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¹³C Isotope-Assisted Methods for Quantifying Glutamine Metabolism in Cancer Cells

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Abstract

Glutamine has recently emerged as a key substrate to support cancer cell proliferation, and the quantification of its metabolic flux is essential to understand the mechanisms by which this amino acid participates in the metabolic rewiring that sustains the survival and growth of neoplastic cells. Glutamine metabolism involves two major routes, glutaminolysis and reductive carboxylation, both of which begin with the deamination of glutamine to glutamate and the conversion of glutamate into α -ketoglutarate. In glutaminolysis, α -ketoglutarate is oxidized via the tricarboxylic acid cycle and decarboxylated to pyruvate. In reductive carboxylation, α -ketoglutarate is reductively converted into isocitrate, which is isomerized to citrate to supply acetyl-CoA for *de novo* lipogenesis. Here, we describe methods to quantify the metabolic flux of glutamine through these two routes, as well as the contribution of glutamine to lipid synthesis. Examples of how these methods can be applied to study metabolic pathways of oncological relevance are provided.

1. INTRODUCTION

In recent years, glutamine has emerged as a central precursor in the metabolism of cancer cells. Not only does glutamine, a nonessential amino acid, serve as the major mechanism of nitrogen transport into cells, but it also supplements glucose as a substantial carbon source via anaplerosis into the tricarboxylic acid (TCA) cycle (Daye & Wellen, 2012; DeBerardinis & Cheng, 2010). Given the necessity of transformed cells to perform elevated macromolecular biosynthesis to continue their growth and invasion within the body, targeting glutamine metabolism represents a promising opportunity for disrupting tumor proliferation (Vander Heiden, 2011).

As has been the case with glucose, mutated genes and malfunctioned signaling pathways in cancers have been found to influence the regulation of glutamine metabolism, including K-Ras (Gaglio et al., 2011; Son et al., 2013), p53 (Hu et al., 2010; Suzuki et al., 2010), and mTOR (Csibi et al., 2013). Most strikingly, c-Myc has been found to elicit “addiction” to the amino acid by inducing the expression of genes involved in glutamine metabolism, such as

the glutamine transporter ASCT2 and glutaminase (GLS) (Gao et al., 2009; Wise et al., 2008).

Once taken up by the cell, glutamine is directed toward protein synthesis or deaminated, typically by GLS; nonproteinogenic glutamate is then converted to α -ketoglutarate via either glutamate dehydrogenase or transamination. After reaching this step, glutamine-derived α -ketoglutarate can be further metabolized along the TCA cycle through two different routes: The first, glutaminolysis, traditionally refers to oxidation of this α -ketoglutarate to malate and subsequent decarboxylation to pyruvate by malic enzyme (ME) or further oxidation to oxaloacetate by malate dehydrogenase. This progression contributes to ATP production through generation of substrates for oxidation in aerobic respiration and enables redox control from NADPH production through ME, formation of precursors for macromolecular biosynthesis such as alanine and pyruvate, or excretion of carbon as lactate by lactate dehydrogenase in Fig. 19.1 (DeBerardinis & Cheng, 2010). The second major route of glutamine metabolism, RC, has been shown to dominate in cell lines under hypoxic stress or disrupted mitochondrial functioning; in these situations, glutamine-derived α -ketoglutarate has been found to preferentially undergo reductive metabolism through the TCA cycle to isocitrate and then citrate, where it can then be converted to acetyl-CoA for lipid synthesis (Metallo et al., 2012; Mullen et al., 2012; Wise et al., 2011). Induction of this pathway has been shown to be controlled by mass action via conditions that perturb the citrate-to- α -ketoglutarate ratio, such as stabilization of the HIF-2 α oncogene and/or oxidative energetic stress, and its activity has been demonstrated both *in vitro* and *in vivo*; targeting glutamine metabolism via GLS inhibition holds promise as a potential therapeutic strategy under these conditions, especially in combination with other anticancer drugs (Fendt et al., 2013a, 2013b; Gameiro et al., 2013).

In recognizing the significance of glutamine anaplerosis and its potentially divergent fates toward meeting the demands of either energy and combating oxidative stress or synthesizing macromolecules, it is necessary to have a means of quantifying these fates experimentally. Stable isotope labeling provides a direct readout of intracellular metabolism, and it can be combined with the known stoichiometry of biochemical pathways to estimate the activity of corresponding enzyme fluxes (Keibler, Fendt, & Stephanopoulos, 2012). We describe here how stable isotope tracers can be used to assess the use of glutamine by cancer cells for survival and proliferation.

2. METHODS

The typical flow of a stable isotopic tracer-assisted study in cancer cells is schemed as in Fig. 19.2. Depending on the goals of each experiment, various factors need to be considered in each of these steps. In this section, we describe and discuss some of the common considerations to be taken during labeling experiments using cultured cancer cells. Although we limit our discussion to *in vitro* cell culture systems, many principles, such as the selection of tracers and analyses of intracellular metabolites, can be extended to *in vivo* animal models in the study of cancer metabolism as well (Bier et al., 1977; Gameiro et al., 2013; Maher et al., 2012; Yuneva et al., 2012). (Despite these parallels, the complexity of

whole-body metabolism and the logistical difficulties of live animal experiments present challenges that make such *in vivo* studies beyond the scope of this chapter.)

2.1. Design of experiment

Experimental design is critical for obtaining useful metabolic information from the cell culture system. In particular, in isotopic labeling studies, the choice of tracer determines the range of possible labeling metabolite patterns and therefore strongly influences the observability as well as accuracy of the estimated intracellular fluxes. A wide collection of stable isotopes is now available, and some investigations have been conducted to assess the effectiveness of different ^{13}C isotopic tracers to determine specific fluxes in mammalian cells, as well as the optimal label or combination of labels that should be used for a particular purpose (Metallo, Walther, & Stephanopoulos, 2009; Walther, Metallo, Zhang, & Stephanopoulos, 2012). Here, we present typical ^{13}C -labeled glutamine tracers that are used to trace the catabolism of glutamine carbons in central carbon metabolism. Generally, $[\text{U-}^{13}\text{C}_5]$ glutamine is a good tracer to effectively evaluate the total contribution of glutamine in the TCA cycle and lipogenesis (Yoo, Stephanopoulos, & Kelleher, 2004). Other partially labeled glutamine tracers such as $[1\text{-}^{13}\text{C}]$ and $[5\text{-}^{13}\text{C}]$ glutamine can also be useful to evaluate the fraction of glutamine that is metabolized through RC (Gameiro et al., 2013). Examples of mass isotopomer distribution (MID) analysis procedures that can be performed using these tracers are provided in Section 3.1.

2.2. Cell culture using stable isotopic tracers

Cell culture is usually performed with commercially available media formulations containing glucose, amino acids, vitamins, and inorganic salts, such as Dulbecco's modified Eagle medium (DMEM) and RPMI-1640. In stable isotope tracer experiments, specialized basal media without glucose, glutamine, and/or any other substrate of interest are used, allowing the use of specific ^{13}C -labeled tracer (typically glucose or glutamine). As is common with cancer cell lines, the culture medium is supplemented with 5–10% fetal bovine serum (FBS), which is complex and contains many unspecified molecules that may dilute ^{13}C -labeling of intracellular metabolites and interfere in cellular metabolism. Thus, in tracer experiments, normal FBS is replaced by dialyzed FBS, in which unspecified small molecules (typical cut-off, 10,000 Da) were minimized to avoid those unwanted effects.

The labeling patterns of intracellular metabolites in a stable isotope experiment are strongly influenced by many factors, including the growth conditions, medium, and particularly the metabolic status of the cells. To obtain reproducibility and comparability, it is of utmost importance not only to carry out the experiments in a consistent manner but also to ensure cells have reached metabolic steady state before quenching and extracting metabolites. Metabolic steady state is the condition in which both extracellular and intracellular metabolic fluxes, as well as intracellular metabolite concentrations, do not change with time. Generally, a constant specific growth rate (either stationary or exponential growth phase) is required for metabolic steady state, although these conditions alone do not guarantee it. Further validations could be performed to ensure that metabolic steady state is established (Ahn & Antoniewicz, 2013; Deshpande, Yang, & Heinzle, 2009).

Similar to but distinct from the concept of metabolic steady state is the isotopic steady state where metabolites reach a steady state in which the isotopic labeling pattern does not change with time. Since the incorporation of ^{13}C atoms into unlabeled metabolites is determined by the biochemical reactions of the metabolic network, it is only possible to reach isotopic steady state after a metabolic steady state has established. In addition, the metabolite pool size has a major impact on how fast it becomes enriched in isotopic label. Owing to various dynamics of isotope incorporation, different metabolites may reach isotopic steady state in distinct timescales. For example, it has been reported that using $[\text{U-}^{13}\text{C}_6]\text{glutamine}$ tracer allowed metabolites in TCA cycle to reach isotopic steady state within 3 h and using $[1,2\text{-}^{13}\text{C}_2]\text{glucose}$ tracer the glycolytic metabolites could reach isotopic steady state within 1.5 h at exponential and stationary phase of cell growth (Ahn & Antoniewicz, 2013).

An example protocol for an isotopic tracer experiment is given below, using the human lung adenocarcinoma epithelial A549 cell line (ATCC, CCL-185). Parameters in the following experimental procedure, such as initial cell density and incubation time with labeled substrate, have been determined from previous studies (Metallo et al., 2012), and preliminary experiments to ensure metabolic and isotopic steady state should be performed if different cell lines are used.

1. Day 0, seed 200,000 cells/well in a 6-well plate (or a 35-mm culture dish) in 2 mL DMEM containing 25 mM glucose, 4 mM glutamine, 10% (v/v) FBS, and 100 U/mL penicillin/streptomycin. Incubate the cells in a humidified incubator controlled at 5% CO_2 and 37 °C. Allow at least 6-h culture to attach cells to the plate.
2. Day 1, remove the growth media completely by aspiration and wash two times with 2 mL phosphate-buffered saline (PBS) (room temperature). Add 2 mL DMEM containing 25 mM glucose, 4 mM $[\text{U-}^{13}\text{C}_5]\text{glutamine}$, 10% (v/v) dialyzed FBS, and 100 U/mL penicillin/streptomycin. Depending on the experimental design, other tracers can be used.
3. Day 2 (24 h after replacing with tracer medium), the cells are ready for the extraction of metabolites.

2.3. Measurement of extracellular glutamine and glutamate

Different cancer cell lines, depending on the oncogenes activated or the tumor suppressors inactivated, have distinct patterns of glutamine metabolism. For example, c-Myc induces the overexpression of GLS in some cancer cells and, as a consequence, increases the glutamine uptake of those cells (Gao et al., 2009; Wise et al., 2008). Measuring consumption of glutamine provides a readout of its total use by cells. In some cases, the glutamine consumption rate is also required as a parameter for metabolic flux analysis (MFA), a methodology that can estimate intracellular metabolic fluxes based on stable isotopes (e.g., ^{13}C -isotopic tracers) and metabolic models (Stephanopoulos, 1999). However, it is beyond the scope of this chapter to describe MFA in great detail, and for further reading the readers are referred to Zamboni (2011).

The concentration of glutamine and glutamate can be measured by numerous ways, for example, enzymatic assay (Pye, Stonier, & McGale, 1978), high-performance liquid chromatography (Tcherkas, Kartsova, & Krasnova, 2001), or mass spectrometry-based analytical methods such as GC- or LC-MS (Darmaun, Manary, & Matthews, 1985; Qu et al., 2002). Here, we use the YSI 7100 Bioanalytical System (YSI Life Science) which applies immobilized enzymes to catalyze the corresponding chemical reactions to measure glutamine and glutamate. Assuming that the cells are in exponential growth phase and the culture has reached its metabolic steady state, that is, all intra- and extracellular fluxes are constant, the specific growth rate μ , specific uptake rate of glutamine q_{Gln} (it has a negative value because glutamine concentration in the medium decreases), and specific production rate of ammonium q_{NH_3} can be determined by solving the following equations as described previously (Glacken, Adema, & Sinskey, 1988).

$$\frac{dX}{dt} = \mu X \quad (19.1)$$

$$\frac{d[\text{Gln}]}{dt} = q_{\text{Gln}} X - k[\text{Gln}] \quad (19.2)$$

$$\frac{d[\text{NH}_3]}{dt} = k[\text{Gln}] + q_{\text{NH}_3} X \quad (19.3)$$

where k is the glutamine decomposition rate constant, which was determined to be approximately 0.0020 h^{-1} under the typical culture condition (Ahn & Antoniewicz, 2011; Ozturk & Palsson, 1990).

If measurements are available at multiple time points, it is possible to validate whether the cells are in exponential growth phase by comparing the specific growth rates or glutamine consumption rates over different periods of time. However, a constant specific growth rate does not necessarily ensure the establishment of metabolic steady state; thus, this validation only provides a partial check of the requirements.

2.4. Extraction of intracellular metabolites

While extracellular metabolite measurements only provide information on those metabolites that are consumed or secreted, intracellular metabolite concentrations contain much richer information on the state of cellular metabolism. However, analyzing for intracellular metabolites poses some extra challenges relatively to measuring extracellular metabolites. First, most intracellular metabolites cannot cross the cell membrane so that their analysis requires that the cells are broken to release the metabolites. Second, metabolites vary in the level of polarity; therefore, it is necessary to apply both polar and nonpolar solvents to extract metabolites once they are released after cell lysis. Third, in the process of cell lysis and metabolite extraction, cells are exposed to extremely harsh conditions and the metabolic steady state may be dramatically perturbed; therefore, an effective quenching procedure is of paramount importance to rapidly stop all enzymatic activities so that concentration of

metabolites is not significantly altered. As such, quenching and extraction methods fall into two main categories dictated by whether the cell culture is in suspension or adherent. Of the various methods that have been developed to address these challenges, we describe one method that is simple and feasible to most biological laboratories. An example protocol for extracting metabolites from a 6-well plate is given below, and it can be modified accordingly. For other methods, the readers are referred to references (Lorenz, Burant, & Kennedy, 2011; Sellick, Hansen, Stephens, Goodacre, & Dickson, 2011).

Example protocol for extraction of metabolites:

1. After taking the 6-well plate from 37 °C incubator, quickly remove the medium by aspiration or store the spent medium at –20 °C freezer until further analysis.
2. Wash the cells with 2 mL of saline solution and remove quickly by aspiration.
Note: Saline rather than PBS is used to avoid the interference of phosphate with the gas chromatography–mass spectrometry (GC-MS) analysis.
3. Place the plate on ice and quickly apply 400 µL of –20 °C methanol directly to the cells to quench metabolism.
4. Add 200 µL of ice-cold distilled water and briefly mix by gentle shaking.
5. Scrape the cells off using a cell scraper.
6. Transfer the methanol–water cell suspension into a microcentrifuge tube containing 400 µL of chloroform.
7. Vortex the microcentrifuge tubes vigorously at 4 °C for 10 min.
8. Separate the two phases by centrifugation at >14,000×g for at least 10 min, preferably at 4 °C. Upper phase (methanol and water) contains polar metabolites and lower phase (chloroform) contains nonpolar metabolites (i.e., lipids). Cell debris, proteins, and nucleic acids are in the middle layer.
9. Transfer the metabolite fraction of interest into a clean microcentrifuge tube without disturbing the middle layer.
10. Dry the metabolite extract in a freeze dryer or a vacuum centrifuge. Alternatively, samples can be dried on an evaporator under air or nitrogen gas flow.

2.5. GC-MS analysis of intracellular metabolites

GC-MS is widely used to analyze intracellular metabolites labeled by ¹³C isotopic tracers (Wittmann, 2002). For GC-MS analysis, the metabolites are chemically derivatized for better volatility in GC separation and analyzed by electron impact ionization in MS. The generated mass spectrum from GC-MS allows us to decipher cellular metabolism (Ahn & Antoniewicz, 2013). A broad spectrum of reagents is available for derivatization (Wittmann, 2007). Here, we provide example protocols of derivatization and GC program for both polar metabolites and fatty acids.

2.5.1 Derivatization of polar metabolites

1. Completely dry samples (in 1.5-mL centrifuge tubes) under air or nitrogen gas flow.

Note: The presence of water will hinder the derivatization reaction!

2. Add 20 μ L of 2 wt% methoxyamine hydrochloride in pyridine (MOX reagent; Thermo Scientific) to the tube and vortex for 5 s.
3. Incubate at 37 $^{\circ}$ C for 90 min on a heating block.
4. Add 25 μ L of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide +1% *tert*-butyldimethylchlorosilane (Thermo Scientific).
5. Incubate at 60 $^{\circ}$ C for 1 h on a heating block.
6. Centrifuge at $>14,000\times g$ for 10 min and transfer the supernatant into GC vials with inserts.

2.5.2 Derivatization of fatty acids

1. Add 500 μ L of 2% sulfuric acid in methanol to dried fatty acid samples. Incubate samples at 60 $^{\circ}$ C for at least 2 h (no more than 8 h).
2. Add 600 μ L hexane and 150–200 μ L saturated NaCl to samples. Vortex (at room temperature) for at least 10 min.
3. Spin samples down for 1 min at $>10,000$ rpm.
4. Transfer 350–400 μ L of upper (hexane) phase into a new microcentrifuge tube without disturbing the lower phase.

Note: The lower phase contains sulfuric acid which will destroy the GC column!

5. Dry samples under air flow for at least 10 min. Add 40–50 μ L hexane and resuspend the samples by vortexing.
6. Centrifuge at $>10,000\times g$ for 1 min. Transfer the samples into GC vials with glass inserts.

2.5.3 GC-MS analysis—The GC-MS system that we use consists of an Agilent 6890N GC equipped with DB-35ms (30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μ m; Agilent J&W Scientific) capillary column and 5975B Inert XL MS system under electron ionization at 70 eV and quadrupole mass analyzer. The MS source and quadrupole were held at 230 and 150 $^{\circ}$ C, respectively. Helium was used as carrier gas at a flow rate of 1 mL/min. Here, we provide GC programs for analyzing polar metabolites and fatty acids (Table 19.1). These programs can also be optimized for different sample types or derivatization methods.

The resulting MID is obtained by integration of ion chromatograms from each set of metabolite mass fragments (Antoniewicz, Kelleher, & Stephanopoulos, 2007a). The MID is characterized as the fractional abundance of mass isotopomers defined by M0, M1 to Mn. Here, M is the base mass of an ion fragment while the following number from 0 to *n* (active carbon number) indicates the mass shift from M (Table 19.2). To account for the

contribution in the labeling of a metabolite that is derived from only the ^{13}C atoms from ^{13}C isotopic tracers, MIDs need to be corrected for natural isotope abundances (Fernandez, Des Rosiers, Previs, David, & Brunengraber, 1996). This is an important step in the correct interpretation of isotopic labeling data. Intracellular metabolites can be identified by searching against libraries of the metabolite's retention time (using the specific GC program) and its characteristic fragmentation pattern (mass spectrum at full range, e.g., m/z 100–600).

3. APPLICATIONS

3.1. Choice of tracers and MID analysis

Various glutamine tracers are available, and the choice depends on which specific pathway or reaction needs to be monitored. Uniformly ^{13}C -labeled glutamine ($[\text{U-}^{13}\text{C}_5]\text{glutamine}$), $[1\text{-}^{13}\text{C}]\text{glutamine}$, and $[5\text{-}^{13}\text{C}]\text{glutamine}$ are good isotopic tracers to analyze the major pathways of glutamine metabolism in mammalian cells.

To trace glutamine catabolism in the TCA cycle, we can use $[\text{U-}^{13}\text{C}_5]$ glutamine and $[1\text{-}^{13}\text{C}]\text{glutamine}$. The $[\text{U-}^{13}\text{C}_5]\text{glutamine}$ tracer transfers *four* ^{13}C atoms to TCA cycle intermediates through oxidation (forward TCA cycle), generating M4 malate, fumarate, and aspartate; whereas it incorporates *five* ^{13}C atoms through RC of α -ketoglutarate, generating M5 citrate, M3 oxaloacetate, M3 aspartate, M3 malate, and M3 fumarate. Acetyl-CoA molecules originating from $[\text{U-}^{13}\text{C}_5]\text{glutamine}$ are fully labeled. Therefore, $[\text{U-}^{13}\text{C}_5]\text{glutamine}$ can be used to trace the overall contribution of glutamine (via RC and glutaminolysis) to lipid synthesis (Fig. 19.3, taken from Gameiro, 2013). Of note, since the glutaminolytic pathway generates M3 pyruvate from $[\text{U-}^{13}\text{C}_5]\text{glutamine}$ (via mitochondrial ME), M5 citrate can in principle be formed via condensation of glutaminolysis-derived acetyl-CoA (M2 AcCoA) with M3 oxaloacetate (not depicted in Fig. 19.3). Thus, the M5 citrate from $[\text{U-}^{13}\text{C}_5]\text{glutamine}$ can be an ambiguous readout of RC activity in cells that heavily rely on complete glutaminolysis (glutamine-to-pyruvate conversion).

To specifically trace the contribution of RC to the TCA cycle, we can use $[1\text{-}^{13}\text{C}]\text{glutamine}$, in which ^{13}C -labeled carbon is lost during oxidation of α -ketoglutarate but is retained on citrate and downstream metabolites through RC activity (Fig. 19.4, taken from Gameiro, 2013). To trace the contribution of RC to lipid synthesis, the $[5\text{-}^{13}\text{C}]\text{glutamine}$ tracer should be used, as the $[1\text{-}^{13}\text{C}]\text{glutamine}$ -derived isotopic label cannot be incorporated in acetyl-CoA through RC. $[5\text{-}^{13}\text{C}]\text{glutamine}$ transfers one ^{13}C atom to acetyl-CoA and fatty acids through RC *only*. Although the $[5\text{-}^{13}\text{C}]\text{glutamine}$ -derived isotopic label is transferred to TCA cycle metabolites oxidatively, it cannot be incorporated in the acetyl-CoA carbon skeleton of TCA cycle metabolites (see the acetyl-CoA moiety highlighted by dashed circles in Fig. 19.4). Consequently, $[5\text{-}^{13}\text{C}]\text{glutamine}$ is specific to trace the RC-to-lipid flux.

It should also be noted that oxidation of $[\text{U-}^{13}\text{C}_5]$ - or $[1\text{-}^{13}\text{C}]\text{glutamine}$ via TCA cycle can yield ^{13}C -labeled CO_2 , which can be further incorporated into some metabolites (e.g., malate) through a carboxylation reaction. Although in a cell culture system this reincorporation of ^{13}C can be considerably diluted by unlabeled bicarbonate in the media

and the high level of unlabeled CO₂ maintained in the incubator, it may become necessary to account for this process if *in vivo* labeling experiments are performed.

3.2. Isotopomer spectral analysis

The principle of isotopomer spectral analysis (ISA) was initially developed by Kelleher and colleagues to quantify *de novo* synthesis of fatty acids using ¹³C-labeled acetate tracers (Kelleher & Masterson, 1992; Kharroubi, Masterson, Aldaghlis, Kennedy, & Kelleher, 1992). ISA provides a general framework to measure the contribution of different sources to the precursor pool (e.g., acetyl-CoA) of a given product (e.g., palmitate); it requires the biochemistry to follow the polymerization principle, with monomeric subunits (the precursors) condensing into a polymer (the product). There are two dimensionless parameters that are determined by the ISA approach: *D*, which is the fractional contribution of ¹³C-enriched atoms of the tracer versus endogenous pathways ($1 - D$) as sources of the precursor (lipogenic acetyl-CoA); *g(t)* indicates the fraction of newly synthesized product in the sample (e.g., palmitate) (Fig. 19.5, taken from Gameiro, 2013). ISA assumes that the precursor mixture rapidly achieves metabolic and isotopic steady state during the incubation with the tracer. A key component of the ISA model is that the sampled product is not required to attain isotopic steady state. The ISA model uses equations to calculate the probability of the appearance of each mass isotopomer (M0, M1, M2, etc.) in the polymer molecule, based on test values for *D* and *g(t)* (Kelleher & Masterson, 1992; Yoo et al., 2004). These probabilities are compared with MID of the polymer measured by GC-MS. A fitting procedure to minimize differences of measured and simulated values outputs a best estimate for *D* and *g(t)*. Here, we performed ISA using the elementary metabolite unit framework-based software *Metran* (Antoniewicz, Kelleher, & Stephanopoulos, 2007b; Young, Walther, Antoniewicz, Yoo, & Stephanopoulos, 2008) on measured palmitate and estimated the percent contribution to lipogenic acetyl-CoA (*D* value) and the percent *de novo* lipogenesis (*g(t)* value), together with their associated confidence intervals. The confidence intervals of *D* and *g(t)* values can be estimated based on measurement errors of the palmitate MID and the metabolic model of ISA (Fig. 19.5) (Antoniewicz, Kelleher, & Stephanopoulos, 2006). Since the precursor acetyl-CoA can come from both glucose-derived pyruvate and glutamine-derived α-ketoglutarate (Fig. 19.4), it is possible to use [U-¹³C₆]glucose and [U-¹³C₅]glutamine to determine their relative contribution to the *de novo* lipogenesis (Gameiro et al., 2013).

For calculation of the absolute *de novo* lipogenic flux (in picomoles of palmitate per cell per hour), the quantity of newly synthesized palmitate can be determined by multiplying the percentage of *de novo* lipogenesis (*g(t)* value) by the total cellular palmitate. Similarly, the absolute flux of a given substrate (e.g., glutamine) to palmitate can be calculated by multiplying the percent contribution to lipogenic acetyl-CoA (the *D* value) by the amount of newly synthesized palmitate and dividing by the integral viable cell density over the course of the experiment.

3.3. Hypoxia case study

A recent example from our lab demonstrates the application of MID analysis and ISA to demonstrate the role of reductive glutamine metabolism in cancer cell *de novo* lipogenesis

under hypoxia (Metallo et al., 2012). After culturing A549 lung adenocarcinoma cells in 1% O₂, we found that, while consumption of glutamine increased relative to 21% O₂ culture conditions, secretion of glutamate into the media did not change (Fig. 19.6A), suggesting an additional biosynthetic role for glutamine activated under hypoxia. To investigate, we first examined the potential fates for glutamine under normoxic conditions, namely oxidative and reductive TCA cycle metabolism. In several cancer cell lines tested, the specific contribution of RC to lipogenesis was estimated by ISA using [5-¹³C]glutamine, which showed that RC, in contrast to glutaminolysis, accounted for the majority of total lipogenic flux from glutamine (determined by ISA using [U-¹³C₅] glutamine) (Fig. 19.6B). We further observed an increase of RC flux in A549 cells under hypoxia, as indicated by a significantly higher contribution of glutamine-derived acetyl-CoA (estimated using [5-¹³C]glutamine) as compared to the total contribution of glucose-derived acetyl-CoA (estimated using [U-¹³C₆]glucose) to lipid synthesis (Fig. 19.6C), as well as a substantial increase in ¹³C label from [1-¹³C]glutamine retained in citrate and downstream metabolites (Fig. 19.6D). Knockdown of the isocitrate dehydrogenase (IDH) isoform encoded by *IDH1* (cytosolic, NADP⁺), but not *IDH2* (mitochondrial, NADP⁺), reduced ¹³C labeling of TCA cycle intermediates in [1-¹³C]glutamine-cultured cells (Fig. 19.6E), demonstrating that IDH1 was responsible for the RC flux under these conditions.

4. SUMMARY

Glutamine has been well known as a central precursor for protein and nucleotide synthesis in proliferating cells. However, in an anaplerotic pathway upregulated in many cancer cells, it can also be converted to α-ketoglutarate and incorporated in the TCA cycle, where it can serve as a supplementary carbon. Strikingly, under conditions of hypoxia or defective mitochondrial function, glutamine can become the major source of lipogenic acetyl-CoA through reductive carboxylation. Given the fact that glutamine has become increasingly important in understanding the metabolism of cancer cells, tools to quantify glutamine metabolism are urgently needed.

Here, we described the method of using stable isotope tracers to study the fate of glutamine and its important role for cancer cell proliferation and survival. First, we described the methods for tracer experiments, measurement of extracellular metabolites, extraction of intracellular metabolites, and GC-MS analysis. Then, we discussed optimal tracer selection for determining glutamine contribution to the TCA cycle via glutaminolysis and RC through MID analysis. Finally, we described the framework of isotopomer spectral analysis to assess the fractional contribution of glutamine to lipid synthesis.

Glutamine appears to be essential for cancer cells beyond the need as a nitrogen source but also as a key metabolite that participates in central carbon metabolism as well as energy production. However, the mechanisms by which glutamine metabolism is regulated are not fully understood. In this chapter, we describe stable isotope-assisted methods that can be applied to investigate the regulation of glutamine catabolism and how glutamine promotes cancer cell proliferation and survival.

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Figure 19.1. Glutamine metabolism in mammalian cells

The figure shows the two major routes for glutamine metabolism in mammalian cells, glutaminolysis and reductive carboxylation. In either pathway, glutamine is first deaminated to glutamate, which is then converted to α -ketoglutarate via glutamate dehydrogenase (GDH) or transamination. In glutaminolysis, α -ketoglutarate is oxidized to malate via the TCA cycle and subsequently decarboxylated to pyruvate via malic enzyme (ME) or oxidized to oxaloacetate via malate dehydrogenase (MDH). In reductive carboxylation, α -ketoglutarate is reductively converted via isocitrate dehydrogenase (IDH) to isocitrate, which is then isomerized to citrate. Abbreviations: LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; CS, citrate synthase; ACL, ATP citrate lyase; ACO, aconitase; OGDH, oxoglutarate dehydrogenase; SDH, succinate dehydrogenase; FH, fumarase; MDH, malate dehydrogenase; GLS, glutaminase.

Figure 19.2. Typical flow of isotopic tracer experiments to study cancer metabolism

The scheme summarizes the main steps in an isotopic tracer-assisted study of cancer metabolism and common factors need to be considered, which are discussed in this section.

Figure 19.3. Carbon atom transition map for [U- $^{13}\text{C}_5$]glutamine

The map illustrates the fate of [U- $^{13}\text{C}_5$]glutamine in the TCA cycle and palmitate (^{12}C atoms are represented by empty circles and ^{13}C -atoms are represented by filled circles). Mass isotopomers generated by RC include M5 citrate (Cit), M3 oxaloacetate (Oac), M3 aspartate (Asp), M3 malate (Mal), and M3 fumarate (Fum). Mass isotopomers generated by oxidative metabolism include M4 Cit, M4 succinate (Suc), M4 Fum, and M4 Oac. Both RC and glutaminolysis (not shown) generate fully labeled acetyl-CoA (M2 AcCoA). For simplicity, labeling patterns arising from molecular symmetry and cellular compartments are not shown. The first round of the TCA cycle is illustrated, and positional labeling is depicted for relevant metabolites.

Figure 19.5. The isotopomer spectral analysis (ISA) method

^{13}C -labeled glucose tracer is used to trace the glucose-to-lipid flux. The glucose-derived ^{13}C atoms and endogenous sources (e.g., acetate and glutamine) contribute to the pool of lipogenic acetyl-CoA. The D parameter indicates the unknown fractional contribution (or relative flux) of the glucose tracer to lipogenic acetyl-CoA. During the incubation with the tracer, palmitate is synthesized from eight molecules of acetyl-CoA. The newly synthesized palmitate mixes with an existing pool of palmitate (prior to tracer addition) and the fraction of *de novo* palmitate synthesis after a period of time, t is $g(t)$. Each bar graph represents the palmitate MID. Preexisting palmitate has a significant M1 enrichment due to natural abundance. The parameters D and $g(t)$ are estimated by fitting the predicted to the measured MID in the sampled palmitate.

Figure 19.6. Hypoxia case study data

Representative data based on authors' previous study on RC and hypoxia. (A) Cell-specific uptake and secretion rates of glutamine and glutamate, respectively, in A549 cells under normoxic and hypoxic conditions. (B) Fractional contribution of total glutamine and RC-metabolized glutamine to lipogenic acetyl-CoA for multiple cancer cell lines, as determined by ISA. (C) Fractional contribution of glucose oxidation and glutamine-derived αKG reduction toward lipid synthesis in A549 cells under normoxic and hypoxic conditions. (D) Fraction of TCA cycle metabolites containing M1 label when cultured in [1-¹³C]glutamine in A549 cells under normoxic and hypoxic conditions. (E) Small-hairpin RNA-induced knockdown of *IDH1* but not *IDH2* expression reduces citrate M1 labeling in various cancer cell lines when cultured in [1-¹³C]glutamine.

Table 19.1

GC programs for polar metabolite and fatty acid samples

Polar metabolites	Fatty acids
Hold at 70 °C for 2 min	Hold at 180 °C for 2 min
Increase to 140 °C at 3 °C/min	Increase to 200 °C at 8 °C/min
Increase to 150 °C at 1 °C/min	Hold at 200 °C for 8 min
Increase to 280 °C at 3 °C/min	Increase to 280 °C at 10 °C/min
Hold at 280 °C for 6.33 min	Hold at 280 °C for 7.5 min

Table 19.2

Mass fragments of intracellular metabolites for GC-MS analysis

Metabolite	Retention time (min)	Mass (<i>m/z</i>)	Carbon atoms	Fragment formula
Pyruvate	8.8	174	1–2–3	C ₆ H ₁₂ O ₃ NSi
Succinate	24.0	289	1–2–3–4	C ₁₂ H ₂₅ O ₄ Si ₂
Fumarate	24.5	287	1–2–3–4	C ₁₂ H ₂₃ O ₄ Si ₂
Malate	32.5	419	1–2–3–4	C ₁₈ H ₃₉ O ₅ Si ₃
Aspartate	33.7	418	1–2–3–4	C ₁₈ H ₄₀ O ₄ NSi ₃
Citrate	44.0	459	1–2–3–4–5–6	C ₂₀ H ₃₉ O ₆ Si ₃
Palmitate	8.5	270	1 to 16	C ₁₇ H ₃₄ O ₂