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Original Research Article

Metabotypes of breast cancer cell lines revealed by non-targeted metabolomics

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ABSTRACT

We present an analysis of intracellular metabolism by non-targeted, high-throughput metabolomics profiling of 18 breast cell lines. We profiled > 900 putatively annotated metabolite ions for > 100 samples collected under both normoxic and hypoxic conditions and revealed extensive heterogeneity across all metabolic pathways and cell lines. Cell line–specific metabolome profiles dominated over patterns associated with malignancy or with the clinical nomenclature of breast cancer cells. Such characteristic metabolome profiles were reproducible across different laboratories and experiments and exhibited mild to robust changes with change in experimental conditions. To extract a functional overview of cell line heterogeneity, we devised an unsupervised metabotyping procedure that for each pathway automatically recognized metabolic types from metabolome data and assigned cell lines. Our procedure provided a condensed yet global representation of cell line metabolism, revealing the fine structure of metabolic heterogeneity across all tested pathways and cell lines. In follow-up experiments on selected pathways, we confirmed that different metabolic types correlated to differences in the underlying fluxes and difference sensitivity to gene knockdown or pharmacological inhibition. Thus, the identified metabotypes recapitulated functional differences at the pathway level. Metabotyping provides a powerful compression of multi-dimensional data that preserves functional information and serves as a resource for reconciling or understanding heterogeneous metabolic phenotypes or response to inhibition of metabolic pathways.

1. Introduction

Cellular metabolism supports core processes such as growth, proliferation, differentiation, migration, and stress resistance. These processes occur through a large but finely coordinated network of biochemical reactions that can catabolize a large number of substrates and fulfill biosynthetic, energetic, and redox requirements in a balanced way. Regulation and coordination of these activities is complex and depends not only on external cues (i.e., nutrients, hormones, cell-to-cell interactions), receptors, and intertwined signaling cascades but also on the metabolic network itself in which metabolites act as integrative signals and drive cellular decision-making (Carey et al., 2015; Wellen et al., 2010).

Such processes are pivotal for cancer cell survival and tumorigenesis (Locasale et al., 2009; Vander Heiden et al., 2011). Solid tumors feature metabolic alterations that generally increase their physiological activity and enable adaptation to changing environments. A common alteration is enhanced glucose uptake and lactate secretion regardless of oxygenation (the Warburg effect), but dependencies on other nutrients are being more frequently reported (Jain et al., 2012; Mayers and Vander Heiden, 2013; Vander Heiden et al., 2009; Yuneva et al., 2007). The metabolic peculiarities of cancer cells are of utmost interest for selective therapy, and several novel drugs targeting metabolism are currently or about to be evaluated in clinic trials (Galluzzi et al., 2013; Zhao et al., 2013). Metabolic alterations in cancer can be the consequence of oncogene activation or loss of tumor suppressors, but genetic alterations within the coding region of metabolic enzymes also have been described (Cairns et al., 2011).

A grand challenge in biomedical research is the heterogeneity that ubiquitously and naturally occurs among and within individuals. Phenotypic heterogeneity arises in normal tissues by environmental differences, differentiation programs, or oscillations coupled to, e.g.,

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cell cycle or circadian clocks. Cellular heterogeneity is further amplified in many diseases that feature increased genomic instability, such as cancer, leading to substantial variety of genetic variants (Parsons et al., 2008; Pleasance et al., 2010; Wood et al., 2007). Such abnormal heterogeneity has severe clinical implications as it translates into differential responses of disease cells to pharmacological treatments and may cause serious complications such as resistance to therapy or relapse (Bedard et al., 2013; Meacham and Morrison, 2013). Cellular heterogeneity manifests also at the metabolic level (Hu et al., 2013), where it can affect cell growth, nutritional requirements, the capacity to endure stress and proliferate in diverse microenvironments, use of alternative pathways, and susceptibility to metabolic perturbations. These factors are crucial for cancer cells to adapt to the demands of proliferation, tumor growth and metastases. As recent studies demonstrate, cancer cells adopt multiple strategies to secure their metabolic demands (Cantor and Sabatini, 2012).

It remains very challenging to investigate metabolic heterogeneity or, more generally, phenotypes solely on the basis of genomic or expression data. The main limitation of such top-down studies is the complexity of predicting how genomic alterations affect enzyme abundance and kinetics and eventually cell-wide metabolic fluxes. Instead, the bottom-up characterization of metabolic phenotypes at the flux or metabolome level is preferable because it provides a direct readout on metabolism. In a milestone study, Jain et al. (2012) reported the characterization of uptake and secretion rates of the NCI60 cancer cell line. In addition to the identification of a ubiquitous dependency on glycine for proliferation, it is striking how many singularities they observed in the uptake and production of metabolites by specific cell lines only.

We wondered how this apparent heterogeneity in the exometabolome translates to the intracellular metabolism of cell lines. We decided to focus on a breast tissue–specific panel of 18 cell lines, 4 of which were non-malignant. These cell lines have been widely phenotyped and characterized (Neve et al., 2006). Studies of the heterogeneity of breast cancer have led to classification into three main clinical groups: i) the estrogen receptor (ER) -positive group, in which the progesterone receptor (PR) is often co-expressed; ii) the human epidermal growth factor receptor 2 (HER2) amplified group; and iii) the triple-negative breast cancer group, lacking ER, PR, or HER2 expression and thus being particularly difficult to treat (Eroles et al., 2012; Weigelt and Reis-Filho, 2009). Recent studies of genomes and transcriptomes have revealed a large diversity of driver mutations among one group of tumors, leading to refinement of these categories (Curtis et al., 2012; Neve et al., 2006; Parker et al., 2009; Reis-Filho and Pusztai, 2011; Shah et al., 2012; Stephens et al., 2012; The Cancer Genome Atlas Network, 2012).

We previously investigated the susceptibility of these breast cell lines to the knockdown of 231 metabolic enzymes and found heterogeneous responses to functional perturbations (Baenke et al., 2015). In the current study, we employed metabolomics to characterize the heterogeneity in intracellular metabolism of the breast cell lines grown *in vitro* under normoxia and hypoxia. Our analysis reveals an extremely heterogeneous and fine-grained landscape of metabolic profiles. We propose an unsupervised metabotyping procedure that automatically recognizes peculiar metabolome profiles within pathways and assigns cell lines to different groups.

2. Material and methods

2.1. Cell culture

Cell lines were obtained from the American Type Culture Collection and LRI Cell Services. All cells were cultured in DMEM: F12 (Gibco 21331), with 2 mM L-glutamine freshly added (Gibco 25030) and 1% penicillin/streptomycin (Gibco 15070). Medium was supplemented with 10% fetal bovine serum (Gibco 10270) for the cancer cell lines and 5% horse serum (Sigma-Aldrich H1270), 20 ng/ml epidermal growth factor (Sigma Aldrich E1257), 0.5 µg/ml hydrocortisone (Calbiochem 3867), 10 µg/ml insulin (Sigma Aldrich I9278) and 100 ng/ml cholera toxin (Sigma Aldrich C8052) for the non-malignant cell lines. When poorer medium was used cell were grown in DMEM (Gibco 11966) supplemented with 17,5 mM of glucose (the same concentration as in DMEM: F12). Cell line were grown at 37 °C with 5% CO_2. For hypoxic culture, oxygen was limited to 0.5%. Cell lines were maintained according to standard protocols, their identity verified via STR sequencing (Microsynth) and tested for mycoplasma infections (Sigma Aldrich D9307).

2.2. Metabolite extract preparation

For all metabolomics experiments, cell lines were seeded in 6 well plates, medium was changed after 24 h, and cell were collected as follows after 48 h. Cell were seeded in order to obtain 70–80% confluency after 48 h (confluency of cell lines is used here as a proxy for volume, i.e. similar confluency equal similar cell volume extracted). At 48 h, medium was removed via aspiration and cells were washed twice with a wash solution (75 mM Ammonium Carbonate, adjusted to pH 7.4 with Acetic Acid). Metabolism was quenched by dipping the bottom of the plate in liquid nitrogen for 1 min. Frozen plates were transferred to −80 °C freezer until further usage.

Extracts were generated by adding twice 0.7 ml of 70% (v:v) ethanol:water at 75 °C to each well and keeping the plate on a hot metallic block (at 75 °C) for 2 min. After the 2 min, extracts were transferred to Eppendorf tubes and kept on ice. The same amount of hot ethanol was added a third time to wash thoroughly the plate. Extracts were then dried at 0.12 mbar in a SpeedVac (Christ, Germany). Dried samples were conserved at −80 °C until measurement.

For labeling experiments, cells were seeded in two 6 well plates. Cells were kept for 96 h in labeled medium (DMEM (Gibco 11966) supplemented with 17,5 mM fully labeled glucose or DMEM: F12 supplemented with 2 mM labeled glutamine) with change of medium at 24 and 72 h. 1 plate per cell line was extracted as mentioned above. To the second plate, 400 µl of 4:4:2 (v:v:v) mix of acetonitrile/methanol/water at −20 °C containing 400 µM of phenylhydrazine for derivatization of pyruvate (Zimmermann et al., 2014) were added. Plates were stored for 10 min at −20 °C and extraction solvent was collected and transferred to Eppendorf tubes and kept on ice. The procedure was repeated a second time, followed by a final wash with the same amount of solvent, before the extracts were dried and conserved as above. The original experiment with 18 cell lines (shown in Fig. 1B) was performed in the lab of AS by FB. Repetitions with a subsets of cell lines (used in Supplementary Fig. 3), uptake and labeling experiment were performed by SD in the lab of NZ. All samples were injected simultaneously as mentioned in the following sections.

2.3. Mass spectrometry measurements

Dried sample were resuspended in 100 µl of deionized water and centrifuged at 20′000 rcf for 30 min at 4 °C. For untargeted metabolomics, clean extracts were plated in 96 well plates and quantification of metabolites was performed by randomized double injection of a total of 10 µl on an Agilent 6550 QTOF instrument (or on an Agilent 6520 QTOF instrument for the serum swap experiment) by flow injection analysis time-of-flight mass spectrometry (Fuhrer et al., 2011). All samples were injected in duplicates. Ions were annotated based on their accurate mass and the Human Metabolome Database (HMDB) (Wishart et al., 2013) reference list allowing a tolerance of 0.001 Da and systematically accounting for numerous expected ions, adducts, and isotopes. Importantly, accurate mass doesn't allow distinguishing between compounds with identical molecular formula and, hence, ions can match multiple chemical formulas. These are