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Original Research Article

Probing the metabolic phenotype of breast cancer cells by multiple tracer stable isotope resolved metabolomics

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$^{13}C_3$-glycerol (PubChem CID:753)
$^{13}C_8$-octanoate (PubChem CID:11939)
lactate (PubChem CID:91435)
ATP
(PubChem CID:5927)
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ABSTRACT

Breast cancers vary by their origin and specific set of genetic lesions, which gives rise to distinct phenotypes and differential response to targeted and untargeted chemotherapies. To explore the functional differences of different breast cell types, we performed Stable Isotope Resolved Metabolomics (SIRM) studies of one primary breast (HMEC) and three breast cancer cells (MCF-7, MDAMB-231, and ZR75-1) having distinct genotypes and growth characteristics, using $^{13}C_6$-glucose, ^{13}C-1+2-glucose, $^{13}C_5$,$^{15}N_2$-Gln, $^{13}C_3$-glycerol, and $^{13}C_8$-octanoate as tracers. These tracers were designed to probe the central energy producing and anabolic pathways (glycolysis, pentose phosphate pathway, Krebs Cycle, glutaminolysis, nucleotide synthesis and lipid turnover). We found that glycolysis was not associated with the rate of breast cancer cell proliferation, glutaminolysis did not support lipid synthesis in primary breast or breast cancer cells, but was a major contributor to pyrimidine ring synthesis in all cell types; anaplerotic pyruvate carboxylation was activated in breast cancer versus primary cells. We also found that glucose metabolism in individual breast cancer cell lines differed between in vitro cultures and tumor xenografts, but not the metabolic distinctions between cell lines, which may reflect the influence of tumor architecture/microenvironment.

1. Introduction

Breast cancer continues to be a major disease afflicting 290,000 people every year in the US, of which ca. 40,000 died (ACS, 2016). Although in recent years, improved surgical techniques, diagnostic procedures and targeted therapeutics such as trastuzumab and anti-estrogen treatment have significantly improved the overall survival rates for those patients who can benefit (Hudis, 2007) (Bliss, Kilburn et al., 2012), recurrence remains a problem. In addition, some forms of breast cancer are refractory to treatment, in particular the triple negative breast cancers and BRCA1 and 2 breast cancers. As with many cancers, 5-year survival rates decrease markedly with increasing stages as the options for treatment become limited (ACS, 2016).

Although numerous genetic lesions that are responsible for the

Abbreviations: DSS, 2,2-dimethyl silapentane-5-sulfonate; ER$^+$, estrogen receptor positive; FID, free induction decay; FT-ICR-MS, fourier transform-ion cyclotron resonance-mass spectrometry; HMEC, human mammary epithelial cell; PPP, Pentose Phosphate Pathway; TCA, trichloracetic acid; SIRM, Stable Isotope Resolved Metabolomics; TME, tumor microenvironment

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development of various subtypes of breast cancers have been identified, for example *BRCA1,2*, *HER2*/neu, among many others (Stephens, Tarpey et al., 2012), the underlying genetics describes only part of the problem, and is therefore insufficient from the diagnostic and treatment standpoints (Graur, Zheng et al., 2013). Cancers are also metabolic diseases, as the metabolic activity of transformed cells is altered to provide advantages in growth, survival and metastasis (Fan et al., 2009; Vander Heiden et al., 2009; Linehan et al., 2010; Le et al., 2012a, 2012b; Liu et al., 2012; Marin-Valencia et al., 2012a; Yuneva et al., 2012). Metabolic reprogramming is now regarded as one of the hallmarks of cancer (Hanahan and Weinberg, 2011). Although the set of genetic lesions, the origin of the transformed cells, and their microenvironment differ among cancer cells (Fan et al., 2009; Marin-Valencia et al., 2012b; Giussani et al., 2015), their metabolism is generally enhanced to provide sufficient metabolic energy and anabolic substrates to drive proliferation, which requires macromolecular biosynthesis. Often, cancer cell metabolism is also adapted to deal with an increasingly hostile extracellular (Gatenby and Gillies, 2008) and intracellular environments, including ROS production from accelerated respiration (Fantin, St-Pierre et al., 2006; Telang et al., 2007; Strovas, McQuaide et al., 2010; Weinberg, Hamanaka et al., 2010; Koppenol, Bounds et al., 2011).

An especially well-attested metabolic adaptation in cultured cancer cells and in tumors is accelerated lactic fermentation even in the presence of oxygen, the so-called Warburg effect. This results not only in increased glucose uptake, but also a diversion of the glycolytically produced pyruvate to lactate, and the co-export of the lactate with a proton, thereby acidifying the extracellular compartment (Tannock and Rotin, 1989; Gillies and Gatenby, 2007). Nevertheless, a significant fraction of the consumed glucose is used anabolically, including synthesis of hexose derivatives (Moseley, Lane et al., 2011), nucleotides and nucleic acids biosynthesis (via the pentose phosphate pathway), the pyrimidine pathway via glucose-and glutamine-derived aspartate (Fan, Tan et al., 2012; Lane and Fan, 2015) the purine pathway via glucose derived serine, glycine, and one-carbon metabolism (Lee, Boros et al., 1998; Kominsky, Klawitter et al., 2009; Possemato, Marks et al., 2011; Fan, Tan et al., 2012; Locasale, 2013; Labuschagne, van den Broek et al., 2014; Lane and Fan, 2015), complex lipid biosynthesis (Swinnen, Brusselmans et al., 2006; Lane, Fan et al., 2009; Metallo, Gameiro et al., 2011), and protein synthesis (cf. Fig. 1). Moreover, glucose is metabolized in the mitochondrial Krebs cycle as mitochondria remain active in many cancer cells, and in fact may respire at a higher rate than the untransformed cell counterparts (Telang et al., 2007; Strovas, McQuaide et al., 2010). The respiratory activity is often, though not always perfectly, coupled to ATP synthesis (Gogvadze, Zhivotovsky et al., 2009) to supplement glycolytically-derived energy. Mitochondria of cancer cells are also active in oxidizing substrates other than glucose-derived pyruvate, notably glutamine (Yuneva et al., 2007; DeBerardinis and Cheng, 2010) and in certain circumstances, fatty acids (Guppy, Leedman et al., 2002). As Fig. 1 shows, the ultimate fate of the glutamine carbon is variable (Mazurek and Eigenbrodt, 2003; DeBerardinis and Cheng, 2010; Fan et al., 2012; Le et al., 2012a, 2012b), though the amide nitrogen is used in nucleobase synthesis, in the hexosamine pathway, or excreted as ammonium ions via glutaminase activity (DeBerardinis and Cheng, 2010; Lane and Fan, 2015).

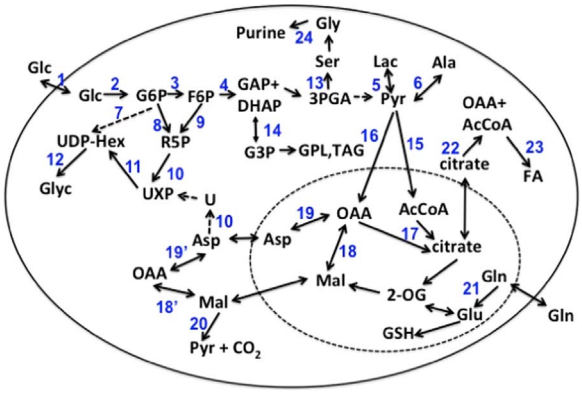

Fig. 1. Carbon fates in central metabolism. Observed fates of glucose and glutamine-derived carbon in breast cancer cells. The inner dashed box represents mitochondrial reactions. 1 glucose transporter; 2 hexokinase; 3 phosphoglucomutase; 4 aldolase; 5 lactate dehydrogenase; 6 alanine amino transferase; 7 glucose/hexosamine pathway; 8 pentose phosphate pathway: oxidative branch; 9 pentose phosphate pathway: non-oxidative branch ; 10, pyrimidine nucleotide synthesis; 11 hexosamine pathway; 12 glycogen synthesis; 13 phosphoglycerate dehydrogenase; 14 glycerol-3-phosphate dehydrogenase 15 pyruvate dehydrogenase; 16 pyruvate carboxylase; 17 citrate synthase; 18, 18' malate dehydrogenase; 19,19' aspartate aminotransferase; 20 malic enzyme; 21 glutaminase; 22 ATP-dependent citrate lyase; 23 fatty acid synthesis; 24 purine biosynthesis.

Although glucose is generally the preferred carbon source for growth and survival for cancer cells by supporting glycolysis and the pentose phosphate pathway, Gln is also important by providing both carbon and nitrogen for pyrimidine and purine biosynthesis. Both of these substrates along with serine, glycerol and fatty acids may also be used for lipid biosynthesis. Here we report the use of multiple stable isotope tracers and SIRM to compare the fates of different carbon sources for lipid and central metabolism in estrogen-sensitive breast cancer cells, a triple negative breast cancer cell, and an untransformed human mammary epithelial cells (HMEC). These ^{13}C-enriched carbon sources helped delineate specific metabolic pathways that are central to proliferation, and therefore enabled understanding of the metabolic basis for differential proliferation among the cell types.

2. Materials and methods

2.1. Materials

Established cancer cell lines (Table 1) were purchased from ATCC. Primary HMEC cells were obtained from Lonza.

Isotopically enriched precursors, [U-^{13}C]-glucose, [^{13}C-1], [^{13}C-2] glucose, [U-^{13}C,^{15}N]-glutamine, [U-^{13}C]-octanoate and [U-^{13}C]-glycerol were purchased from Sigma Isotec (St. Louis, MO), and were at > 99% isotope purity.

2.2. Cell growth and growth curves

Cells were cultured in RPMI containing 5.5 mM glucose +4 mM Gln in which either the glucose contained ^{13}C at all or specific position (C1 or C2) or the glutamine contained ^{13}C and ^{15}N at all positions. When present, [U-^{13}C]-octanoate or [U-^{13}C]-glycerol were added to a final concentration of 2 mM in the cell media. Cell growth was monitored by trypan blue exclusion and counting viable cells.

2.3. Mouse Xenografts

NOD/SCID Gamma (NSG) mice were obtained from Jackson Laboratories (Bar Harbor, Maine), were caged and fed according to

Table 1
Cells used for Stable Isotope Resolved Metabolomics (SIRM) analysis.

Cell Designation	optimal [E2]/ nM	Doubling time/h	Tumorigenic?
HMEC	–	48	N
MDA-MB-231	–	18–20	Y
MCF-7	10	30	Y
ZR-75–1	80	80	Y

E2: 17-β-estradiol; HMEC: primary human mammary epithelial cells, MDA-MB-231: a triple negative breast carcinoma cell; MCF-7, ZR-75-1: estrogen-receptor positive breast carcinoma cells