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Original Article

Metabolic re-wiring of isogenic breast epithelial cell lines following epithelial to mesenchymal transition

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ABSTRACT

Epithelial to mesenchymal transition (EMT) has implications in tumor progression and metastasis. Metabolic alterations have been described in cancer development but studies focused on the metabolic re-wiring that takes place during EMT are still limited. We performed metabolomics profiling of a breast epithelial cell line and its EMT derived mesenchymal phenotype to create genome-scale metabolic models descriptive of both cell lines. Glycolysis and OXPHOS were higher in the epithelial phenotype while amino acid anaplerosis and fatty acid oxidation fueled the mesenchymal phenotype. Through comparative bioinformatics analysis, PPAR-γ1, PPAR- γ2 and AP-1 were found to be the most influential transcription factors associated with metabolic re-wiring. *In silico* gene essentiality analysis predicts that the LAT1 neutral amino acid transporter is essential for mesenchymal cell survival. Our results define metabolic traits that distinguish an EMT derived mesenchymal cell line from its epithelial progenitor and may have implications in cancer progression and metastasis. Furthermore, the tools presented here can aid in identifying critical metabolic nodes that may serve as therapeutic targets aiming to prevent EMT and inhibit metastatic dissemination.
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Introduction

Epithelial to mesenchymal transition (EMT) is a process where cells of epithelial origin lose their polarity and cell–cell adhesion and change their phenotype to mesenchymal-like cells. EMT is a fundamental process in embryonic development allowing cells to detach from the newly formed epithelium and migrate to other parts of the developing embryo [1,2]. Once they have reached their destination, these epithelial-derived mesenchymal cells revert to their original phenotype via mesenchymal to epithelial transition (MET) and take part in establishing new tissues and organs.

Many carcinomas are known to revive these mechanisms during cancer invasion and metastasis. EMT shifts the phenotype of the polarized epithelial cell, bound to its neighbors and extracellular matrix via tight junctions, desmosomes and E-cadherin, to an invasive mesenchymal phenotype that exists largely without direct cell–cell contacts or defined cell polarity. The ability to separate from neighboring cells and penetrate into the surrounding tissues

Abbreviations: EMT, Epithelial to mesenchymal transition; MET, Mesenchymal to epithelial transition; RNA-seq, RNA sequencing; UPLC-MS, Ultra-performance liquid chromatography mass-spectrometry; GEM, Genome scale metabolic model; FBA, Flux balance analysis; GPR, Gene-Protein-Reaction association; TF, Transcription factor.

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is believed to be an important initiating step in tumor metastasis [3]. EMT also acts in tumor progression by providing increased resistance to apoptotic agents [4], and by producing supporting tissues that enhance the malignancy of the central tumor [5]. As such, EMT confers on epithelial cells precisely the set of traits that would empower them to disseminate from primary tumors and seed metastases [6].

Metabolic alterations have been shown to play a role in determining cellular phenotypes. For example, knock-down of fructose-1,6-bisphosphatase has been shown to induce EMT in basal-like breast cancer cells [7], while knockdown of ATP citrate lyase has been shown to revert the EMT phenotype in non-small cell lung carcinoma cells [8]. Mutations or epigenetic changes that cause accumulation of certain metabolites have also been shown to induce EMT [9]. While these findings show that metabolic alterations are important for induction and maintenance of a mesenchymal phenotype; studies of the global metabolic changes that occur during EMT are still limited.

Genome scale metabolic models (GEMs) provide descriptions of metabolic phenotypes that can be queried computationally through constraint-based modeling [10]. The building of GEMs and their application to the analysis of metabolism of diverse biological processes is well established [11]. EMT metabolism has so far mainly been investigated through more targeted cell- and molecular biology based approaches. Previous efforts show that systems based analysis, in particular constraint based modeling methods, may provide important insights into EMT metabolism [9,12,13].

Here, we describe the metabolic phenotype of the immortalized, breast epithelial cell line, D492. We compare the metabolic phenotype of D492 to that of its mesenchymal "daughter" cell line, D492M. D492M cells were isolated after a spontaneous EMT event in D492 cells in 3D co-culture with endothelial cells [14]. Although neither of these cell lines are tumorigenic in an *in vivo* setting, they provide an interesting model system to investigate EMT without the need for external stimulation or genetic manipulation. Ultra performance liquid chromatography Mass Spectrometry (UPLC-MS) on spent media was used to quantify uptake and secretion of 43 selected metabolites. These data, coupled with microarray and RNA sequencing expression profiles, were used to build GEMs descriptive of metabolism in the two cell lines. We validated the computationally proposed metabolic phenotypes through enzymatic assays of intracellular ATP, NADH, and glutathione levels together with mitochondrial functionality assays. The GEMs were then used to predict enzymatic reactions and pathways of importance for the metabolic re-programming that occurs during EMT. Some of these reactions, in particular the large neutral amino acid transporter LAT1, appear important for cancer remission following breast cancer treatment. The results represent the construction of the first curated GEMs descriptive of metabolism pre- and post EMT. As such they serve as tools for future investigation of EMT metabolism. Furthermore, the results highlight how GEMs can be applied to the integrated analysis of polyomics data and propose metabolic biomarkers of importance for EMT and metastasis.

Materials and methods

Cell culture

D492 and D492M cells were cultured on collagen coated surfaces or in reconstituted basement membrane (Matrigel, Corning) in H14 serum-free medium as previously described [14] at 37 °C, 5% CO_2. H14 is a fully defined medium consisting of a DMEM/F12 base with 250 ng/ml insulin, 10 mg/ml transferrin, 2.6 ng/ml sodium selenite, 10^{-10} M estradiol, 1.4×10^{-6} M hydrocortisone, 5 mg/ml prolactin and 10 ng/ml EGF. Proliferation and ATP concentration was measured with a CellTiter Glo™ assay (Promega). Cell volume was calculated based on diameter measurements obtained from a Countess cell counting instrument (Invitrogen). For UPLC-MS, cells were seeded in triplicates into 24-well plates in 400 μL H14 medium at 15,000 cells/cm^2. Media was collected from cultures after 24 and 48 h along with cell-free controls, centrifuged to remove cellular debris and stored at −80 °C until further analysis.

Cell profiling

Total RNA sequence profiles were obtained for D492 and D492M as outlined in supplementary methods (**Additional file 2**). Microarray expression profiles of D492 and D492M cells were obtained from [14]. Medium metabolites were measured using an established metabolomics pipeline. Both the metabolite isolation procedure and metabolomic pipeline analysis were adapted from Paglia et al., 2014 [15]. The methods are explained in SI Materials and Methods. To account for differences in cell weight and growth rate, the measured metabolite concentrations were normalized to cell weight and growth rates as previously described [16]. Seahorse XFe-96 metabolic extracellular flux analyzer (Seahorse Biosciences) was used to measure the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR). See SI Materials and methods for details. Intracellular NAD^+/NADH and GSH/GSSG were assayed with respective Glo™ kits from Promega. Additional glucose and lactate measurements were performed in an ABL 90 blood gas analyzer (Radiometer, Brønshøj, Denmark).

Generation of a breast tissue specific metabolic model

RNA-seq data from the D492 and D492M cells were used to create a breast tissue specific model from the human metabolic reconstruction RECON2 [17] as follows. All genes with expression values exceeding a fixed cut-off value in either the D492 or D492M data sets that were also present in RECON2 were identified. The gene-protein-reaction rules (GPRs) of RECON2 were then used to identify the associated metabolic reactions and the FASTCORE model building algorithm [18] used to build a functional metabolic network from the list of reactions. The resulting network, referred to as the EMT model, was manually curated in order to ensure that no major pathways and metabolites were missing. Details of the model construction are provided in SI Materials and methods.

Construction and analysis of the D492 and D492M GEMs

The EMT model was used to create models of the epithelial D492 cells and mesenchymal D492M cells. Random sampling was first used to estimate flux ranges of all the reactions in the EMT model. While the RNA-seq data had considerably more coverage, it lacked the necessary replicates. Therefore, the microarray expression data was used to constrain the EMT model to simulate the effects of up and downregulated genes on reaction flux. This gave rise to models EPI (epithelial) and MES (mesenchymal) once metabolomics constraints had been applied. The two models generated in this manner therefore had the same stoichiometry but different constraints on reaction fluxes as defined by differential expression of metabolic genes and extracellular metabolomics measurements. Deriving the two models from a common model was done because the D492M cells initially arose from D492 cells and this facilitated both model curation and subsequent model comparisons. See SI Material and methods for details.

Analysis of the EPI and MES models

We used flux balance analysis (FBA) [19,20] and the generic biomass reaction present in RECON2 to predict the maximum growth rates of the EPI and MES models. Random sampling [21] was used to compare the two. An optimization algorithm was used to identify targets for transforming the epithelial phenotype into the mesenchymal phenotype and vice-versa. The algorithm works by relaxing flux bounds for reactions in one model in order to obtain a flux distribution that is as close as possible to the flux distribution in the other [13]. The genes corresponding to these reactions were identified via the GPRs and the associated transcription factors then located in SABiosciences' proprietary database (http://www.sabiosciences.com/chipqpcrsearch). Gene essentiality analysis was performed on the EPI and MES models by simulating single gene knockouts with FBA.

Results

D492M cells show reduced size and growth rate compared to D492 cells

In order to accurately calculate metabolic uptake and secretion rates morphological parameters including cell size and weight, and proliferation rates were established. In 2D culture, D492 cells display the typical cobblestone morphology of epithelial cells while D492M cells have acquired the spindle-like phenotype of mesenchymal cells, characterized by multiple membrane protrusions (Fig. 1A**, top row**). In 3D culture, D492 cells form organized, branching structures while D492M form colonies that have lost polarity and cell–cell contact (Fig. 1A**, bottom row**). Growth rate measurements in 2D indicated population doubling times of 25 h for D492 cells and

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