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Expanding the Concepts and Tools of Metabolic Engineering to Elucidate Cancer Metabolism

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Abstract

The metabolic engineer's toolbox, comprising stable isotope tracers, flux estimation and analysis, pathway identification, and pathway kinetics and regulation, among other techniques, has long been used to elucidate and quantify pathways primarily in the context of engineering microbes for producing small molecules of interest. Recently, these tools are increasingly finding use in cancer biology due to their unparalleled capacity for quantifying intracellular metabolism of mammalian cells. Here we review basic concepts that are used to derive useful insights about the metabolism of tumor cells, along with a number of illustrative examples highlighting the fundamental contributions of these methods to elucidating cancer cell metabolism. This area presents unique opportunities for metabolic engineering to expand its portfolio of applications into the realm of cancer biology and help develop new cancer therapies based on a new class of metabolically derived targets.

Stable isotopic tracers and other tools of Metabolic Engineering

Metabolic engineers are naturally interested in the metabolic phenotype of cells. By measuring actual intracellular fluxes (or pathway reaction rates), they generate valuable insights about metabolic control that enables identification of kinetic limiting steps and targets for genetic modification that will contribute most effectively to the development of desirable biochemical properties.¹ In this effort, metabolic engineers rely on macroscopic balances complemented by stable isotopic tracers to study metabolic networks and estimate pathway fluxes. Stable isotopic tracers are compounds that are labeled with at least one stable (i.e. non-radioactive) atom, such as carbon-13 (¹³C), that can be metabolically consumed by cells. Examples include [U-¹³C₆] glucose, in which all six carbon atoms have been labeled, and [5-¹³C] glutamine, in which only the fifth carbon is labeled. Catabolism of the tracer generates a mixture of intracellular metabolites of varying states of enrichment (e.g. M0 with no labeled atoms, M1 with one labeled atom, etc.), whose fractional distribution is known as the Mass Isotopomer Distribution (MID).

MIDs serve as fingerprints of the pathway(s) that interconvert metabolites; furthermore, quantitative measurements of MIDs can be used to obtain estimates of metabolic fluxes.^{2–4} Through an increasingly more prevalent set of analytical technologies, including GC/MS, LC/MS/MS, and NMR, signals directly proportional to the enrichment can be distinguished and quantified, enabling calculation of the MIDs of a large number of intracellular metabolites. MIDs of various metabolites are very information-rich and can be analyzed to derive significant conclusions about intracellular metabolism. In addition, when combined with a stoichiometric model that details the atom transitions in the network reactions, as well as a set of extracellular flux measurements, MIDs can be used to successfully estimate the

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set of intracellular fluxes in the system under consideration. We do not intend to review here methods developed for flux determination collectively known as Metabolic Flux Analysis (MFA). This subject is an active area of metabolic engineering and has produced many fundamental contributions,^{1,5-9} as well as insightful applications.⁹⁻¹³ We note that these techniques have effectively offered metabolic engineers a window into intracellular metabolism. For more than a decade, they have used ¹³C-labeled substrates to generate MIDs and perform MFA, which has greatly assisted them in the rational identification of target enzymes for genetic manipulation.^{3,14} In this effort, researchers were assisted by concepts of kinetics and regulation of metabolic pathways,¹⁵⁻¹⁸ as well as distribution of metabolic control¹⁹⁻²³ and identification of the collection of pathways responsible for converting a substrate to products. Recently, these same technologies have found renewed use in the context of biomedicine, and biologists have used them with great success to reveal the potential of cancer (as well as other diseases) to alter the cellular metabolic landscape.

Cancer and Metabolism

Historically, cancer has been viewed almost exclusively in the context of being a genetic disease. Since the realization in the late 1970s and early 1980s that cancer resulted from the mutation of endogenous proto-oncogenes and tumor suppressor genes, much effort has been spent on identifying oncogenes and understanding their mechanism of manifesting the transformed cell phenotype.²⁴ It has become clear that the associated uncontrolled proliferation and capacity for invasion result from dysregulation of a carefully controlled network of signaling pathways that normally function to maintain a delicate balance of growth and differentiation.²⁵ While biologists, aided by the widespread dissemination of tools such as recombinant DNA, cell-wide transcriptional and proteomic measurements, creative methods of constructing numerous genetic backgrounds, and generation of knock-out animals, have been successful in identifying the innumerable variety of genetic lesions responsible for the oncogenic phenotype, progress is lacking in understanding cancer cell metabolism, an often-overlooked yet amazingly fundamental aspect of cancer cell physiology.

In normal, healthy cells, careful regulation of metabolism is crucial for managing their growth; as with cell cycle checkpoints and other control mechanisms, cancerous transformation causes a destabilization of metabolic regulation.²⁶⁻²⁹ Within the past few years, a redirection of focus on the reprogramming of cell metabolism has emerged as a promising avenue in the quest for more effective clinical anticancer strategies. Many of the most prominent and commonly expressed oncoproteins, such as K-Ras and Myc, have traditionally been considered “undruggable” because of their insensitivity to small molecule inhibitors, the major structural drug class approved to treat cancer.²⁴ The low molecular weights of these compounds enable them to diffuse across the cell membrane to inhibit intracellular proteins, but typically limits their targets to enzymes with well-defined catalytic clefts that provide accessible hydrophobic pockets for substrate binding.³⁰ The tumorigenic capabilities of many major oncoproteins, however, strongly rely upon protein-protein interactions that extend over multiple points of contact along flat molecular surfaces, making them largely refractory to small-molecule drug compounds.³¹⁻³³ However, many well-characterized metabolic enzymes possess catalytic clefts and thus accessible drug-binding pockets, making them much more effective and practical targets of therapeutic inhibitors.³⁴ As well, because the actions of metabolic enzymes are largely downstream of other molecular events, the possibility of side-effects is minimized compared to those of more upstream signaling pathway components, making them a safer drug target.^{35,36} With these facts in mind, it is worth noting that there is precedent for anti-cancer drugs that target metabolism; antifolate drugs, which inhibit crucial nucleic acid synthesis enzymes, have

proven to be among the most enduring and effective chemotherapeutics, successfully targeting a wide range of cancers for more than half a century.³⁴

Besides the practical aspect of cancer drug development, however, the mind-frame and tools of metabolic engineering can play a central role in elucidating basic biochemical mechanisms and helping resolve some long-standing problems in cancer metabolism. A case in point is Otto Warburg's 1924 discovery that tumor cells perform aerobic glycolysis, consuming glucose and producing lactate at significantly elevated rates even under well-oxygenated conditions.^{37,38} Despite important questions as to the biochemical origins of the Warburg effect raised by this classic and widespread (although not universal) behavior of cancer cells, it is only recently that some new insights have been obtained through the critical use of stable isotopes.^{29,39} This lack of progress is not surprising when one considers the means employed to-date for observing cellular metabolism, which has largely been limited to bulk measurements of glucose consumption, lactate production, and cell growth. In contrast to the very advanced methods of cell and molecular biology employed for the generation and study of sophisticated genetic backgrounds, methods employed for assessing the metabolism and overall physiology of cells have been rather rudimentary. This is rapidly changing with the widespread adoption of the metabolic engineering toolkit in cancer metabolism research.

Recent investigations have revealed a number of direct metabolic targets of prominent oncogenes,^{26,27,29} enabling us to generate a general overview of cancer metabolism (Figure 1). For example, it has been shown that stabilization of the alpha subunit of hypoxia-inducible factors (HIFs) is accompanied by expression of glucose transporters, glycolytic enzymes, lactate dehydrogenase A, and pyruvate dehydrogenase kinase 1 (PDK1), among others.⁴⁰⁻⁴⁴ Similar information begins to arise about the action of most all of the other oncogenes. While this constitutes an important advance, this type of information is presently fragmented and incomplete. Furthermore, these findings are the result of *ad hoc* investigations and not the product of systematic metabolic analysis as would be obtained by a comprehensive determination and analysis of the fluxes of central carbon metabolism impacted by oncogene activation. This is one of the benefits that a systems-level analysis of metabolism, which is a central tenet of metabolic engineering, can contribute to cancer metabolism research.

It should be noted that flux determination is not the only element that metabolic engineering can contribute to cancer research. Through flux determination, kinetics and regulation of the corresponding enzymatic reactions can be studied and conclusions can be derived about the distribution of kinetic control in various enzymes and pathways.^{19,21,22} Furthermore, methods for enumerating candidate pathways leading to the synthesis of key metabolites are important for elucidating the biochemical mechanism(s) induced by oncogene action, and pathway thermodynamics can aid in assessing the feasibility of various routes for metabolite synthesis.^{5,45} Metabolomics techniques, which detect and quantify levels of all measureable small-molecule metabolites on a systems-wide level, have also recently come into practice, and they have been used successfully to identify additional novel biomarkers and cancer-specific alterations in metabolism.^{46,47}

Another important point is that data generated by the above methods are most informative when they are used *collectively* rather than individually. For example, the metabolome, though information-rich, provides an incomplete description of metabolic changes and is generally difficult to interpret as alterations in metabolite concentrations can occur for various reasons. Rather than attempting to make inferences from metabolite levels alone, it would be most useful to complement metabolite data with direct measures of pathway activity as measured by the pathway fluxes. Similarly, because a large flux through a

pathway can be the result of high activity of the corresponding enzymes and/or the accumulation of corresponding metabolites, identification of pathway fluxes *and* metabolite levels most differentially changed in cancerous versus normal cells provides a more informative means for identifying novel cancer-specific therapeutic targets. In essence, this cancer metabolism problem is identical to the metabolic engineer's, and, as one would expect, the tools developed for engineering overproducing microbes are just as applicable to elucidating metabolic pathways in a biomedical context (Fig. 2). In particular, stable isotopes and MFA provide a pathway-specific resolution of general metabolism that is impossible using traditional biochemical measures of metabolism.

Finally, just as modulating most enzymes in a pathway is ineffective for enhancing product synthesis, similarly modulating the activity of many enzymes in a metabolic pathway that is affected by an oncogene does not necessarily constitute an effective therapeutic strategy. Unresponsive enzymes are bad targets both for product synthesis and cancer therapy. Identifying specific enzymes controlling pathway flux is therefore critical for developing effective therapies. This is the main reason for invoking kinetics and in understanding the distribution of kinetic control in metabolic pathways, which is the subject of Metabolic Control Analysis and a centerpiece of metabolic engineering.^{19,22}

Observing cancer metabolism by the use of stable isotopic tracers

Of the various tools of metabolic engineering, the one that has found widespread use to-date in studies of cancer metabolism is the use of stable isotopes. Stable isotopic tracers and MFA enable cancer biologists to effectively peer into intracellular metabolism and detect changes in metabolite fluxes that were until recently unobservable. Although these tools have just recently begun to see use in the cancer context, they have long been a regular component in the toolkit of physiologists studying hyperglycemia, diabetes, and obesity.^{48–52} With regard to tracers, metabolic studies in the 20th century most commonly employed radioisotope tracers, but since the 1970s, they have been gradually replaced with stable isotopic labeled tracers, owing to their safety and the increasing affordability of detection technologies such as GC/MS, LC/MS/MS, and NMR.⁵³ In the context of measuring metabolism, radioisotopes were also limited by their inability to determine differences in enrichment; unlike stable isotopes, which generate MIDs that differentially show the full spectrum of enrichment states for each metabolite, radioisotopes, in producing only a single readout (specific activity, an indicator of the rate of decay), do not enable the extent of enrichment to be quantified.

The key ideas underlying the use of ¹³C isotopic tracers in delineating the nature of metabolic networks are exemplified by the case of using [3-¹³C] glucose to determine the extent to which pyruvate carboxylase (PC) contributes to citrate synthesis (Fig. 3a). Following glycolysis, this tracer will give rise to [1-¹³C] pyruvate, which upon carboxylation by PC, will generate labeled oxaloacetate (as well as malate, fumarate, and succinate by equilibrium and aspartate via transamination of oxaloacetate); however, upon oxidation of [1-¹³C] pyruvate to acetyl-CoA (AcCoA), the ¹³C label on pyruvate is lost as CO₂, and the AcCoA contributes to the formation of unlabeled tricarboxylic acid (TCA) cycle intermediates. Thus, the fraction of M1-labeled TCA cycle intermediates, as shown in the bar graph, provides a measure of activity through the PC pathway. Other examples, that will be discussed later, include the use of uniformly labeled glutamine, which incorporates label into citrate only when glutamine is used as a precursor, to determine the total amount of citrate formed from glutamine (Fig. 3b); the use of [1-¹³C] glutamine, which incorporates label into citrate only under reductive TCA cycle metabolism, to determine the fraction of citrate formed from glutamine by reductive carboxylation (Fig. 3c); and the use of [5-¹³C] glutamine, which incorporates label into lipogenic AcCoA only under reductive TCA cycle

metabolism, to determine the fraction of lipids synthesized from glutamine via reductive carboxylation (Fig 3d).

Despite the utility of raw MID data in assessing pathway activity, the culmination of the use of stable isotopic tracers is flux determination by MFA, which is now a basic component of metabolic engineering.^{1,2,54} Additionally, a number of other advanced techniques have been developed that employ the unique advantages of isotopic tracers to quantify fluxes and pathway contributions in the context of metabolic disorders. For instance, Isotopomer Spectral Analysis (ISA; also known as mass isotopomer distribution analysis), developed in the early 1990s, is a special form of nonstationary MFA that fits measured lipid (e.g. palmitate) MID data with a least-squares model that describes isotopic enrichment as a function of two key parameters in lipid synthesis reactions, the fractional contribution of tracer to synthesized lipids and the fraction of measured lipids that has been synthesized *de novo* since the beginning of the experiment (known respectively as *D* and *g(t)*).^{55,56} Though first used primarily to study lipid synthesis and gluconeogenesis in the contexts of hepatocyte function and metabolic diseases,^{57,58} ISA has proved especially applicable in cancer metabolism to quantify pathway contributions to lipogenesis, which has emerged as a major proliferative target.^{59,60}

Also of note from the 1990s is a series of studies by Blanch and Clark that focused on the resolution of intracellular metabolism in hybridomas using ¹³C NMR.^{61–63} These experiments, which sought to connect metabolic pathway activity with antibody production under various growth conditions, used label enrichment patterns from ¹³C-labeled glucose and glutamine in conjunction with extracellular metabolite measurements, oxygen consumption rates, and redox balances to comprehensively quantify intracellular fluxes and pathway contributions. An early juxtaposition of metabolic engineering and tumor cells, this series of investigations foreshadowed the rich trend that would later emerge in using stable isotope labels to assess alterations in cancer cell metabolism.

Now, with the significance of cancer metabolism in full consideration, various labs have developed and optimized metabolic engineering tools for application in mammalian cells for biomedical purposes.⁶⁴ For instance, several groups have developed sensitivity-based methods for determining confidence intervals in MFA, which, by imparting a measure of goodness-of-fit to the calculated values of fluxes, are as critical as the fluxes themselves.^{65–67} As well, a number of metabolic engineering groups have introduced algorithms designed to minimize the number of variables (and hence computation time) needed to model MIDs for isotopomer simulation in MFA.^{68,69} In addition, our group has developed a novel technique, non-targeted fate detection (NTFD), which combines stable isotopes with GC/MS-based metabolomics to enable the quantification of all targets, known and unknown, downstream of a labeled tracer; this opens the possibility of detecting downstream metabolites of a substrate and novel biomarkers in diseased cells.⁷⁰ Specific to mammalian cells, we have catalogued the major tracer types according to the confidence intervals of estimated fluxes and optimized their precision for each network within central carbon metabolism.^{71,72}

Examples

We provide now several examples illustrating the use of stable isotopes and MFA in elucidating the metabolic phenotype in cancer. Results have generated a good deal of excitement in cancer biology due to the unveiling, after a very long time, of basic biochemical mechanisms and the identification of new drug metabolic targets in the process.

Discovery of Novel Metabolic Pathways

A very recent example of the discovery of a novel oncogenic metabolic phenotype is the reductive carboxylation of α -ketoglutarate to isocitrate under conditions of mitochondrial stress. Several groups independently demonstrated that hypoxia or disruption of cellular respiration through mutation/inhibition of electron transport chain (ETC) enzymes promotes reductive glutamine metabolism for proliferation in a large variety of cancer cell lines.^{73–76} In contrast to glucose oxidation, which was shown to be the primary source of AcCoA for lipogenesis under ambient oxygen levels in cells with functional ETCs, glutamine reduction was shown to dominate formation of lipogenic AcCoA in hypoxic or ETC-compromised cells. These changes were mirrored in “pseudohypoxic” renal cell carcinoma cells that lacked functional von-Hippel-Lindau protein, a negative regulator of the HIF transcription factor, implicating HIF, a known promoter of tumorigenesis, in reductive carboxylation. With fatty acid synthesis emerging as a major target for limiting proliferation, these results impact formulation of alternative therapeutic strategies.

Macroscopically, using traditional biochemical metabolic measures, hypoxic or ETC-compromised cells undergoing extensive reductive carboxylation show little evidence of the pathway (aside from a modest increase in glutamine uptake in the case of hypoxic cells). Hence, it is difficult to imagine how this mechanism could have been determined without isotopic tracers, particularly the resolution of enrichment afforded by stable ^{13}C tracers. In this case, it was the dramatic increase in label contribution of enriched glutamine relative to enriched glucose to TCA cycle metabolites (e.g. M1 metabolites from $[1-^{13}\text{C}]$ glutamine vs. M2 metabolites from $[\text{U}-^{13}\text{C}_6]$ glucose) that first indicated a major shift. In addition, the conclusions regarding the fractional substrate contributions to lipogenesis, which were derived from ISA and ^{13}C NMR, would have been challenging to make solely with radioactive tracers. MFA provided additional confirmation by indicating a substantial increase in α -ketoglutarate to isocitrate flux under the mentioned hypoxic conditions.^{73,75}

Another recently uncovered metabolic pathway of cancer cells is the proliferation- and oncogenesis-associated use (as evidenced by flux elevation) of serine biosynthesis. Although this pathway had previously been shown to be unusually active in various cancers, its metabolic underpinnings remained unknown.^{77–79} A series of recent papers linked this behavior with amplification or high expression of phosphoglycerate dehydrogenase (PHGDH), the enzyme responsible for shunting the glycolytic intermediate 3-phosphoglycerate toward serine biosynthesis, in breast cancer and melanoma.^{80,81} Both groups identified PHGDH as a commonly amplified gene in these cancers and showed that cells which harbored multiple copies required its expression for proliferation. Locasale et al. demonstrated its ability to modulate glycolytic flux and to induce a number of morphogenic changes characteristic in tumorigenesis. Possemato et al. additionally demonstrated its significance in interacting with other metabolic pathways, most directly α -ketoglutarate generation, and subsequently indicated that these external pathways were likely responsible for the proliferative abilities of PHGDH.

In this instance, each group reached its conclusions from opposite directions, but ^{13}C -labeled tracers were fundamental in allowing both to do so. Locasale et al. began from a metabolomic perspective; after culturing cells in $[\text{U}-^{13}\text{C}_6]$ glucose, they determined via NMR that levels of glycine, a product of serine biosynthesis, were among the highest of all detected cellular metabolites. Additionally, they used LC/MS/MS to detect substantial label in a number of serine biosynthesis intermediates, indicating a substantial redirection of glycolytic flux. These findings then led them to observe PHGDH amplification to be a frequent mutation in melanoma through a false-discovery rate screen of genes from pooled cancer samples; further experimentation enabled them to infer PHGDH's relevance in proliferation and oncogenesis. Possemato et al., on the other hand, began by using a small-

hairpin RNA (shRNA) functional genomics screen that revealed PHGDH to be essential for tumorigenesis in a breast cancer cell line. Following the identification of markers that showed correlation between PHGDH amplification and aggressiveness in breast cancers, its shRNA-mediated knockdown demonstrated the requirement of serine biosynthesis pathway functionality for proliferation in amplified cells even in serine-rich media. This was confirmed by MFA with [U-¹³C₆] glucose, which established that in amplified cells, a substantial portion of glycolytic flux (8-9%, comparing to only 1-2% in non-amplified cells) was directed toward serine biosynthesis. Furthermore, LC/MS detection of enriched metabolites from cells grown in [U-¹³C₅]-glutamine provided the surprising conclusion that shRNA-mediated knockdown of PHGDH in amplified cells resulted in a drop in α-ketoglutarate level more dramatic than for any other metabolite, including serine. These findings demonstrate the complex nature of and sometimes unexpected conclusions from metabolic interconnectivity. With interplay between glycolysis, TCA anaplerosis, amino acid metabolism, and likely other side pathways, stable isotopic tracers and MFA turn the investigation of metabolic systems into a tractable problem. It should be noted that similar tools are lacking in analyzing data from and elucidating the ever-expanding interconnectivity of signaling pathways and other cell networks. The metabolic engineering toolkit makes these questions far more tractable in the context of metabolism.

Metabolic Response to Oncogenes and Tumor Environment

Myc and Ras are among the most common drivers of oncogenic transformation, with their mutation found in an estimated 15-30% and 25% respectively of all human cancers.^{24,82} Although their expression has previously been linked to metabolic targets,^{83,84} it has been only recently, through the use of stable isotope technologies, that their precise, system-wide influence on pathways has been quantified in the context of physiological tumorigenesis.

In one study, oncogenic K-Ras was used to transform non-tumorigenic 3T3 fibroblast cells, and the effects of this transformation on metabolic flux were observed.⁸² Transformation was shown to increase glycolysis but decrease entry of pyruvate into the TCA cycle via PDH. Concomitantly, flux of glutamine toward the TCA cycle increased, as did its contribution toward amino acid and nucleotide synthesis; thus, K-Ras transformation was shown to effectively “decouple” glucose and glutamine metabolism. This shift was accompanied by a sensitization of cells to aminotransferase inhibition and an increase in mortality to glutamate dehydrogenase inhibition, likely due to precursor biosynthesis disruption and antioxidant deprivation, identifying additional strategies that may be effective against K-Ras-driven tumors.

Throughout this investigation, MFA, as well as MID-based inference, was used extensively to determine system-wide flux response to K-Ras transformation.⁸² For instance, MFA using [U-¹³C₅] glutamine coupled with extracellular flux measurements showed increases in glycolytic flux and glutamine anaplerosis with a decrease in PDH flux. MIDs mirrored these changes; decreases in M2 pyruvate and glutamate from [U-¹³C₆] glucose and an increase in M4 aspartate (derived from oxaloacetate) from [U-¹³C₅] glutamine indicated a glucose-to-glutamine shift in substrate contribution under transformation. The increased role of glutamine was highlighted by NTFD with [α-¹⁵N] glutamine, which showed an increase in labeling of amino acids and adenine. Although this investigation was relatively straightforward and looked simply at the metabolic implications of the fundamental process of transformation, ¹³C-label-mediated techniques such as MFA and NTFD revealed an abundance of information on system-wide pathway contributions that has enabled further inference into its oncogenic mechanisms and possible therapeutic vulnerabilities.

Another study specifically considered the metabolic consequences of oncogene expression in the context of tumor microenvironment. Grassian et al., investigated the metabolic

consequences of extracellular matrix (ECM) detachment, a mechanism necessary for the metastatic invasion of tumor cells, in a breast cancer cell line, MCF-10A, and found that loss of attachment resulted in significant decreases in glycolytic, pentose phosphate pathway, and TCA cycle fluxes, with a disproportionately lowered flux through PDH.⁸⁵ Overexpression of ErbB2 (also known as HER2/Neu), which is amplified in approximately 25% of breast tumors, partially rescued PDH flux through decreasing expression of pyruvate dehydrogenase kinase (PDK) 4, a potent modulator of PDH activity. Subsequently, these changes were found to be mediated by Erk signaling, which in turn was determined to become activated in response to epidermal growth factor. PDK4 expression was shown to restrict lipogenesis, ATP synthesis, and proliferation, demonstrating an intriguing result that runs counter to other findings that have linked PDK restriction to tumor regression.^{86–88}

MFA with [1,2-¹³C₂] glucose was performed on cells both attached and detached from the ECM, with ErbB2 both at normal and overexpressed levels. In combination with ratios of fractionally labeled M2, M3, and M4 citrate, glutamate, fumarate, and aspartate to M2 pyruvate, which provide normalized measures of flux from pyruvate to the TCA cycle, MFA indicated a substantial decrease in PDH flux, even when compared to lowered activity through other estimated fluxes, under ECM-free conditions; these same measures showed a reverse trend upon ErbB2 overexpression, and it was this behavior that directed the authors' focus toward observing PDK levels. As they pursued connections with other known signaling nodes and identified the role of the Erk pathway, they continued to use labeling data to assess PDH activity.⁸⁵ In this case, through identifying oncogenic alterations in biochemical pathways that would be invisible using traditional quantitative techniques, MFA provided valuable findings about how signaling pathways may modulate metabolism to mediate proliferation.

Another investigation by Le et al.⁸⁹ also illustrated how stable isotopic tracers could prove essential for the elucidation of glutamine metabolism in the context of cancer. Previous groups had shown that expression of Myc led to dependence on or “addiction” to glutamine, but little was known about the metabolism of Myc-transformed cells under conditions characteristic of the tumor microenvironment, which is oxygen- and glucose-poor.^{90,91} The authors cultured a Myc-inducible Burkett lymphoma model and found that, though Myc normally promoted entry of glucose to the TCA cycle, hypoxia reversed this trend via PDK activation, even with Myc turned on. Myc was found to also promote glutamine flux into the TCA cycle, even enabling growth and proliferation of cells cultured in glucose-free conditions. Further experiments showed that glutamine strongly contributed to ATP and antioxidant production, and they demonstrated the capability of glutaminase inhibition to slow proliferation and induce cell death in Myc-driven cancers.

In this study, the authors used stably labeled tracers to quantify the contributions of the two major substrates used by cancer cells, glucose and glutamine. For instance, their observation that M2 labeling of TCA cycle intermediates was elevated under aerobic Myc activation and depressed under hypoxia in [U-¹³C₆] glucose-cultured cells enabled them to reach conclusions regarding PDH activity. Furthermore, their observation that labeling in glutamate and TCA cycle intermediates increased under Myc activation in [U-¹³C₆, ¹⁵N₂] glutamine-cultured cells motivated them to investigate the role of glutamine in Myc-driven cancer cells.⁸⁹ Significant levels of M6 citrate, formed through the malic enzyme, pyruvate carboxylate, and ATP-citrate lyase reactions, indicated the presence of glucose-independent TCA cycling; successful cell proliferation under glucose-free conditions validated the notion of Myc-overexpressing cells driving precursor biosynthesis and ATP generation through glutamine metabolism. Further analysis of elevated levels of enriched glutathione, a potent antioxidant, in cells cultured in labeled glutamine relative to labeled glucose also supported

this, indicating the unique capability of glutamine to combat production of reactive oxygen species, a major hazard for cells especially under hypoxic conditions.^{92,93}

Although observing cells in culture under conditions that mimic the tumor microenvironment (low glucose, oxygen-deprived) has played a key role in the advancement of our understanding of tumor metabolism, cell culture systems are a poor substitute for studying tumors *in vivo*. Mice xenografts serve as superior models for studying the oncogene-induced reprogramming of metabolism. Thus, Yuneva et al.⁹⁴ examined the metabolic profiles of liver tumors driven by the Myc and Met oncogenes in mice and found drastic differences in the behavior of each. In particular, Myc was found to drive increased flux of glucose and glutamine toward lactate production and TCA cycle replenishment, whereas Met was found to drive conversion of glucose to glutamine while producing lactate, glutamate, and TCA intermediates and consuming glutamine in quantities almost identical to those seen in normal tissue. These findings confirm that the metabolic profile of tumors is highly dependent on the identity of initiating oncogene. As well, in contrast to liver tumors, lung tumors induced by Myc were shown to possess increased glutamine synthesis relative normal tissue, demonstrating that, even in tumors initiated by the same oncogenes, the originating tissue type is a key factor in determining the metabolic phenotype.

Throughout this study, stable isotopic tracers were fundamental in identifying the metabolic pathways activated by the expressed oncogenes. For each mouse, either [U-¹³C₆] glucose or [U-¹³C₅] glutamine was injected to visualize downstream products by NMR.⁹⁴ With peak intensities indicating alterations in pathway contributions in tumors relative to normal tissue controls, these experiments were integrated with mRNA, protein, and metabolite abundance measurements to explain the differential metabolic profiles for each condition. As in previous cases, the precise fates of exogenous glucose and glutamine would be indeterminable without stable isotope labels to determine their placement.

Conclusion

The previous examples demonstrate that stable isotope tracers, MFA, and other tools traditionally developed and used by metabolic engineers have found productive use in elucidating cancer metabolism. Essentially, they have opened a window into the cell and enabled us, for the first time, to comprehensively visualize system-wide metabolic activity that goes beyond the simple recording of net changes of a few extracellular metabolites. The discernment of the fluxome adds a major compendium to the library of omics sets, providing a more immediate readout of cell physiology than the genome, transcriptome, proteome, or metabolome.⁹⁵

As well, their use has helped bring about the recent explosion of research directed toward uncovering the metabolic phenotype of traditionally non-metabolic diseases such as cancer. Although the onset of cancer may be genetic in origin, there is vast interplay between each of the two worlds, with genes coding for metabolic enzymes potentially serving as proto-oncogenes and tumor suppressor genes and genetic lesions triggering a rewiring of metabolism to mediate cell proliferation and evade apoptosis.^{26,96–98}

Ultimately, one of the fundamental goals of biomedical research is to discover and explore new potential treatments. In this sense, the ability to quantify metabolic pathways and assess the comprehensive response of pathways to various therapeutic strategies opens a wide range of possibilities for discovery of new cancer drugs. Although a long road remains ahead, with few novel metabolic inhibitors currently past the preclinical stage, past successes of similar approaches provide reason for cautious optimism about the ultimate success of these methods.³⁴ In any event, much as they have proven invaluable in metabolic

engineering, stable isotopic tracers have become firmly ingrained in biomedical research as new tools essential for elucidating the metabolism of cancer.

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Figure 1.

A schematic overview of cancer cell metabolism. Oncogenes and tumor suppressor genes (blue) mutated in cancer reprogram metabolism to support elevated proliferation and enhanced survival, increasing flux (wide arrows) through pathways such as aerobic glycolysis, lipogenesis, and nucleotide/NADPH synthesis (products in green). This leads certain enzymes (red) to have particular oncological significance, as they may be distinctly activated, expressed, or mutated in cancer cells. This figure is courtesy of Christian Metallo (University of California, San Diego).

Figure 2.

Both metabolic engineering and cancer cell metabolism involve the reprogramming of physiological metabolic pathways for some alternative purpose; for metabolic engineers, this purpose is typically the overproduction of some chemical, while for cancer cells, it is primarily accelerated proliferation. Tools such as stable isotopic tracers, metabolic flux analysis, metabolic control analysis, and thermodynamic pathway analysis can be used to identify key pathways in each; in metabolic engineering, these are reactions that contribute most to the intended product formation (and are hence potential targets of genetic modification), and in cancer metabolism, these are reactions that most drastically contribute to the tumor cell phenotype (and are hence potential targets of therapeutic inhibition).

Figure 3.

¹³C isotopic tracers can help identify the differential contributions of fluxes to metabolic pathways. Cells are cultured in the presence of labeled media; after a prolonged period, their metabolites are harvested, analyzed using GC/MS (or another isotope-sensitive detection instrument), and catalogued into mass isotopomer distributions (MIDs). (a) Contribution of pyruvate carboxylase (PC) flux to the TCA cycle can be measured by culturing cells in [3-¹³C] glucose, which is eventually metabolized to [1-¹³C] pyruvate. Because the labeled carbon is retained only in the PC reaction, quantification of M1 (singly-enriched) TCA cycle intermediates from the generated MIDs enables estimation of PC activity. (b) In addition, M4 and M5 citrate levels from [U-¹³C₅] glutamine-cultured cells can be used to determine the total amount of citrate synthesized from glutamine. (c) The fractional contribution of reductive carboxylation to citrate formation is directly given by the M1 citrate isotopomer in [1-¹³C] glutamine-cultured cells, and likewise, (d) its contribution to lipid synthesis by the palmitate MID in [5-¹³C] glutamine-cultured cells.