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Review of metabolic pathways activated in cancer cells as determined through isotopic labeling and network analysis

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Abstract

Cancer metabolism has emerged as an indispensable part of contemporary cancer research. During the past 10 years, the use of stable isotopic tracers and network analysis have unveiled a number of metabolic pathways activated in cancer cells. Here, we review such pathways along with the particular tracers and labeling observations that led to the discovery of their rewiring in cancer cells. The list of such pathways comprises the reductive metabolism of glutamine, altered glycolysis, serine and glycine metabolism, mutant isocitrate dehydrogenase (IDH) induced reprogramming and the onset of acetate metabolism. Additionally, we demonstrate the critical role of isotopic labeling and network analysis in identifying these pathways. The alterations described in this review do not constitute a complete list, and future research using these powerful tools is likely to discover other cancer-related pathways and new metabolic targets for cancer therapy.

Keywords

Cancer metabolism; isotopic labeling analysis

Introduction

Genetically, cancer is characterized by gain of functions of oncogenes and loss of functions of tumor suppressor genes (Hanahan & Weinberg, 2011). As dictated by such genetic modifications, cancer cells usually exhibit uncontrolled proliferation, meaning cell growth is independent of external stimulation or suppression. In order to fuel the continuous reproduction of cancer cells, a stable supply of anabolic building blocks, reducing equivalents and energy carriers is required (Vander Heiden et al., 2009) (Vander Heiden et al., 2011) (Locasale, 2013).

Cancer cells have evolved to acquire rewired enzymatic mechanisms to extract building blocks and energy from substrates. One of the major features of this reprogrammed cellular metabolism was first observed by Otto Warburg in early 20th century. The fact that cancer

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cells preferentially convert pyruvate to lactate from glucose even under aerobic conditions has been persistently perplexing to biologists (Warburg, 1956). Warburg referred to his observation as aerobic glycolysis, describing the inputs and outputs of the “black box” while leaving the underlying cellular mechanisms unresolved. Limited by the techniques available at the time, only hypotheses could be made. Warburg suspected that impaired mitochondrial function might be responsible for inducing aerobic glycolysis. Yet even at this time of writing, the Warburg effect is still not fully understood. Further, it was not until the beginning of the 21st century – and the use of isotopic tracers for the fine dissection of central carbon metabolic pathways, the development of molecular biology and recombinant DNA technology, and the maturation of systems-level approaches to study biology – that researchers began to explore the full ramifications of metabolic rewiring.

In contrast to Warburg’s analysis in which he treated glycolysis as an isolated system, modern researchers are able to dissect the pathway into different segments controlled by various glycolytic enzymes. Studies on many of these enzymes have generated a plethora of significant findings in the effort to elucidate the conundrum of the Warburg effect and topics beyond (Vander Heiden et al., 2010) (Christofk et al., 2008). In addition, focus has shifted beyond glucose to also include the extensive profiling of extracellular fluxes of different nutrients, amino acids such as glutamine, serine and glycine, as well as other unconventional substrates like acetate (Pacold et al., 2016) (Possemato et al., 2011) (Metallo et al., 2011) (Schug et al., 2015) (Mashimo et al., 2014) (Comerford et al., 2014). Finally, research on various aspects in cancer metabolism suggests the coordination of multiple metabolic rewiring phenomena. For instance, the cross-talk between glycolysis, serine metabolism, and the tricarboxylic acid cycle (TCA) anaplerosis suggest that the complexity of metabolic reprogramming extends beyond single pathways and is a multidimensional phenomenon (Vander Heiden et al., 2010) (Possemato et al., 2011).

Of great significance in the exploration of cancer metabolism is isotopic labeling and network analysis. To quantitatively characterize the metabolic phenotype of mammalian cells, ^{13}C isotopic labeling and metabolic flux analysis (MFA) are widely used (Sauer, 2006) (Hiller & Metallo, 2013) (Weindl et al., 2016). MFA is based on a comprehensive stoichiometric model of all biochemical reactions within a specific set of metabolic network. To decipher the internal structure of this network within a cell, the crosstalk between the cell and its environment has to be quantified. Cells exchange nutrients and other biochemical species with the environment. The influxes of nutrients and the effluxes of waste species or natural products are referred to as extracellular fluxes. Extracellular fluxes can be measured experimentally in a fairly straightforward manner. The intracellular fluxes can be estimated with a pseudo-steady-state assumption that the concentrations of all intracellular metabolites of interest remain unchanged during the course of flux analysis. The mass balance of each metabolite within the cell in conjunction with the quantified extracellular fluxes leads to a system of equations that can be solved to reflect the distribution of intracellular fluxes within cells. As one of the most widely used tools to fully specify the metabolic system, isotopic labeling becomes an essential technique to couple extracellular flux measurements in order to solve the flux values within a metabolic network. When cells are fed with isotopically labeled metabolites, the intracellular intermediates acquire specific labeling patterns through biochemical reactions that convert the labeled metabolites into those intermediates. The

reaction mechanisms dictate how labeled atoms get transferred into intracellular species, rendering a unique isotopic profile for each metabolite within the network. Throughout the research highlights listed in this paper, we have summarized multiple cases where isotopic labeling and network analysis have provided a robust platform to draw convincing conclusions.

The Warburg effect

Among all metabolic rewiring phenomena, the Warburg effect is the most commonly observed in cancer cells. The reasoning of the appearance of the Warburg effect is still being actively researched. Yet some hypotheses consistent with the traits and special needs of cancer cells have been proposed. One possible explanation is that the cost of synthesizing the TCA cycle enzymes may exceed the corresponding marginal gain of ATP through respiration, so that cells can extract more free energy when metabolizing substrates solely through glycolysis (Basan et al., 2015). Also, despite their active contribution to angiogenesis, cancer cells are subject to variations of oxygen supply under physiological conditions. In this case, aerobic glycolysis confers cancer cells with an internal resistance to such fluctuations since it functions invariantly under both hypoxic and normoxic conditions (Hsu & Sabatini, 2008). It is also possible that, besides decoupling glycolysis from the physiological oxygen level, aerobic glycolysis may also assist cancer cells to escape from apoptosis. One argument in support of this hypothesis is that decreased TCA flux and corresponding electron transport chain activity lead to attenuated depolarization of the mitochondrial membrane potential, inhibiting the release of the apoptosis effector cytochrome c (Bonnet et al., 2007). Without mitochondrial release of cytochrome c, the apoptosome cannot be assembled and apoptosis is repressed (Riedl & Salvesen, 2007). Metabolically, it has been proposed that elevated glucose consumption induced by the Warburg effect could promote anabolic reactions in serine biosynthesis, pentose phosphate pathway (PPP), *de novo* lipogenesis, one-carbon metabolism (Liberti & Locasale, 2016), etc. In addition, the Warburg effect is believed to be capable of modulating cell signaling in favor of tumorigenesis through reactive oxygen species (ROS), histone acetylation and deacetylation (Liberti & Locasale, 2016). Finally, others have proposed that lactate, the product of aerobic glycolysis may be responsible for resistance to chemotherapies (Fang et al., 2006). Increased lactate in the microenvironment can upregulate several transporters and enzymes that are critical for toxin clearance in cancer cells (Hsu & Sabatini, 2008).

With the rapid progress of recent research, more metabolic rewiring phenomena have been observed, and the diversity and sophistication of cancer metabolism research have exceeded the centrality of the Warburg effect. An overview of our current understanding of the metabolic network critical in cancer metabolism, as described in this manuscript, is illustrated in Figure 1.

Metabolic alterations along glycolysis

High expression of the M2 isoform of pyruvate kinase (PKM2) has been observed in cancerous and healthy proliferative cells. This preferential selective expression of PKM2 is also believed to be essential for the Warburg effect (Christofk et al., 2008). PKM2 exhibits

reduced catalytic activity compared to PKM1, but surprisingly, the elevation of glycolytic flux is associated with this “weaker” isoform. Contrary to the conventional view of a one-directional linear glycolytic pathway, it has been hypothesized that there is an alternative glycolytic step in which the generation of ATP may be decoupled from pyruvate kinase (PK) activity. In this model, phosphoenolpyruvate (PEP) transfers its phosphate group to the enzyme phosphoglycerate mutase 1 (PGAM1), generating pyruvate independent of PK (Vander Heiden et al., 2010). Because phosphorylation of PGAM1 is required for its activation, this process may further promote the production of PEP, constituting a positive feedback loop which further increases overall glycolytic flux (Fothergill-Gilmore & Watson, 1990). In addition, this feedback loop may also increase the ratio of 3-phosphoglycerate (3PG) to 2-phosphoglycerate (2PG), which promotes serine biosynthesis pathway that branches at 3PG (de Koning et al., 2003) (Vander Heiden et al., 2011). A schematic of this alternative glycolytic pathway is illustrated in Figure 2.

Additionally, the M2 isoform of PK is inhibited by tyrosine-phosphorylated proteins such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR). This inhibition is hypothesized to increase the accumulation of PEP and 3PG (Hitosugi et al., 2010) (Vander Heiden et al., 2010) (Christofk et al., 2008). This bottleneck may then subsequently lead to elevated anabolic metabolism through *de novo* serine synthesis. The alternative pathway may be preferential for sustaining a high rate of glycolysis since ATP allosterically inhibits phosphofructokinase (PFK) at a high ATP/AMP ratio (Vander Heiden et al., 2010).

Similar to the PKM2-mediated glycolytic pathway in the context of serine biosynthesis, cancer cells must also coordinate their metabolic network to generate nucleotide precursors in support of fast proliferation. To this end, it has been demonstrated that some tumor cells are able to upregulate glucose uptake and enhance non-oxidative PPP due to the presence of KRAS oncogene (Ying et al., 2012). In fact, it has also been proposed that cancer cells could promote the synthesis of ribose 5-phosphate (R5P) through non-oxidative PPP (Ahn et al., n.d.). The enhanced reverse flux along glycolysis is mediated by the aldolase and transaldolase reactions.

It was observed that the magnitude of oxidative PPP flux (relative to glycolytic flux) is reduced under hypoxia. On the other hand, as an alternative pathway to supply cancer cells with nucleotide biosynthetic precursors, the non-oxidative PPP increases to produce more R5P. Mechanistically, the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) plays a critical role in this metabolic shift. Hypoxia-mediated reduction of the NAD^+/NADH ratio results in a decrease in GAPDH activity. In contrast, the expression of the other lower glycolytic enzymes is elevated upon stabilization of hypoxia-inducible factors. As a result, GAPDH creates a bottleneck at the lower glycolytic pathway, enabling accumulation of high concentrations of upper glycolytic metabolites, which in turn increase the reversible non-oxidative PPP reactions toward nucleotide biosynthesis via mass action. This is shown in Figure 3 (Ahn et al., n.d.).

In fact, GAPDH plays a critical role in balancing nucleotide biosynthesis via PPP and energy production via the TCA cycle; as such, modulation of GAPDH activity alters the

viability of cancer cells. This is supported by a recent report that the uptake of dehydroascorbate (DHA), the oxidized form of vitamin C, is able to inhibit GAPDH and lead to energy crisis in colorectal cancer (CRC) cells containing mutations in KRAS and BRAF. The CRC cells similarly exhibited high rates of glycolysis and the non-oxidative PPP. The “bottleneck” role of GAPDH was shown to be vital in this trade-off between ATP generation and nucleotide biosynthesis. The inhibition caused by DHA increased oxidative PPP flux at the expense of the pyruvate entry into TCA cycle, depriving cancer cells of robust energy support as evidenced by an elevated AMP/ATP ratio (Yun et al., 2015).

To quantitatively derive the aforementioned conclusions, the isotopic labeling tracer 1,2-¹³C₂-glucose was employed. Entry of this tracer into the oxidative PPP results in a loss of the carbon in the 1-position as ¹³CO₂, which contributes triose phosphates that contain one fewer ¹³C label than those that bypass the PPP via upper glycolytic reactions. Through comparison of the M2/M1 isotopomer ratio of these metabolites, the relative magnitude of glycolysis (Figure 4a) and oxidative PPP (Figure 4b) can be estimated. Similarly, the oxidative PPP also yields one fewer ¹³C labeled carbon in the ribose products than the non-oxidative PPP following nucleotide biosynthesis, so the M1/M2 ratio of the RNA-derived ribose can be used to compare the relative contributions of the oxidative and non-oxidative PPPs (Ahn et al., n.d.).

Reductive metabolism of glutamine

The Warburg effect is associated with significant inefficiency of substrate utilization, as predominant conversion to lactate results in a smaller fraction of glucose-derived carbon skeletons used for anabolism or high-yield ATP production via respiration. Considering the fact that cancer cells require a robust supply of energy and anabolic precursors, alternative pathways must be activated to compensate for the reduced supply from aerobic glycolysis. Reductive metabolism of glutamine is one of the metabolic reprogramming switches used to achieve this objective.

Glutamine has a special significance in central carbon metabolism since it is the second most consumed carbon source following glucose in some cells and the most abundant amino acid in serum (Smith, 1990). Previously, it was discovered that a reductive metabolic pathway from glutamine exists in adipocytes and is responsible for a substantial *de novo* fatty acid synthesis (Yoo et al., 2008). It was further demonstrated that this reductive pathway was active in numerous cancer cell lines (Metallo et al., 2011). Extracellular glutamine can be internalized through cytoplasmic glutamine transporters. Following the conversion of glutamine to glutamate via glutaminase, glutamate can be transformed to α-ketoglutarate via transamination reactions. The key step then takes place as α-ketoglutarate is carboxylated into isocitrate with the condensation of one molecule of carbon dioxide. This reaction is catalyzed by isocitrate dehydrogenases (IDHs) and is NADP⁺ dependent (Metallo et al., 2011) (Wise et al., 2011) (Mullen et al., 2012). Further research demonstrates that this behavior is associated with elevation of the α-ketoglutarate to citrate ratio (Fendt et al., 2013) (Gameiro et al., 2013). The citrate molecules produced through this mechanism can be cleaved in the cytosol into oxaloacetate and acetyl-CoA in the presence of the enzyme ATP citrate lyase. Acetyl-CoA is then ready to be recruited for fatty acid synthesis. It is

worth mentioning that, however, some labeling experiments on fatty acid from glutamine tracers suggest exchange flux rather than net reductive metabolism of glutamine (Fan et al., 2013). Still, cancer and proliferating cells are capable of switching their carbon source from glucose to glutamine for lipogenesis especially under hypoxic conditions. As the amount of building blocks for fatty acids decreases from the upstream glycolytic pathway, cells use an alternative substrate to replenish the need for biosynthesis, exhibiting a unique way of metabolic rewiring. Although many of these findings are based on tissue culture experiments, which usually artificially increases the concentration of glutamine, we anticipate that the physiological relevance of this phenomenon still holds, as suggested by comparable results obtained through *in vivo* labeling experiments (Gameiro et al., 2013). Indeed, glutamine consumption rate is persistently high in some cancer cell lines (Wise & Thompson, 2011) (Hensley et al., 2013), and the so-called glutamine addiction reflects the anabolic significance of the aforementioned reductive metabolism.

Isotopic labeling and network analysis contributed to this work, as both 1-¹³C- and U-¹³C₅-glutamine provided quantitative information about the magnitude of reductive carboxylation (Zhang et al., 2014). In the case of 1-¹³C-glutamine, the only heavily labeled carbon corresponds to the carboxyl group, which is lost as CO₂ during the oxidative decarboxylation reaction in the TCA cycle. Only through the reductive pathway is the ¹³C labeled carbon retained in the intracellular pools of citrate, oxaloacetate, malate, fumarate and aspartate. Alternatively, similar information can be inferred from the uniformly labeled tracer U-¹³C₅-glutamine. All the ¹³C labeled carbon atoms are retained through the reductive pathway after the condensation of unlabeled carbon dioxide at the carboxylation step (Figure 5a). However, through oxidative reactions along the TCA cycle, the ¹³C labeled carbon atoms are successively lost as carbon dioxide, leading to reduced labeling patterns for all TCA metabolites (Figure 5b and 5c) (Metallo et al., 2011). On a relative basis, the ratio of oxidative TCA flux to reductive carboxylation flux can be compared by evaluating the M4/M5 ratio of citrate under the condition of strong glutamine influx. In addition, the combination of the labeling results from 5-¹³C- and U-¹³C₅-glutamine enables estimation of the fraction of lipids generated through the carboxylation mechanism. The acetyl-CoA molecules generated by these two glutamine tracers yield different labeling patterns during *de novo* lipogenesis. U-¹³C₅-glutamine produces fully labeled acetyl-CoA whereas only one carbon atom per molecule of acetyl-CoA is labeled following the addition of 5-¹³C-glutamine (Metallo et al., 2011). These differential labeling results form the basis for quantification of the reductive flux from glutamine.

Isocitrate dehydrogenase (IDH) mutations

IDHs are normally responsible for the interconversion between isocitrate and α-ketoglutarate. There are three isoforms of IDH enzymes. Mutations in cytosolic isocitrate dehydrogenase (IDH1) and mitochondrial isocitrate dehydrogenase (IDH2) are observed in glioblastomas and leukemias (Mi et al., 2009) (Cohen et al., 2013) (Mardis et al., 2009). The mutations are usually heterozygous with only one allele of normal IDH exchanged for its oncogenic version.

Since these IDH enzymes participate in cellular functions in the form of heterodimers, earlier analyses have focused on the dominant negative effect of these heterozygous mutations on the metabolic balance between isocitrate and α -ketoglutarate (Zhao et al., 2009) (Ward et al., 2010). However, recent studies revealed another critical metabolic alteration due to IDH mutations: The intracellular level of 2-hydroxyglutarate increases upon expression of R132 or R172 mutants of IDH1 and IDH2, respectively (Dang et al., 2009) (Ward et al., 2010). The oxidative direction of IDH enzymes is concomitant with NADPH production. Consistent with this mechanism, both IDH1 and IDH2 mutants result in significant reduction of the rate of NADPH formation in the presence of isocitrate. Interestingly, the consumption rate of NADPH increased when the starting substrate was exchanged to α -ketoglutarate which can be reductively transformed to 2-hydroxyglutarate via mutated IDH enzymes. Since the production of 2-hydroxyglutarate is correlated with brain and blood tumor cells harboring IDH mutations, it is proposed that 2-hydroxyglutarate functions as a oncometabolite (Ward et al., 2010). It is also believed that accumulation of 2-hydroxyglutarate can elevate the level of intracellular ROS (Latini et al., 2003) (Kölker et al., 2002). Cellular redox homeostasis is further disrupted since the formation of ROS can aggravate the effect of reduced NADPH production due to decreased flux from isocitrate to α -ketoglutarate. In addition, 2-hydroxyglutarate is able to impede histone methylation, which plays an essential role in the temporal regulation of gene expression (Lu et al., 2012). The delay of cell differentiation resulting from the accumulation of 2-hydroxyglutarate enables fast proliferation of undifferentiated progenitor cells, which promotes tumorigenesis of gliomas and leukemias (Ward et al., 2010).

So far, the metabolic rewiring due to IDH mutations is unique in the sense that it creates an accumulation of a single metabolite 2-hydroxyglutarate, which triggers cascades of events stimulating tumorigenesis. However, IDH mutations also lead to redistribution of substrates into different metabolic pathways, exhibiting another level of metabolic reprogramming. As discussed previously, the reductive carboxylation from α -ketoglutarate to isocitrate is catalyzed by IDH1 (Metallo et al., 2011). IDH1 mutations have been demonstrated to hinder the reductive metabolism in glutamine, leading to reduced proliferation under hypoxia and electron transport chain inhibition (Grassian et al., 2014). Furthermore, the isotopic labeling experiment using U- $^{13}\text{C}_5$ - glutamine shows a reduction of the M5/M4 ratio for citrate in cells treated with IDH2 siRNAs, supporting the possibility that IDH2 may be essential for mitochondrial reductive carboxylation (Ward et al., 2010). These data collectively show that IDH mutations can also inhibit proliferation through suppression of reductive carboxylation reactions under certain contexts.

Serine and glycine metabolism

One of the major categories of chemotherapeutic drugs is antifolates, which target one-carbon metabolism, a complex metabolic network involving folate and methionine cycles (Farber et al., 1948). Serine and glycine metabolism links one-carbon and central carbon metabolism, which has led to its emergence as a significant topic of study. In addition to being imported from the extracellular environment, serine can also be synthesized *de novo* in a linear pathway (Figure 6). The glycolytic intermediate 3PG can be transformed to 3-phosphohydroxypyruvate (P-pyruvate) via the enzyme phosphoglycerate dehydrogenase

(PHGDH) in conjunction with NAD^+ (Deberardinis, 2011). P-pyruvate is converted to phosphoserine via the transamination reaction catalyzed by phosphoserine aminotransferase. The enzyme phosphoserine phosphatase removes the phosphate group and yields serine (Pacold et al., 2016).

The conversion between serine and glycine is reversibly catalyzed by serine hydroxymethyl transferases (SHMTs). Glycine is produced along with cytosolic 5,10-methylenetetrahydrofolate (5,10- CH_2 -THF) from serine, supplying methyl groups for DNA methylation and nucleotide biosynthesis (Amelio et al., 2014). In addition, glycine is an essential precursor for glutathione, an important antioxidant protecting cellular machinery from ROS (Ruiz-Ramírez et al., 2014).

Copy number amplification of the PHGDH gene has been observed in breast cancers and melanomas (Locasale et al., 2011). The tumorigenicity of PHGDH also brings attention since it is likely to promote metastasis in mouse models (Pollari et al., 2011). PHGDH suppression does not affect intracellular serine levels, but it decreases the levels of α -ketoglutarate, presumably since the serine biosynthetic pathway produces equimolar amounts of serine and α -ketoglutarate via P-pyruvate transamination. In cells with high PHGDH expression, about half of the anaplerotic flux from glutamine has been attributed to the serine synthesis pathway (Possemato et al., 2011). Through a series of metabolic and transcriptional studies, researchers have demonstrated that the regulation of PHGDH and other key enzymes in the serine biosynthetic pathway is achieved by the transcription factor nuclear factor erythroid-2-related factor 2 (NRF) in non-small cell lung cancer (DeNicola et al., 2015).

In addition to likely facilitating TCA anaplerosis, serine and glycine synthesis also regulates nucleotide biosynthesis via critical modulations on one-carbon metabolism. Cytosolic 5,10- CH_2 -THF can be combined with glycine to form serine by the catalysis of cytosolic serine hydroxymethyl transferase (SHMT1) (Renwick et al., 1998) (Garrow et al., 1993). However, 5,10- CH_2 -THF is also essential for nucleotide biosynthesis. In addition to synthesizing serine intracellularly, the serine pathway is also responsible for inhibiting SHMT1 activity in order to push one-carbon metabolites toward the direction of nucleotide synthesis. These hypotheses may help explain the observation that nucleotide synthesis from glucose-derived and extracellular serine is attenuated under PHGDH inhibition (Pacold et al., 2016). Furthermore, metabolic reprogramming also takes place inside mitochondria. The mitochondrial serine hydroxymethyl transferase (SHMT2) is believed to be responsible for the defense mechanism against hypoxic stress. Another mitochondrial enzyme ALDH1L2 which generates NADPH at the expense of consuming one-carbon metabolites could protect cells against oxidative stress. The upregulation of these enzymes imparts cancer cells with enhanced antioxidant defense (Ducker & Rabinowitz, 2016).

Isotopic labeling plays an important role in this analysis. The amount of glucose-derived serine and glycine can be quantified through examining their M3 and M2 labeling fractions, respectively, in cells treated with $\text{U-}^{13}\text{C}_6$ -glucose (Pacold et al., 2016). By following the biochemical pathways that produce dTMP and AMP, rates of nucleotide biosynthesis can also be determined. In addition, the striking discovery that the serine synthesis pathway

inhibits one-carbon metabolism is also corroborated by isotopic labeling experiments. In the presence of exogenous uniformly labeled serine, the labeling fractions of AMP and dTMP decrease in their M1-4 and M1 pools, respectively (Pacold et al., 2016).

Acetate metabolism and alternative sources of acetyl-CoA

Mammalian cell metabolism has been primarily focused on glucose and glutamine, presumably due to their high rates of consumption (Hosios & Vander Heiden, 2014). Recently, various research work has shown that some unconventional metabolites could be utilized as carbon sources in certain types of cancer cells. For instance, alanine could replenish the TCA cycle in pancreatic stellate cells in the context of pancreatic ductal adenocarcinoma (Sousa et al., 2016). In addition, the tumor cells harboring non-small cell lung cancer is believed to be capable of consuming lactate as nutrient to fuel cell metabolism (Hensley et al., 2016). In the realm of microbial cell metabolism, acetate has been demonstrated to be a robust substrate for normal cell growth and lipid production (Smith & Mah, 1980) (Barbosa et al., 2001) (Liu et al., 2016). Recently, it was discovered that acetate is able to substitute, at least partially, for the roles of glucose and glutamine to fuel cancer cell metabolism under hypoxia, particularly for *de novo* lipogenesis (Schug et al., 2015).

Under normoxic conditions, the majority of cellular acetyl-CoA is derived from glucose or glutamine. In contrast, the total contribution from glucose and glutamine to acetyl-CoA decreases significantly under hypoxia (Kamphorst et al., 2013), whereas acetate at physiological levels may contribute up to 20%–50% of the acetyl-CoA pool (Kamphorst et al., 2014). In another independent parallel experiment, the isotopic labeling analysis in tumors from patients with brain cancer showed that more than half of the acetyl-CoA pool is made from sources other than glucose (Maher et al., 2012). The anaplerotic flux of acetyl-CoA synthesis from glutamine has been shown to be minimal in mouse brain tumors (Mashimo et al., 2014). The key enzyme that appears to control this metabolic rewiring is acetyl-CoA synthetase 2 (ACSS2) (Comerford et al., 2014) (Schug et al., 2015). Although physiological levels of acetate exhibit great variability (50–600 μ M), the acetate concentration in the blood stream could reach millimolar level in chronic alcoholics (Hosios & Vander Heiden, 2014) (Nuutinen et al., 1985) (Korri et al., 1985). From another perspective, *in vivo* recycling and the circulation system may function in a way to recapture acetate released from protein deacetylation (Lyssiotis & Cantley, 2014), suggesting the physiological significance of acetate metabolism. We expect tissues where such recapturing events are active to exhibit even more dysregulated acetate metabolism, which may potentially be validated through additional *in vivo* labeling work.

Acetate may also serve as a critical metabolite interconnecting glycolysis, the pentose phosphate pathway and *de novo* lipogenesis. Recent work suggests that the enzyme transketolase-like 1 (TKTL1) may resemble its bacterial counterpart phosphoketolase (Heath et al., 1958) (Schramm et al., 1958) in terms of its capability of cleaving xylulose-5-phosphate into GAP and acetate (Diaz-Moralli et al., 2016). GAP is diverted back to glycolysis while acetate can be utilized to replenish acetyl-CoA for lipid synthesis. Thus, in addition to being supplied through reductive glutamine metabolism and exogenous acetate, acetyl-CoA may be produced from TKTL1-mediated cleavage in cancer cells. Collectively,

these sources may represent alternative means of replenishing acetyl-CoA for *de novo* lipogenesis despite reduced production due to the Warburg effect.

The above results again are derived from various types of isotopic labeling experiments. For the general study of acetate metabolism, the percentage labeling of acetyl-CoA from U-¹³C₆-glucose or U-¹³C₅- glutamine was shown to dominate over other sources under normoxia. The percentage labeling of acetyl-CoA from labeled amino acids were also shown to constitute a minor portion under normoxia. However, the contributions from glucose and glutamine were demonstrated to decrease significantly under hypoxia. With these quantitative results, the authors concluded that acetate is the dominant source of labeling for acetyl-CoA under hypoxic conditions (Kamphorst et al., 2014) (Mashimo et al., 2014). In addition, the TKTL1 activity was suggested through a 1,2-¹³C₂-glucose labeling experiment in which the labeling pattern of lactate was altered following genetic knockdown in a manner consistent with the presence of the cleavage reaction catalyzed by TKTL1 (Diaz-Moralli et al., 2016).

Oncogene-specific metabolic rewiring

Decades of modern cancer research have identified many genes that promote tumorigenesis. With contemporary knowledge of cell biology, the mechanisms surrounding protein regulation have been further elucidated. Recent efforts to consolidate our understanding on cellular functions of oncogenes and various metabolic alterations have shed light on links between metabolism and the genetic origins of cancer.

One of the most extensively studied oncogenes is KRAS. It is involved in the mitogen-activated protein (MAP) kinase pathway, functioning as a messenger to deliver proliferative signals to downstream targets (Dhillon et al., 2007). Upon stimulation from upstream receptor tyrosine kinases (RTKs), the RAS-GDP complex is switched to its activated RAS-GTP form, which subsequently can activate downstream MAP kinases and promote cellular growth (Pearson et al., 2001). The mutant KRAS oncogene encodes the RAS protein which stays at its activated GTP-bound form, thus constitutively transducing growth signals and conferring cells with uncontrolled proliferation (Bos, 1989). In order to sustain deregulated doubling of cellular contents, KRAS promotes glucose consumption as well as glutamine utilization (Gaglio et al., 2011) (Son et al., 2013). Although the oxidative TCA cycle flux is reduced upon mutant KRAS induction, the glycolytic flux together with various anabolic fluxes extracting nitrogen from glutamine are elevated. It has been proposed that upon KRAS transformation, cells adapt themselves to rely on glucose for energy support and glutamine for the supply of anabolic building blocks (Gaglio et al., 2011). Further research has elucidated the primary metabolic route of glutamine-derived glutamate in KRAS-reprogrammed pancreatic ductal adenocarcinoma cells. Instead of converting glutamate to α -ketoglutarate via deamination through glutamate dehydrogenase, the KRAS mutation has been shown to favor the transamination reaction through which aspartate is produced. Mitochondrial aspartate may be then transported into the cytosol after which NADPH is generated through subsequent reactions. This altered metabolism is believed to be beneficial to maintain the redox status conducive for deregulated proliferation (Son et al., 2013). Concomitantly, anabolism can be further reinforced through increased flux along the non-

oxidative PPP to generate precursors for nucleotide biosynthesis in pancreatic ductal adenocarcinoma cells (Ying et al., 2012).

In addition to KRAS, the oncogene c-Myc also promotes deregulated proliferation. As an example of the intricacy of cell signaling pathways, the KRAS-mediated MAP kinase pathway also stabilizes c-Myc protein and thus further enhances growth signals (Sears et al., 2000). Metabolically, c-Myc has been demonstrated to also support aerobic glycolysis (Dang et al., 2008). Outside of the centrality of the Warburg effect, researchers have shown that c-Myc also plays a critical role in altered glutamine metabolism in cancer cells. As a transcription factor, c-Myc inhibits the expression of microRNAs which downregulate mitochondrial glutaminase responsible for converting glutamine to glutamate (Gao et al., 2009). As mentioned previously, glutaminase catalyzes this crucial catabolic reaction, which fuels cancer cells with anaplerotic flux from glutamine. As the Warburg effect directs a significant portion of glucose toward lactate, anaplerosis from glutamine may hold immense significance in maintaining an ample supply of energy, anabolic building blocks and reducing equivalents. Furthermore, c-Myc is also capable of replenishing glutamine through epigenetic modulations of *de novo* glutamine synthesis in many cell lines. DNA demethylation can be triggered by c-Myc to upregulate the expression of glutamine synthetase to facilitate the production of glutamine from glutamate and ammonia (Bott et al., 2015). Moreover, c-Myc is believed to be able to suppress proline synthesis, which potentially functions as a tumor suppressing pathway, strengthening the role of c-Myc as a potent oncogene (Liu et al., 2012) (Polyak et al., 1997).

Concluding remarks

We reviewed recently elucidated metabolic pathways in cancer cells, as determined by stable isotopic labeling and network analysis. The detailed metabolic network illustrating this is shown in Figure 7. The compendium of our knowledge of cancer metabolism has passed its infancy, owing to our improved ability to resolve metabolic activity at the molecular and enzymatic level, the diversity of substrates and the coordination of different metabolic pathways. Research on the effect of enzymatic activities (Vander Heiden et al., 2010) (Mullen et al., 2012) (Grassian et al., 2014) (Pacold et al., 2016), mass action kinetics (Fendt et al., 2013) and cofactor ratios on biochemical reaction systems (Ahn et al., n.d.) has strengthened our fundamental understanding of this field. Studies on the use of unconventional substrates may have brought us closer to approximating the physiological environment that nurtures cancer cells. The interconnection of pathways imparts us with a global systematic view of cancer metabolism. The recent progress made in this field also contributes to the joint effort to find the causalities and connections between signaling pathways and metabolic behaviors. Taken together, such advancement will hopefully direct us toward the discovery of innovative therapeutic targets for cancer treatment.

The pathways described above do not provide an absolutely exhaustive picture of metabolic rewiring in cancer cells, and it is likely that additional pathways that we have not yet identified are activated during tumorigenesis. In this effort, we believe that isotopic labeling using stable ^{13}C -, ^2H -, ^{18}O -, ^{15}N - and ^{35}S -based tracers will facilitate the discovery and investigation of these metabolic alterations.

One area largely underdeveloped in this field is the study of metabolic reprogramming inside cellular compartments such as mitochondria. The main reason for this is the limited capability to dissect metabolic pathways with the current techniques. We expect this to be a promising area as new methods are being developed to probe the intricacy of sub-cellular metabolic activities. One excellent example is the ^2H -isotopic labeling work performed to resolve the NADPH metabolism in different cellular compartments (Lewis et al., 2014). Other alternative approaches such as organelle isolation and coupled database analysis also demonstrate great potential to investigate compartmentalized metabolism (Chen et al., 2016).

In addition, recent research on amino acid metabolism has increasingly demonstrated an important role of substrates other than glucose and glutamine. Quantification of the composition of cells argues that amino acids contributed to the majority of the cell mass in proliferating mammalian cells. Although the consumption rates of glucose and glutamine are usually the highest, it is amino acids that eventually provide most of the anabolic building blocks for fast growing cells (Hosios et al., 2016). Aside from the significance of glutamine, serine and glycine metabolism, it was discovered that the amino acid aspartate also plays a vital role in proliferating cells. Aspartate production requires the supply of electron acceptors to be fully functional. Electron acceptors can be provided by compounds such as pyruvate or α -ketobutyrate to rescue growth when the electron transport chain is compromised. The work demonstrates the dependence of proliferation on aspartate synthesis and its connection with electron transport chain (Sullivan et al., 2015) (Birsoy et al., 2015).

Another emerging area is *in vivo* labeling analysis. While most previous work has been conducted in cultured cells, researchers have begun to investigate metabolic behaviors subject to different physiological conditions. Studies on lung cancer have demonstrated that the tumor microenvironment exerts discernible effects on the metabolic phenotypes of cells, especially with respect to glutamine metabolism (Davidson et al., 2016). In addition, the heterogeneity in tumor tissues has been shown to confer cells with altered substrate utilization preference, such as the onset of lactate metabolism, in non-small cell lung cancer (Hensley et al., 2016).

The value of the research we reviewed stems not only from the overarching objective to elucidate basic cancer biochemistry, but also the profound implications for cancer therapy. Novel pharmaceutical targets are being identified concomitant with the theoretical development in cancer metabolism. This was recently reviewed in an article highlighting the clinical effort to implement IDH inhibitors in cancer treatment (Garber, 2016). We believe that the combination of glucose and glutamine targeting, potentially in conjunction with restricting the supply of other substrates, may be able to enhance the effectiveness of the overall chemotherapy.

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Highlights

- Key metabolic rewiring phenomena in cancer cells are reviewed.
- Isotopic labeling and network analysis proves to be a powerful tool for deciphering metabolic reprogramming.
- Research in rewired metabolism provides new therapeutic targets for cancer treatment.

Figure 1. A global overview of the metabolic network critical in cancer cells

Glucose is the most highly consumed carbon substrate, and its entry initiates glycolysis, which contains several anabolic arms. The pentose phosphate pathway (PPP) directs glucose-derived glucose-6-phosphate (G6P) toward oxidative PPP where NADPH is generated. From ribulose-5-phosphate (Ru5P), the non-oxidative PPP continues, supplying building blocks for nucleotide biosynthesis and reversibly exchanging metabolites with glycolysis via fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (GAP). Serine and glycine metabolism branches from 3-phosphoglycerate (3PG) and connects central carbon metabolism with one-carbon metabolism. The end product pyruvate can be converted to lactate or can enter the TCA cycle in the form of acetyl-CoA, which can also be derived from acetate under certain conditions. Glutamine is metabolized to glutamate and then α -ketoglutarate, which can enter the TCA cycle for replenishing anaplerosis or be reductively metabolized via a carboxylation reaction to fuel de novo lipogenesis. Various pathways are altered to sustain the uncontrolled proliferation exhibited by cancer cells. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; GAP, glyceraldehyde-3-phosphate; 3PG, 3-phosphoglycerate; R5P, ribose-5-phosphate; Akg, α -ketoglutarate; Cit, citrate; Ac-CoA, acetyl-CoA; Oxa, oxaloacetate.

Figure 2. The PKM2-mediated glycolytic pathway

The M2 isoform of pyruvate kinase (PKM2), which is preferentially expressed by cancer cells, has been hypothesized to promote an alternative catalytic activity that does not produce ATP, in contrast to PKM1. This potential mechanism would likely contribute to continual activation of phosphofructokinase (PFK), which is inhibited by ATP. Consequently, glycolytic flux may be enhanced. This alternative mechanism involves transfer of the phosphate group from phosphoenolpyruvate (PEP) to phosphoglycerate mutase 1 (PGAM1), which activates it. This positive feedback mechanism may drive the enhanced production of 2-phosphoglycerate (2PG), PEP and eventually enhance the overall PGAM1 activity. Through this mechanism, the generation of pyruvate may become partially decoupled from ATP production, which could enable a high rate of glycolysis. Activation and inhibition of enzyme activity are denoted by arrows and blunt arrows, respectively. The relative strengths of metabolic pathways and activation/inhibition effects are shown by the thickness of the arrows.

Figure 3. The GAPDH-mediated reversal of glycolytic flux

Under hypoxic condition, GAPDH activity is inhibited while the expression of other upper glycolytic enzymes is elevated. Due to the accumulation of high concentrations of upper glycolytic metabolites and the reversibility of the non-oxidative PPP, more ribose-5-phosphate (R5P) is synthesized to promote nucleotide biosynthesis. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; 1,3-BPG, 1,3-bisphosphoglycerate. The relative strengths of metabolic pathways and activation/inhibition effects are shown by the thickness of the arrows. The figure is adapted from Ahn et al. (under review).

Figure 4a

Figure 4b

Figure 4. The isotopic labeling pattern generated by 1,2-¹³C₂-glucose

(a) Following exclusive use of the glycolytic pathway, the M2-labeled pattern retains until fructose-1,6-bisphosphate (FBP). The aldolase-catalyzed cleavage reaction produces M2-labeled dihydroxyacetone (DHAP) and unlabeled glyceraldehyde-3-phosphate (GAP). The

labeling pattern is eventually inherited by pyruvate, yielding an equimolar mixture of M2-labeled and unlabeled pyruvate. **(b)** This labeling pattern can be best explained by analyzing three molecules of 1,2-¹³C₂-glucose going through the oxidative PPP. The first step in glycolysis transforms all 1,2-¹³C₂-glucose into M2-labeled glucose-6-phosphate (G6P). The oxidative PPP releases one molecule of heavy labeled carbon dioxide per molecule of 1,2-¹³C₂ G6P. The resulting three molecules of M1-labeled ribulose-5-phosphate (Ru5P) have equal chances of going through three distinct biochemical reactions. The transketolation and transaldolation reactions lead to one molecule of M2-labeled fructose-6-phosphate (F6P), one molecule of M1-labeled F6P, and one molecule of unlabeled glyceraldehyde-3-phosphate (GAP). These metabolite intermediates are then diverted back to the glycolytic pathway. One molecule of M2-labeled G6P generates equimolar mixture of M2-labeled and unlabeled pyruvate. Similarly, one molecule of M1-labeled G6P generates an equimolar mixture of M1-labeled and unlabeled pyruvate. The unlabeled GAP molecule yields one molecule of unlabeled pyruvate. Therefore, theoretically, if all 1,2-¹³C₂-glucose goes through the oxidative PPP exclusively, there would be 20% of M2-labeled pyruvate, 20% of M1-labeled pyruvate and 60% of unlabeled pyruvate.

Figure 5a

Figure 5b

Figure 5c

Figure 5. The isotopic labeling pattern generated by U-¹³C₅- glutamine

(a) The isotopically labeled U- $^{13}\text{C}_5$ - glutamine is converted to glutamate via glutaminase. Transamination or deamination reactions then take place to transform glutamate to α -ketoglutarate. M5-labeled citrate is formed in the mitochondria and cytosol through reductive carboxylation by isocitrate dehydrogenases (IDHs). This citrate is cleaved into oxaloacetate and acetyl-CoA, and this heavy labeled acetyl-CoA is employed for de novo lipogenesis. The reaction network in cytosol further generates M3-labeled oxaloacetate, aspartate, malate and fumarate. The figure is adapted from Metallo et al. (2011). (b) In the first cycle of oxidative metabolism of U- $^{13}\text{C}_5$ - glutamine, the uniform labeling pattern no longer holds at citrate, which is generated by consolidating unlabeled acetyl-CoA with M4 labeled oxaloacetate. The beginning of the second cycle primes at M3-labeled α -ketoglutarate (in the blue box) after the release of a heavy labeled carbon dioxide. When there is a strong influx of glutamine, the labeling pattern of the first cycle dominates. (c) Starting from the M3 labeled α -ketoglutarate (in the blue box), the release of another heavy labeled carbon dioxide further reduces the labeling pattern to generate M2-labeled succinate and fumarate. Due to the molecular asymmetry of malate and oxaloacetate, two different forms of M2-labeled compounds are generated in equimolar ratio. After the consolidation of unlabeled acetyl-CoA and M2-labeled oxaloacetate, M2-labeled citrate is generated. The labeling pattern of the second cycle is less important when there is a strong glutamine influx.

Figure 6. Serine and glycine metabolism

The de novo serine biosynthesis pathway branches from glycolysis at 3-phosphoglycerate (3PG). The enzyme phosphoglycerate dehydrogenase (PHGDH) oxidizes 3PG to phosphohydroxypyruvate (P-pyruvate) in the presence of cofactor NAD⁺. P-pyruvate is then converted to phosphoserine (P-ser) by phosphoserine aminotransferase 1 (PSAT1). Serine is eventually synthesized after the last step of dephosphorylation by phosphoserine phosphatase (PSPH). Glycine metabolism is linked to serine metabolism through serine hydroxymethyltransferase (SHMT). This reversible reaction is critical in bridging glycolytic substrates with compounds in one-carbon metabolism. The figure is adapted from Possemato et al. (2011) and Pacold et al. (2016).

Figure 7. Detailed summary of rewired metabolic pathways in cancer cells

Metabolic reprogramming along glycolysis, reductive carboxylation of glutamine, serine and glycine metabolism, IDH mutations and TKTL1-mediated acetate metabolism are shown in a detailed depiction. Glc, glucose; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; 1,3-BPG, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; Ru5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; Xu5P, xylulose-5-phosphate; P-Pyr, 3-phosphohydroxypyruvate; P-Ser, phosphoserine; Ser, serine; Gly, glycine; 5,10-CH₂-THF, 5,10-methylenetetrahydrofolate; THF, tetrahydrofolate; Ac-CoA, acetyl-CoA; Cit, citrate; I-Cit, isocitrate; Akg, α-ketoglutarate; Suc, succinate; Fum, fumarate; Mal, malate; Oxa, oxaloacetate; Gln, glutamine; Glu, glutamate; Asp, aspartate; 2-Hg, 2-hydroxyglutarate. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PKM2, pyruvate kinase M2 isoform; PGAM1, phosphoglycerate mutase 1; TKTL1, transketolase-like 1; PHGDH, phosphoglycerate dehydrogenase; PSAT1, phosphoserine aminotransferase 1; PSPH, phosphoserine phosphatase; SHMT, serine hydroxymethyl transferase; PDH, pyruvate dehydrogenase; IDH1, isocitrate dehydrogenase 1; IDH2, isocitrate dehydrogenase 2.