, K., Vuckovic, S., et al. (2000). The role of dendritic cells in the innate immune system. *Microbes Infect.* 2, 257–272. doi: 10.1016/S1286-4579(00)00302-6

Cleeter, M. W., Cooper, J. M., Darley-Usmar, V. M., Moncada, S., and Schapira, A. H. (1994). Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. *FEBS Lett.* 345, 50–54. doi: 10.1016/0014-5793(94)00424-2

Cogliati, S., Frezza, C., Soriano, M. E., Varanita, T., Quintana-Cabrera, R., Corrado, M., et al. (2013). Mitochondrial cristae shape determines respiratory chain supercomplexes assembly and respiratory efficiency. *Cell* 155, 160–171. doi: 10.1016/j.cell.2013.08.032

Cubillos-Ruiz, J. R., Silberman, P. C., Rutkowski, M. R., Chopra, S., Perales-Puchalt, A., Song, M., et al. (2015). ER stress sensor XBP1 controls anti-tumor immunity by disrupting dendritic cell homeostasis. *Cell* 161, 1527–1538. doi: 10.1016/j.cell.2015.05.025

Du, X., Wen, J., Wang, Y., Karmaus, P. W. F., Khatamian, A., Tan, H., et al. (2018). Hippo/Mst signalling couples metabolic state and immune function of CD8α^+ dendritic cells. *Nature* 558, 141–145. doi: 10.1038/s41586-018-0177-0

Everts, B., Amiel, E., Huang, S. C. C., Smith, A. M., Chang, C. H., Lam, W. Y., et al. (2014). TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKε supports the anabolic demands of dendritic cell activation. *Nat. Immunol.* 15, 323–332. doi: 10.1038/ni.2833

Everts, B., Amiel, E., Van Der Windt, G. J. W., Freitas, T. C., Chott, R., Yarasheski, K. E., et al. (2012). Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells. *Blood* 120, 1422–1431. doi: 10.1182/blood-2012-03-419747

Everts, B., and Pearce, E. J. (2014). Metabolic control of dendritic cell activation and function: recent advances and clinical implications. *Front. Immunol.* 5:203. doi: 10.3389/fimmu.2014.00203

Förstermann, U., and Sessa, W. C. (2012). Nitric oxide synthases: regulation and function. *Eur. Heart J.* 33, 829–837. doi: 10.1093/eurheartj/ehr304

Ganeshan, K., and Chawla, A. (2014). Metabolic regulation of immune responses. *Annu. Rev. Immunol.* 32, 609–634. doi: 10.1146/annurev-immunol-032713-120236

Ginhoux, F., Guilliams, M., and Naik, S. H. (2016). Dendritic cell and macrophage nomenclature and classification. *Front. Immunol.* 7:168. doi: 10.3389/fimmu.2016.00168

Guak, H., Al Habyan, S., Ma, E. H., Aldossary, H., Al-Masri, M., Won, S. Y., et al. (2018). Glycolytic metabolism is essential for CCR7 oligomerization and dendritic cell migration. *Nat. Commun.* 9:2463. doi: 10.1038/s41467-018-04804-6

Guilliams, M., Dutertre, C. A., Scott, C. L., McGovern, N., Sichien, D., Chakarov, S., et al. (2016). Unsupervised high-dimensional analysis aligns dendritic cells across tissues and species. *Immunity* 45, 669–684. doi: 10.1016/j.immuni.2016.08.015

Guilliams, M., Ginhoux, F., Jakubzick, C., Naik, S. H., Onai, N., Schraml, B. U., et al. (2014). Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nat. Rev. Immunol.* 14, 571–578. doi: 10.1038/nri3712

Hammer, G. E., and Ma, A. (2013). Molecular control of steady-state dendritic cell maturation and immune homeostasis. *Annu. Rev. Immunol.* 31, 743–791. doi: 10.1146/annurev-immunol-020711-074929

Helft, J., Böttcher, J., Chakravarty, P., Zelenay, S., Huotari, J., Schraml, B. U., et al. (2015). GM-CSF mouse bone marrow cultures comprise a heterogeneous population of CD11c$^+$ MHCII$^+$ macrophages and dendritic cells. *Immunity* 42, 1197–1211. doi: 10.1016/j.immuni.2015.05.018

Hemmi, H., and Akira, S. (2005). TLR signalling and the function of dendritic cells. *Chem. Immunol. Allergy* 86, 120–135. doi: 10.1159/000086657

Herber, D. L., Cao, W., Nefedova, Y., Novitskiy, S. V., Nagaraj, S., Tyurin, V. A., et al. (2010). Lipid accumulation and dendritic cell dysfunction in cancer. *Nat. Med.* 16, 880–886. doi: 10.1038/nm.2172

Houten, S. M., Violante, S., Ventura, F. V., and Wanders, R. J. A. (2016). The biochemistry and physiology of mitochondrial fatty acid β-oxidation and its genetic disorders. *Annu. Rev. Physiol.* 78, 23–44. doi: 10.1146/annurev-physiol-021115-105045

Ibrahim, J., Nguyen, A. H., Rehman, A., Ochi, A., Jamal, M., Graffeo, C. S., et al. (2012). Dendritic cell populations with different concentrations of lipid regulate tolerance and immunity in mouse and human liver. *Gastroenterology* 143, 1061–1072. doi: 10.1053/j.gastro.2012.06.003

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., et al. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176, 1693–1702. doi: 10.1084/jem.176.6.1693

Jantsch, J., Chakravortty, D., Turza, N., Prechtel, A. T., Buchholz, B., Gerlach, R. G., et al. (2008). Hypoxia and hypoxia-inducible factor-1 modulate lipopolysaccharide-induced dendritic cell activation and function. *J. Immunol.* 180, 4697–4705. doi: 10.4049/jimmunol.180.7.4697

Krawczyk, C. M., Holowka, T., Sun, J., Blagih, J., Amiel, E., DeBerardinis, R. J., et al. (2010). Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood* 115, 4742–4749. doi: 10.1182/blood-2009-10-249540

Krishnaswamy, J. K., Chu, T., and Eisenbarth, S. C. (2013). Beyond pattern recognition: NOD-like receptors in dendritic cells. *Trends Immunol.* 34, 224–233. doi: 10.1016/j.it.2012.12.003

Land, S. C., and Tee, A. R. (2007). Hypoxia-inducible factor 1α is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif. *J. Biol. Chem.* 282, 20534–20543. doi: 10.1074/jbc.M611782200

Lawless, S. J., Kedia-Mehta, N., Walls, J. F., McGarrigle, R., Convery, O., Sinclair, L. V., et al. (2017). Glucose represses dendritic cell-induced T cell responses. *Nat. Commun.* 8:15620. doi: 10.1038/ncomms15620

Liberti, M. V., and Locasale, J. W. (2016). The warburg effect: how does it benefit cancer cells? *Trends Biochem. Sci.* 41, 211–218. doi: 10.1016/j.tibs.2015.12.001

Lunt, S. Y., and Vander Heiden, M. G. (2011). Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu. Rev. Cell Dev. Biol.* 27, 441–464. doi: 10.1146/annurev-cellbio-092910-154237

Lutz, M. B., Kukutsch, N., Ogilvie, A. L., Rößner, S., Koch, F., Romani, N., et al. (1999). An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* 223, 77–92. doi: 10.1016/S0022-1759(98)00204-X

Maroof, A., English, N. R., Bedford, P. A., Gabrilovich, D. I., and Knight, S. C. (2005). Developing dendritic cells become "lacy" cells packed with fat and glycogen. *Immunology* 115, 473–483. doi: 10.1111/j.1365-2567.2005.02181.x

Meng, Z., Moroishi, T., and Guan, K.-L. (2016). Mechanisms of Hippo pathway regulation. *Genes Dev.* 30, 1–17. doi: 10.1101/gad.274027.115

Merad, M., Sathe, P., Helft, J., Miller, J., and Mortha, A. (2013). The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu. Rev. Immunol.* 31, 563–604. doi: 10.1146/annurev-immunol-020711-074950

Murphy, K. M. (2016). Transcriptional control of dendritic cell development. *Annu. Rev. Immunol.* 34, 93–119. doi: 10.1146/annurev-immunol-032713-120204

Naik, S. H., Proietto, A. I., Wilson, N. S., Dakic, A., Schnorrer, P., Fuchsberger, M., et al. (2005). Cutting edge: generation of splenic $CD8^+$ and $CD8^-$ dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *J. Immunol.* 174, 6592–6597. doi: 10.4049/jimmunol.174.11.6592

Oakes, S. A., and Papa, F. R. (2015). The role of endoplasmic reticulum stress in human pathology. *Annu. Rev. Pathol. Mech. Dis.* 10, 173–194. doi: 10.1146/annurev-pathol-012513-104649

Oberkampf, M., Guillerey, C., Mouriès, J., Rosenbaum, P., Fayolle, C., Bobard, A., et al. (2018). Mitochondrial reactive oxygen species regulate the induction of $CD8^+$ T cells by plasmacytoid dendritic cells. *Nat. Commun.* 9:2241. doi: 10.1038/s41467-018-04686-8

O'Neill, L. A. J., Kishton, R. J., and Rathmell, J. (2016). A guide to immunometabolism for immunologists. *Nat. Rev. Immunol.* 16, 553–565. doi: 10.1038/nri.2016.70

O'Neill, L. A. J., and Pearce, E. J. (2016). Immunometabolism governs dendritic cell and macrophage function. *J. Exp. Med.* 213, 15–23. doi: 10.1084/jem.20151570

Pantel, A., Teixeira, A., Haddad, E., Wood, E. G., Steinman, R. M., and Longhi, M. P. (2014). Direct type I IFN but Not MDA5/TLR3 activation of dendritic cells is required for maturation and metabolic shift to glycolysis after poly IC stimulation. *PLoS Biol.* 12:e1001759. doi: 10.1371/journal.pbio.1001759

Pearce, E. J., and Everts, B. (2015). Dendritic cell metabolism. *Nat. Rev. Immunol.* 15, 18–29. doi: 10.1038/nri3771

Qiu, C. C., Lee, M. H., and Gallucci, S. (2017). Modulation of the energy metabolism inhibits plasmacytoid dendritic cell activation and delays onset of autoantibody production in murine models of systemic lupus erythematosus. *J. Immunol.* 198(Suppl. 1):80.12. doi: 10.1016/j.neuron.2014.12.052

Rehman, A., Hemmert, K. C., Ochi, A., Jamal, M., Henning, J. R., Barilla, R., et al. (2013). Role of fatty-acid synthesis in dendritic cell generation and function. *J. Immunol.* 190, 4640–4649. doi: 10.4049/jimmunol.1202312

Saxton, R. A., and Sabatini, D. M. (2017). mTOR signaling in growth, metabolism, and disease. *Cell* 168, 960–976. doi: 10.1016/j.cell.2017.02.004

Segura, E. (2016). Review of mouse and human dendritic cell subsets. *Methods Mol. Biol.* 1423, 3–15. doi: 10.1016/S0264-410X(02)00612-6

Soliman, G. A. (2011). The integral role of mTOR in lipid metabolism. *Cell Cycle* 10, 861–862. doi: 10.4161/cc.10.6.14930

Stelzner, K., Herbert, D., Popkova, Y., Lorz, A., Schiller, J., Gericke, M., et al. (2016). Free fatty acids sensitize dendritic cells to amplify TH1/TH17-immune responses. *Eur. J. Immunol.* 46, 2043–2053. doi: 10.1002/eji.201546263

Stüve, P., Minarrieta, L., Erdmann, H., Arnold-Schrauf, C., Swallow, M., Guderian, M., et al. (2018). De novo fatty acid synthesis during mycobacterial infection is a prerequisite for the function of highly proliferative T cells, but not for dendritic cells or macrophages. *Front. Immunol.* 9:495. doi: 10.3389/fimmu.2018.00495

Thwe, P. M., Pelgrom, L., Cooper, R., Beauchamp, S., Reisz, J. A., D'Alessandro, A., et al. (2017). Cell-intrinsic glycogen metabolism supports early glycolytic reprogramming required for dendritic cell immune responses. *Cell Metab.* 26, 558–567. doi: 10.1016/j.cmet.2017.08.012

Um, S. H., Frigerio, F., Watanabe, M., Picard, F., Joaquin, M., Sticker, M., et al. (2004). Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* 431, 200–205. doi: 10.1038/nature02866

Wang, Y., Huang, G., Zeng, H., Yang, K., Lamb, R. F., and Chi, H. (2013). Tuberous sclerosis 1 (Tsc1)-dependent metabolic checkpoint controls development of dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 110, E4894–E4903. doi: 10.1073/pnas.1308905110

Watts, C., West, M. A., and Zaru, R. (2010). TLR signalling regulated antigen presentation in dendritic cells. *Curr. Opin. Immunol.* 22, 124–130. doi: 10.1016/j.coi.2009.12.005

Wu, D., Sanin, D. E., Everts, B., Chen, Q., Qiu, J., Buck, M. D., et al. (2016). Type 1 interferons induce changes in core metabolism that are critical for immune function. *Immunity* 44, 1325–1336. doi: 10.1016/j.immuni.2016.06.006

Xie, X., Zhang, D., Zhao, B., Lu, M.-K., You, M., Condorelli, G., et al. (2011). I B kinase and TANK-binding kinase 1 activate AKT by direct phosphorylation. *Proc. Natl. Acad. Sci. U.S.A.* 108, 6474–6479. doi: 10.1073/pnas.1016132108

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The Sweet Surrender: How Myeloid Cell Metabolic Plasticity Shapes the Tumor Microenvironment

Je Lin Sieow[1,2†], Sin Yee Gun[1†] and Siew Cheng Wong[1,2]*

[1] Singapore Immunology Network, A*STAR, Singapore, Singapore, [2] School of Biological Sciences, Nanyang Technological University, Singapore, Singapore

Immune cells are one of the most versatile cell types, as they can tailor their metabolic activity according to their required function. In response to diverse environmental cues, immune cells undergo metabolic reprogramming to support their differentiation, proliferation and pro-inflammatory effector functions. To meet a dramatic surge in energetic demand, immune cells rewire their metabolism to utilize aerobic glycolysis. This preferential use of glycolysis even under aerobic conditions is well established in tumor cells, and is known as the "Warburg effect." Tumor cells avidly use glucose for aerobic glycolysis, thereby creating a nutrient-starved microenvironment, outcompeting T cells for glucose, and directly inhibiting T-cell anti-tumoral effector function. Given that both immune and tumor cells use similar modes of metabolism in the tumor stroma, it is imperative to identify a therapeutic window in which immune-cell and tumor-cell glycolysis can be specifically targeted. In this review, we focus on the Warburg metabolism as well as other metabolic pathways of myeloid cells, which comprise a notable niche in the tumor environment and promote the growth and metastasis of malignant tumors. We examine how differential immune-cell activation triggers metabolic fate, and detail how this forbidding microenvironment succeeds in shutting down the vigorous anti-tumoral response. Finally, we highlight emerging therapeutic concepts that aim to target immune-cell metabolism. Improving our understanding of immunometabolism and immune-cell commitment to specific metabolic fates will help identify alternative therapeutic approaches to battle this intractable disease.

Keywords: tumor immunology, macrophages, myeloid derived suppressor cell (MDSC), immunometabolism, immunotharapy, glycolysis

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University of Pittsburgh, United States

****Correspondence:***
Siew Cheng Wong
wong_siew_cheng@immunol.a-star.edu.sg

† These authors have contributed equally to this work

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IMMUNOMETABOLISM: A RE-EMERGING FIELD

Immunometabolism describes the interplay between immunologic and metabolic processes, and can be interpreted in two possible ways: (1) the role of metabolic changes in immune cells in influencing cellular functions and broader immunity (Buck et al., 2017), and (2) the role of immune cells in directing metabolism in organs or the whole organism (Pearce and Pearce, 2013). Here, we focus on the former and discuss how changes in the metabolic profile of immune cells can have an impact in cancer.

Immune cells respond to environmental cues (e.g., infection, tissue injury, cellular stress and tumor invasion) to assume a wide variety of functional states. Immune cells are a vital component of the body's defense against disease and are important in maintaining tissue homeostasis. Upon encountering a stimulus, immune cells may migrate, proliferate, secrete cytokines and undergo apoptosis. Different immune-cell subsets utilize different metabolic pathways to generate the required cellular energy and biosynthetic macromolecules to fulfill their effector functions when mounting a host immune response.

Thus far, the metabolic pathways utilized by T cells have been the most well studied and recently reviewed (Bettonville et al., 2016; Bantug et al., 2018). As such, we focus primarily on myeloid cell metabolism, as these cells represent a substantial component of the innate immune system. Similar to cells of the adaptive immune system, myeloid cells undergo robust metabolic reprogramming upon activation and stimulation (Geeraerts et al., 2017; Stienstra et al., 2017). Myeloid cells are highly plastic, they can exhibit wide metabolic heterogeneity depending on the types of stimuli and microenvironment they encounter (Biswas, 2015). This diverse metabolic response gives rise to numerous phenotypes and polarization states that dictate downstream immune responses. Here, we highlight the metabolic pathways used by myeloid cells in the cancer setting, and discuss some of the current strategies to target myeloid-cell metabolism and improve the efficacy of cancer immunotherapies.

IMMUNE CELL METABOLIC PATHWAYS

Metabolic reprogramming is a hallmark of cancer (Hanahan and Weinberg, 2011). For tumor cells to thrive, they must reprogram their metabolic profiles to fuel their energy needs according to the microenvironment, and to promote their proliferation, survival and differentiation (Boroughs and DeBerardinis, 2015). One of the main metabolic pathways used by tumor cells is aerobic glycolysis, widely known as the Warburg effect (Sica et al., 2017). Likewise, immune cells can also utilize multiple metabolic pathways for energy production. For example, immune cells can undergo either glycolysis and/or oxidative phosphorylation (OXPHOS) to produce adenosine triphosphate (ATP) for their functional requirements. During glycolysis, cells uptake glucose in the environment via glucose transporters (GLUT), and convert it into pyruvate and ATP. To maintain glycolytic flux, cells convert pyruvate to lactate to regenerate nicotinamide adenine dinucleotide (NAD+). An intermediate molecule of glycolysis is glucose-6-phosphate (G6P) — the first molecule of the pentose phosphate pathway (PPP). The PPP consists of an oxidative and a non-oxidative branch (Patra and Hay, 2014). The oxidative branch generates reducing equivalents, such as nicotinamide adenine dinucleotide phosphate (NADPH) and ribose-5-phosphate (R5P) (Patra and Hay, 2014) while the non-oxidative branch coverts glycolytic intermediates, such as fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (G3P) into pentose phosphates. The reversible nature of enzymes in the non-oxidative branch enables the PPP to utilize glycolysis according to the metabolic demands of a cell (Cho et al., 2018). By contrast, OXPHOS is an oxygen-driven process that produces 36 ATP per glucose molecule (O'Neill et al., 2016). OXPHOS takes place when energy precursors derived from acetyl-CoA generate and feed electrons into the electron transport chain (ETC), leading to phosphorylation of ADP to produce ATP.

Cells can also use glutamine or fatty acids as nutrients to fuel the TCA cycle (Geeraerts et al., 2017). For example, glutamine can be converted into α-ketoglutarate, an intermediate of the TCA cycle, while the fatty acid oxidation (FAO) pathway degrades fatty acids into acetyl-CoA for ATP production.

OXPHOS is a highly efficient pathway for ATP production, and is the preferred metabolic pathway utilized by cells with high energy demands (O'Neill et al., 2016). Conversely, glycolysis yields only two ATP per glucose molecule. Although glycolysis is not the most efficient way to produce energy, high glycolytic rates allow cells to produce sufficient energy and intermediates to fuel their growth and functional demands. Tumor cells are a typical cell-type that uses this approach, switching from OXPHOS to glycolysis even in the presence of oxygen. The Warburg effect enables tumor cells to adapt to their microenvironment for their survival and proliferation (Hanahan and Weinberg, 2011; Ward and Thompson, 2012).

Besides cellular energy and ATP production, intermediates produced from the different metabolic pathways, such as glycolysis, the PPP and the TCA cycle are also necessary for fatty acid and amino acid synthesis. For example the PPP and TCA cycles generate NADPH and citrate for fatty acid synthesis, respectively (O'Neill et al., 2016). The unique model of metabolism that each cell uses is dependent on their microenvironment and external stimuli.

Mechanistically, conceptual progress has been made in understanding the signaling pathways that underlie immune cell metabolism reprogramming. One such pathway is the mTOR pathway, which regulates various important cell processes such as protein synthesis, cell growth and metabolism (Saxton and Sabatini, 2017). Specifically, myelopoiesis requires mTOR signaling and loss of mTOR dampens innate immune responses against *Listeria monocytogenes* infection (Karmaus et al., 2017). Inhibition of the mTOR pathway with rapamycin in both human monocytes and dendritic cells prevented the anti-inflammatory effect and Th1 responses of glucocorticoids (Weichhart et al., 2011). The mTOR pathway is also a key orchestrator of myeloid cell effector responses to nutrient availability and cellular energy requirements, driving an increase in glucose utilization during glycolysis (Covarrubias et al., 2015). HIF-1α induces the over-expression of several glycolytic proteins including glucose transporters (i.e., GLUT1 and GLUT3), and enzymes such as hexokinase-1 (HK1), HK2 and LDHA in cancer cells (Marin-Hernandez et al., 2009). Likewise in macrophages, HIF1α enhances glycolytic pathway activity and lowers OXPHOS rate (Wang et al., 2017; Li et al., 2018). In cases where tumor growth exceeds the ability of the host's vascular system to supply the tumor microenvironment with sufficient oxygen, hypoxic regions are established that induce HIF-1α activation and instruct cancer cells to utilize glucose causing an increase in lactate release (Eales et al., 2016).

THE TUMOR MICROENVIRONMENT AND MYELOID CELLS

The tumor microenvironment consists of a mix of tumor, immune and stromal cells, all of which contribute to shaping the pro-inflammatory state and promoting tumor initiation, progression and metastasis (Whiteside, 2008) (**Figure 1**).

Macrophage Polarization in the Tumor Microenvironment

Macrophages are a prominent immune subset involved in many homeostatic and immune functions. These cells are highly plastic and thus can perform a wide diversity of functions (Wynn et al., 2013). Classical (M1) macrophages are activated primarily by IFN-γ and/or lipopolysaccharide (LPS), and produce pro-inflammatory cytokines, nitric oxide or reactive oxygen intermediates (ROI) to mount an immune response against bacteria and viruses. Alternative (M2) macrophages are activated by cytokines, such as interleukin (IL)-4 and IL-10. These macrophages are mainly associated with wound healing and tissue repair (Wynn et al., 2013).

Depending on the external stimuli, microenvironment and types of cytokine present, these myeloid cells can polarize into specialized subsets (Wynn et al., 2013). For example in prostate cancer, milk fat globule-EGF factor 8 (MFG-E8) secreted by tumor cells facilitates macrophage efferocytosis – a process of removing apoptotic cells and also promotes M2 polarization (Soki et al., 2014). Furthermore, the hypoxic microenvironment, created by highly glycolytic tumor cells, also triggers macrophage polarization toward an anti-inflammatory phenotype (Leblond et al., 2016). As such, strategies that can re-polarize these tumor-associated macrophages (TAMs) toward an anti-tumoral phenotype are advantageous for targeting tumor cells.

Using a murine model of pancreatic ductal adenocarcinoma (PDAC), Zhu and colleagues highlighted that TAMs are of heterogeneous origin (Zhu et al., 2017). They found that circulating blood monocyte-derived TAMs have a potent role in antigen presentation whereas tissue resident embryonically derived TAMs are preferentially involved in tissue repair and remodeling (Zhu et al., 2017). Recent studies observed that tumor-conditioned macrophages (TCMs) exhibit a mixed M1 and M2 phenotype (Penny et al., 2016), which underlies their capability to promote tumor progression and metastasis, whilst retaining their anti-tumoral function in the presence of tumor-targeting monoclonal antibodies (Grugan et al., 2012). Such tumor antigen-targeting antibody-dependent cellular phagocytosis is observed to be more superior in TCMs and M1 (IFNγ + LPS) macrophages as compared to M2 macrophages (IL-4 + IL-13) (Grugan et al., 2012). Besides tumor-targeting antibodies, a soluble SIRPα-Fc protein could also promote Fcγ receptor-mediated phagocytosis of cancer cells by macrophages (Lin G.H.Y. et al., 2017). Specifically, this soluble SIRPα-Fc triggers both M1 (IFNγ + LPS) and M2 (IL-10 + TGF-β) macrophages to significantly increase phagocytosis of lymphoma cells as compared to other macrophages populations (Lin G.H.Y. et al., 2017). A possible explanation for the difference in phagocytic nature of M2 (compared to M1) in both studies could be due to the use of unique M2 polarizing signals, i.e., IL-4 and IL-13 versus IL-10 and TGF-β. Again, these studies demonstrated that macrophages are plastic in nature and their phenotypes strongly dictate their functions. The diverse spectrum of differential TAM activation states in the tumor microenvironment highlights the potential of targeting and re-polarizing their pro-tumoral phenotype toward an anti-tumoral phenotype to augment tumor progression and metastasis.

Metabolic Sensing of Macrophages Shapes Their Functional Phenotype

Numerous studies have suggested that the metabolic pathways used by macrophages regulate their immune function (Lampropoulou et al., 2016; Mills and O'Neill, 2016; Wang et al., 2017). Macrophages undergo metabolic adaptations to survive the harsh tumor microenvironment, resulting in differential phenotypes and downstream effector functions. M1 macrophages up-regulate glycolysis leading to the accumulation of succinate, while M2 macrophages up-regulate OXPHOS and FAO (Mills and O'Neill, 2016).

Metabolites generated during macrophage activation can impact and shape the inflammatory immune response. For instance, succinate, a metabolic product of the citric acid cycle accumulates in inflammatory macrophages to drive HIF-1α stabilization and IL-1β production (Tannahill et al., 2013). This metabolite, when secreted into the extracellular space, can further stimulate IL-1β secretion by macrophages in an autocrine and paracrine manner (Littlewood-Evans and Sarret, 2016). On the other hand, as a negative feedback mechanism, itaconate generated by LPS-stimulated macrophages suppresses inflammatory macrophages either by blocking succinate dehydrogenase-mediated oxidation (Lampropoulou et al., 2016) or by alkylation of the cysteine residue on repressor protein kelch-like ECH-associated protein 1 (KEAP1) to release transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) from proteasomal degradation (Mills et al., 2018). This led to the eventual downregulated expression of HIF-1α and reduced production of pro-inflammatory proteins. Taken together, these extracellular metabolites can have a critical influence on macrophage activation and polarization. This phenomenon influences the way in which macrophages direct the immune response in the tumor microenvironment.

Glycolysis and the Warburg Effect

Decades of work have focused on tumor-cell metabolism, but it was not until the last decade did we learn that the highly glycolytic nature of tumor cells results in a nutrient-limited microenvironment (Hanahan and Weinberg, 2011; Cantor and Sabatini, 2012; Ward and Thompson, 2012). Tumor-infiltrating macrophages are forced to compete with tumor cells particularly for glucose, and thus undergo reprogramming in their energy requirements and changes in their glucose metabolism (Netea-Maier et al., 2018). As such, macrophages alter their cellular bioenergetics to promote their re-polarization to a pro-inflammatory phenotype.

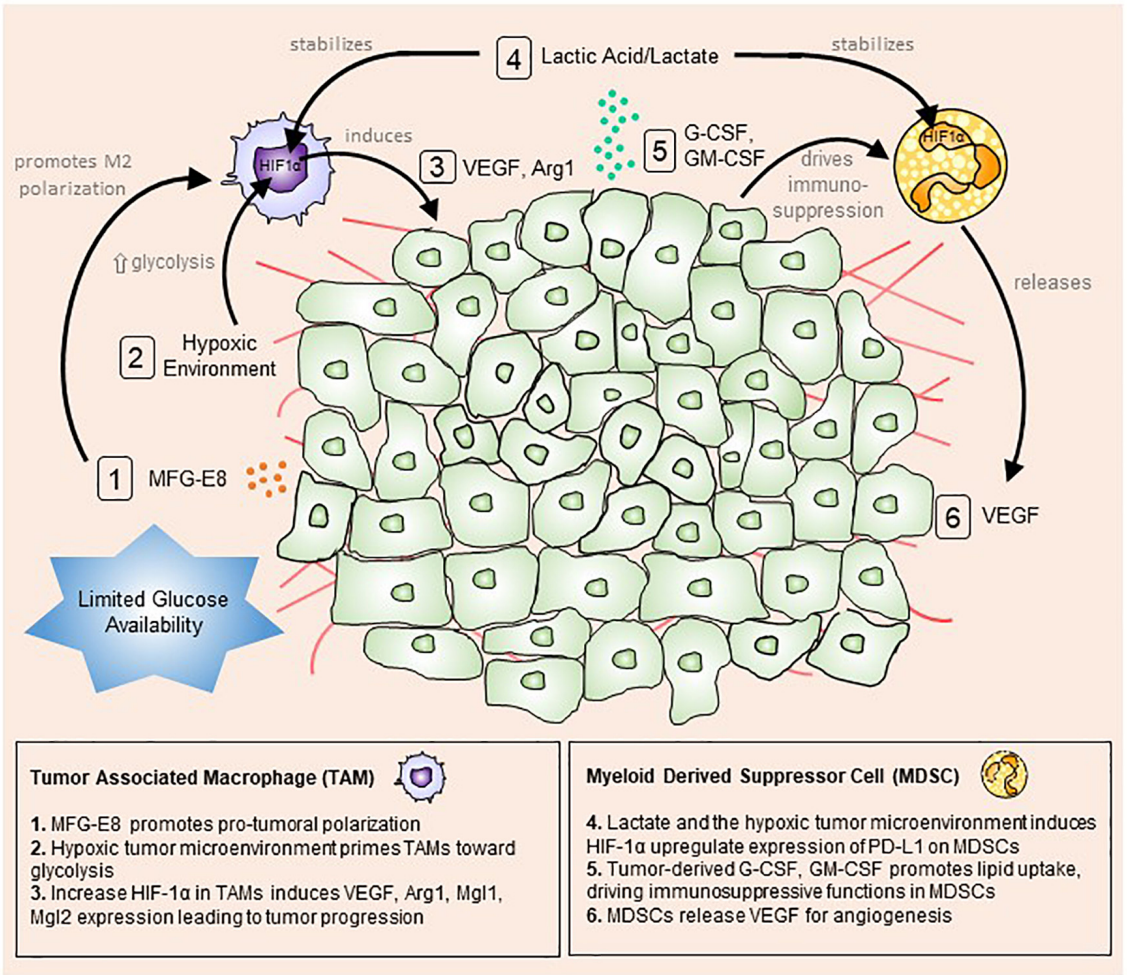

FIGURE 1 | The tumor microenvironment primes myeloid cells toward a pro-tumoral phenotype. Tumor cells actively uptake surrounding glucose to drive aerobic glycolysis and fuel their growth and proliferation. This mode of metabolism creates a microenvironment with limited available glucose and oxygen. Stressed tumor cells undergo apoptosis and produce milk fat globule-EGF factor 8 protein (MFG-E8), which promotes alternative (M2) macrophage polarization (1). At the same time, hypoxic conditions trigger macrophages to up-regulate hypoxia-inducible factor 1-alpha (HIF-1α), promoting a glycolytic switch (2). Lactic acid/lactate, the by-product of glycolysis, stabilizes HIF1α in tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells MDSCs (4). Increased HIF1α expression in TAMs enhances vascular endothelial growth factor (VEGF) and arginase 1 (Arg1) expression and secretion, which feedbacks to tumor cells to boost tumor progression (3). Conversely, tumor-derived granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) up-regulate lipid transport receptors to increase lipid metabolism and drive immunosuppressive functions in MDSCs (5). In turn, MDSCs release VEGF and cathepsin to induce angiogenesis and vasculogenesis (6).

HIF-1α over-expression in macrophages upregulates glycolytic genes, such as pyruvate dehydrogenase kinase 1 (Pdk1), phosphoglycerate kinase 1 (Pgk1), glucose transporter 1 (Glut1), glucokinase (Gck) and pyruvate kinase isozymes M2 (Pkm2) (Wang et al., 2017). This resulted in an active glycolytic phenotype and decreases mitochondrial oxidation in these HIF-1α-overexpressing macrophages (Wang et al., 2017). LPS-activated monocytes reduce oxygen consumption rates and increase, to some degree, anaerobic glycolysis. However, as lactate concentration increases, LPS-induced glycolysis is abrogated in conjunction with down-regulation of pro-inflammatory markers, such as IL-1β, IL-6, IL-12β and CD40 in bone marrow-derived macrophages and peritoneal macrophages (Errea et al., 2016). In line with this, lactate derived from the PDAC cell line Panc-1, also promotes M2-like polarization of THP-1 derived macrophages (Ye et al., 2018). In addition, blocking glycolysis with 2-deoxyglucose (2DG) — a competitive inhibitor of hexokinase 2 (HK2) that catalyzes the rate-limiting step of glycolysis — disrupts glycolytic flux in activated monocytes and impedes TNFα secretion (Dietl et al., 2010). Our group previously showed that PDAC tumor-conditioned macrophages, differentiated from CD14+ human peripheral blood monocytes, exhibit a pro-metastatic phenotype (Penny et al., 2016). In contrast to macrophages differentiated in media of human pancreatic normal epithelia, PDAC tumor-conditioned macrophages promoted angiogenesis,

enhanced epithelial-to-mesenchymal transition and increased the extravasation of PDAC cells out of blood vessels (Penny et al., 2016). Interestingly, PDAC-differentiated macrophages also showed an increased glycolytic capacity, and 2DG treatment abrogated the pro-tumoral function of these cells (Penny et al., 2016). These findings suggest that changes in the metabolic profiles of monocytes and macrophages have an important role in the functional output of these cells toward a pro-metastatic phenotype.

During aerobic glycolysis (the Warburg effect), pyruvate is converted to lactic acid by lactate dehydrogenase A (LDHA). Lactic acid, an end product of both aerobic and anaerobic glycolysis, activates vascular endothelial growth factor (VEGF), transforming growth factor β (TGF-β), and HIF-1α in oxidative tumor cells (Dietl et al., 2010). At the same time, lactic acid released by glycolytic cancer cells into the tumor microenvironment also stabilize HIF-1α expression in bone-marrow derived macrophages (Dietl et al., 2010). Colegio and colleagues further demonstrated that HIF-1α expression in tumor macrophages induce M2-associated genes such as VEGF, arginase 1 (Arg1), macrophage galactose-type lectin-1 (Mgl1) and macrophage galactose-type lectin-2 (Mgl2) in a colon carcinoma mouse model. Human breast cancer cell line-derived lactate also up-regulate the expression of the M2 markers CD163 and CD206, and down-regulate the M1 marker HLA-DRα in human THP-1 monocytic cells (Lin S. et al., 2017). In the same study, they also reported that chemokine CCL5 is up-regulated, and this specific CCL5–CCR5 axis can govern cancer cell metastasis *in vivo* and *in vitro* (Lin S. et al., 2017). These data highlights the pivotal role that metabolic derivatives have in remodeling the immune system, by acting on the differentiation, recruitment, activation and polarization of tumor-infiltrating macrophages.

A study by Wenes et al. (2016) showed that nutrients abundance (such as glucose) within the tumor microenvironment could affect blood vessels formation. Using *in vivo* mouse models for subcutaneous Lewis Lung carcinoma, orthotopic breast cancer and spontaneous mammary tumor, they observed that REDD1, an inhibitor of mTORC1 is highly upregulated in TAMs, particularly those located in hypoxic regions. Depletion of REDD1 in these macrophages greatly enhanced their glycolysis and they competed with neighboring endothelial cells for glucose. The restriction in glucose availability for the endothelial cells, in turn, stabilizes the vascular network to inhibit metastasis. These findings are in contrast to those reported by our group, where inhibiting glycolysis in TAMs resulted in a decrease in their pro-metastatic phenotype (Penny et al., 2016). At first glance, it appears that Wenes and colleagues drew a contradictory conclusion to that of our group; however, many experimental differences may account for this inconsistency. In our *in vitro* study, peripheral blood monocytes from healthy donors were stimulated with tumor-conditioned media under normoxic conditions to derive TCMs (Penny et al., 2016). On the other hand, Wenes et al. (2016) utilized an *in vitro* system where bone-marrow derived macrophages were stimulated with conditioned media and incubated in either normoxic or hypoxic culture conditions. Another major difference in these two studies is the glycolytic state of the TCMs: the study by Penny et al. (2016) was blocking macrophage glycolysis while the REDD1-deficient macrophages in the study by Wenes et al. (2016) further enhances their glycolysis. Despite contradictory results, these studies nevertheless provide some insight as to how metabolic rewiring in TAMs can affect tumor metastasis and progression. As such, current studies and strategies are targeted toward the glycolysis of macrophages to skew TAMs away from a pro-tumoral toward an anti-tumoral phenotype to impart a positive impact on tumor outcome.

Amino Acid Metabolism

Targeting amino acid metabolism in cancer cells has the potential to confer metabolic control and regulation of the tumor microenvironment. Amino acid metabolic enzymes are regulated by tumor suppressors and oncogenes, and have thus been exploited as targets for cancer treatment (Ananieva, 2015). The different macrophage polarized states exhibit readily distinguishable modes of L-arginine (L-Arg) metabolism, giving rise to differential macrophage functions (Rath et al., 2014). For example, IFNγ and LPS-stimulated macrophages up-regulate inducible nitric oxide synthase (iNOS), which catalyzes the conversion of L-Arg into nitric oxide (NO) and L-citrulline. On the other hand, lL-4-polarized macrophages up-regulate Arg1, which catalyzes the conversion of L-Arg to L-ornithine, and polyamine synthesis (Modolell et al., 1995). NO release by macrophages contributes to TAM anti-tumoral activity, whereas polyamines promote tumor-cell growth and progression (Chang et al., 2001). Macrophage Arg1 expression also enhances tumor-cell growth and suppresses tumor cytotoxicity by inhibiting NO production (Chang et al., 2001). Indeed, Arg1 expression by TAMs mediates T-cell immunosuppression (Kusmartsev and Gabrilovich, 2005). Taken together, data suggest that L-Arg metabolism in macrophages can either enhance tumor cell growth by providing tumors with polyamines or suppress cytotoxicity of tumor cells by reducing NO production. Depending on the macrophage polarization state in the tumor microenvironment, amino acid metabolism can have differential effects on tumor progression.

Lipid Metabolism

Tumor-associated fatty acid synthase (FASN) is a key lipogenic enzyme that catalyzes the terminal steps of fatty acid biogenesis and confers a growth and survival advantage to cancer cells (Menendez and Lupu, 2007). Cancer cells adapt and undergo changes in their lipid metabolism; they acquire a lipogenic phenotype by expressing high levels of monoacylglycerol lipase (MAGL), which regulates the pro-tumorigenic lipid network that supports tumor progression (Nomura et al., 2010). Similar to tumor cells, macrophages also alter their lipid metabolism in response to microenvironmental stimuli (Dennis et al., 2010). For example, IL-4-activated macrophages, but not IFNγ-activated or LPS-activated mouse macrophages up-regulate fatty acid uptake and FAO (Odegaard and Chawla, 2011). In the context of the tumor microenvironment, FASN expression by TAMs polarized cells toward a pro-tumoral phenotype expressing IL-10 (Park et al., 2015). FASN was also shown to be an upstream regulator of peroxisome proliferator-activated receptor gamma (PPAR)-β/δ

in myeloid cells, and myeloid cell-specific PPAR-β/δ knockout reduced tumor burden (Park et al., 2015). Other reports have shown that myeloid cells in tumor-bearing hosts possess high levels of triglycerides and cytoplasmic lipid droplets compared to cells from tumor-free mice and healthy individuals (Herber et al., 2010). These findings suggest that by altering lipid metabolism and the lipid levels in professional antigen-presenting cells, the functional activity and anti-tumoral immune response can be restored. Targeting TAM metabolism in tumor growth may thus be an important molecular mechanism in directing their anti-tumoral activity.

CHARACTERISTICS OF MYELOID-DERIVED SUPPRESSOR CELLS IN TUMOR SITES

The role that myeloid-derived suppressor cells (MDSCs) have in regulating tumor progression is well recognized (Gabrilovich et al., 2012; Ugel et al., 2015). MDSCs represent a heterogeneous population of early myeloid progenitors, immature granulocytes, macrophages and dendritic cells at different stages of differentiation that are distinct from mature myeloid cells, and can be functionally described by their strong immunosuppressive properties (Gabrilovich et al., 2012). MDSCs strongly expand as a result of perturbed hematopoiesis in pathological diseases, such as chronic inflammation and cancer, and were originally characterized in tumor-bearing mice as having a CD11b+Gr1+ phenotype (Ugel et al., 2015). MDSCs accumulate in the blood, bone marrow, and the peripheral lymphoid organs (including the lymph nodes and the spleen of tumor-bearing mice), where they have a causative role in promoting immune suppression and thus tumor progression (Ugel et al., 2015). MDSCs may potentially serve as a cellular target to control tumor cell growth.

MDSCs are comprised of two major subsets: monocytic MDSCs (M-MDSCs) and granulocytic MDSC (G-MDSCs). Both subsets can suppress the cytotoxic activity of cytotoxic CD8+ T lymphocytes (CTLs) and natural killer (NK) cells (Gabrilovich et al., 2012). In mice, M-MDSCs are classified as CD11b+Ly6ChighLy6G- cells, while G-MDSCs are classified as CD11b+Ly6ClowLy6G+ cells. In humans, M-MDSCs are CD33+CD14+CD15-HLA-DRlow while G-MDSCs are CD33+CD14-CD15+CD66b+HLA-DR−/low (Bronte et al., 2016). Despite extensive research on these immune cells, the cellular definition of MDSCs subsets remains to be elucidated. At the morphological level, M-MDSCs and G-MDSCs are identical to monocytes and granulocytes, respectively and there are no specific markers that can unequivocally differentiate them. Consequently, researchers are reluctant to use the current MDSC nomenclature to identify myeloid cells with immuno-suppressive capabilities (Coffelt et al., 2016; Porta et al., 2018).

Over the course of inflammation, neutrophils engage in various cell–cell interactions with other immune cells, such as macrophages, dendritic cells and lymphocytes (Mantovani et al., 2011). Neutrophils are innate immune cells involved in the first line of defense at the site of infection, and account for up to 60% of all leukocytes in the circulation. Neutrophils were traditionally seen as terminally differentiated effector cells that have a major role in microbial immunity and acute inflammation (Rosales, 2018). However, it now seems that these short-lived cells can function as immunosuppressive cells in the chronic, progressive disease such as cancer (Nagaraj et al., 2010; Rosales, 2018). Neutrophils secrete cytokines and myeloperoxidase, which is involved in monocyte and macrophage recruitment (Kolarova et al., 2013). Various studies have shown that tumor associated neutrophils (TANs) and their myeloid precursors, G-MDSCs, have important roles in tumor progression (Gregory and Houghton, 2011; Fridlender et al., 2012).

The tumor microenvironment polarizes TAMs toward a pro-tumoral M2-like phenotype, capable of promoting epithelial-to-mesenchymal transition in early pancreatic initiation and development (Helm et al., 2014a). Similar to macrophages, neutrophil plasticity has also been reported with data supporting a skewing of neutrophil phenotypes (Fridlender et al., 2009). TANs acquire a pro-tumoral phenotype to become N2-like neutrophils. These N2-like neutrophils favor tumor initiation and progression by releasing VEGF for angiogenesis, and expressing arginase to suppress cytotoxic T-cell activity (Galdiero et al., 2013). This polarization is largely dependent on TGF-β: during TGF-β blockade, neutrophils acquire an anti-tumor phenotype to become N1-like TANs (Fridlender et al., 2009). Anti-tumor N1-like TANs produce elevated amounts of tumor necrosis factor alpha (TNF-α), macrophage inflammatory proteins-1 alpha (MIP-1α), hydrogen peroxide and NO that are cytotoxic to tumor cells (Jablonska et al., 2010).

In a recent study, Sagiv et al. (2015) distinguished circulating murine neutrophils according to their density. The researchers characterized high-density neutrophils (HDNs) as N1-like cells and circulating low-density neutrophils (LDNs) as N2-like pro-tumoral cells. LDNs can be further subdivided into mature and immature cells, with the immature cells being referred to as G-MDSCs previously (Sagiv et al., 2015). Taken together, these data implicate neutrophil plasticity in mediating cancer. There is now major interest in understanding and characterizing neutrophil infiltration and polarization in cancer progression.

METABOLIC ALTERATIONS OF TUMOR-INFILTRATING MDSCs

The high concentration of lactate produced when tumor cells undergo Warburg metabolism as well as the hypoxic tumor microenvironment promote tumor progression by modifying the anti-tumoral immune response through priming tumor-infiltrating neutrophils toward an immunosuppressive state resulting in the induction of MDSCs (Husain et al., 2013b). In turn, MDSCs inhibit CTLs and NK cell activities (Husain et al., 2013a; Umansky et al., 2016). MDSCs promote immune system dysfunction either by (1) depriving T cells of essential metabolites, such as arginine, tryptophan, and cysteine; (2) interfering with T-cell migration and stimulation; or (3) activating other pro-tumoral immune cells, such as regulatory T cells or TAMs (Rodriguez et al., 2017).

A study conducted by Corzo and colleagues showed that tumor-infiltrating MDSCs suppressed both antigen-specific and non-specific T-cell activity, which was accompanied by up-regulation of Arg1 and iNOS, and down-regulation of NADPH oxidase and reactive oxygen species (ROS) (Corzo et al., 2010). The mechanism for regulating the function and differentiation of MDSCs in the tumor microenvironment involves the transcriptional factor HIF-1α (Corzo et al., 2010). Moreover, lactate-induced HIF-1α contributes to suppressing adaptive immunity by promoting immuno-suppression via inducing expression of programmed death-ligand 1 (PD-L1) on MDSCs (Noman et al., 2014). The hypoxic tumor microenvironment causes selective up-regulation of PD-L1 on splenic MDSCs and PD-L1 blockade could enhance MDSC-mediated T-cell activation, accompanied by the concomitant down-regulation of immuno-suppressive cytokines IL-6 and IL-10 secreted by MDSCs.

Another key sensors of cellular energy metabolism is adenosine monophosphate-activated protein kinase (AMPK) (Long and Zierath, 2006). AMPK is activated by various stimuli, such as hypoxia and oxidative stress. In the context of MDSCs, murine cells exposed to OSU-53, a PPAR-inactive derivative that stimulates AMPK kinase modulated their function (Trikha et al., 2016). Specifically, increased AMPK phosphorylation reduced NO production, inhibited MDSC migration, and reduced IL-6 levels. This role for AMPK in regulating murine MDSC effector functions by dampening their immunosuppressive functions hence promoting T-cell proliferation may represent a novel role for AMPK in metabolic reprogramming of MDSCs (Trikha et al., 2016).

Glycolysis and the Warburg Effect

Tumor cells derived lactic acid (the final product of glycolysis) mediated by HIF-1α can acts as an immunosuppressive metabolite and direct differential myeloid cell functions such as M2-like polarization (Colegio et al., 2014). Neutrophils rely almost exclusively on glycolysis and are strongly committed to anaerobic glycolysis for energy production (Pelletier et al., 2014) and effector functions, such as respiratory burst and chemotaxis (Jun et al., 2014). Neutrophils exhibit very low rates of OXPHOS due to the presence of only a few mitochondria per cell (Borregaard and Herlin, 1982). A study conducted by Azevedo et al. (2015) showed how PPP and glycolysis contribute to the formation of neutrophil extracellular traps (NETs). They demonstrated that a metabolic shift toward PPP is essential, as glucose-6-phosphate dehydrogenase (G6PD) release will fuel NADPH oxidase activity to ultimately produce superoxide (SO) that induces NET formation (Azevedo et al., 2015).

Both M-MDSCs and G-MDSCs in tumor-bearing mice exhibit higher rates of glycolysis compared to their normal mature cell counterparts in healthy mice. By up-regulating glycolytic genes in response to tumor-derived factors and down-regulating ROS production to protect from apoptosis, MDSCs can accumulate in tumors (Jian et al., 2017). Phosphoenolpyruvate, a glycolytic metabolite, is also involved in MDSC proliferation and survival status (Jian et al., 2017).

The glucocorticoid receptor (GR) is expressed by almost every cellular organism and is involved in regulating the genes that control energy metabolism and the immune response (Liao et al., 2014; Lu et al., 2017). GR signaling suppresses HIF-1α and regulates MDSC function via HIF-1α-dependent glycolysis, thus revealing a role for GR-HIF-1α axis in the metabolism and suppressive activities of MDSCs (Lu et al., 2017). These studies indicate that the regulation of glycolysis and its metabolites are able to direct downstream MDSC effector functions.

Amino Acid Metabolism

The depletion of arginine through Arg1 was the first T-cell suppressive mechanism described in G-MDSCs, as these cells are the major source of Arg1 (Rodriguez et al., 2004, 2009). MDSC activation is initiated in response to IFNγ produced by anti-tumoral T cells in the tumor microenvironment (Wu et al., 2012). Once activated, MDSCs uptake large amounts of l-Arg by inducing the cationic amino acid transporter 2 (Cat2), Arg1 and iNOS (Cimen Bozkus et al., 2015). Rodriguez et al. (2004) showed that L-Arg depletion by G-MDSCs blocks CD3zeta expression in T cells resulting in the inhibition of antigen-specific proliferation (Rodriguez et al., 2004). As such, Arg1 production by G-MDSCs in the tumor microenvironment may represent a target for tumor evasion. Besides targeting Arg1, Cat2 ablation was shown to block L-Arg uptake, and as a result, impairs MDSC immunosuppressive and pro-tumoral activities (Cimen Bozkus et al., 2015).

Myeloid-derived suppressor cells can also sequester L-cysteine, causing its deprivation in the microenvironment (Srivastava et al., 2010). L-cysteine deprivation decreases the expression of CD3zeta and inhibits T-cell proliferation (Srivastava et al., 2010). As a result, MDSCs can effectively block T-cell activation by sequestering cysteine, as T cells lack the cystathionase required to convert methionine to cysteine.

The mTOR-signaling pathway can sense a decrease in amino acid metabolism and metabolites making it an important checkpoint that governs cell-cycle entry (Chantranupong et al., 2016). In the context of a tumor, restraining mTOR-mediated T-cell responses by depleting amino acids in the microenvironment may prevent the anti-tumoral immune response (Chantranupong et al., 2016). Besides mTOR, general control non-derepressible 2 (GCN2) kinase expressed by T cells, detects and responds to the immuno-regulatory signals generated by indoleamine 2,3-dioxygenase (IDO), and inhibits T-cell proliferation (Munn et al., 2005). MDSCs express high levels of IDO in response to IFNγ production and activation in the tumor microenvironment, creating yet another mechanism that drives their immunosuppressive phenotype during tumor progression (Pinton et al., 2016).

The multifaceted regulation and suppression of anti-tumoral T-cell responses by MDSC L-Arg metabolism provides several potential areas for therapeutic intervention. One example is the enzymatic depletion of L-Arg in cancer patients via the administration of a pegylated form of the catabolic enzyme arginase I (peg-Arg I), which has shown some therapeutic potential (Hernandez et al., 2010). Metabolites derived from L-Arg metabolism, such as cysteine and tryptophan, are also important regulators of MDSC immunosuppressive activity

(Wu et al., 2012). Several studies have also revealed crucial roles for arginine, serine and glycine in driving T-cell proliferation and anti-tumoral activity (Stachlewitz et al., 2000; Geiger et al., 2016; Ma et al., 2017), however, their roles in mediating MDSC immunosuppressive activity remains to be explored.

Lipid Metabolism

Another important metabolic pathway utilized by tumor cells is lipid metabolism, where lipid β-oxidation is one of the most efficient ways to generate ATP and fuel the cellular energy needs of tumor cells (Nieman et al., 2011; Li and Kang, 2017). Lipids and lipoprotein metabolites in the tumor microenvironment are important mediators of immune-cell function. For example, tumor-resident dendritic cells accumulate oxidized lipoproteins via scavenger receptor-mediated internalization and form lipid droplets (Ramakrishnan et al., 2014; Cubillos-Ruiz et al., 2015). Also, expression of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) by MDSCs enable these cells to specifically associate with endoplasmic reticulum (ER) stress and lipid metabolism, which posses potent immuno suppressive activity promoting T-cell suppressive functions (Condamine et al., 2016). More recently, it has been shown in MDSCs that PPAR-γ has an important role in neutral lipid metabolism signaling controlled by lysosomal acid lipase (Zhao et al., 2016). Zhao et al. (2016) showed that enhanced PPAR-γ activity restrains ROS production by G-MDSCs, thereby impairing cancer cell proliferation and metastasis. In addition, increased exogenous lipid uptake and FAO causes tumor-infiltrating MDSCs to undergo both metabolic and functional reprogramming to become highly immunosuppressive cells (Al-Khami et al., 2017). Tumor-derived granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) together with signal transducer and activator of transcription 3 and 5 (STAT3 and STAT5) signaling induces lipid transport receptor up-regulation, which increases lipid uptake in the tumor microenvironment (Al-Khami et al., 2017). Inhibiting STAT3 or STAT5 signaling or knockout of CD36, a fatty acid translocase, can prevent lipid metabolism and thus the immunosuppressive functions of MDSCs in the tumor environment. This effect results in a CTL-dependent reduction in tumor kinetics.

Taken together, tumor-derived metabolites and fatty acid derivatives in the tumor environment can reprogram and cause downstream functional changes in MDSCs. As fatty acids are largely involved in tumor progression and survival, including important roles in providing the necessary energy and macromolecules for membrane synthesis (Liu et al., 2017), approaches that target lipid metabolism may have promise in rewiring the pro-tumoral phenotype of MDSCs toward a more favorable anti-tumoral one.

In summary, we consider that understanding the immunometabolism of MDSCs is of pivotal and crucial importance, as targeting their metabolic profiles will widen the therapeutic armamentarium for cancer patients. Reprogramming the downstream suppressive effector functions of MDSCs within the tumor microenvironment may be a novel target for tumor resistance.

MYELOID CELLS INFLUENCE ANTI-TUMORAL IMMUNITY

Anti-tumoral immune responses are mainly directed by cytotoxic CD8+ T cells and NK cells. However, the tumor microenvironment is often unsupportive of these cancer-killing cells, with TAMs and MDSCs having a critical role in suppressing their cytolytic functions (**Figure 2**).

TAMs on T Cells

Tumor-associated macrophages repress anti-tumoral cellular activity mainly through (1) suppressing polyclonal T-cell proliferation and (2) promoting an immunosuppressive T-cell phenotype in the tumor. Macrophages are the most abundant population in the tumor microenvironment, and have a crucial role in regulating the T-cell response. Unfortunately, both M1-like ($MHCII^{high}$) and M2-like ($MHCII^{low}$) TAMs are poor antigen presenters and are ineffective at stimulating naïve T-cell proliferation (Movahedi et al., 2010). In the hypoxic tumor stroma, up-regulated HIF-1α and STAT1 expression in TAMs (Doedens et al., 2010) triggers NO secretion (Kusmartsev and Gabrilovich, 2005; Movahedi et al., 2010) and induces arginase activity respectively to induce T-cell apoptosis (Kusmartsev and Gabrilovich, 2005) and halt their expansion. Even if successful activation occurs, proinflammatory CD69+ T cells were found to promote tumor progression. Crosstalk between CD69+ T cells and TAMs up-regulates IL-12, IFNγ and TNF-α, which collectively potentiate IDO expression in TAMs to suppress antigen-specific T-cell expansion (Zhao et al., 2012).

Tumor-associated macrophages also help tumor cells evade immuno-surveillance by suppressing T-cell activity. Regulatory T cells (T_{reg}) are immunosuppressive cells that are critical for maintaining self-tolerance, especially in autoimmune diseases. In the context of cancer, the presence of T_{reg} is often a negative prognostic factor with respect to overall survival (Waniczek et al., 2017). Su and colleagues demonstrated that T_{reg} develop from naïve CD4+ T cells *in situ*. More importantly, their study using a human breast cancer xenograft mouse model showed that naïve T-cell recruitment is directed by CCL18 produced by TAMs (Su et al., 2017). It has also been reported that $CD163^{high}$ TAMs induce blood-derived CD4+ T cells to secrete less IL-2 and more TGF-β, IL-10 and IL-4 (Dannenmann et al., 2013). Besides promoting T_{reg} function, CD163+ TAMs (M1-like) located around tumor/connective tissue also strongly secrete IL-10 and express more PD-L1 compared to other TAM subsets found within the tumor (Kubota and Moriyama, 2017). Collectively, TAMs hijack T-cell physiological regulatory mechanisms to create a pro-tumoral microenvironment that promotes tumor progression.

The other arm of host anti-tumor immune response in human cancer is mediated by NK cells. A previous study by Mattiola and colleagues highlighted the effect of different macrophage subsets on NK-cell activity (Mattiola et al., 2015). They found that M1 macrophages specifically promoted IL-23 and

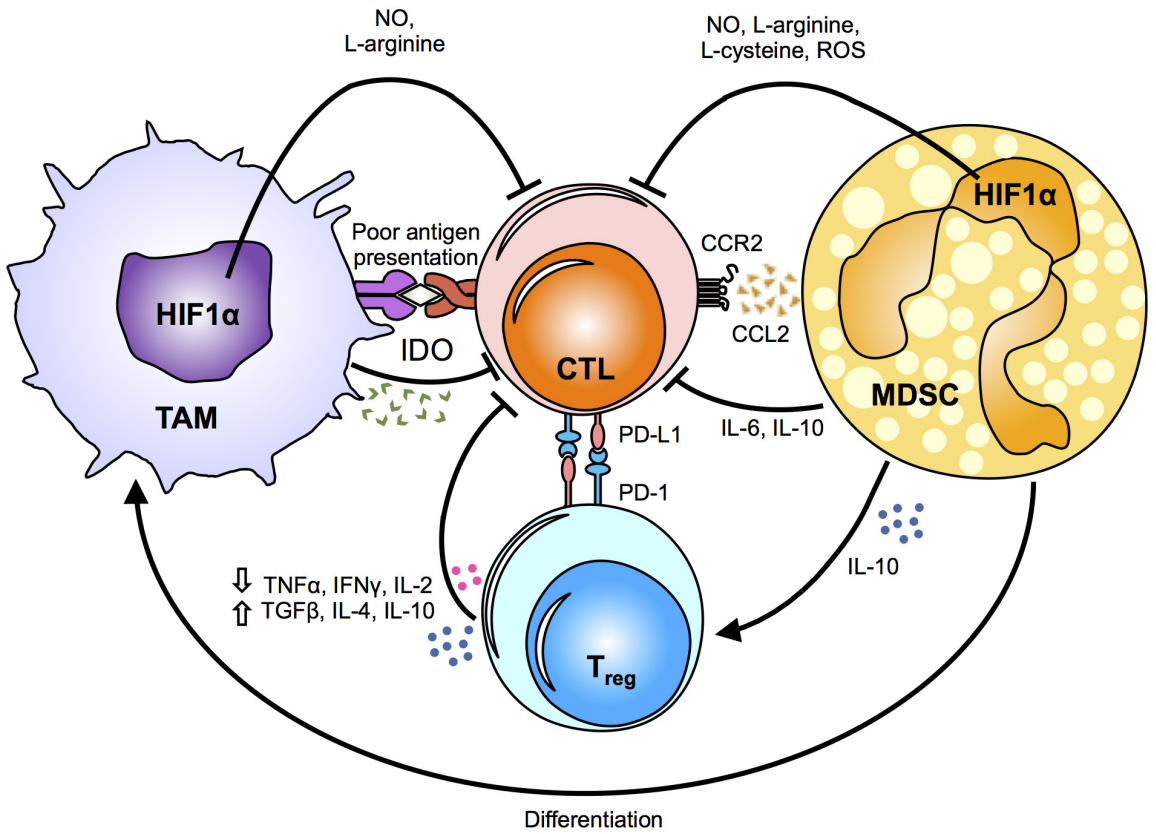

FIGURE 2 | Mechanisms of myeloid cell suppression in the tumor. The tumor microenvironment primes myeloid cells, such as tumor-associated macrophages (TAMs) and myeloid derived suppressor cells (MDSCs), by driving their function towards a pro-tumoral phenotype. The metabolic fates and alterations in myeloid cells caused by changes in the hypoxic tumor microenvironment induce the release of metabolic intermediates, such as nitric oxide (NO), L-arginine, L-cysteine and reactive oxygen species (ROS), which in turn modulate cytotoxic T-lymphocyte (CTL) effector responses. Notably, indoleamine 2,3-dioxygenase (IDO) secretion suppresses antigen-specific T-cell expansion. Chemokines and cytokines secreted by TAMs and MSDCs directly and/or indirectly inhibit the anti-tumoral cytotoxic responses of CTLs by inducing regulatory T cells (T_{regs}). In addition, PD-1 up-regulation on T_{regs} renders them as highly immunosuppressive cells. The critical role of TAMs and MDSCs in suppressing the anti-tumoral responses of CTLs supports that changes in their metabolic profile can influence the release of various cytokines and metabolic intermediates, and ultimately affect tumor growth, metastasis and drug resistance. HIF1α, hypoxia-inducible factor 1-alpha; TNFα, tumor necrosis factor alpha; IFNγ, Interferon gamma; IL, interleukin; TGFβ, transforming growth factor beta; CCR2, C-C chemokine receptor type 2; CCL2, chemokine (C-C motif) ligand 2; PD-L1, programmed death-ligand 1; PD-1, programmed cell death protein 1.

IFN-β-dependent up-regulation of NKG2D, IL-1β-dependent up-regulation of NKp44, and trans-presentation of IL-15. These synergistic cellular mechanisms lead to enhance NK cell cytotoxicity (Mattiola et al., 2015). Conversely, co-culture of TAMs and NK cells stimulate macrophages to produce immunosuppressive TGF-β which induced exhaustion of NK cells characterized by decreased IFNγ, TNFα, and Ki-67 expression, and an overall reduction in NK-cell activation and cytotoxicity (Krneta et al., 2017; Peng et al., 2017). Evidently, contact between TAMs and NK cells downplays their cytotoxic function and promotes a skew toward a pro-tumoral response.

MDSCs on T Cells

As discussed, MDSCs exert immune suppression via multiple mechanisms, such as NO production (Li et al., 2015), L-Arg depletion (Abdelaal et al., 2017) and L-cysteine sequestration (Srivastava et al., 2010). It seems that MDSCs essentially limit nutrient availability and deprive T cells of the energy source required to support their function. Interestingly, reactive nitrogen species produced by MDSCs induce post-translational modifications in CCL2 that decrease its affinity to CCR2 and its chemoattractant effects on CD8+ T cells, but not myeloid cells, into the tumor (Molon et al., 2011). Even after overcoming the hurdle of tumor infiltration, cell–cell contact between MDSCs and tumor-infiltrating lymphocytes inhibits effector-cell differentiation that is independent of activation status, IL-2 production or T-cell receptor signaling (Raber et al., 2016). Moreover, the hypoxic tumor stroma stabilizes HIF-1α in MDSCs promoting their rapid cellular differentiation into TAMs (Corzo et al., 2010). As such, intratumoral T cells not only endure nutrient deprivation, but are surrounded by immunosuppressive MDSCs and TAMs that douse them with suppressive cytokines. IL-10 secretion by immunosuppressed T cells up-regulates PD-L1 expression on MDSCs, in turn leading to T-cell dysfunction (Pinton et al., 2016).

TUMOR ASSOCIATED MYELOID CELL UNDERMINES EFFICACY OF CHEMOTHERAPY

TAMs in Chemotherapy

Conventional cancer modalities, such as radiotherapy and chemotherapy, focus on killing dividing tumor cells. As scientists gather more information on cancer immunology, it has become apparent that TAMs are chemoprotective against these cancer-cell-centered treatments. Untargeted anti-neoplastic treatment not only eradicates cancer cells, but also impact surrounding normal tissue and immune cells. As discussed, macrophages are the most abundant immune cell populating the tumor tissue; they are exceptionally plastic cells that can differentiate into either pro-inflammatory or suppressive phenotypes depending on the external stimuli and internal metabolic state. In response to cancer-cell killing compounds, macrophages vigorously infiltrate the tumor and secrete tumorigenic factors, such as cathepsin (Shree et al., 2011; Alishekevitz et al., 2016), angiogenic factors (Alishekevitz et al., 2016) and chemokines (Welford et al., 2011), to promote tumor re-growth and metastasis. Cathepsin also secreted by MDSCs, further contribute to pro-tumoral recovery (Bruchard et al., 2013). Instead of supporting antigen presentation for a pro-tumoral response, cathepsin directly interacts with the NLRP3–IL-1β signaling pathway to induce IL-17 secretion by CD4+ T cells (Bruchard et al., 2013). This effect promotes angiogenesis and tumor progression. Chemotherapy also induces cancer cells to secrete more inflammatory IL-6 and prostaglandin E2, which drives monocyte differentiation toward tumor-promoting macrophages (Dijkgraaf et al., 2013). IL-6 can further coordinate with milk-fat globule-epidermal growth factor-VIII produced by macrophages to trigger STAT3 and Sonic Hedgehog pathways in cancer stem cells, thus amplifying their drug resistance (Jinushi et al., 2011). Oncolytic treatment also induces macrophages to produce large amounts of IL-10, which suppresses the anti-tumoral CD8+ T-cell response (Ruffell et al., 2014). Notably, most studies have described chemoprotective macrophages as having an M2-like phenotype, highlighting the importance of the macrophage phenotype in the outcome of cancer-cell mediated treatment.

MDSCs in Chemotherapy

Myeloid-derived suppressor cells exhibit a clear immunosuppressive profile that restricts the efficacy of chemotherapy and often correlates with poor prognosis (Kawano et al., 2015; Takeuchi et al., 2015; Wesolowski et al., 2016). Cancer cells secrete more GM-CSF in response to chemotherapy, which induces monocyte differentiation into MDSCs. These newly differentiated MDSCs can suppress T-cell proliferation (Takeuchi et al., 2015). A significantly higher percentage of MDSCs after a completed round of chemotherapy is associated with a poor response and disease progression (Koinis et al., 2016). Limiting the MDSC population during adoptive immunotherapy using cytokine-induced killer cells has been shown to drastically improve survival in patients diagnosed with metastatic renal cancer, advanced pancreatic cancer and metastatic melanoma (Wang et al., 2016).

MYELOID CELLS INTERFERE WITH IMMUNOTHERAPY

A greater understanding of onco-immunology will arm scientists with better knowledge as to how to mobilize the immune system in an effective fight against cancer. Immunotherapy has attracted vast attention as scientists began to demonstrate its efficacy in treating patients who are unresponsive to traditional anti-cancer methods. The landscape of cancer treatment has thus dramatically transformed with immunotherapy being used in many cases as a first-line treatment alongside conventional chemotherapy for highly refractory disease. In 2017, the number of immunotherapeutics on clinical trial reached a record high, with 467 ongoing studies registered just in the United States (Schmidt, 2017). Of these trials, T cells are the most commonly targeted immune cell type. Unfortunately, cancer cells readily secrete immunosuppressive compounds and express checkpoint ligands, thereby signaling to T cells to halt all killing activities. To circumvent this anti-T cell response, current therapeutic strategies aim to bolster anti-tumor T-cell activity with checkpoints inhibitors as a frontline cancer immunotherapy (Sharma et al., 2017).

TAMs in Immunotherapy

Tumor-associated macrophages can impede the efficacy of checkpoint immunotherapy: *in vivo* imaging clearly showed that TAMs uptake anti-PD-1 monoclonal antibodies in tumor-bearing mice, thereby limiting the therapeutic antibody effect on PD1-expressing tumor-infiltrating CD8+ T cells (Arlauckas et al., 2017). In addition, evidence supports that reprogramming TAMs can alter efficacy of checkpoint drugs. For example, selective pharmacologic targeting of either gamma isoform of phosphoinositide 3-kinase (PI3Kγ) (De Henau et al., 2016; Kaneda et al., 2016), FcγR (Arlauckas et al., 2017), colony-stimulating factor 1 (CSF1R) (Zhu et al., 2014) or enzyme Arg1 (Steggerda et al., 2017) on myeloid cells suppresses their interaction with checkpoint drugs, thereby synergizing with T-cell targeted therapy to ensure effective targeting by checkpoint inhibitors. Killing TAMs seems an obvious solution to relieve immunosuppression in the tumor stroma. However, Zhu et al. (2014) demonstrated that treatment with CSF1R inhibitor, which effectively depletes TAMs, up-regulated PD-L1 expression on tumor cells and CTLA4 on T cells. Hence, depletion of TAMs essentially limits the anti-tumoral effects. Moreover, macrophage ablation could be detrimental to the host by increasing susceptibility to infection. Given that M2-like TAMs also function to control tumor growth under certain conditions (Grugan et al., 2012; Lin G.H.Y. et al., 2017), total macrophage ablation would not necessarily improve tumor outcomes. Instead, we propose taking advantage of their repolarization properties and enlisting these TAMs to fight against cancer.

MDSCs in Immunotherapy

Similar to TAMs, MDSCs are obstructive to immunotherapy. An enhanced level of MDSCs was detected in patients with metastatic renal cancer, pancreatic cancer (Wang et al., 2016), colorectal cancer (Kanterman et al., 2014), metastatic pediatric sarcomas (Highfill et al., 2014) and non-small cell lung cancer (Delaunay et al., 2018). Combinational treatment with both immunotherapy and drugs targeting MDSC depletion drastically improved survival outcomes in cancer patients (Wang et al., 2016). Analysis attributed this favorable clinical outcome to reduced numbers of intratumoral MDSCs (Wang et al., 2016), which relieves T-cell suppression (Tongu et al., 2015). In a preclinical renal cell carcinoma mouse model, Rayman and colleagues observed that depleting MDSCs with TKI, sunitinib together with checkpoint blockade by anti-PD1 antibody co-treatment resulted in significantly more CD8+ T cells in the tumor (Rayman et al., 2015). This is accompanied by an increased production of pro-inflammatory IFNγ and granzyme B (Guan et al., 2017). More importantly, a high percentage of tumor-infiltrating CD8+ T cells expressed CD107a, which is involved in the cytotoxic killing of tumor target cells (Rayman et al., 2015). Other approaches such as monoclonal antibody therapy against CXCR2 (Highfill et al., 2014), CCL2 (Wang et al., 2018), or IL-18 (Guan et al., 2017) to inhibit MDSCs trafficking into tumors also successfully induces tumor regression upon anti-PD1 treatment. These studies suggest that resistance to immune-checkpoint blockade might be alleviated by therapeutic strategies that reprogram dominant myeloid responses.

TARGETING WARBURG METABOLISM IN MYELOID CELLS

As we have illustrated, myeloid cells, which are often immunosuppressive, have a diverse impact on cancer development, ranging from tumor progression to efficacy of cancer therapy. Therefore, it is imperative that we target these immune subsets during anti-cancer therapy. Rather than aiming for complete ablation and compromising our body to pathogens, re-polarizing myeloid cells to adopt an anti-tumoral profile is a promising avenue to explore. This way, macrophages can also be exploited to participate in and promote an anti-tumoral response. Most importantly, M2 macrophages can re-polarize into M1 macrophages in response to certain cytokines in the microenvironment (Davis et al., 2013). For example, blocking the CSF-1/CSF-1R axis or targeting the pattern recognition receptor MARCO causes a phenotypic shift from M2-like macrophages to M1-like macrophages resulting in increased tumor immunogenicity (Georgoudaki et al., 2016; Quaranta et al., 2018). In a glioblastoma multiforme mouse model, reduced expression of M2 markers accompanied with impaired pro-tumoral function was also observed (Pyonteck et al., 2013). In addition, sorafenib, a multikinase inhibitor, can reverse the immunosuppressive cytokine profile in tumor-conditioned macrophages, promoting them to elicit a more favorable anti-tumoral response (Edwards and Emens, 2010).

Administration of IFNγ, a stimulant of M1 polarization, achieved a favorable clinical outcome, with increased tumor cytotoxicity of TAMs in patients diagnosed with ovarian carcinoma (Allavena et al., 1990; Colombo et al., 1992). This clearly demonstrated the feasibility of immune remodeling in clinical settings. Given that the tumor microenvironment can condition myeloid cells toward a pro-tumoral phenotype, scientists can also take advantage of this plastic nature to reprogram myeloid cells toward an anti-tumoral phenotype that boost tumor control, hence improving treatment outcomes.

In general, pro-inflammatory M1 macrophages favor glycolysis whereas pro-wound healing M2 macrophages rely on oxidative metabolism (Galván-Peña and O'Neill, 2014). In the setting of the tumor microenvironment, pro-inflammatory M1-like macrophages target cancer cells and control tumor progression while pro-healing M2 macrophages promote tissue repair, assisting tumor growth (Helm et al., 2014b). However, TAMs do not simply display a distinct M1 or M2 profile. Rather, TAMs typically exhibit a mixed phenotype and several studies have demonstrated that glycolysis is essential to sustain these immunosuppressive cells (Helm et al., 2014b). Both our group and Zhao et al. (2017) reported that 2DG treatment can impede tumor growth *in vitro* and *in vivo* and transform TAMs into exhibiting an anti-tumoral phenotype (Penny et al., 2016). As cancer cells exhibit similar metabolic signature as pro-tumoral TAMs, currently available glycolytic drugs that target cancer cells might be relevant to reprogramming TAMs.

Glycolytic inhibitors, such as 3-bromopyruvate (3-BP) (Yun et al., 2009), MJE3 (Evans et al., 2005), 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) (Clem et al., 2008), 3-dihydroxy-6-methyl-7-(phenylmethyl)-4-propylnaphthalene-1-carboxylic acid (FX11) (Le et al., 2010), and dichloroacetate (DCA) (Sutendra and Michelakis, 2013) target HK2, PFKFB3, PGAM1, LDHA and PDH, respectively in cancer cells to effectively suppress tumor growth. Genotoxic exposure induced by cisplatin blocks Glut1 and Glut3 expression on cancer cells, leading to a suppressed glycolytic rate and enhanced oxygen consumption (Zhou et al., 2002). Cisplatin is an appropriate drug to use since both glucose transporters are expressed on differentiated macrophages (Folco et al., 2011). HK2 is the first rate-limiting enzyme of the glycolytic pathway and silencing HK2 in hepatocellular carcinoma inhibits glucose flux to pyruvate and lactate, leading to cell death (DeWaal et al., 2018). Based on this concept, Ko et al. (2004) discovered 3-BP as an "incredible" anticancer agent that effectively eradicates all cancer growth in rodents within a short treatment regimen (Ko et al., 2004). TAMs harvested from tumor-bearing mice treated with 3-BP displayed enhanced tumoricidal activity and pro-inflammatory cytokine (IL-1, TNFα) production. However, TAM culture with 3-BP alone did not induce any changes in TAM cytotoxic function (Yadav et al., 2018) despite inhibiting the first step of glycolysis (Errea et al., 2016). Clearly, glycolytic drugs that are effective toward cancer cells might not necessarily be as effective toward TAMs. Further studies are now required to validate the effectiveness of these drugs on TAMs.

The ideal anti-cancer therapy would be to specifically eradicate tumor cells with minimal effect on non-neoplastic populations

in the body. This feature is even more critical for glycolytic inhibitors since glycolysis is a metabolic pathway utilized by almost all mammalian cells. For example 3-BP, the most "promising" anti-cancer drug mentioned earlier, was associated with causing death in at least three cancer patients (DutchNews, 2016). Early *in vitro* and *in vivo* studies reported encouraging results showing the ability of 3-BP to inhibit tumor growth (Isayev et al., 2014; Liu et al., 2014; Gandham et al., 2015; Valenti et al., 2015; Zou et al., 2015), yet only two clinical trials, i.e., the studies on fibrolamellar hepatocellular carcinoma (Ko et al., 2012) and metastatic melanoma (El Sayed et al., 2014) were performed to further validate its effectiveness in human malignancies or examine the toxicity of the compound. *In vitro* studies using mouse/rat hepatocytes (Sobotka et al., 2016) and primary rat astrocytes (Ehrke et al., 2015) reported a dose-dependent toxicity of 3-BP (at doses $\geq$ 50 μM for hepatocytes and 100 μM for astrocytes) on non-neoplastic cells. In another study by Rodrigues and colleagues, they reported that 3-BP promotes a metabolic switch in embryonic stem cells resulting in the loss of pluripotency. However, the use of this drug alone is unlikely to drive these stem cells toward specific differentiation fates (Rodrigues et al., 2015). These *in vitro* data clearly showed that 3-BP can impact on healthy tissues, particularly those that rely heavily on glycolysis. As such, using the same glycolytic inhibitors to target myeloid cells will also encounter the exact same adverse effects. Therefore, delivery of drugs specifically to the tumor will be the game-changer for these glycolytic drugs to progress form bench to bedside.

CONCLUDING REMARKS

Otto Warburg introduced the concept of Warburg metabolism, whereby tumor cells rely on glycolysis to support uncontrolled growth and proliferation (Warburg et al., 1927). We now know that tumor associated myeloid cells can also use this metabolic pathway. Under the influence of the tumor microenvironment, infiltrating myeloid cells undergo metabolic reprogramming to develop a new set of cellular functions. As these myeloid cells are responsive to environmental cues and are abundant within the tumor, exposure to metabolic drugs may prove to be an effective avenue to reshape these cells to kill cancer cells and alleviate T-cell suppression. Notably, the tumor stroma changes throughout cancer development, which also means that these dynamic myeloid cells must adopt unique metabolic states during cancer progression. There may be a therapeutic window when myeloid cells have nested within the tumor stroma but the fibrotic capsule has yet to form around the tumor. With growing interest in immunometabolism, continuous improvements in understanding the various metabolic pathways will provide new avenues to design formulated drugs that specifically target cancer cells and tumor associated myeloid cells in the future. In order to design strategies around this concept, improvements in metabolism-based therapeutics will be essential. Ultimately, immune-metabolism is only one arm of the cancer treatment strategy. Combinational treatment with targeted immunotherapy is recommended to successfully fight cancer.

AUTHOR CONTRIBUTIONS

JS, SG, and SW conceptualized the content and wrote the manuscript. JS and SG prepared the figures.

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REFERENCES

Abdelaal, A. M., Bian, Z., Culpepper, C., and Liu, Y. (2017). Induction of arginase-1 in MDSC requires exposure to CD3/CD28 activated T cells. *J. Immunol.* 198:154.13.

Alishekevitz, D., Gingis-Velitski, S., Kaidar-Person, O., Gutter-Kapon, L., Scherer, S. D., Raviv, Z., et al. (2016). Macrophage-induced lymphangiogenesis and metastasis following paclitaxel chemotherapy is regulated by VEGFR3. *Cell Rep.* 17, 1344–1356. doi: 10.1016/j.celrep.2016.09.083

Al-Khami, A. A., Zheng, L., Del Valle, L., Hossain, F., Wyczechowska, D., Zabaleta, J., et al. (2017). Exogenous lipid uptake induces metabolic and functional reprogramming of tumor-associated myeloid-derived suppressor cells. *Oncoimmunology* 6:e1344804. doi: 10.1080/2162402X.2017.1344804

Allavena, P., Peccatori, F., Maggioni, D., Erroi, A., Sironi, M., Colombo, N., et al. (1990). Intraperitoneal recombinant gamma-interferon in patients with recurrent ascitic ovarian carcinoma: modulation of cytotoxicity and cytokine production in tumor-associated effectors and of major histocompatibility antigen expression on tumor cells. *Cancer Res.* 50, 7318–7323.

Ananieva, E. (2015). Targeting amino acid metabolism in cancer growth and anti-tumor immune response. *World J. Biol. Chem.* 6, 281–289. doi: 10.4331/wjbc.v6.i4.281

Arlauckas, S. P., Garris, C. S., Kohler, R. H., Kitaoka, M., Cuccarese, M. F., Yang, K. S., et al. (2017). In vivo imaging reveals a tumor-associated macrophage–mediated resistance pathway in anti–PD-1 therapy. *Sci. Transl. Med.* 9:eaal3604. doi: 10.1126/scitranslmed.aal3604

Azevedo, E. P., Rochael, N. C., Guimaraes-Costa, A. B., de Souza-Vieira, T. S., Ganilho, J., Saraiva, E. M., et al. (2015). A metabolic shift toward pentose phosphate pathway is necessary for amyloid fibril- and Phorbol 12-Myristate 13-Acetate-induced Neutrophil Extracellular Trap (NET) Formation. *J. Biol. Chem.* 290, 22174–22183. doi: 10.1074/jbc.M115.640094

Bantug, G. R., Galluzzi, L., Kroemer, G., and Hess, C. (2018). The spectrum of T cell metabolism in health and disease. *Nat. Rev. Immunol.* 18, 19–34. doi: 10.1038/nri.2017.99

Bettonville, M., D'Aria, S., and Braun, M. Y. (2016). Metabolic programming in chronically stimulated T cells: lessons from cancer and viral infections. *Eur. J. Immunol.* 46, 1574–1582. doi: 10.1002/eji.201546176

Biswas, S. K. (2015). Metabolic reprogramming of immune cells in cancer progression. *Immunity* 43, 435–449. doi: 10.1016/j.immuni.2015.09.001

Boroughs, L. K., and DeBerardinis, R. J. (2015). Metabolic pathways promoting cancer cell survival and growth. *Nat. Cell Biol.* 17, 351–359. doi: 10.1038/ncb3124

Borregaard, N., and Herlin, T. (1982). Energy metabolism of human neutrophils during phagocytosis. *J. Clin. Invest.* 70, 550–557. doi: 10.1172/JCI110647

Bronte, V., Brandau, S., Chen, S. H., Colombo, M. P., Frey, A. B., Greten, T. F., et al. (2016). Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nat. Commun.* 7:12150. doi: 10.1038/ncomms12150

Bruchard, M., Mignot, G., Derangere, V., Chalmin, F., Chevriaux, A., Vegran, F., et al. (2013). Chemotherapy-triggered cathepsin B release in myeloid-derived suppressor cells activates the Nlrp3 inflammasome and promotes tumor growth. *Nat. Med.* 19, 57–64. doi: 10.1038/nm.2999

Buck, M. D., Sowell, R. T., Kaech, S. M., and Pearce, E. L. (2017). Metabolic instruction of immunity. *Cell* 169, 570–586. doi: 10.1016/j.cell.2017.04.004

Cantor, J. R., and Sabatini, D. M. (2012). Cancer cell metabolism: one hallmark, many faces. *Cancer Discov.* 2, 881–898. doi: 10.1158/2159-8290.CD-12-0345

Chang, C. I., Liao, J. C., and Kuo, L. (2001). Macrophage arginase promotes tumor cell growth and suppresses nitric oxide-mediated tumor cytotoxicity. *Cancer Res.* 61, 1100–1106.

Chantranupong, L., Scaria, S. M., Saxton, R. A., Gygi, M. P., Shen, K., Wyant, G. A., et al. (2016). The CASTOR proteins are arginine sensors for the mTORC1 pathway. *Cell* 165, 153–164. doi: 10.1016/j.cell.2016.02.035

Cho, E. S., Cha, Y. H., Kim, H. S., Kim, N. H., and Yook, J. I. (2018). The pentose phosphate pathway as a potential target for cancer therapy. *Biomol. Ther.* 26, 29–38. doi: 10.4062/biomolther.2017.179

Cimen Bozkus, C., Elzey, B. D., Crist, S. A., Ellies, L. G., and Ratliff, T. L. (2015). Expression of cationic amino acid transporter 2 is required for myeloid-derived suppressor cell-mediated control of T cell immunity. *J. Immunol.* 195, 5237–5250. doi: 10.4049/jimmunol.1500959

Clem, B., Telang, S., Clem, A., Yalcin, A., Meier, J., Simmons, A., et al. (2008). Small-molecule inhibition of 6-phosphofructo-2-kinase activity suppresses glycolytic flux and tumor growth. *Mol. Cancer Ther.* 7, 110–120. doi: 10.1158/1535-7163.mct-07-0482

Coffelt, S. B., Wellenstein, M. D., and de Visser, K. E. (2016). Neutrophils in cancer: neutral no more. *Nat. Rev. Cancer* 16, 431–446. doi: 10.1038/nrc.2016.52

Colegio, O. R., Chu, N. Q., Szabo, A. L., Chu, T., Rhebergen, A. M., Jairam, V., et al. (2014). Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature* 513, 559–563. doi: 10.1038/nature13490

Colombo, N., Peccatori, F., Paganin, C., Bini, S., Brandely, M., Mangioni, C., et al. (1992). Anti-tumor and immunomodulatory activity of intraperitoneal IFN-gamma in ovarian carcinoma patients with minimal residual tumor after chemotherapy. *Int. J. Cancer* 51, 42–46. doi: 10.1002/ijc.2910510109

Condamine, T., Dominguez, G. A., Youn, J. I., Kossenkov, A. V., Mony, S., Alicea-Torres, K., et al. (2016). Lectin-type oxidized LDL receptor-1 distinguishes population of human polymorphonuclear myeloid-derived suppressor cells in cancer patients. *Sci. Immunol.* 1:aaf8943. doi: 10.1126/sciimmunol.aaf8943

Corzo, C. A., Condamine, T., Lu, L., Cotter, M. J., Youn, J. I., Cheng, P., et al. (2010). HIF-1alpha regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment. *J. Exp. Med.* 207, 2439–2453. doi: 10.1084/jem.20100587

Covarrubias, A. J., Aksoylar, H. I., and Horng, T. (2015). Control of macrophage metabolism and activation by mTOR and Akt signaling. *Semin. Immunol.* 27, 286–296. doi: 10.1016/j.smim.2015.08.001

Cubillos-Ruiz, J. R., Silberman, P. C., Rutkowski, M. R., Chopra, S., Perales-Puchalt, A., Song, M., et al. (2015). ER stress sensor XBP1 controls anti-tumor immunity by disrupting dendritic cell homeostasis. *Cell* 161, 1527–1538. doi: 10.1016/j.cell.2015.05.025

Dannenmann, S. R., Thielicke, J., Stöckli, M., Matter, C., von Boehmer, L., Cecconi, V., et al. (2013). Tumor-associated macrophages subvert T-cell function and correlate with reduced survival in clear cell renal cell carcinoma. *Oncoimmunology* 2:e23562. doi: 10.4161/onci.23562

Davis, M. J., Tsang, T. M., Qiu, Y., Dayrit, J. K., Freij, J. B., Huffnagle, G. B., et al. (2013). Macrophage M1/M2 polarization dynamically adapts to changes in cytokine microenvironments in cryptococcus neoformans infection. *mBio* 4:e00264-13. doi: 10.1128/mBio.00264-13

De Henau, O., Rausch, M., Winkler, D., Campesato, L. F., Liu, C., Cymerman, D. H., et al. (2016). Overcoming resistance to checkpoint blockade therapy by targeting PI3Kgamma in myeloid cells. *Nature* 539, 443–447. doi: 10.1038/nature20554

Delaunay, M., Guibert, N., Lusque, A., Farella, M., Boubekeur, N., Gouin, S., et al. (2018). Baseline circulating myeloid-derived suppressor cells and response to PD-1 inhibitor in non-small cell lung cancer patients. *J. Clin. Oncol.* 36:145. doi: 10.1200/JCO.2018.36.5_suppl.145

Dennis, E. A., Deems, R. A., Harkewicz, R., Quehenberger, O., Brown, H. A., Milne, S. B., et al. (2010). A mouse macrophage lipidome. *J. Biol. Chem.* 285, 39976–39985. doi: 10.1074/jbc.M110.182915

DeWaal, D., Nogueira, V., Terry, A. R., Patra, K. C., Jeon, S.-M., Guzman, G., et al. (2018). Hexokinase-2 depletion inhibits glycolysis and induces oxidative phosphorylation in hepatocellular carcinoma and sensitizes to metformin. *Nat. Commun.* 9:446. doi: 10.1038/s41467-017-02733-4

Dietl, K., Renner, K., Dettmer, K., Timischl, B., Eberhart, K., Dorn, C., et al. (2010). Lactic acid and acidification inhibit TNF secretion and glycolysis of human monocytes. *J. Immunol.* 184, 1200–1209. doi: 10.4049/jimmunol.0902584

Dijkgraaf, E. M., Heusinkveld, M., Tummers, B., Vogelpoel, L. T., Goedemans, R., Jha, V., et al. (2013). Chemotherapy alters monocyte differentiation to favor generation of cancer-supporting M2 macrophages in the tumor microenvironment. *Cancer Res.* 73, 2480–2492. doi: 10.1158/0008-5472.can-12-3542

Doedens, A. L., Stockmann, C., Rubinstein, M. P., Liao, D., Zhang, N., DeNardo, D. G., et al. (2010). Macrophage expression of hypoxia-inducible factor-1 alpha suppresses T-cell function and promotes tumor progression. *Cancer Res.* 70, 7465–7475. doi: 10.1158/0008-5472.can-10-1439

DutchNews (2016). *"Dutch woman dies at German alternative cancer clinic", in: Health*. Available at: http://www.dutchnews.nl/news/archives/2016/08/dutch-woman-di

Eales, K. L., Hollinshead, K. E., and Tennant, D. A. (2016). Hypoxia and metabolic adaptation of cancer cells. *Oncogenesis* 5:e190. doi: 10.1038/oncsis.2015.50

Edwards, J. P., and Emens, L. A. (2010). The multikinase inhibitor sorafenib reverses the suppression of IL-12 and enhancement of IL-10 by PGE(2) in murine macrophages. *Int. Immunopharmacol.* 10, 1220–1228. doi: 10.1016/j.intimp.2010.07.002

Ehrke, E., Arend, C., and Dringen, R. (2015). 3-bromopyruvate inhibits glycolysis, depletes cellular glutathione, and compromises the viability of cultured primary rat astrocytes. *J. Neurosci. Res.* 93, 1138–1146. doi: 10.1002/jnr.23474

El Sayed, S. M., Mohamed, W. G., Seddik, M. A., Ahmed, A. S., Mahmoud, A. G., Amer, W. H., et al. (2014). Safety and outcome of treatment of metastatic melanoma using 3-bromopyruvate: a concise literature review and case study. *Chin. J. Cancer* 33, 356–364. doi: 10.5732/cjc.013.10111

Errea, A., Cayet, D., Marchetti, P., Tang, C., Kluza, J., Offermanns, S., et al. (2016). Lactate inhibits the pro-inflammatory response and metabolic reprogramming in murine macrophages in a GPR81-independent manner. *PLoS One* 11:e0163694. doi: 10.1371/journal.pone.0163694

Evans, M. J., Saghatelian, A., Sorensen, E. J., and Cravatt, B. F. (2005). Target discovery in small-molecule cell-based screens by in situ proteome reactivity profiling. *Nat. Biotechnol.* 23, 1303–1307. doi: 10.1038/nbt1149

Folco, E. J., Sheikine, Y., Rocha, V. Z., Christen, T., Shvartz, E., Sukhova, G. K., et al. (2011). Hypoxia but not inflammation augments glucose uptake in human macrophages: implications for imaging atherosclerosis with 18Fluorine-Labeled 2-Deoxy-D-Glucose positron emission tomography. *J. Am. Coll. Cardiol.* 58, 603–614. doi: 10.1016/j.jacc.2011.03.044

Fridlender, Z. G., Sun, J., Kim, S., Kapoor, V., Cheng, G., Ling, L., et al. (2009). Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* 16, 183–194. doi: 10.1016/j.ccr.2009.06.017

Fridlender, Z. G., Sun, J., Mishalian, I., Singhal, S., Cheng, G., Kapoor, V., et al. (2012). Transcriptomic analysis comparing tumor-associated neutrophils with granulocytic myeloid-derived suppressor cells and normal neutrophils. *PLoS One* 7:e31524. doi: 10.1371/journal.pone.0031524

Gabrilovich, D. I., Ostrand-Rosenberg, S., and Bronte, V. (2012). Coordinated regulation of myeloid cells by tumours. *Nat. Rev. Immunol.* 12, 253–268. doi: 10.1038/nri3175

Galdiero, M. R., Bonavita, E., Barajon, I., Garlanda, C., Mantovani, A., and Jaillon, S. (2013). Tumor associated macrophages and neutrophils in cancer. *Immunobiology* 218, 1402–1410. doi: 10.1016/j.imbio.2013.06.003

Galván-Peña, S., and O'Neill, L. A. J. (2014). Metabolic reprograming in macrophage polarization. *Front. Immunol.* 5:420. doi: 10.3389/fimmu.2014.00420

Gandham, S. K., Talekar, M., Singh, A., and Amiji, M. M. (2015). Inhibition of hexokinase-2 with targeted liposomal 3-bromopyruvate in an ovarian tumor

spheroid model of aerobic glycolysis. *Int. J. Nanomedicine* 10, 4405–4423. doi: 10.2147/ijn.s82818

Geeraerts, X., Bolli, E., Fendt, S. M., and Van Ginderachter, J. A. (2017). Macrophage metabolism as therapeutic target for cancer, atherosclerosis, and obesity. *Front. Immunol.* 8:289. doi: 10.3389/fimmu.2017.00289

Geiger, R., Rieckmann, J. C., Wolf, T., Basso, C., Feng, Y., Fuhrer, T., et al. (2016). L-arginine modulates T cell metabolism and enhances survival and anti-tumor activity. *Cell* 167, 829–842.e13. doi: 10.1016/j.cell.2016.09.031

Georgoudaki, A. M., Prokopec, K. E., Boura, V. F., Hellqvist, E., Sohn, S., Ostling, J., et al. (2016). Reprogramming tumor-associated macrophages by antibody targeting inhibits cancer progression and metastasis. *Cell Rep.* 15, 2000–2011. doi: 10.1016/j.celrep.2016.04.084

Gregory, A. D., and Houghton, A. M. (2011). Tumor-associated neutrophils: new targets for cancer therapy. *Cancer Res.* 71, 2411–2416. doi: 10.1158/0008-5472.CAN-10-2583

Grugan, K. D., McCabe, F. L., Kinder, M., Greenplate, A. R., Harman, B. C., Ekert, J. E., et al. (2012). Tumor-associated macrophages promote invasion while retaining Fc-dependent anti-tumor function. *J. Immunol.* 189, 5457–5466. doi: 10.4049/jimmunol.1201889

Guan, Y., Zhang, R., Peng, Z., Dong, D., Wei, G., and Wang, Y. (2017). Inhibition of IL-18-mediated myeloid derived suppressor cell accumulation enhances anti-PD1 efficacy against osteosarcoma cancer. *J. Bone Oncol.* 9, 59–64. doi: 10.1016/j.jbo.2017.10.002

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674. doi: 10.1016/j.cell.2011.02.013

Helm, O., Held-Feindt, J., Grage-Griebenow, E., Reiling, N., Ungefroren, H., Vogel, I., et al. (2014a). Tumor-associated macrophages exhibit pro- and anti-inflammatory properties by which they impact on pancreatic tumorigenesis. *Int. J. Cancer* 135, 843–861. doi: 10.1002/ijc.28736

Helm, O., Held-Feindt, J., Schäfer, H., and Sebens, S. (2014b). M1 and M2: there is no "good" and "bad"—How macrophages promote malignancy-associated features in tumorigenesis. *Oncoimmunology* 3:e946818. doi: 10.4161/21624011.2014.946818

Herber, D. L., Cao, W., Nefedova, Y., Novitskiy, S. V., Nagaraj, S., Tyurin, V. A., et al. (2010). Lipid accumulation and dendritic cell dysfunction in cancer. *Nat. Med.* 16, 880–886. doi: 10.1038/nm.2172

Hernandez, C. P., Morrow, K., Lopez-Barcons, L. A., Zabaleta, J., Sierra, R., Velasco, C., et al. (2010). Pegylated arginase I: a potential therapeutic approach in T-ALL. *Blood* 115, 5214–5221. doi: 10.1182/blood-2009-12-258822

Highfill, S. L., Cui, Y., Giles, A. J., Smith, J. P., Zhang, H., Morse, E., et al. (2014). Disruption of CXCR2-mediated MDSC tumor trafficking enhances anti-PD1 efficacy. *Sci. Transl. Med.* 6:237ra267. doi: 10.1126/scitranslmed.3007974

Husain, Z., Huang, Y., Seth, P., and Sukhatme, V. P. (2013a). Tumor-derived lactate modifies antitumor immune response: effect on myeloid-derived suppressor cells and NK cells. *J. Immunol.* 191, 1486–1495. doi: 10.4049/jimmunol.1202702

Husain, Z., Seth, P., and Sukhatme, V. P. (2013b). Tumor-derived lactate and myeloid-derived suppressor cells: linking metabolism to cancer immunology. *Oncoimmunology* 2:e26383. doi: 10.4161/onci.26383

Isayev, O., Rausch, V., Bauer, N., Liu, L., Fan, P., Zhang, Y., et al. (2014). Inhibition of glucose turnover by 3-bromopyruvate counteracts pancreatic cancer stem cell features and sensitizes cells to gemcitabine. *Oncotarget* 5, 5177–5189. doi: 10.18632/oncotarget.2120

Jablonska, J., Leschner, S., Westphal, K., Lienenklaus, S., and Weiss, S. (2010). Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model. *J. Clin. Invest.* 120, 1151–1164. doi: 10.1172/JCI37223

Jian, S. L., Chen, W. W., Su, Y. C., Su, Y. W., Chuang, T. H., Hsu, S. C., et al. (2017). Glycolysis regulates the expansion of myeloid-derived suppressor cells in tumor-bearing hosts through prevention of ROS-mediated apoptosis. *Cell Death Dis.* 8:e2779. doi: 10.1038/cddis.2017.192

Jinushi, M., Chiba, S., Yoshiyama, H., Masutomi, K., Kinoshita, I., Dosaka-Akita, H., et al. (2011). Tumor-associated macrophages regulate tumorigenicity and anticancer drug responses of cancer stem/initiating cells. *Proc. Natl. Acad. Sci. U.S.A.* 108, 12425–12430. doi: 10.1073/pnas.1106645108

Jun, H. S., Weinstein, D. A., Lee, Y. M., Mansfield, B. C., and Chou, J. Y. (2014). Molecular mechanisms of neutrophil dysfunction in glycogen storage disease type Ib. *Blood* 123, 2843–2853. doi: 10.1182/blood-2013-05-502435

Kaneda, M. M., Messer, K. S., Ralainirina, N., Li, H., Leem, C. J., Gorjestani, S., et al. (2016). PI3Kgamma is a molecular switch that controls immune suppression. *Nature* 539, 437–442. doi: 10.1038/nature19834

Kanterman, J., Sade-Feldman, M., Biton, M., Ish-Shalom, E., Lasry, A., Goldshtein, A., et al. (2014). Adverse immunoregulatory effects of 5FU and CPT11 chemotherapy on myeloid-derived suppressor cells and colorectal cancer outcomes. *Cancer Res.* 74, 6022–6035. doi: 10.1158/0008-5472.can-14-0657

Karmaus, P. W. F., Herrada, A. A., Guy, C., Neale, G., Dhungana, Y., Long, L., et al. (2017). Critical roles of mTORC1 signaling and metabolic reprogramming for M-CSF–mediated myelopoiesis. *J. Exp. Med.* 214:jem.20161855. doi: 10.1084/jem.20161855

Kawano, M., Mabuchi, S., Matsumoto, Y., Sasano, T., Takahashi, R., Kuroda, H., et al. (2015). The significance of G-CSF expression and myeloid-derived suppressor cells in the chemoresistance of uterine cervical cancer. *Sci. Rep.* 5:18217. doi: 10.1038/srep18217

Ko, Y. H., Smith, B. L., Wang, Y., Pomper, M. G., Rini, D. A., Torbenson, M. S., et al. (2004). Advanced cancers: eradication in all cases using 3-bromopyruvate therapy to deplete ATP. *Biochem. Biophys. Res. Commun.* 324, 269–275. doi: 10.1016/j.bbrc.2004.09.047

Ko, Y. H., Verhoeven, H. A., Lee, M. J., Corbin, D. J., Vogl, T. J., and Pedersen, P. L. (2012). A translational study "case report" on the small molecule "energy blocker" 3-bromopyruvate (3BP) as a potent anticancer agent: from bench side to bedside. *J. Bioenerg. Biomembr.* 44, 163–170. doi: 10.1007/s10863-012-9417-4

Koinis, F., Vetsika, E. K., Aggouraki, D., Skalidaki, E., Koutoulaki, A., Gkioulmpasani, M., et al. (2016). Effect of first-line treatment on myeloid-derived suppressor cells' subpopulations in the peripheral blood of patients with non-small cell lung cancer. *J. Thorac. Oncol.* 11, 1263–1272. doi: 10.1016/j.jtho.2016.04.026

Kolarova, H., Klinke, A., Kremserova, S., Adam, M., Pekarova, M., Baldus, S., et al. (2013). Myeloperoxidase induces the priming of platelets. *Free Radic. Biol. Med.* 61, 357–369. doi: 10.1016/j.freeradbiomed.2013.04.014

Krneta, T., Gillgrass, A., Poznanski, S., Chew, M., Lee, A. J., Kolb, M., et al. (2017). M2-polarized and tumor-associated macrophages alter NK cell phenotype and function in a contact-dependent manner. *J. Leukoc. Biol.* 101, 285–295. doi: 10.1189/jlb.3A1215-552R

Kubota, K., and Moriyama, M. (2017). CD163(+)CD204(+) tumor-associated macrophages contribute to T cell regulation via interleukin-10 and PD-L1 production in oral squamous cell carcinoma. *Sci. Rep.* 7:1755. doi: 10.1038/s41598-017-01661-z

Kusmartsev, S., and Gabrilovich, D. I. (2005). STAT1 signaling regulates tumor-associated macrophage-mediated T cell deletion. *J. Immunol.* 174, 4880–4891. doi: 10.4049/jimmunol.174.8.4880

Lampropoulou, V., Sergushichev, A., Bambouskova, M., Nair, S., Vincent, E. E., Loginicheva, E., et al. (2016). Itaconate links inhibition of succinate dehydrogenase with macrophage metabolic remodeling and regulation of inflammation. *Cell Metab.* 24, 158–166. doi: 10.1016/j.cmet.2016.06.004

Le, A., Cooper, C. R., Gouw, A. M., Dinavahi, R., Maitra, A., Deck, L. M., et al. (2010). Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. *Proc. Natl. Acad. Sci. U.S.A.* 107, 2037–2042. doi: 10.1073/pnas.0914433107

Leblond, M. M., Gérault, A. N., Corroyer-Dulmont, A., MacKenzie, E. T., Petit, E., Bernaudin, M., et al. (2016). Hypoxia induces macrophage polarization and re-education toward an M2 phenotype in U87 and U251 glioblastoma models. *Oncoimmunology* 5, e1056442. doi: 10.1080/2162402X.2015.1056442

Li, C., Wang, Y., Li, Y., Yu, Q., Jin, X., Wang, X., et al. (2018). HIF1α-dependent glycolysis promotes macrophage functional activities in protecting against bacterial and fungal infection. *Sci. Rep.* 8:3603. doi: 10.1038/s41598-018-22039-9

Li, H., Dai, F., Peng, Q., Gan, H., Zheng, J., Xia, Y., et al. (2015). Myeloid-derived suppressor cells suppress CD4(+) and CD8(+) T cell responses in autoimmune hepatitis. *Mol. Med. Rep.* 12, 3667–3673. doi: 10.3892/mmr.2015.3791

Li, Z., and Kang, Y. (2017). Lipid metabolism fuels cancer's spread. *Cell Metab.* 25, 228–230. doi: 10.1016/j.cmet.2017.01.016

Liao, J., Wang, X., Bi, Y., Shen, B., Shao, K., Yang, H., et al. (2014). Dexamethasone potentiates myeloid-derived suppressor cell function in prolonging allograft

survival through nitric oxide. *J. Leukoc. Biol.* 96, 675–684. doi: 10.1189/jlb.2HI1113-611RR

Lin, G. H. Y., Chai, V., Lee, V., Dodge, K., Truong, T., Wong, M., et al. (2017). TTI-621 (SIRPalphaFc), a CD47-blocking cancer immunotherapeutic, triggers phagocytosis of lymphoma cells by multiple polarized macrophage subsets. *PLoS One* 12:e0187262. doi: 10.1371/journal.pone.0187262

Lin, S., Sun, L., Lyu, X., Ai, X., Du, D., Su, N., et al. (2017). Lactate-activated macrophages induced aerobic glycolysis and epithelial-mesenchymal transition in breast cancer by regulation of CCL5-CCR5 axis: a positive metabolic feedback loop. *Oncotarget* 8, 110426–110443. doi: 10.18632/oncotarget.22786

Littlewood-Evans, A., and Sarret, S. (2016). GPR91 senses extracellular succinate released from inflammatory macrophages and exacerbates rheumatoid arthritis. *J. Exp. Med.* 213, 1655–1662. doi: 10.1084/jem.20160061

Liu, Q., Luo, Q., Halim, A., and Song, G. (2017). Targeting lipid metabolism of cancer cells: a promising therapeutic strategy for cancer. *Cancer Lett.* 401, 39–45. doi: 10.1016/j.canlet.2017.05.002

Liu, Z., Zhang, Y. Y., Zhang, Q. W., Zhao, S. R., Wu, C. Z., Cheng, X., et al. (2014). 3-Bromopyruvate induces apoptosis in breast cancer cells by downregulating Mcl-1 through the PI3K/Akt signaling pathway. *Anticancer Drugs* 25, 447–455. doi: 10.1097/cad.0000000000000081

Long, Y. C., and Zierath, J. R. (2006). AMP-activated protein kinase signaling in metabolic regulation. *J. Clin. Invest.* 116, 1776–1783. doi: 10.1172/JCI29044

Lu, Y., Liu, H., Bi, Y., Yang, H., Li, Y., Wang, J., et al. (2017). Glucocorticoid receptor promotes the function of myeloid-derived suppressor cells by suppressing HIF1alpha-dependent glycolysis. *Cell Mol. Immunol.* 15, 618–629. doi: 10.1038/cmi.2017.5

Ma, E. H., Bantug, G., Griss, T., Condotta, S., Johnson, R. M., Samborska, B., et al. (2017). Serine is an essential metabolite for effector T cell expansion. *Cell Metab.* 25, 482. doi: 10.1016/j.cmet.2017.01.014

Mantovani, A., Cassatella, M. A., Costantini, C., and Jaillon, S. (2011). Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat. Rev. Immunol.* 11, 519–531. doi: 10.1038/nri3024

Marin-Hernandez, A., Gallardo-Perez, J. C., Ralph, S. J., Rodriguez-Enriquez, S., and Moreno-Sanchez, R. (2009). HIF-1alpha modulates energy metabolism in cancer cells by inducing over-expression of specific glycolytic isoforms. *Mini Rev. Med. Chem.* 9, 1084–1101. doi: 10.2174/138955709788922610

Mattiola, I., Pesant, M., Tentorio, P. F., Molgora, M., Marcenaro, E., Lugli, E., et al. (2015). Priming of human resting NK Cells by autologous M1 macrophages via the engagement of IL-1beta, IFN-beta, and IL-15 pathways. *J. Immunol.* 195, 2818–2828. doi: 10.4049/jimmunol.1500325

Menendez, J. A., and Lupu, R. (2007). Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat. Rev. Cancer* 7, 763–777. doi: 10.1038/nrc2222

Mills, E. L., and O'Neill, L. A. (2016). Reprogramming mitochondrial metabolism in macrophages as an anti-inflammatory signal. *Eur. J. Immunol.* 46, 13–21. doi: 10.1002/eji.201445427

Mills, E. L., Ryan, D. G., Prag, H. A., Dikovskaya, D., Menon, D., Zaslona, Z., et al. (2018). Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1. *Nature* 556, 113–117. doi: 10.1038/nature25986

Modolell, M., Corraliza, I. M., Link, F., Soler, G., and Eichmann, K. (1995). Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. *Eur. J. Immunol.* 25, 1101–1104. doi: 10.1002/eji.1830250436

Molon, B., Ugel, S., Del Pozzo, F., Soldani, C., Zilio, S., Avella, D., et al. (2011). Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. *J. Exp. Med.* 208, 1949–1962. doi: 10.1084/jem.20101956

Movahedi, K., Laoui, D., Gysemans, C., Baeten, M., Stange, G., Van den Bossche, J., et al. (2010). Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. *Cancer Res.* 70, 5728–5739. doi: 10.1158/0008-5472.can-09-4672

Munn, D. H., Sharma, M. D., Baban, B., Harding, H. P., Zhang, Y., Ron, D., et al. (2005). GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. *Immunity* 22, 633–642. doi: 10.1016/j.immuni.2005.03.013

Nagaraj, S., Schrum, A. G., Cho, H. I., Celis, E., and Gabrilovich, D. I. (2010). Mechanism of T cell tolerance induced by myeloid-derived suppressor cells. *J. Immunol.* 184, 3106–3116. doi: 10.4049/jimmunol.0902661

Netea-Maier, R. T., Smit, J. W. A., and Netea, M. G. (2018). Metabolic changes in tumor cells and tumor-associated macrophages: a mutual relationship. *Cancer Lett.* 413, 102–109. doi: 10.1016/j.canlet.2017.10.037

Nieman, K. M., Kenny, H. A., Penicka, C. V., Ladanyi, A., Buell-Gutbrod, R., Zillhardt, M. R., et al. (2011). Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nat. Med.* 17, 1498–1503. doi: 10.1038/nm.2492

Noman, M. Z., Desantis, G., Janji, B., Hasmim, M., Karray, S., Dessen, P., et al. (2014). PD-L1 is a novel direct target of HIF-1alpha, and its blockade under hypoxia enhanced MDSC-mediated T cell activation. *J. Exp. Med.* 211, 781–790. doi: 10.1084/jem.20131916

Nomura, D. K., Long, J. Z., Niessen, S., Hoover, H. S., Ng, S. W., and Cravatt, B. F. (2010). Monoacylglycerol lipase regulates a fatty acid network that promotes cancer pathogenesis. *Cell* 140, 49–61. doi: 10.1016/j.cell.2009.11.027

Odegaard, J. I., and Chawla, A. (2011). Alternative macrophage activation and metabolism. *Annu. Rev. Pathol.* 6, 275–297. doi: 10.1146/annurev-pathol-011110-130138

O'Neill, L. A., Kishton, R. J., and Rathmell, J. (2016). A guide to immunometabolism for immunologists. *Nat. Rev. Immunol.* 16, 553–565. doi: 10.1038/nri.2016.70

Park, J., Lee, S. E., Hur, J., Hong, E. B., Choi, J. I., Yang, J. M., et al. (2015). M-CSF from cancer cells induces fatty acid synthase and PPARbeta/delta activation in tumor myeloid cells, leading to tumor progression. *Cell Rep.* 10, 1614–1625. doi: 10.1016/j.celrep.2015.02.024

Patra, K. C., and Hay, N. (2014). The pentose phosphate pathway and cancer. *Trends Biochem. Sci.* 39, 347–354. doi: 10.1016/j.tibs.2014.06.005

Pearce, E. L., and Pearce, E. J. (2013). Metabolic pathways in immune cell activation and quiescence. *Immunity* 38, 633–643. doi: 10.1016/j.immuni.2013.04.005

Pelletier, M., Billingham, L. K., Ramaswamy, M., and Siegel, R. M. (2014). Extracellular flux analysis to monitor glycolytic rates and mitochondrial oxygen consumption. *Methods Enzymol.* 542, 125–149. doi: 10.1016/B978-0-12-416618-9.000071-8

Peng, L. S., Zhang, J. Y., Teng, Y. S., Zhao, Y. L., Wang, T. T., Mao, F. Y., et al. (2017). Tumor-Associated Monocytes/Macrophages Impair NK-Cell Function via TGFbeta1 in Human Gastric Cancer. *Cancer Immunol. Res.* 5, 248–256. doi: 10.1158/2326-6066.cir-16-0152

Penny, H. L., Sieow, J. L., Adriani, G., Yeap, W. H., See Chi Ee, P., San Luis, B., et al. (2016). Warburg metabolism in tumor-conditioned macrophages promotes metastasis in human pancreatic ductal adenocarcinoma. *Oncoimmunology* 5:e1191731. doi: 10.1080/2162402X.2016.1191731

Pinton, L., Solito, S., Damuzzo, V., Francescato, S., Pozzuoli, A., Berizzi, A., et al. (2016). Activated T cells sustain myeloid-derived suppressor cell-mediated immune suppression. *Oncotarget* 7, 1168–1184. doi: 10.18632/oncotarget.6662

Porta, C., Sica, A., and Riboldi, E. (2018). Tumor-associated myeloid cells: new understandings on their metabolic regulation and their influence in cancer immunotherapy. *FEBS J* 285, 717–733. doi: 10.1111/febs.14288

Pyonteck, S. M., Akkari, L., Schuhmacher, A. J., Bowman, R. L., Sevenich, L., Quail, D. F., et al. (2013). CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nat. Med.* 19, 1264–1272. doi: 10.1038/nm.3337

Quaranta, V., Rainer, C., Nielsen, S. R., Raymant, M. L., Ahmed, M. S., Engle, D. D., et al. (2018). Macrophage-derived granulin drives resistance to immune checkpoint inhibition in metastatic pancreatic cancer. *Cancer Res.* 78, 4253–4269. doi: 10.1158/0008-5472.CAN-17-3876

Raber, P. L., Sierra, R. A., Thevenot, P. T., Shuzhong, Z., Wyczechowska, D. D., Kumai, T., et al. (2016). T cells conditioned with MDSC show an increased anti-tumor activity after adoptive T cell based immunotherapy. *Oncotarget* 7, 17565–17578. doi: 10.18632/oncotarget.8197

Ramakrishnan, R., Tyurin, V. A., Veglia, F., Condamine, T., Amoscato, A., Mohammadyani, D., et al. (2014). Oxidized lipids block antigen cross-presentation by dendritic cells in cancer. *J. Immunol.* 192, 2920–2931. doi: 10.4049/jimmunol.1302801

Rath, M., Muller, I., Kropf, P., Closs, E. I., and Munder, M. (2014). Metabolism via arginase or Nitric Oxide synthase: two competing arginine pathways in macrophages. *Front. Immunol.* 5:532. doi: 10.3389/fimmu.2014.00532

Rayman, P., Diaz-Montero, C. M., Yang, Y., Rini, B., Elson, P., and Finke, J. (2015). Modulation of immune cell infiltrate with sunitinib to improve anti-PD1 therapy in preclinical tumor model. *J. Immunother. Cancer* 3(Suppl. 2), 310–310. doi: 10.1186/2051-1426-3-S2-P310

Rodrigues, A. S., Pereira, S. L., Correia, M., Gomes, A., Perestrelo, T., and Ramalho-Santos, J. (2015). Differentiate or Die: 3-bromopyruvate and pluripotency in mouse embryonic stem cells. *PLoS One* 10:e0135617. doi: 10.1371/journal.pone.0135617

Rodriguez, P. C., Ernstoff, M. S., Hernandez, C., Atkins, M., Zabaleta, J., Sierra, R., et al. (2009). Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. *Cancer Res.* 69, 1553–1560. doi: 10.1158/0008-5472.CAN-08-1921

Rodriguez, P. C., Ochoa, A. C., and Al-Khami, A. A. (2017). Arginine metabolism in myeloid cells shapes innate and adaptive immunity. *Front. Immunol.* 8:93. doi: 10.3389/fimmu.2017.00093

Rodriguez, P. C., Quiceno, D. G., Zabaleta, J., Ortiz, B., Zea, A. H., Piazuelo, M. B., et al. (2004). Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res.* 64, 5839–5849. doi: 10.1158/0008-5472.CAN-04-0465

Rosales, C. (2018). Neutrophil: a cell with many roles in inflammation or several cell types? *Front. Physiol.* 9:113. doi: 10.3389/fphys.2018.00113

Ruffell, B., Chang-Strachan, D., Chan, V., Rosenbusch, A., Ho, C. M., Pryer, N., et al. (2014). Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells. *Cancer Cell* 26, 623–637. doi: 10.1016/j.ccell.2014.09.006

Sagiv, J. Y., Michaeli, J., Assi, S., Mishalian, I., Kisos, H., Levy, L., et al. (2015). Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. *Cell Rep.* 10, 562–573. doi: 10.1016/j.celrep.2014.12.039

Saxton, R. A., and Sabatini, D. M. (2017). mTOR signaling in growth, metabolism, and disease. *Cell* 169, 361–371. doi: 10.1016/j.cell.2017.03.035

Schmidt, C. (2017). The benefits of immunotherapy combinations. *Nature* 552, S67–S69. doi: 10.1038/d41586-017-08702-7

Sharma, P., Hu-Lieskovan, S., Wargo, J. A., and Ribas, A. (2017). Primary, adaptive, and acquired resistance to cancer immunotherapy. *Cell* 168, 707–723. doi: 10.1016/j.cell.2017.01.017

Shree, T., Olson, O. C., Elie, B. T., Kester, J. C., Garfall, A. L., Simpson, K., et al. (2011). Macrophages and cathepsin proteases blunt chemotherapeutic response in breast cancer. *Genes Dev.* 25, 2465–2479. doi: 10.1101/gad.180331.111

Sica, A., Strauss, L., Consonni, F. M., Travelli, C., Genazzani, A., and Porta, C. (2017). Metabolic regulation of suppressive myeloid cells in cancer. *Cytokine Growth Factor Rev.* 35, 27–35. doi: 10.1016/j.cytogfr.2017.05.002

Sobotka, O., Endlicher, R., Drahota, Z., Kuèera, O., Rychtrmoc, D., Raad, M., et al. (2016). Impaired mitochondrial functions contribute to 3-bromopyruvate toxicity in primary rat and mouse hepatocytes. *J. Bioenerg. Biomembr.* 48, 363–373. doi: 10.1007/s10863-016-9674-8

Soki, F. N., Koh, A. J., Jones, J. D., Kim, Y. W., Dai, J., Keller, E. T., et al. (2014). Polarization of prostate cancer-associated macrophages is induced by milk fat globule-EGF factor 8 (MFG-E8)-mediated efferocytosis. *J. Biol. Chem.* 289, 24560–24572. doi: 10.1074/jbc.M114.571620

Srivastava, M. K., Sinha, P., Clements, V. K., Rodriguez, P., and Ostrand-Rosenberg, S. (2010). Myeloid-derived suppressor cells inhibit T-cell activation by depleting cystine and cysteine. *Cancer Res.* 70, 68–77. doi: 10.1158/0008-5472.CAN-09-2587

Stachlewitz, R. F., Li, X., Smith, S., Bunzendahl, H., Graves, L. M., and Thurman, R. G. (2000). Glycine inhibits growth of T lymphocytes by an IL-2-independent mechanism. *J. Immunol.* 164, 176–182. doi: 10.4049/jimmunol.164.1.176

Steggerda, S. M., Bennett, M. K., Chen, J., Emberley, E., Huang, T., Janes, J. R., et al. (2017). Inhibition of arginase by CB-1158 blocks myeloid cell-mediated immune suppression in the tumor microenvironment. *J. Immunother. Cancer* 5:101. doi: 10.1186/s40425-017-0308-4

Stienstra, R., Netea-Maier, R. T., Riksen, N. P., Joosten, L. A. B., and Netea, M. G. (2017). Specific and complex reprogramming of cellular metabolism in myeloid cells during innate immune responses. *Cell Metab.* 26, 142–156. doi: 10.1016/j.cmet.2017.06.001

Su, S., Liao, J., Liu, J., Huang, D., He, C., Chen, F., et al. (2017). Blocking the recruitment of naive CD4+ T cells reverses immunosuppression in breast cancer. *Cell Res.* 27, 461–482. doi: 10.1038/cr.2017.34

Sutendra, G., and Michelakis, E. D. (2013). Pyruvate dehydrogenase kinase as a novel therapeutic target in oncology. *Front. Oncol.* 3:38. doi: 10.3389/fonc.2013.00038

Takeuchi, S., Baghdadi, M., Tsuchikawa, T., Wada, H., Nakamura, T., Abe, H., et al. (2015). Chemotherapy-derived inflammatory responses accelerate the formation of immunosuppressive myeloid cells in the tissue microenvironment of human pancreatic cancer. *Cancer Res.* 75, 2629–2640. doi: 10.1158/0008-5472.can-14-2921

Tannahill, G. M., Curtis, A. M., Adamik, J., Palsson-McDermott, E. M., McGettrick, A. F., Goel, G., et al. (2013). Succinate is an inflammatory signal that induces IL-1β through HIF-1α. *Nature* 496, 238–242. doi: 10.1038/nature11986

Tongu, M., Harashima, N., Tamada, K., Chen, L., and Harada, M. (2015). Intermittent chemotherapy can retain the therapeutic potential of anti-CD137 antibody during the late tumor-bearing state. *Cancer Sci.* 106, 9–17. doi: 10.1111/cas.12568

Trikha, P., Plews, R. L., Stiff, A., Gautam, S., Hsu, V., Abood, D., et al. (2016). Targeting myeloid-derived suppressor cells using a novel adenosine monophosphate-activated protein kinase (AMPK) activator. *Oncoimmunology* 5:e1214787. doi: 10.1080/2162402X.2016.1214787

Ugel, S., De Sanctis, F., Mandruzzato, S., and Bronte, V. (2015). Tumor-induced myeloid deviation: when myeloid-derived suppressor cells meet tumor-associated macrophages. *J. Clin. Invest.* 125, 3365–3376. doi: 10.1172/JCI80006

Umansky, V., Blattner, C., Gebhardt, C., and Utikal, J. (2016). The Role of Myeloid-Derived Suppressor Cells (MDSC) in cancer progression. *Vaccines* 4:36. doi: 10.3390/vaccines4040036

Valenti, D., Vacca, R. A., and de Bari, L. (2015). 3-Bromopyruvate induces rapid human prostate cancer cell death by affecting cell energy metabolism, GSH pool and the glyoxalase system. *J. Bioenerg. Biomembr.* 47, 493–506. doi: 10.1007/s10863-015-9631-y

Wang, T., Liu, H., Lian, G., Zhang, S. Y., Wang, X., and Jiang, C. (2017). HIF1alpha-induced glycolysis metabolism is essential to the activation of inflammatory macrophages. *Mediators Inflamm.* 2017:9029327. doi: 10.1155/2017/9029327

Wang, Y., Zhang, X., Yang, L., Xue, J., and Hu, G. (2018). Blockade of CCL2 enhances immunotherapeutic effect of anti-PD1 in lung cancer. *J. Bone Oncol.* 11, 27–32. doi: 10.1016/j.jbo.2018.01.002

Wang, Z., Liu, Y., Zhang, Y., Shang, Y., and Gao, Q. (2016). MDSC-decreasing chemotherapy increases the efficacy of cytokine-induced killer cell immunotherapy in metastatic renal cell carcinoma and pancreatic cancer. *Oncotarget* 7, 4760–4769. doi: 10.18632/oncotarget.6734

Waniczek, D., Lorenc, Z., Śnietura, M., Wesecki, M., Kopec, A., and Muc-Wierzgoñ, M. (2017). Tumor-associated macrophages and regulatory T cells infiltration and the clinical outcome in colorectal cancer. *Arch. Immunol. Ther. Exp.* 65, 445–454. doi: 10.1007/s00005-017-0463-9

Warburg, O., Wind, F., and Negelein, E. (1927). The metabolism of tumors in the body. *J. Gen. Physiol.* 8, 519–530. doi: 10.1085/jgp.8.6.519

Ward, P. S., and Thompson, C. B. (2012). Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. *Cancer Cell* 21, 297–308. doi: 10.1016/j.ccr.2012.02.014

Weichhart, T., Haidinger, M., Katholnig, K., Kopecky, C., Poglitsch, M., Lassnig, C., et al. (2011). Inhibition of mTOR blocks the anti-inflammatory effects of glucocorticoids in myeloid immune cells. *Blood* 117, 4273–4283. doi: 10.1182/blood-2010-09-310888

Welford, A. F., Biziato, D., Coffelt, S. B., Nucera, S., Fisher, M., Pucci, F., et al. (2011). TIE2-expressing macrophages limit the therapeutic efficacy of the vascular-disrupting agent combretastatin A4 phosphate in mice. *J. Clin. Invest.* 121, 1969–1973. doi: 10.1172/jci44562

Wenes, M., Shang, M., Di Matteo, M., Goveia, J., Martin-Perez, R., Serneels, J., et al. (2016). Macrophage metabolism controls tumor blood vessel morphogenesis and metastasis. *Cell Metab.* 24, 701–715. doi: 10.1016/j.cmet.2016.09.008

Wesolowski, R., Duggan, M., Stiff, A., Trikha, P., Schoenfield, L., Abdel-Rasoul, M., et al. (2016). Abstract P4-09-18: Characterization of circulating myeloid derived suppressor cells and cytokines in patients undergoing neo-adjuvant chemotherapy for breast cancer. *Cancer Research* 76(4 Suppl.):P4-09-18-P04-09-18. doi: 10.1158/1538-7445.sabcs15-p4-09-18

Whiteside, T. L. (2008). The tumor microenvironment and its role in promoting tumor growth. *Oncogene* 27, 5904–5912. doi: 10.1038/onc.2008.271

Wu, T., Sun, C., Chen, Z., Zhen, Y., Peng, J., Qi, Z., et al. (2012). Smad3-deficient CD11b(+)Gr1(+) myeloid-derived suppressor cells prevent allograft rejection via the nitric oxide pathway. *J. Immunol.* 189, 4989–5000. doi: 10.4049/jimmunol.1200068

Wynn, T. A., Chawla, A., and Pollard, J. W. (2013). Macrophage biology in development, homeostasis and disease. *Nature* 496, 445–455. doi: 10.1038/nature12034

Yadav, S., Pandey, S. K., Goel, Y., Kujur, P. K., Maurya, B. N., Verma, A., et al. (2018). Protective and recuperative effects of 3-bromopyruvate on immunological, hepatic and renal homeostasis in a murine host bearing ascitic lymphoma: implication of niche dependent differential roles of macrophages. *Biomed. Pharmacother.* 99, 970–985. doi: 10.1016/j.biopha.2018.01.149

Ye, H., Zhou, Q., Zheng, S., Li, G., Lin, Q., Wei, L., et al. (2018). Tumor-associated macrophages promote progression and the Warburg effect via CCL18/NF-kB/VCAM-1 pathway in pancreatic ductal adenocarcinoma. *Cell Death Dis.* 9:453. doi: 10.1038/s41419-018-0486-0

Yun, J., Rago, C., Cheong, I., Pagliarini, R., Angenendt, P., Rajagopalan, H., et al. (2009). Glucose deprivation contributes to the development of KRAS pathway mutations in tumor cells. *Science* 325, 1555–1559. doi: 10.1126/science.1174229

Zhao, Q., Chu, Z., Zhu, L., Yang, T., Wang, P., Liu, F., et al. (2017). 2-Deoxy-d-glucose treatment decreases anti-inflammatory m2 macrophage polarization in mice with tumor and allergic airway inflammation. *Front. Immunol.* 8:637. doi: 10.3389/fimmu.2017.00637

Zhao, Q., Kuang, D.-M., Wu, Y., Xiao, X., Li, X.-F., Li, T.-J., et al. (2012). Activated CD69 < sup > + < /sup > T cells foster immune privilege by regulating ido expression in tumor-associated macrophages. *J. Immunol.* 188, 1117–1124. doi: 10.4049/jimmunol.1100164

Zhao, T., Du, H., Blum, J. S., and Yan, C. (2016). Critical role of PPARgamma in myeloid-derived suppressor cell-stimulated cancer cell proliferation and metastasis. *Oncotarget* 7, 1529–1543. doi: 10.18632/oncotarget.6414

Zhou, R., Vander Heiden, M. G., and Rudin, C. M. (2002). Genotoxic exposure is associated with alterations in glucose uptake and metabolism. *Cancer Res.* 62, 3515–3120.

Zhu, Y., Herndon, J. M., Sojka, D. K., Kim, K. W., Knolhoff, B. L., Zuo, C., et al. (2017). Tissue-resident macrophages in pancreatic ductal adenocarcinoma originate from embryonic hematopoiesis and promote tumor progression. *Immunity* 47, 323–338.e6. doi: 10.1016/j.immuni.2017.07.014

Zhu, Y., Knolhoff, B. L., Meyer, M. A., Nywening, T. M., West, B. L., Luo, J., et al. (2014). CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T-cell checkpoint immunotherapy in pancreatic cancer models. *Cancer Res.* 74, 5057–5069. doi: 10.1158/0008-5472.CAN-13-3723

Zou, X., Zhang, M., Sun, Y., Zhao, S., Wei, Y., Zhang, X., et al. (2015). Inhibitory effects of 3-bromopyruvate in human nasopharyngeal carcinoma cells. *Oncol. Rep.* 34, 1895–1904. doi: 10.3892/or.2015.4147

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Beyond the Warburg Effect: How Do Cancer Cells Regulate One-Carbon Metabolism?

Adam Rosenzweig[1,2], *John Blenis*[1,2] *and Ana P. Gomes*[1,2*]

[1] *Meyer Cancer Center, Weill Cornell Medicine, New York, NY, United States,* [2] *Department of Pharmacology, Weill Cornell Medicine, New York, NY, United States*

Altered metabolism in cancer cells is critical for tumor growth. One of the most notable aspects of this metabolic reprogramming lies in one-carbon metabolism. Cells require one-carbon units for nucleotide synthesis, methylation reactions, and for the generation of reducing cofactors. Therefore, the ability to rewire and fine-tune one-carbon metabolism is essential for the maintenance of cellular homeostasis. In this review, we describe how the major nutrient, energy, and redox sensors of the cell play a significant role in the regulation of flux through one-carbon metabolism to enable cell fate decisions. We will also discuss how dysregulated oncogenic signaling hijacks these regulatory mechanisms to support and sustain high rates of proliferation and cell survival essential for tumor growth.

Keywords: one carbon metabolism, cancer, metabolic reprogramming, folate cycle, methionine cycle, metabolic regulation

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***Correspondence:**
Ana P. Gomes
and2049@med.cornell.edu

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INTRODUCTION

One-carbon metabolism encompasses a broad range of biosynthetic reactions that occur in the cytoplasm and the mitochondria which are essential for maintaining cellular homeostasis. These pathways catabolize different carbon sources to derive one-carbon (methyl) units to be utilized in fundamental cellular functions (Ducker and Rabinowitz, 2017). Due to the specific manner in which one-carbon units are obtained and utilized, one-carbon metabolism serves as an integrative pathway, relating many nutrients to one another. Flux through one-carbon metabolism must remain plastic for cells to regulate levels of the related nutrients in response to ever-changing intra- and extracellular conditions. One-carbon metabolism provides cells with the building blocks, as well as the reducing power, necessary to maintain high rates of proliferation, and therefore is key in supporting cancer. In this review, we discuss how cells regulate flux through one-carbon metabolism and its implications for tumorigenesis.

ONE-CARBON METABOLISM, INTEGRATOR OF NUTRIENT STATUS?

One-carbon metabolism is essential in cellular physiology as it functions as an integrator of the nutritional status of cells. One-carbon units are derived from different nutrients inputs and generate various molecular outputs that serve as building blocks for biosynthesis, methylation and redox reactions.

One-carbon units are largely derived from the non-essential amino acids serine and glycine (Kalhan and Hanson, 2012). Both serine and glycine can be obtained exogenously or synthesized from other carbon sources. Serine can be created *de novo* from glucose through a series of enzymes which convert 3-phosphoglycerate (3PG) into serine [phosphoglycerate dehydrogenase (PHDGH), phosphoserine aminotransferase 1 (PSAT1), and phosphoserine phosphatase (PSPH) –referred hereafter as the serine synthesis pathway (SSP)] (Locasale, 2013). Glycine can be produced from serine or threonine (Wang et al., 2009). Hypothetically, both serine and glycine can equally donate one-carbon groups, however, the actual contribution of serine and glycine is far more complex. Serine is believed to be the more significant one-carbon unit donor, but glycine can also contribute one-carbon units through oxidation by the glycine cleavage system (GCS), though in lesser quantities than the serine-to-glycine conversion (Tedeschi et al., 2013). It is clear that glycine catabolism is important for one-carbon metabolism, however the relative contribution of glycine vs. serine is still debated. Several reports showed that cancer cells fail to consume glycine when serine is abundant (Maddocks et al., 2013; Labuschagne et al., 2014), while others have shown a significant upregulation in glycine consumption (Jain et al., 2012). It is likely that the relative contribution of either serine or glycine to fuel one-carbon metabolism is dependent on cell type and environment.

One-carbon units are utilized in two pathways: the folate cycle and the methionine cycle (**Figure 1**). In the folate cycle, folic acid is reduced by dihydrofolate reductase (DHFR) to the biologically active tetrahydrofolate (THF) (Newman and Maddocks, 2017). In this reduced form, one-carbon units from serine and glycine can be transferred by serine hydroxymethyltransferase (SHMT) and glycine decarboxylase (GLDC; of the glycine cleavage system [GCS]), respectively, onto THF forming methyl-THF. Once methylated, THF can undergo a series of redox transformations by the multi-functional enzyme methylenetetrahydrofolate dehydrogenase (MTHFD1/2/1L), which has cytosolic and mitochondrial isoforms (Lewis et al., 2014; for detailed information on the folate cycle see Ducker and Rabinowitz, 2017). In the methionine cycle, homocysteine is re-methylated using a one-carbon unit from methyl-THF to form methionine via methionine synthase (MS) (Yang and Vousden, 2016). Demethylation of S-adenosyl-methionine (SAM) yields S-adenosyl-homocysteine (SAH), which is then converted to homocysteine, completing the cycle. The crosstalk between the folate and methionine cycles goes beyond the re-methylation of homocysteine, as *de novo* ATP synthesis powered by the folate cycle directly contributes to the formation of SAM, a

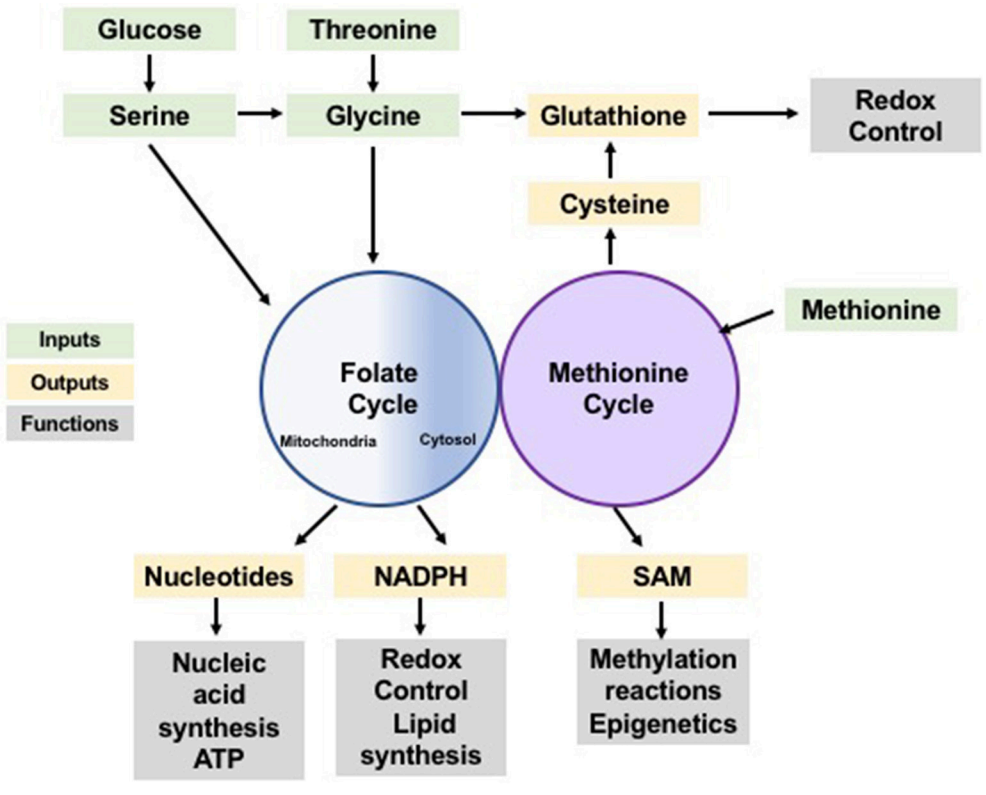

FIGURE 1 | One-carbon metabolism as a cellular process integrating nutrient status and availability. Glucose and amino acids input to the folate and methionine cycles (green) contributing with one-carbon units which can be used in anabolic synthesis of many building blocks, reducing species and co-factors (yellow). These synthesis products support a variety of cellular functions (gray) including synthesis of biomolecules, redox control and post-translational modification, sustaining cellular homeostasis.

critical donor to multiple methylation reactions (Maddocks et al., 2016). Furthermore, *de novo* generation of ATP by the folate cycle is likely to be indispensable in maintaining energetic homeostasis, particularly in conditions of high energy demand such as sustaining high rates of proliferation.

It is noteworthy that the folate cycle functions in the cytosol and in the mitochondria, although most proliferating cells rely primarily on the mitochondrial pathway (Tibbetts and Appling, 2010; Ducker et al., 2016). This compartmentalization is hypothesized to be a mechanism by which cells can decouple one-carbon metabolism from glycolysis (Ducker and Rabinowitz, 2017). One-carbon metabolism produces a large quantity of NADH, and localization to the mitochondria preserves cytosolic NAD^+ which is necessary for glycolysis reactions (Ducker and Rabinowitz, 2017). The mitochondrial pathway may also convey an energetic advantage over the cytosolic. Evidence suggests that excess formate resulting from serine metabolism is exported from the mitochondria, and with each formate exported, an ADP molecule is phosphorylated to ATP (Meiser et al., 2016). Whether these two branches of the folate cycle are redundant or not is still controversial. Some groups have shown that the cytosolic folate cycle cannot compensate for loss of the mitochondrial pathway (Celardo et al., 2017), others have shown the opposite (Ducker et al., 2016). It is likely that the existence or the lack of redundancy between these arms of the folate cycle is also context dependent (Ducker et al., 2016).

Together the folate and methionine cycles mediate the redistribution of one-carbon groups derived from nutrients into the production of purine nucleotides (Tedeschi et al., 2013), glutathione, (Zhou et al., 2017), ATP, and NADPH; to control cell fate and maintain homeostasis (Tedeschi et al., 2013; Maddocks et al., 2016). Yet, the influence of one-carbon metabolism goes beyond energy currency and redox power. SAM plays a significant role in epigenetics, in post-translational modifications, and in signaling pathways through its contribution to methylation reactions (Finkelstein, 1990; Su et al., 2016).Therefore, one-carbon metabolism not only dispenses carbon atoms to various acceptor molecules, but it also integrates nutrient status with epigenetic, energetic, and redox statuses to maintain cellular homeostasis (Rowe and Lewis, 1973; **Figure 1**).

NUTRIENT AND ENERGY SENSORS KEEP TABS ON ONE-CARBON METABOLISM

The mammalian target of rapamycin (mTOR) is a critical rheostat for the maintenance of metabolic balance and tightly regulates many aspects of metabolism (Gomes and Blenis, 2015). When nutrient availability is high, mTOR is activated, promoting anabolic reactions to sustain growth and proliferation. mTOR is a major regulator of one-carbon metabolism. One of the main effectors of mTOR for metabolic regulation is the activating transcription factor 4 (ATF4). When cellular serine levels are low, ATF4 is activated, leading to its binding to the promoter of the genes encoding SSP enzymes (Ye et al., 2012). This then drives their expression and consequentially increases serine pools (Ye et al., 2012). Moreover, mTOR signaling through ATF4 also regulates the expression of MTHFD2, stimulating the mitochondrial branch of the folate cycle (Ben-Sahra et al., 2016). Highlighting the importance of mTOR for one-carbon metabolism, another transcription factor that acts downstream of mTOR, FOXK1, is found to regulate the SSP, SHMT2, and MTHFD1L (He et al., 2018). As the forward flux of the folate cycle occurs in the mitochondria, the various forms of methylated THF are synthesized and then must be translocated to contribute to anabolism (Brosnan et al., 2015; Ducker et al., 2016). In the case of 10-formyl THF, it has been shown that the complex of enzymes which receive its one-carbon units, called the purinosome, is formed in the cytosol and colocalizes with the mitochondria under purine deficiency to expedite the synthesis of purine molecules (Zhao et al., 2013; Chan et al., 2015). This colocalization is thought to be mTOR dependent (French et al., 2016). Interestingly, one-carbon metabolism also signals back to mTOR as SAM was recently found to directly regulate mTORC1 activity (Gu et al., 2017).

One-carbon metabolism provides units to support growth and proliferation when nutrient availability and energy levels are high. Conversely, when these factors are limited, a brake is needed to slow down anabolic reactions and conserve energy. Such a mechanism relies on the activation of the AMP-activated kinase (AMPK). AMPK is the energy sensor of the cell; when ATP levels are high, AMPK is inhibited allowing anabolic reactions to transpire. When ATP levels are low and AMP levels rise, AMPK is activated, inhibiting anabolism and promoting catabolic reactions to restore ATP levels (Gomes and Blenis, 2015). Interestingly, AMPK has recently been shown to downregulate the expression of MTHFD1/2/1L through the PGC-1α/ERRα axis (Audet-Walsh et al., 2016). This suppression limits the flux of one-carbon units from serine and glycine to the products of one-carbon metabolism, thereby conserving energy.

Together, these observations suggest that cells tightly regulate flux through one-carbon metabolism. This regulation is established based upon the energetic status of the cell and creates the metabolic flexibility necessary to maintain homeostasis (**Figure 2**).

REDOX AND OXYGEN SENSING MECHANISMS AND ONE-CARBON METABOLISM REGULATION

Under physiological conditions, the balance between generation and elimination of reactive oxygen species (ROS) maintains the proper function of redox sensitive pathways. When redox homeostasis is disturbed, oxidative stress contributes to disease development and can also lead to aberrant cell death. Therefore, eukaryotic cells have evolved systems to tightly regulate redox balance (Panieri and Santoro, 2016). Key components of these systems are the cofactors NADH and NADPH. NADPH plays a crucial role in the cell as it provides the reducing power that enables lipid synthesis and nucleotide synthesis, and the oxidation-reduction involved in detoxification of ROS (Panieri and Santoro, 2016). Recently, one-carbon metabolism has gained recognition as a main regulator of NADPH levels in the cells

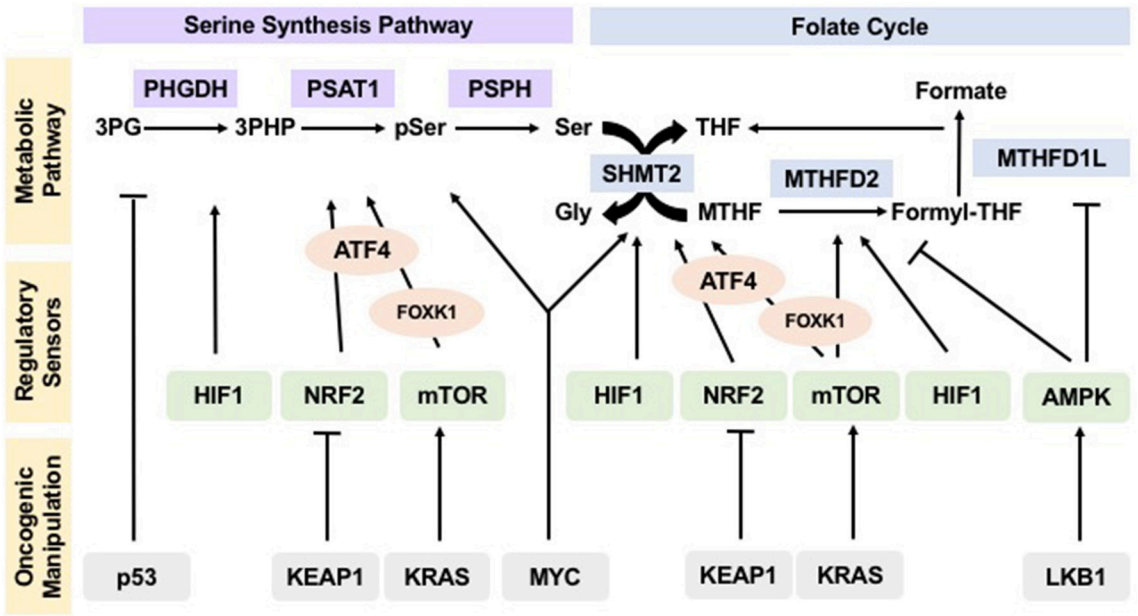

FIGURE 2 | Oncogenes and tumor suppressors manipulate regulation of one-carbon metabolism pathways in cancer to drive tumorigenesis. Sensors of nutrient and energy levels, mTOR and AMPK, and sensors of redox potential, HIF1 and NRF2, regulate the different steps in one-carbon metabolism to ensure proper flux. In cancers, oncogenes KEAP1, KRAS, and MYC, as well as tumor suppressors p53 and LKB1, manipulate these regulatory sensors, thus affecting the operation and flux of pathways within one-carbon metabolism and allowing for their hyperactivity to sustain uncontrolled proliferation and tumorigenesis.

through the action of the MTHFD enzymes (Fan et al., 2014). Depletion of either the cytosolic or mitochondrial MTHFD enzymes resulted in decreased cellular NADPH/NADP+ and increased sensitivity to oxidative stress (Fan et al., 2014).

The nuclear factor erythroid-derived 2 (NRF2) coordinates an evolutionarily conserved transcriptional activation pathway that mediates antioxidant and detoxification responses, which is activated in response to oxidative stress. NRF2 has been shown to lead to the upregulation of the SSP and SHMT enzymes, through induction of ATF4 (DeNicola et al., 2015). This transcriptional upregulation works to promote serine flux through the folate cycle, consequently increasing the production of NADPH and powering cells with the reducing equivalents necessary to detoxify ROS (DeNicola et al., 2015). Moreover, the hypoxia inducible factor 1 (HIF1), which senses cellular oxygen levels, has also been shown to act as an activator of the SSP, mediating transcription of the genes involved in the SSP as well as SHMT2 (Iyer et al., 1998; Samanta et al., 2014). This allows the cells to build redox power to combat the build-up of ROS generated by hypoxia (Fan et al., 2014).

Besides increasing NAD(P)H production, one-carbon flux induced by these oxygen and redox sensing pathways also contributes to the maintenance of the oxidative balance through generation of glutathione (Locasale, 2013; Lu et al., 2015). Glutathione is a tripeptide of glutamate, glycine, and cysteine and is a powerful antioxidant molecule (Ballatori et al., 2009). The increased serine production and catabolism induced by HIF1 and NRF2 leads to an increase in glycine as a byproduct of serine catabolism. It also contributes to an increase in cysteine which is a product of the trans-sulfuration pathway, thus resulting in higher levels of glutathione, facilitating rapid ROS detoxification (DeNicola et al., 2015).

This evidence asserts that not only are one-carbon units important to maintain redox balance, but also that the master regulators of redox remodeling participate in regulating flux through one-carbon metabolism ensuring a feedback mechanism that keeps homeostasis (**Figure 2**).

ONE-CARBON METABOLISM AT THE ROOT OF CARCINOGENESIS

The indispensability of one-carbon metabolism in carcinogenesis is well demonstrated by many established cancer therapies. For decades, methotrexate has been used as a standard of care treatment for cancer patients (Newman and Maddocks, 2017). Methotrexate is in the class of drugs called anti-folates and is an inhibitor of DHFR, which prevents THF production and halts the folate cycle (Osborn et al., 1958). Another commonly used anti-cancer treatment, 5-fluorouracil (5-FU), is known to inhibit thymidylate synthase (TYMS), which catalyzes the transfer of a one-carbon unit from methylene-THF onto dUMP to make dTMP (Longley et al., 2003). The efficacy of methotrexate and 5-FU in the clinic demonstrates that many cancers are dependent on one-carbon metabolism.

More recently, it has been established that the repression of tumor suppressor genes by methylation, which is dependent on SAM levels, is a key-initiating event for many cancers (Kulis

and Esteller, 2010). Oncogenic KRAS mutations have been shown to increase regional DNA methylation due to increased SAM obtained via one-carbon metabolism, resulting in increased tumor growth (Kottakis et al., 2016). *De novo* serine synthesis has also been identified as a metabolic vulnerability of many cancers, including non-small cell lung cancer (NSCLC), breast cancer, and melanoma (Locasale et al., 2011; Possemato et al., 2011; DeNicola et al., 2015). Increased expression of the enzymes involved in the SSP is frequently observed in these types of cancers, and overexpression of PHGDH in non-tumorigenic breast cells is sufficient to develop a cancerous phenotype (Locasale et al., 2011). Chemical and genetic inhibition of the SSP is sufficient to abrogate proliferation of cancer cells in culture and to reduce xenograft tumor burden (Possemato et al., 2011; Mullarky et al., 2016; Pacold et al., 2016). Additionally, increased activity of SHMT, has been observed in multiple cancers and shown to be critical for tumor formation (Jain et al., 2012; Ye et al., 2014). In fact, in the absence of SHMT and the SSP, tumors become addicted to exogenous serine and serine starvation reduces cancer cell proliferation and tumor growth (Maddocks et al., 2013, 2016; Labuschagne et al., 2014).

Glycine metabolism has also been shown to be important in cancer development and growth. The main enzyme in the glycine cleavage pathway, GLDC, is upregulated in lung-tumor initiating cells as well as glioblastomas, and sustained GLDC hyperactivity has been shown to be critical for tumorigenesis (Zhang et al., 2012). In a mouse embryonic fibroblast cell line, overexpression of GLDC alone was sufficient to induce tumorigenesis (Zhang et al., 2012). There is evidence suggesting that the GCS supports tumor survival by reducing toxicity due to accumulation of glycine (Kim et al., 2015). When there is an excess of glycine in a cell, such as when SHMT is rapidly converting serine to glycine, this alternative metabolic pathway becomes active and subverts the toxic accumulation of glycine while producing one carbon units (Kim et al., 2015). This evidence implicates glycine catabolism as a driver of carcinogenesis both by driving one-carbon metabolism and protecting from glycine toxicity. Additionally, interfering with the mitochondrial folate cycle by suppression of MTHFD1L also has a potent anti-tumor effect, further supporting the essential nature of the mitochondrial folate cycle for carcinogenesis (Ducker et al., 2016; Lee et al., 2017). In this scenario, it is possible that the lack of redundancy between the cytosolic and mitochondrial folate pathways is linked to hyperactivity of the GCS, which is solely mitochondrial.

ONCOGENES HIJACK SENSING MECHANISMS TO SUSTAIN ONE-CARBON METABOLISM

In many cancer types, proto-oncogenes have their function altered, thus contributing to hijacking of the regulatory mechanisms that preserve homeostasis in healthy cells (Gomes and Blenis, 2015). Given that one-carbon metabolism is regulated by nutrient, energy, oxygen, and redox sensors working concertedly to keep homeostasis, the idea that oncogenes or loss of tumor suppressors manipulate one-carbon flux in tumorigenesis is attractive.

In support of this idea, the oncogene MYC has been shown to hijack regulatory pathways to increase flux through one-carbon metabolism in different cancers. For example, C-MYC induces SSP activity under nutrient deficient conditions in liver carcinomas (Sun et al., 2015). In neuroblastoma, N-MYC causes a HIF1-dependent induction of SHMT2 under hypoxic conditions (Ye et al., 2014). Additionally, loss of function mutations in the tumor suppressor KEAP1 have been shown to power one-carbon metabolism in NSCLCs (DeNicola et al., 2015). KEAP1 is the suppressor of NRF2. When its function is lost, a NRF2-induced upregulation of ATF4 triggers the SSP (Kansanen et al., 2013).

Oncogenic mutations promoting constitutive KRAS activation, one of the most common occurrences in cancer, correlates with increased expression of folate cycle enzymes (Moran et al., 2014). Oncogenic KRAS leads to aberrant activation of mTOR, which regulates both the SSP and the folate cycle (Gomes and Blenis, 2015; Ben-Sahra et al., 2016). Loss of another tumor suppressor, the AMPK activator liver kinase B1 (LKB1), is prevalent in KRAS mutant tumors (Kottakis et al., 2016). This suggests that along with stimulating mTOR, KRAS may also increase one-carbon metabolism through subversion of AMPK's inhibitory effects in the folate cycle.

The tumor suppressor p53 is also known to play a key role in one-carbon metabolism regulation. p53 is responsible for halting the cell cycle in conditions of stress, preventing uncontrolled proliferation (Maddocks et al., 2013). Loss of p53 is common in many types of cancer, conveying a survival advantage by allowing carcinogenic cells to replicate regardless of stress (Kruiswijk et al., 2015). Activation of p53 by non-genotoxic stresses in non-cancerous cells represses the expression of, PHGDH, to promote apoptosis (Ou et al., 2015). In tumors, loss of p53 causes addiction to serine (Maddocks et al., 2013). Consequently, serine starvation has been shown to considerably decrease growth of these tumors (Maddocks et al., 2013). So, it is interesting that, in cancers where p53 remains active, serine starvation leads to activation of p53, triggering cell cycle arrest. This phenomenon allows cells to channel serine into glutathione synthesis rather than the production of building blocks, therefore allowing cell survival (Maddocks et al., 2013; Kruiswijk et al., 2015). These paradoxical roles of p53 in regulating serine synthesis demonstrate the complexity of one-carbon metabolism regulation and the role it plays in different physiological conditions.

It is notable that the majority of evidence regarding oncogenic regulation of one-carbon metabolism lies in the upregulation of *de novo* serine synthesis. Upregulation of the SSP allows for flexibility to fuel various downstream pathways. Additionally, this phenomenon can be explained by the Warburg effect, where glucose consumption and oxidation become dysregulated to allow for rapid growth and proliferation (Liberti and Locasale, 2016). An increase in glycolysis leads to the accumulation of its intermediates, including the precursor for *de novo* serine synthesis, 3PG. By upregulating the SSP and other enzymes necessary for one-carbon metabolism, glucose-derived carbon can be shunted to a process capable of producing a variety of biomolecules and redox species (Lunt and Vander Heiden, 2011).

This, along with upregulation of other glucose-derived pathways, is key for tumor growth and proliferation.

These examples demonstrate that activation and/or loss of oncogenes and tumor suppressors override the control of sensing mechanisms and drive flux through one-carbon metabolism, allowing tumors to thrive (**Figure 2**).

CONCLUSIONS

As a growing body of evidence supports the key role of one-carbon metabolism in cancer, it becomes of interest to expand our knowledge on how one-carbon metabolism is regulated. Here, we propose that one-carbon metabolism integrates the nutrient, energetic, and redox statuses of cells, and that flux through associated pathways is fine-tuned to reflect said status and to ensure cellular homeostasis. Taking into consideration the critical role of one-carbon metabolism as a producer of reducing power and building blocks, as well as its part in regulating substrates for epigenetic and post-translational modifications, an important line of questioning emerges. What determines preferential utilization of the mitochondrial vs. the cytosolic folate cycle? Can we take advantage of one-carbon metabolism for the development of more efficacious cancer therapies? Additionally, can we use one-carbon metabolism as a predictor of responsiveness to chemotherapies such as methotrexate and 5-FU? Is one-carbon metabolism and its remarkable flexibility responsible for the development of drug resistance? The answers to these questions are still largely unknown but may prove vital for advances in precision medicine and the treatment of cancer.

AUTHOR CONTRIBUTIONS

APG conceived, wrote and edited the manuscript. AR wrote and JB edited the manuscript.

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REFERENCES

Audet-Walsh, É., Papadopoli, D. J., Gravel, S. P., Yee, T., Bridon, G., Caron, M., et al. (2016). The PGC-1alpha/ERRalpha axis represses one-carbon metabolism and promotes sensitivity to anti-folate therapy in breast cancer. *Cell Rep.* 14, 920–931. doi: 10.1016/j.celrep.2015.12.086

Ballatori, N., Krance, S. M., Notenboom, S., Shi, S., Tieu, K., and Hammond, C. L. (2009). Glutathione dysregulation and the etiology and progression of human diseases. *Biol. Chem.* 390, 191–214. doi: 10.1515/BC.2009.033

Ben-Sahra, I., Hoxhaj, G., Ricoult, S. J. H., Asara, J. M., and Manning, B. D. (2016). mTORC1 induces purine synthesis through control of the mitochondrial tetrahydrofolate cycle. *Science* 351, 728–733. doi: 10.1126/science.aad0489

Brosnan, M. E., MacMillan, L., Stevens, J. R., and Brosnan, J. T. (2015). Division of labour: how does folate metabolism partition between one-carbon metabolism and amino acid oxidation? *Biochem. J.* 472, 135–146. doi: 10.1042/BJ20150837

Celardo, I., Lehmann, S., Costa, A. C., Loh, S. H., and Miguel Martins, L. (2017). dATF4 Regulation of mitochondrial folate-mediated one-carbon metabolism is neuroprotective. *Cell Death. Differ.* 24, 638–648. doi: 10.1038/cdd.2016.158

Chan, C. Y., Zhao, H., Pugh, R. J., Pedley, A. M., French, J., Jones, S. A., et al. (2015). Purinosome formation as a function of the cell cycle. *Proc. Natl. Acad. Sci. U.S.A.* 112, 1368–1373. doi: 10.1073/pnas.1423009112

DeNicola, G. M., Chen, P. H., Mullarky, E., Sudderth, J. A., Hu, Z., Wu, D., et al. (2015). NRF2 regulates serine biosynthesis in non-small cell lung cancer. *Nat. Genet.* 47, 1475–1481. doi: 10.1038/ng.3421

Ducker, G. S., Chen, L., Morscher, R. J., Ghergurovich, J. M., Esposito, M., Teng, X., et al. (2016). Reversal of cytosolic one-carbon flux compensates for loss of the mitochondrial folate pathway. *Cell Metab.* 24, 640–641. doi: 10.1016/j.cmet.2016.09.011

Ducker, G. S., and Rabinowitz, J. D. (2017). One-carbon metabolism in health and disease. *Cell Metab.* 25, 27–42. doi: 10.1016/j.cmet.2016.08.009

Fan, J., Ye, J., Kamphorst, J. J., Shlomi, T., Thompson, C. B., and Rabinowitz, J. D. (2014). Quantitative flux analysis reveals folate-dependent NADPH production. *Nature* 510, 298–302. doi: 10.1038/nature13236

Finkelstein, J. D. (1990). Methionine metabolism in mammals. *J. Nutr. Biochem.* 1, 228–237. doi: 10.1016/0955-2863(90)90070-2

French, J. B., Jones, S. A., Deng, H., Pedley, A. M., Kim, D., Chan, C. Y., et al. (2016). Spatial colocalization and functional link of purinosomes with mitochondria. *Science* 351, 733–737. doi: 10.1126/science.aac6054

Gomes, A. P., and Blenis, J. (2015). A nexus for cellular homeostasis: the interplay between metabolic and signal transduction pathways. *Curr. Opin. Biotechnol.* 34, 110–117. doi: 10.1016/j.copbio.2014.12.007

Gu, X., Orozco, J. M., Saxton, R. A., Condon, K. J., Liu, G. Y., Krawczyk, P. A., et al. (2017). SAMTOR is an S-adenosylmethionine sensor for the mTORC1 pathway. *Science* 358, 813–818. doi: 10.1126/science.aao3265

He, L., Gomes, A. P., Wang. X., Yoon, S. O., Lee. G., Nagiec, M. J., et. al. (2018). mTORC1 promotes metabolic reprogramming by the suppression of gsk3-dependent foxk1 phosphorylation. *Mol. Cell* 70, 949–960. doi: 10.1016/j.molcel.2018.04.024

Iyer, N. V., Kotch, L. E., Agani, F., Leung, S. W., Laughner, E., Wenger, R. H., et al. (1998). Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1 alpha. *Genes. Dev.* 12, 149–162. doi: 10.1101/gad.12.2.149

Jain, M., Nilsson, R., Sharma, S., Madhusudhan, N., Kitami, T., Souza, A. L., et al. (2012). Metabolite profiling identifies a key role for glycine in rapid cancer cell proliferation. *Science* 336, 1040–1044. doi: 10.1126/science.1218595

Kalhan, S. C., and Hanson, R. W. (2012). Resurgence of serine: an often neglected but indispensable amino acid. *J. Biol. Chem.* 287, 19786–19791. doi: 10.1074/jbc.R112.357194

Kansanen, E., Kuosmanen, S. M., Leinonen, H., and Levonen, A. L. (2013). The Keap1-Nrf2 pathway: mechanisms of activation and dysregulation in cancer. *Redox Biol.* 1, 45–49. doi: 10.1016/j.redox.2012.10.001

Kim, D., Fiske, B. P., Birsoy, K., Freinkman, E., Kami, K., Possemato, R. L., et al. (2015). SHMT2 drives glioma cell survival in the tumor microenvironment but imposes a dependence on glycine clearance. *Nature* 520, 363–367. doi: 10.1038/nature14363

Kottakis, F., Nicolay, B. N., Roumane, A., Karnik, R., Gu, H., Nagle, J. M., et al. (2016). LKB1 loss links serine metabolism to DNA methylation and tumorigenesis. *Nature* 539, 390–395. doi: 10.1038/nature20132

Kruiswijk, F., Labuschagne, C. F., and Vousden, K. H. (2015). p53 in survival, death and metabolic health: a lifeguard with a licence to kill. *Nat. Rev. Mol. Cell Biol.* 16, 393–405. doi: 10.1038/nrm4007

Kulis, M., and Esteller, M. (2010). DNA methylation and cancer. *Adv. Genet.* 70, 27–56. doi: 10.1016/B978-0-12-380866-0.60002-2

Labuschagne, C. F., van den Broek, N. J., Mackay, G. M., Vousden, K. H., and Maddocks, O. D. (2014). Serine, but not glycine, supports one-carbon metabolism and proliferation of cancer cells. *Cell Rep.* 7, 1248–1258. doi: 10.1016/j.celrep.2014.04.045

Lee, D., Xu, I. M., Chiu, D. K., Lai, R. K., Tse, A. P., Lan Li, L., et al. (2017). Folate cycle enzyme MTHFD1L confers metabolic advantages in hepatocellular carcinoma. *J. Clin. Invest.* 127, 1856–1872. doi: 10.1172/JCI90253

Lewis, C. A., Parker, S. J., Fiske, B. P., McCloskey, D., Gui, D. Y., Green, C. R., et al. (2014). Tracing compartmentalized NADPH metabolism in the cytosol and mitochondria of mammalian cells. *Mol. Cell* 55, 253–263. doi: 10.1016/j.molcel.2014.05.008

Liberti, M. V., and Locasale, J. W. (2016). The warburg effect: how does it benefit cancer cells? *Trends Biochem. Sci.* 41, 211–218. doi: 10.1016/j.tibs.2015.12.001

Locasale, J. W. (2013). Serine, glycine and one-carbon units: cancer metabolism in full circle. *Nat. Rev. Cancer* 13, 572–583. doi: 10.1038/nrc3557

Locasale, J. W., Grassian, A. R., Melman, T., Lyssiotis, C. A., Mattaini, K. R., Bass, A. J., et al. (2011). Phosphoglycerate dehydrogenase diverts glycolytic flux and contributes to oncogenesis. *Nat. Genet.* 43, 869–874. doi: 10.1038/ng.890

Longley, D. B., Harkin, D. P., and Johnston, P. G. (2003). 5-fluorouracil: mechanisms of action and clinical strategies. *Nat. Rev. Cancer* 3, 330–338. doi: 10.1038/nrc1074

Lu, H., Samanta, D., Xiang, L., Zhang, H., Hu, H., Chen, I., et al. (2015). Chemotherapy triggers HIF-1-dependent glutathione synthesis and copper chelation that induces the breast cancer stem cell phenotype. *Proc. Natl. Acad. Sci. U.S.A.* 112, E4600–E4609. doi: 10.1073/pnas.1513433112

Lunt, S. Y., and Vander Heiden, M. G. (2011). Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu. Rev. Cell Dev. Biol.* 27, 441–464. doi: 10.1146/annurev-cellbio-092910-154237

Maddocks, O. D., Berkers, C. R., Mason, S. M., Zheng, L., Blyth, K., Gottlieb, E., et al. (2013). Serine starvation induces stress and p53-dependent metabolic remodelling in cancer cells. *Nature* 493, 542–546. doi: 10.1038/nature11743

Maddocks, O. D., Labuschagne, C. F., Adams, P. D., and Vousden, K. H. (2016). Serine metabolism supports the methionine cycle and dna/rna methylation through de novo atp synthesis in cancer cells. *Mol. Cell* 61, 210–221. doi: 10.1016/j.molcel.2015.12.014

Meiser, J., Tumanov, S., Maddocks, O., Labuschagne, C. F., Athineos, D., Van Den Broek, N., et al. (2016). Serine one-carbon catabolism with formate overflow. *Sci. Adv.* 2:E1601273. doi: 10.1126/sciadv.1601273

Moran, D. M., Trusk, P. B., Pry, K., Paz, K., Sidransky, D., and Bacus, S. S. (2014). KRAS mutation status is associated with enhanced dependency on folate metabolism pathways in non-small cell lung cancer cells. *Mol. Cancer Ther.* 13, 1611–1624. doi: 10.1158/1535-7163.MCT-13-0649

Mullarky, E., Lucki, N. C., Beheshti Zavareh, R., Anglin, J. L., Gomes, A. P., Nicolay, B. N., et al. (2016). Identification of a small molecule inhibitor of 3-phosphoglycerate dehydrogenase to target serine biosynthesis in cancers. *Proc. Natl. Acad. Sci. U.S.A.* 113, 1778–1783. doi: 10.1073/pnas.1521548113

Newman, A. C., and Maddocks, O. D. K. (2017). One-carbon metabolism in cancer. *Br. J. Cancer* 116, 1499–1504. doi: 10.1038/bjc.2017.118

Osborn, M. J., Freeman, M., and Huennekens, F. M. (1958). Inhibition of dihydrofolic reductase by aminopterin and amethopterin. *Proc. Soc. Exp. Biol. Med.* 97, 429–431. doi: 10.3181/00379727-97-23764

Ou, Y., Wang, S. J., Jiang, L., Zheng, B., and Gu, W. (2015). p53 Protein-mediated regulation of phosphoglycerate dehydrogenase (PHGDH) is crucial for the apoptotic response upon serine starvation. *J. Biol. Chem.* 290, 457–466. doi: 10.1074/jbc.M114.616359

Pacold, M. E., Brimacombe, K. R., Chan, S. H., Rohde, J. M., Lewis, C. A., Swier, L. J., et al. (2016). A PHGDH inhibitor reveals coordination of serine synthesis and one-carbon unit fate. *Nat. Chem. Biol.* 12, 452–458. doi: 10.1038/nchembio.2070

Panieri, E., and Santoro, M. M. (2016). ROS homeostasis and metabolism: a dangerous liason in cancer cells. *Cell Death Dis.* 7:e2253. doi: 10.1038/cddis.2016.105

Possemato, R., Marks, K. M., Shaul, Y. D., Pacold, M. E., Kim, D., Birsoy, K., et al. (2011). Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. *Nature* 476, 346–350. doi: 10.1038/nature10350

Rowe, P. B., and Lewis, G. P. (1973). Mammalian folate metabolism. regulation of folate interconversion enzymes. *Biochemistry* 12, 1962–1969.

Samanta, D., Gilkes, D. M., Chaturvedi, P., Xiang, L., and Semenza, G. L. (2014). Hypoxia-inducible factors are required for chemotherapy resistance of breast cancer stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 111, E5429–E5438. doi: 10.1073/pnas.1421438111

Su, X., Wellen, K. E., and Rabinowitz, J. D. (2016). Metabolic control of methylation and acetylation. *Curr. Opin. Chem. Biol.* 30, 52–60. doi: 10.1016/j.cbpa.2015.10.030

Sun, L., Song, L., Wan, Q., Wu, G., Li, X., Wang, Y., et al. (2015). cMyc-mediated activation of serine biosynthesis pathway is critical for cancer progression under nutrient deprivation conditions. *Cell Res.* 25, 429–444. doi: 10.1038/cr.2015.33

Tedeschi, P. M., Markert, E. K., Gounder, M., Lin, H., Dvorzhinski, D., Dolfi, S. C., et al. (2013). Contribution of serine, folate and glycine metabolism to the ATP, NADPH and purine requirements of cancer cells. *Cell Death Dis.* 4:E877. doi: 10.1038/cddis.2013.393

Tibbetts, A. S., and Appling, D. R. (2010). Compartmentalization of Mammalian folate-mediated one-carbon metabolism. *Annu. Rev. Nutr.* 30, 57–81. doi: 10.1146/annurev.nutr.012809.104810

Wang, J., Alexander, P., Wu, L., Hammer, R., Cleaver, O., and McKnight, S. L. (2009). Dependence of mouse embryonic stem cells on threonine catabolism. *Science* 325, 435–439. doi: 10.1126/science.1173288

Yang, M., and Vousden, K. H. (2016). Serine and one-carbon metabolism in cancer. *Nat. Rev. Cancer* 16, 650–662. doi: 10.1038/nrc.2016.81

Ye, J., Fan, J., Venneti, S., Wan, Y. W., Pawel, B. R., Zhang, J., et al. (2014). Serine catabolism regulates mitochondrial redox control during hypoxia. *Cancer Discov.* 4, 1406–1417. doi: 10.1158/2159-8290.CD-14-0250

Ye, J., Mancuso, A., Tong, X., Ward, P. S., Fan, J., Rabinowitz, J. D., et al. (2012). Pyruvate kinase M2 promotes de novo serine synthesis to sustain mTORC1 activity and cell proliferation. *Proc. Natl. Acad. Sci. U.S.A.* 109, 6904–6909. doi: 10.1073/pnas.1204176109

Zhang, W. C., Shyh-Chang, N., Yang, H., Rai, A., Umashankar, S., Ma, S., et al. (2012). Glycine decarboxylase activity drives non-small cell lung cancer tumor-initiating cells and tumorigenesis. *Cell* 148, 259–272. doi: 10.1016/j.cell.2011.11.050

Zhao, H., French, J. B., Fang, Y., and Benkovic, S. J. (2013). The purinosome, a multi-protein complex involved in the de novo biosynthesis of purines in humans. *Chem. Commun.* 49, 4444–4452. doi: 10.1039/c3cc41437j

Zhou, X., He, L., Wu, C., Zhang, Y., Wu, X., and Yin, Y. (2017). Serine alleviates oxidative stress via supporting glutathione synthesis and methionine cycle in mice. *Mol. Nutri. Food Res.* 61:262. doi: 10.1002/mnfr.201700262

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