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Altered glutamine metabolism exposes EMT derived mesenchymal cells to PI3K/Akt/mTOR pathway inhibition

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Abstract

Background: Epithelial-to-mesenchymal transition (EMT) is a fundamental developmental process with strong implications in cancer progression. Understanding the metabolic alterations associated with EMT may open new avenues of treatment and prevention.

Methods: We utilize ^{13}C carbon analogs of glucose and glutamine to examine difference in internal flux patterns of metabolites in central carbon and lipid metabolism following EMT in breast epithelial cell lines. Furthermore, an isotopomer spectral analysis, ^{13}C-metabolic flux analysis and weighted correlation network analysis are utilized for investigation of the alterations in metabolic functionality following EMT in breast.

Results: There are inherent differences in metabolic profiles before and after EMT. We observed EMT-dependent re-routing of TCA-cycle flux characterized by increased mitochondrial *IDH2*-mediated reductive carboxylation of glutamine to lipid biosynthesis with a concomitant lowering of glycolytic rates and glutamine-dependent glutathione (GSH) generation. Our network approach identified specific subtype of cancer drugs that are significantly associated with GSH abundance and we confirmed this *in vitro*.

Conclusions: EMT-linked alterations in GSH synthesis modulate the sensitivity of breast epithelial cells to PI3K/Akt/mTOR inhibitors.

Keywords: Metabolomics; Epithelial-to-mesenchymal transition; Fluxomics; Network analysis; Breast cancer

Background

Epithelial to mesenchymal transition (EMT) is a fundamental developmental process where tightly bound epithelial cells differentiate into migratory mesenchymal cells that can relocate into adjacent or distant tissues. This process is vital for tissue restructuring during embryonic development and is also necessary for proper wound healing in adult tissue. EMT has strong implications in cancer progression and metastasis where primary tumor cells of epithelial origin can take on a motile phenotype with the ability to migrate through the body and establish secondary

Metabolic reprogramming is recognized as one of the ten cancer hallmarks as proposed by Hanahan and Weinberg [2]. In contrast to rapidly dividing cancer cells,

a mesenchymal phenotype faces a different set of metabolic requirements whose relation to malignant transformation has been intensely studied and associated with enhanced glycolysis, increased glutaminolysis, nucleotide metabolism and abnormal choline metabolism [3, 4, 5]. Quantitative understanding of the metabolic requirements of mesenchymal cells is however lacking, particularly the changes to the turnover and quantity of metabolites involved in xenobiotic clearance, i.e. the drug response of cells. Cancer cells that undergo EMT have increased resistance to various drugs [6, 7, 8], which indicates that the xenobiotic clearance of the cells is altered. There are three steps involved in the metabolism of xenobiotics: 1) Modification, 2) Conjugation and 3) Excretion. Conjugation involves the binding of special metabolites (e.g. glutathione, UDP-glucuronate, PAPS, S-adenosylmethionine) to a xenobiotic compound, which leads to the assumption that the availability of these particular metabolites within cells influences the activity of the drug. Therefore, accurate metabolic measurements of EMT may contribute to better understanding of the drug resistance of cancer cells and lead to novel therapeutic approaches aimed at eliminating metastatic cancer cells.

We have previously used both ultra-performance liquid chromatography coupled mass spectrometry (UPLC-MS) and NMR to study EMT and cancer metabolism [9, 10, 11]. Integrated analyses of these metabolomics data with transcriptomic and proteomic data within genome-scale metabolic models predicted metabolic differences that occur following EMT in breast epithelium [11]. These included alterations to glycolysis, the pentose phosphate pathway TCA flux and fatty acid synthesis. Although these models provided useful insights into metabolic alterations associated with EMT, they lacked accuracy in predicting internal fluxes in a quantitative manner in the compartmentalized central carbon metabolism.

In order to better understand the metabolite flux changes that accompany EMT we characterized the internal flow of metabolites in D492 breast epithelial cells and their mesenchymal variant, D492M, to provide a detailed, quantitative analysis of metabolic reprogramming following EMT in breast. We performed stable isotope tracing of ^{13}C labelled glucose and two separate ^{13}C labelled glutamine analogs. UPLC-MS and NMR were used to measure label incorporation into metabolites associated with central carbon

metabolism and lipid biosynthesis. Next, we utilized these data to perform ^{13}C-metabolic flux analysis (13C-MFA) and isotopomer spectral analysis (ISA) to provide computational predictions of 1) absolute flux values based on measured uptake rates of different carbon sources, 2) fractions of lipogenic acetyl-CoA from each carbon source and 3) fractions of newly synthesized fatty acids. We subsequently performed shRNA lentiviral silencing of key genes to further elucidate their role in EMT metabolic re-programming. Finally, using an integrated network analysis of the NCI-60 Human Tumor Cell Line panel and an untargeted metabolomic analysis, we investigate how the EMT-dependent re-routing of central carbon metabolism affects drug responsiveness in D492 and D492M cells.

Methods

Cell culture

D492 and D492M cells were cultured in DMEM/F12-based medium H14 at 37C in 5% CO_2 as previously described [12]. For the labeling experiments, the cells were fed with medium containing 100% ^{13}C labelled glutamine at the 1 or 5 position (Cambridge Isotope Laboratories, Inc.) or ^{13}C labelled glucose at the 1 and 2 positions (Cambridge Isotope Laboratories, Inc.).

Lentiviral shRNA production and transduction

HEK293T cells were transfected using TurboFect transfection reagent (Thermo Fisher) at 80% confluency in T25 cell culture vessels. The cells were then incubated at 37°C and 5% CO_2. Viral supernatant was collected at two timepoints, the first being after 48 hours in culture, and the second 72 hours after changing medium at the first timepoint. The viral supernatant was filtered through a 0.45 μm filter using a syringe and stored at -20°C until usage. The lentiviral vectors were acquired from GeneCopoeia. They contained an shRNA construct for the selective targeting of *IDH2*. The construct was based on a psi-LVRH1MH vector with an mCherry fluorescent reporter, resistance against hygromycin B and the identical hairpin sequence TCAAGAG. The target sequence was *IDH2* 5′ GTACAAGGCCACAGACTTTGT-3′ . The D492 and D492M cell lines were transduced using 1 mL of filtered viral supernatant at 70% confluency and incubated at 37°C and 5% CO_2 for 24 hours, at which timepoint the medium was changed to fresh H14 medium. After

further 48 hours, the cells were grown in medium containing hygromycin B (200 μg/mL) for three weeks to selectively grow cells containing the shRNA construct.

Real-Time PCR

Whole-cell RNA was extracted using Tri-Reagent (Thermo Fisher Scientific, AM9738). Reverse transcription was performed using High-Capacity cDNA Reverse Transcription kit (Thermo Fisher, 4368814). The expression of the genes *IDH1* and *IDH2* was measured, where *ACTB* (Beta-actin) and *POLR2A* were used as endogenous reference genes. The primers for *IDH1*, *IDH2* and *ACTB* were designed using the Primer3 software in the Benchling website (https://benchling.com). The primer sequences are shown in Additional file 1. The expression of *IDH2* and *IDH1* was assessed using real-time PCR (qPCR). Real-time quantitative PCR reactions were carried out using Luna Universal qPCR Master Mix (New England Biolabs) according to manufacturer' s instcutions on a BioRad CFX384 Touch™ Real Time System (BioRad). Gene expression levels were determined using CFX Manager Software (BioRad) and differences in relative expression were estimated with the $2^{\Delta\Delta Ct}$ method

Proliferation assay

Cells were seeded in quadruplicates in 48 well plates (10.000 cells/well). They were grown in a large chamber incubation system (PeCon) at 37° C in 5% CO_2 and imaged for 12-72 hours using Leica DMI6000B. Images of cells were opened with

Fiji [13], where the cells were counted with the help of an in-house script. The slope of the best fitting line through log-transformed cell number over time was used to represent proliferation rate.

Detection of intracellular $NADP^+$ and NADPH

$NADP^+$ and NADPH were measured using NADP/NADPH-Glo™ Assay (G9071, Promega, Madison, WI). Cells were seeded in triplicates in opaque 96 well plates (10.000 cells/well) and incubated at 37C in 5% CO_2. After 24 hours, the medium was removed, cells were washed with cold PBS and then supplemented with 50μL and 50μL 1% DTAB in 0.2N NaOH solution to induce cell lysis. Next steps were according to manufacturer' s protocol. The luminescence was measured 50 minutes
after addition of the NADP-NADPH-Glo™ Detection reagent in SpectraMax M3 Microplate Reader from Molecular Devices (San Jose, CA, USA).

Nuclear magnetic resonance (NMR)

For NMR analysis, D492 and D492M cells were cultured in T225 flasks in supplemented DMEM/F12 until they reached approximately 70% confluency. Cells were then fed with either 1,2-^{13}C glucose or 1-^{13}C glutamine for 6 hours. Parallels without ^{13}C tracers were also cultured. Culture medium was collected after incubation. Methanol extracts from glucose- and glutamine-labelled cells were prepared as described previously [14]. The cell extracts were freeze dried prior to NMR analysis. For NMR, freeze-dried cell extracts were dissolved in 600 μL D_2O in PBS while culture medium (500 μL) was diluted with in D_2O-based PBS (100 μL). NMR analysis was performed using a 600 MHz Bruker Avance III NMR spectrometer (Bruker Biospin GmbH, Germany), equipped with a 5 mm QCI Cryoprobe with integrated, cooled preamplifiers for ^{1}H, ^{2}H and ^{13}C. Proton spectra were acquired at 300 K using 1D NOESY (Bruker: noesygppr1d) with presaturation and spoiler gradients as previously described [15]. The spectra were collected with 32 scans and 4 dummy scans. The acquisition time was 2.73 s and relaxation delay 4 s, measuring the FID via collection of 64 K complex data points. The ^{1}H spectra were Fourier transformed with a 0.3 Hz exponential line broadening and the chemical shift was calibrated to alanine at 1.48 ppm. ^{1}H spectra from D492 (n=5) and D492M (n=6) cells were transferred to MATLAB R2017a for multivariate data analysis. The spectra were baseline corrected using asymmetric least squares method [16] and peak aligned using icoshift [17]. The water peak and areas in the spectra with contamination and noise

only were removed. All spectra were mean normalized and mean centered. Principal component analysis (PCA) was performed using PLS toolbox v8.2.1 (Eigenvector Research). Proton decoupled ^{13}C spectra (Bruker: zgpg30) were acquired using a power gated decoupling sequence with a 30° pulse angle as described in Bettum et al. [18]. The spectra were collected with either 4K (for 1,2-^{13}C-glucose) or 16 K (for 1-^{13}C-glutamine) scans and 16 dummy scans. The acquisition time was 1.65 s, relaxation delay 0.5 s, measuring the FID via collection of 96 K complex data points over a sweep width of 197.175 ppm. The ^{13}C spectra were Fourier transformed with a 3.0 Hz exponential line broadening and the chemical shift was calibrated to the 3-^{13}C-alanine peak at 19.0 ppm or 1-^{13}C-glutamine peak at 176.4 ppm. ^{13}C-labelled metabolites downstream from the tracers were identified by comparing ^{13}C spectra with natural abundance spectra acquired under the same conditions. Levels of selected metabolites in the extracts were semi-quantitatively assessed by integration of resonance signals using TopSpin

4.0.8 (Bruker Biospin GmbH, Germany) after correcting for natural abundance levels. The ^{13}C spectra were normalized to the total area under the curve (AUC) in the ^{1}H spectra acquired from the same sample.

Metabolomics

Sample extraction

Polar (lipids) and non-polar metabolites were extracted from cell cultures by methanol/chloroform/water extraction. Cells were harvested in ice-cold methanol, vortexed vigorously and let stand on ice for 10 minutes. Equal amounts of water and chloroform were added to a final composition of 1:1:1 (CH_3OH:H_2O:$CHCl_3$), vortexed and left to stand overnight at 4°C. The organic phase (lipids) was collected into a glass vial and dried in a stream of N_2 and stored under N_2 at -80° C until analysis. The aqueous phase (polar metabolites) was stored at -80° C and evaporated in a miVac concentrator (SP scientific, Warminster PA, USA) before analysis.

UPLC-MS

Before UPLC-MS analysis, the organic phase was reconstituted in MTBE before a methanol solution containing 1M NaOH was added (10:1 v/v, respectively). This was incubated for 3.5 hours at 37°C, when 1 μL formic acid was added (to neutralize the solution), the samples were dried in a stream of N_2 and then resuspended in isopropanol:ACN:H_2O (6:9:1, v/v/v). The aqueous phase (metabolites) were reconstituted in isopropanol:ACN:H_2O (2:1:1, v/v/v). Ultra-performance liquid chromatography (UPLC) (Acquity, Waters, Manchester, UK) was coupled with a quadrupole-time of flight mass spectrometer (Synapt G2, Waters, Manchester, UK). For the lipid samples, chromatographic separation was achieved as previously described [19]. For the metabolomic samples, chromatographic separation was achieved by hydrophilic interaction liquid chromatography (HILIC) using an Acquity amide column, 1.7 μm (2.1 x 150 mm) (Waters, Manchester, UK). All samples were analyzed in positive ionization and negative ionization mode using acidic and basic chromatographic conditions. In positive mode and in negative acidic conditions, mobile phase A was 100% ACN and B was 100% H_2O both containing 0.1% formic acid. The following elution gradient was used: 0 min 99% A; 7 min 30% A; 7.1 min 99% A; 10 min 99% A. In negative mode basic conditions, mobile phase A contained ACN:sodium

bicarbonate 10 mM (95:5) and mobile phase B contained ACN:sodium bicarbonate 10 mM (5:95). The following elution gradient was used: 0 min 99% A; 6 min 30% A; 6.5 min 99% A; 10 min 99 % A. In all conditions, the flow rate was 0.4 mL/min, the column temperature was 45°C, and the injection volume was 3.5 μL. The mass spectrometer was operated using a capillary voltage of 1.5 kV, the sampling cone and the extraction cone were of 30 V and 5 V. The cone and the desolvation gas flow were 50 L/h and 800 L/h, while the source and desolvation gas temperature were 120 and 500°C. MS spectra were acquired in centroid mode from m/z 50 to 1000 using scan time of 0.3 s. Leucine enkephalin (2 ng/μL) was used as lock mass (m/z 556.2771 and 554.2615 in positive and negative experiments respectively). A typical analytical block consisted of the following: pooled QC samples to equilibrate the system; calibrators; samples and spiked pooled QC samples; calibrators.

Data analysis

TargetLynx (v4.1, Waters) was used to integrate chromatograms of all isotopologues of the metabolites of interest. Ion chromatograms were extracted using a window of
0.02 mDa which was centered on the expected m/z for each targeted isotopologue. The output was a mass distribution vector (MDV) describing the relative amount of each detected isotopologue of the metabolite. Ion chromatograms of isotopologues of interest extracted and corrected for abundance of naturally occuring isotopes using the IsoCor software [20]. When calculating the total contribution of carbon sources to metabolites, we used the following equation [21]:

$$Total\ Contribution = \frac{\sum_{i=0}^{n} i \cdot m_i}{n} \quad (1)$$

Where n is the number of C atoms in the metabolite, i represents the isotopologues and m is the relative fraction of the isotopologues.

In order to evaluate the percentage of glucose that enters the pentose phoshate pathway, we utilized a formula from Lee *et al.* [22]:

$$PPP_{cycle} = \frac{m1/m2}{3 + m1/m2} \quad (2)$$

In equation 2, m_1 and m_2 are the fractional abundances of M+1 and M+2 lactate isotopologues, respectively, (e.g. from Fig. 1).

Isotopomer spectral analysis

To determine the fractional contribution of glutamine and glucose to fatty acid biosynthesis, we used the convoluted isotopic spectral analysis algorithm [23]. The chosen proxy for fatty acid labeling was palmitate (16:0). The MDV vector for palmitate was corrected for natural abundance using IsoCor. The corrected MDVs for palmitate were imported into MATLAB where the analysis was carried out.

^{13}C-Metabolic flux analysis

Flux of intracellular reactions was determined using ^{13}C-Metabolic flux analysis (13C-MFA). A metabolic network was constructed using the Isotopomer Network Compartmental Analysis (INCA) software

package [24] in MATLAB. This network represents the central carbon metabolism and describes the transition of carbons between each biochemical reaction therein. For determination of flux within each cell line (D492 and D492M), parallel labeling data from cells fed with 1,2-^{13}Cglucose, 1-^{13}C-glutamine and 5-^{13}C-glutamine were used for simultaneous fitting of our network. More specifically, the 6-hour timepoints were used. In our analysis, the following assumptions were made:

1 The cells are in the exponential growth phase, a phase where metabolic steady state is present.
2 CO_2 is not recycled.
3 Serine one-carbon metabolism was not included. We saw that glucose contribution to glutathione was minimal, indicating that the glycine used for GSH generation is not derived from glucose.

4 Isotopic steady state of ^{13}C-glutamineand 1,2-^{13}C-glucose-derived TCA cycle intermediates has been reached after 6 hours of incubation.

The flux of individual reactions was estimated relative to uptake of glutamine. The actual flux value (units of μmol/gDW/hour) was then found by multiplying by measured glutamine uptake rates of the cells from [11]. The sum of squared residuals (SSR) was minimized between experimentally determined MIDs of 7 metabolites and simulated fluxes in the network. Goodness-of-fit was determined using the chi-square test. The fit was accepted within the 95% confidence interval of the chi-square distribution.

Weighted correlation network analysis

Drug sensitivity data were gathered from the NCI-60 Human Tumor Cell Lines [25] using the *rcellminer* R-package [26]. We focused specifically on FDA-approved drugs and cells that were contained within the independent dataset from Ortmayr et al. [27] so after removal of drugs with more than 5% missing values, the final size of the drug sensitivity matrix was 240 drugs x 51 cell lines. To construct a network of drug-sensitivities, a weighted correlation network analysis was employed using the *WGCNA* R-package. A soft threshold value of 7 was used to obtain a scalefree network topology (R^2 = 0.97). Highly correlated modules that had an average distance < 0.7 were merged. The eigenvectors (first principal components) for each module were identified and used to test association of drug-modules with metabolite levels. The correlation of the drug modules to metabolite levels of the cells within the NCI-60 (from [27]) was calculated. An R-script for the whole analysis is in additional file 2.

Drug treatment assays

Cells were seeded (3000 cells/well) in white 96 well plates (Costar) and maintained at 37°C in 5% CO_2. 24 hours later, drugs were added together with or without buthionine sulphoximine (BSO (B2515, Sigma Aldrich)) with DMSO for controls,

and 72 hours later cell viability was evaluated. The viability was evaluated by measuring cells metabolic activity by CellTiter-Glo (CTG) assay (Promega, Madison, WI), adding CTG assay mix directly to the wells in a 1:1 ratio. After 10 min, luminescence was measured

by VICTOR X3 Multiplate reader (Perkin Elmer, MA). The drugs tested were mTOR inhibitor everolimus (Sigma Aldrich), PI3K inhibitor LY-294002 (Cell Signaling Technology, Danvers, MA), taxane drug paclitaxel (Fresenius Kabi, Halden, Norway) and the macrolid mTOR inhibitor rapamycin (Selleck Chemicals, Houston, TX).

Detection of intracellular glutathione abundance

Cells were seeded (2000 cells/well) in white 384 well plates (Greiner, Bio-One) and maintained at 37°C in 5% CO_2. 24 hours later, BSO was added (medium for controls) and 24 hours later, glutathione measurement was performed by using

GSH-Glo Glutathione Assay kit (V6911, Promega, Madison, WI) in accordance with the manufacturer's protocol.

Statistical analysis

A Student' s t-test was employed for comparison of two treatments, and in the case of non-parametric data, a Mann-Whitney U-test was used. One-way ANOVA was used to compare data from three or more treatments. The asterisks in each figure represent the p-values (* < 0.05, ** < 0.01, *** < 0.001). Data were assumed to be normally distributed unless otherwise stated in figure legends. Statistical analysis and image generation was carried out in the R environment [28] using the *ggplot2* and *ggpubr* packages. All data are presented as mean + standard deviation.

Results

Glycolysis rates determine the pentose phosphate shunt in the D492 EMT cell model

Principal component analysis of ^{1}H spectra show that that there is a clear difference in the overall metabolic profile of D492 and D492M cells. The score and loading plot from PCA (Additional file 3, A and B), indicate that D492 cells have more intracellular isoleucine, leucine, valine, alanine, arginine, glutathione, myo-inositol, asparagine, proline, AMP, ADP, ATP, tyrosine, phenylalanine, and NAD^{+}, and less glutamine, glutamate, phosphocholine, glycine, threonine, glucose, fumarate, NADP and NADH. The rate of glucose uptake and lactate secretion are higher in the epithelial phenotype of D492 cells compared to the mesenchymal phenotype [11] indicative of enhanced glycolysis in D492. Our previous findings could not adequately estimate flux through the pentose phosphate pathway (PPP) or the directionality of reversible reactions. Here, we used 1,2-^{13}C glucose to distinguish between glycolytic and PPP flux as previously described [29]. A schematic overview of label distribution from 1,2-^{13}C glucose into central carbon metabolism can be seen in Fig. 1A. Label incorporation from glucose into lactate was higher in D492 epithelial cells than in D492M mesenchymal cells (Fig. 1B), indicating that the glycolytic flux is reduced after EMT in D492 cells. The flux of glucose through the oxidative phase of the PPP was estimated from the labeling profiles of lactate and showed that label incorporation of M+1 isotopologue is low in both cell lines (Additional file 4). Using equation 2, we estimated that only 2% of carbons that are metabolized in the oxidative PPP return to glycolysis through reductive PPP in both phenotypes. Therefore,

D492 had an overall higher flux into the oxidative PPP due to higher glucose intake. As a result, reduction of NADP to NADPH via the oxidative phase of the PPP in D492M cells is dampened.

Glutamine fuels citrate and lipogenic acetyl-CoA production via reductive carboxylation following EMT in D492 cells

TCA cycle activity within the D492 and D492M cells was assessed by analyzing ^{13}C label enrichment in TCA-cycle metabolites from ^{13}C labelled glucose and glutamine. The labeling profiles of citrate revealed that more citrate is derived from glucose through the activity of citrate synthase (*CS*) in D492 epithelial cells than D492M mesenchymal cells (Fig. 1C), in agreement with differences in glycolytic activity, NMR measurements of 4-^{13}C-glutamate (Additional file 3C) and glucose-derived label in glutamate (Additional file 4). However, a higher total contribution of glucose to malate was seen in the mesenchymal phenotype (Fig. 1D), suggestive of increased flux through pyruvate carboxylase (PC) to replenish the TCA cycle post EMT.

Unlike the flux rate differences observed within glycolysis, these results indicated significant changes to the flux network topology following EMT.

Citrate is used for energy production following its oxidation in the TCA cycle or as a precursor for lipids [30]. Based on the reduced synthesis of citrate from glucose, we hypothesized that the generation of citrate in D492M could be more dependent on another carbon source the amino acid glutamine. Glutamine the second most consumed carbon source after glucose in D492 and D492M cells [11] and we found that it is essential for their proliferation (Additional file 5). The overall contribution of glutamine to the TCA cycle was analyzed by label incorporation from ^{13}C glutamine to citrate. By utilizing either 1-^{13}C or 5-^{13}C-labelled glutamine analogs (Fig. 2A), we quantified both the reductive and oxidative flow of carbons from glutamine within the TCA cycle [31] (Additional files 6-7). D492 epithelial cells primarily oxidized glutamine in the TCA cycle while the D492M mesenchymal cells had significantly higher flux through reductive carboxylation to citrate (Fig. 2B and C), confirming our hypothesis of increased reliance on glutamine for citrate synthesis following EMT. Furthermore, there was higher incorporation of glutamine into glutathione (Fig. 2D).

We next investigated the transfer of labelled carbons to fatty acids from glucose and glutamine to further determine the metabolic fate of citrate. To this end we specifically looked at the label incorporation from both 1,2-^{13}C-glucose and 5-^{13}Cglutamine into palmitic acid (C16:0). The contribution of glucose to fatty acid synthesis was relatively higher in D492, whereas the contribution of glutamine to fatty acids was higher in D492M (Fig. 3A and B). These results are consistent with the increased contribution of glutamine to the citrate pool following EMT. Isotopomer spectral analysis (ISA) of labelled palmitic acid revealed that the pool of lipogenic acetyl-CoA derived from glucose was significantly diminished while the glutamine-derived pool is increased following EMT in D492 epithelial cells (Fig. 3C).

Metabolic re-routing following EMT affects redox metabolism in D492 cells

The synthesis of fatty acids requires both acetyl-CoA from a carbon source and reducing potential in the form of NADPH. The spatial difference in requirement of reducing potential (cytosol vs.

mitochondria) and increased reductive carboxylation following EMT (Fig. 2B) along with the diminished net oxidative PPP flux led us to hypothesize that the alternate EMT metabolic phenotypes contribute differently to redox homeostasis. Measurement of NADPH/NADP+ revealed an increase in NADPH availability in the mesenchymal phenotype (Fig. 3D). Isotopologue enrichment revealed significantly increased proline synthesis that is glutamine and NADPH dependent in D492M cells (Fig. 3E). No glutathione labeling was observed when the D492 or D492M cells were cultured with 1,2-^{13}C-glucose (Additional file 4), but there was significant contribution from ^{13}C-glutamine, indicating that glutamine is directed towards glutathione synthesis in both cells, but significantly less so in D492M cells (Fig. 3F), further supported by NMR experiments (Additional file 3D). The relative increase in NADPH levels and drop in total production and abundance of glutathione reflect changes to redox balance on account of the alternate metabolic phenotypes (i.e. metabotypes) following EMT in breast epithelial-derived D492.

To put the results from the ^{13}C tracing experiments into quantitative context, we combined our results with the cell-specific uptake rates from our previous study [11] via ^{13}C metabolic flux analysis (13C-MFA) [32]. 13C-MFA from D492 and D492M cells allowed the differences in the flow of carbons within central carbon metabolism before and after EMT to be calculated. Differences in calculated flux values between D492 and D492M are shown in Fig. 3G. Absolute flux values are shown in additional file 8. The 13C-MFA confirmed higher glycolytic flux and glucose-dependent citrate generation in D492 cells. In contrast, reductive carboxylation, proline synthesis and both cytosolic and mitochondrial malic enzyme were shown to carry relatively higher flux in D492M cells along with lowered glutathione synthesis and reversed malate-aspartate shuttle.

IDH2 knockdown increases glucose-dependent fatty acid synthesis

Based on the difference in reductive carboxylation (Fig. 2B and 3G) and recent literature [33, 34], we hypothesized that isocitrate dehydrogenase (IDH) would contribute to the discrimination between the D492 and D492M flux phenotypes through glutamine consumption and redox balance. RNA sequencing and proteomic data from the cell lines clearly showed that one isoform of IDH in particular was higher in abundance in D492M cell lines compared to D492. This was the mitochondrial $NADPH^+$-dependent isocitrate dehydrogenase IDH2 (Fig. 4A) which indicated that the increased reductive carboxylation in D492M could depend on the increased expression of this isoform. After knocking down the mRNA levels of IDH2 using shRNA lentiviral transduction, we investigated its metabolic and morphological effects on both cell lines. When the $IDH2$ gene was silenced (Fig. 4B), the mRNA levels from the cytosolic isoform $IDH1$ were increased (Fig. 4C). There were no morphological differences observed when $IDH2$ expression was diminished in neither D492 nor D492M cells (Fig. 4D). However, the $IDH2$-KD D492 cells showed reduced proliferation compared to the wildtype D492 cells (Fig. 4E) while no growth reduction was observed in D492M cells upon knockdown of $IDH2$. Knockdown of $IDH2$ inhibited reductive carboxylation of glutamine (Fig. 4F) despite the observed upregulation of $IDH1$. This in turn resulted in upregulated glycolytic flux in both cell lines as measured by lactate and citrate labeling patterns of $IDH2$-silenced cell lines (Additional file 9). This

difference in glycolytic activity was reflected in the ISA of labeling profiles of palmitic acid, which showed that the fractional contribution of glucose to palmitate was increased in the IDH2-deficient cells, along with lowering of glutamine-derived lipogenic acetyl-CoA (Fig. 4G). Taken together, these results demonstrate a fundamental role of IDH2 in the increased reductive carboxylation of glutamine for lipogenesis following EMT of D492 cells.

Alteration in redox metabotype drives sensitivity to PI3K/Akt/mTOR inhibition

The metabolic phenotypes characterized by different glycolytic rates and altered carbon source preference for TCA, changes to total concentrations and synthesis rates of proline and glutathione are reminiscent of cancer stem cell metabotypes [35, 36]. Due to glutathione' s role in drug resistance in various cell types [37, 38, 39], we hypothesized that metabolic rerouting centered around alternate utilization of glutamine-derived glutamate for glutathione synthesis would contribute to the different drug sensitivities of D492 and D492M.

To identify drugs that are selectively affected by glutathione concentrations within cells, we performed an integrated network analysis of I) drug sensitivity profiles within the NCI-60 Human Tumor Cell Line database [25] and II) untargeted metabolomic analysis of NCI-60 cell lines from Ortmayr et al. [27]. The network analysis revealed that FDA-approved drugs in the NCI-60 are grouped into 6 intracorrelated drug modules (Fig. 5A). The black module represents tyrosine kinase inhibitors (n=12), the blue module is PI3K-, Aktand mTOR inhibitors, along with arsenic trioxide (A_2O_3) and acetalax, the brown module includes histone deacetylase (HDAC) inhibitors (n=12), the green module is purine analogs (n=6) and the red module is the largest and includes DNA alkylating agents, taxanes, topoisomerase inhibitors and anthracyclines (n=111). The turquoise module is protein kinase inhibitors (n=14).

Interestingly, both the reduced and oxidized forms of glutathione were negatively correlated with the blue module (PI3K-, Aktand mTOR inhibitors), the members of which are shown in Fig. 5B, which implies that higher glutathione concentration is associated with lower sensitivity to drugs belonging to that module. Other conjugation metabolites (i.e. UDP-glucuronate and S-adenosylmethionine) did not correlate significantly with this module. We examined the effects of selected drugs within the blue and red modules to see if they would have different effects on D492 and D492M. D492M was more sensitive to LY-294002, everolimus and rapamycin from the blue module (Fig. 5C), but also to paclitaxel, a taxane drug belonging to the red module. In order to establish a functional link between glutathione and the blue module drugs, we co-treated D492 and D492M cells with buthionine sulphoximine (BSO), an inhibitor of the rate-limiting enzyme glutamate-cysteine ligase (GCL) in glutathione synthesis (Fig. 5D-E), and either everolimus (Fig. 5F) or paclitaxel (Fig. 5G). We saw that the sensitivity of both D492 and D492M to everolimus could be enhanced by co-treatment with BSO, whereas these effects were not observed when the cells were co-treated with paclitaxel and BSO. Together, these data suggest that glutathione availability directly affects sensitivity to drugs that affect the PI3K/Akt/mTOR pathway.

Discussion

D492 and D492M cells represent only two of the numerous phenotypes within the spectrum of EMT [40]. Herein, we have thoroughly characterized the central carbon metabolic activity of these cell types using ^{13}C-labelled carbon sources along with metabolic flux analysis, isotopomer spectral analysis, and both *in silico* and *in vitro* drug-sensitivity analyses.

IDH2 plays a key role in EMT in breast

The data from labelled glucose and glutamine experiments indicate that D492M cells rely on reductive carboxylation of glutamine to citrate via isocitrate dehydrogenase activity. Transcriptomic and proteomic data from D492 and D492M cells confirm that the predominant EMT-associated form of isocitrate dehydrogenase is the mitochondrial $NADP^+$-dependent IDH2, with D492M cells showing significantly higher levels (Fig. 4A). Knockdown of *IDH2* by lentiviral shRNA induction caused a marked reduction of reductive carboxylation of glutamine to citrate in D492M

cells. The citrate pool was replenished by funneling glucose-derived acetyl-CoA into the mitochondria (Fig. 4G). The decrease in citrate labeling from 1-^{13}C-glutamine (Fig. 4F) indicates that in these cells, reductive carboxylation primarily takes place within the mitochondria, as opposed to in the cytosol via *IDH1* activity. In addition, D492 epithelial cells metabolize glutamine oxidatively in the TCA cycle that eventually provides oxaloacetate to citrate synthase. Interestingly, the knockdown of *IDH2* and the subsequent re-routing of glutamine metabolism reduces the availability of glutamine for glutathione production in D492 epithelial cells (Additional files 10 and 11). The labeling profiles for glutamate, oxidized glutathione and proline were altered upon knockdown of *IDH2* in D492M with increased glutamine-dependent labeling of glutamate and glutathione but reduced the labeling of proline. Finally, the incorporation of glutamine carbons into fatty acids from reductive carboxylation is replaced by glucose carbons when the IDH2 levels are lowered in both cell lines (Fig. 4G).

Our findings highlight the role of IDH2 in the increased reductive carboxylation following EMT in breast. However, we cannot exclude the importance of IDH1 in this context. It is reasonable to assume that the reductive carboxylation works through IDH1 in the cytosol, and citrate is subsequently transported into the mitochondria where IDH2 takes part in its ultimate oxidation (as proposed by *Jiang et al.* [33]). When IDH2 levels are diminished, the activity of this pathway is inevitably halted. Nevertheless, our results demonstrate that *IDH2* knockdown significantly affects the reductive carboxylation of glutamine to citrate and ultimately fatty acids which unequivocally establishes a crucial, functional role of the mitochondrial isoform in this process.

Alterations in reductive carboxylation and redox metabolism follow EMT in breast

Our results show that glutamine-derived citrate is being utilized for fatty acid synthesis in the D492 cell model, but the reliance on this pathway is enhanced following EMT. We show that there is a concurrent increase in NADPH/NADP$^+$ ratio and proline synthesis along with a decrease in glutathione synthesis (Fig. 3D-F). *Jiang et al.* have previously shown that anchorage-independent growth relies on increased reductive carboxylation and subsequent mitigation of mitochondrial ROS [33]. The role of proline in anchorage-independent growth has also been demonstrated by *Elia et al.*, who

showed that its degradation and cycling is higher in breast cancer cells grown in 3D culture than in 2D [41], which ultimately altered the NADPH/$NADP^+$ balance. Phang hypothesized that this could be due to the fact that proline is being directed away from protein synthesis and towards redox regulation [42], a pathway that proline has previously been shown to take part in [43]. More recently, *Liu et al.* showed that under hypoxic conditions, proline synthesis and reductive carboxylation, both of which require NADPH, act as alternative bins for electrons so that electron transfer can occur for cellular proliferation [36]. These studies fit well with our observations, where D492M cells indeed have lower oxygen consumption rate [11] and show higher activity of aforementioned pathways. More specifically, the higher NADPH availability of D492M is reflected in increased reductive carboxylation and proline synthesis in the cells. Furthermore, D492M cells display a concomitant lowering of glutathione synthesis and its overall abundance, a phenomenon shown to occur when EMT-inducing transcription factor Snail is overexpressed in MCF7 breast cancer cells [44].

Diminished glutathione abundance potentiates sensitivity to PI3K/Akt/mTOR inhibitors

We have previously reported a reduction in oxidative phosphorylation following EMT in D492 cells. This causes a metabolic shift towards anaplerosis and upregulation of pathways receiving otherwise ETC-directed electrons (i.e. proline synthesis and reductive carboxylation) [36] and decreased flux of glutamine towards glutathione synthesis. Glutathione is the most abundant non-protein thiol in animal cells, and it plays a crucial role in the conjugation phase of xenobiotic metabolism. This leads to increased water-solubility of foreign compounds such as drugs and reduced efficacy [45, 46].

Due to the clear differences we observed in glutathione synthesis and overall abundance between D492 and D492M cells (Fig. 3 and additional file 3B), we hypothesized that this would result in altered drug sensitivity of the cells. Integrated network analysis suggested that D492M cells are more affected than D492 by drugs that specifically target mTOR, PI3K and Akt along with acetalax and A_2O_3, a drug known to be affected by glutathione abundance in cancer cells [37] (Fig. 5A-B). Furthermore, the lack of a significant relationship of the other metabolites shown, S-adenosylmethionine and UDP-glucuronate, which are also known to partake in the conjugation to xenobiotic compounds, suggests that these drugs are specifically affected by glutathione availability. We tested several drugs that target mTOR, PI3K and Akt and found that D492M cells were more sensitive than D492 cells (Fig. 5C). Furthermore, we showed that by manipulating the glutathione levels within the cells via BSO treatment (Fig. 5E), we could increase the sensitivity to these drugs (Fig. 5F), but the same manipulation did not affect the sensitivity to paclitaxel, a microtubule stabilizer and mitotic inhibitor (Fig. 5G). These results indicate that glutathione availability primarily affects drugs that target the PI3K/Akt/mTOR pathway. In recent years, studies have shown a direct relationship between the PI3K/Akt/mTOR signaling pathway and oxidative stress response [47, 48, 49]. Furthermore, the PI3K/Akt/mTOR pathway has been shown to be highly involved in the EMT process and chemoresistance of ovarian cancer cells and melanoma [50, 51].

Lien et al. elegantly showed how glutathione synthesis is stimulated by the PI3K/Akt signaling pathway [47] which is consistent with our findings that high GSH levels reduce sensitivity to PI3K/Akt/mTOR inhibition. When glutathione synthesis is inhibited (e.g. through BSO), the intracellular glutathione pool is quickly depleted. Inhibition of PI3K/Akt/mTOR activity under these circumstances inhibits glutathione synthesis and reduces viability. Collectively, our results introduce a valuable mechanistic insight into the altered drug sensitivity following EMT and support previous findings that glutathione depletion in combination with PI3K/Akt/mTOR inhibitors may specifically target the metastatic potential and/or stemness of cancer cells.

Conclusions

In summary, we have defined alterations in central carbon metabolism of a breast epithelial cell model of EMT using ^{13}C carbon tracing and flux analysis. We show that glutamine metabolism is re-routed towards reductive carboxylation to fuel fatty acid synthesis following EMT due to activity of the mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (*IDH2*). This leads to decreased glutathione production and disrupted redox balance within the cells. Integrated network analysis of the NCI-60 Human Tumor Cell Line database revealed negative correlation between intracellular glutathione levels and sensitivity to PI3K/Akt/mTOR inhibitors. Depletion of intracellular glutathione levels specifically sensitized cells to PI3K/Akt/mTOR pathway inhibitors. Our results highlight a potential metabolic weakness of EMT derived cells that may be exploited in anti-metastatic treatment.

List of Abbreviations

EMT: Epithelial-to-mesenchymal transition

MFA: Metabolic flux analysis

GSH: Glutathione (reduced)

mTOR: Mammalian target of rapamycin

PI3K: Phosphoinositide 3-kinase

NADP: Nicotinamide adenine dinucleotide phosphate

NAD: Nicotinamide adenine dinucleotide

NMR: Nuclear magnetic resonance

UPLC/MS: Ultra performance liquid chromatography/mass spectrometry

BSO: Buthionine sulphoximine

IDH: Isocitrate dehydrogenase

TCA: Tricarboxylic acid

PPP: Pentose phosphate pathway

Declarations

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Availability of data and materials
All raw data/models/methods used and/or analysed in the study are available from the corresponding author on reasonable request. The NCI-60 drug treatment data are available through the *rcellminer* R-package.

Competing interests
The authors declare that they have no competing interests.

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Author's contributions
STK, OR and SH conceived and designed this study. STK, SH, AS, KS and MTG performed and analyzed the results of all experiments. QW oversaw running the proteomic method. FJ and OR oversaw running the UPLC-MS method. MTG and SAM oversaw running the NMR method. All authors contributed to interpreting the data and edited the manuscript. OR and SH supervised the work carried out in this study. All authors read and approved the final manuscript.

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Figures

Figure 1 Glucose metabolism of D492 and D492M. A) An atom transition map of glucose metabolism showing a part of the metabolic fates of 1,2-^{13}C glucose within mammalian cells, where the ^{13}C-isotopes are shown in green. Metabolites in bold are measured in our experiment. Parallel lines through the arrows indicates one or more steps not shown. Total contribution of glucose to B) lactate, C) citrate and D) malate was measured after culturing of D492 and D492M with 1,2-^{13}C-glucose for 6 hours. Results are presented as mean + std (n=3).

Figure 2 Glutamine metabolism of D492 and D492M. A) Atom transition map of glutamine metabolism showing the different metabolic fates of 1-^{13}C glutamine (red) and 5-^{13}C glutamine (blue) within mammalian cells. Metabolites in bold are measured in our experiments. Parallel lines through the arrows indicates one or more steps not shown. B) Reductive carboxylation of D492 and D492M, as indicated by the fractional abundance of the M+1 isotopologue of citrate normalized to the M+1 isotopologue of glutamate from cells cultured with 1-^{13}C-glutamine for 6 hours. C) Total fraction of citrate originating from glutamine in D492 and D492M, as indicated by the fractional abundance of the M+1 isotopologue of citrate normalized to the M+1 isotopologue of glutamate from cells cultured with 5-^{13}C-glutamine for 6 hours. D) Total fraction of GSH originating from glutamine in D492 and D492M, as indicated by the fractional abundance of the M+1 isotopologue of citrate normalized to the M+1 isotopologue of glutamate from cells cultured with 1-^{13}C-glutamine for 6 hours. Results are presented as mean + std (n=3).

Figure 3 Fatty acid and redox metabolism are altered following EMT. A) Label enrichment in palmitate after culturing the cells with 1,2-^{13}C-glucose for 24 hours. B) Label enrichment in palmitate after culturing the cells with 5-^{13}C-glutamine for 24 hours. C) Results from ISA showing fraction of lipogenic acetyl-CoA derived from glucose and glutamine. D) NADPH/NADP$^+$ ratios in D492 and D492M cells after 24 hours in culture (n=4). E) Proline synthesis in D492 and D492M cells cultured with 5-^{13}C-glutamine for 6 hours (n=3). F) Glutathione synthesis from glutamine in D492 and D492M in cells cultured with 1-^{13}C-glutamine for 6 hours (n=3). G) Relative flux differences according to steady-state metabolic ^{13}C flux analysis using INCA. Different pathways are indicated in grey. For both D492 and D492M models, the expected 95% confidence interval for the sum-of-squared residuals (SSR) for 108 degrees-of-freedom was 81.1 - 138.7. Both models had lower SSR than expected (44.5 and 100.8 for D492 and D492M, respectively). Results in A-F are presented as mean + std.

Figure 4 Effects of IDH2 knockdown in D492 and D492M cells. A) Relative levels of different isoforms of isocitrate dehydrogenase in D492M compared to D492. X-axis represents mRNA levels from RNA sequencing whereas the Y-axis represents protein levels from proteomic profiling of the cells. Results are depicted as mean + sem (n=3). B,C) Real-Time PCR from D492, D492M and their *IDH2* -silenced counterpart cell lines show that the shRNA transduction cause B) significantly decreased expression of the *IDH2* gene and a C) concurrent increase in expression of *IDH1* (mean + std, n=3). D) Effects of *IDH2* knockdown on D492 and D492M morphology. Phase-contrast images of D492-WT, D492M-WT, D492-IDH2 and D492M-IDH2 cells. E) Proliferation of D492, D492-IDH2, D492M and D492M-IDH2 cell lines (mean + sem, n=4). F) Effect of *IDH2* silencing on reductive carboxylation of D492 and D492M, shown by the fractional abundance of the M+1 isotopologue of citrate normalized to the M+1 isotopologue of glutamate from cells cultured with 1-^{13}C-glutamine for 6 hours. G) Results from ISA showing fraction of lipogenic acetyl-CoA (D) derived from glucose and glutamine.

Figure 5 Glutathione levels regulate sensitivity to PI3K-, AKTand mTOR inhibitors. A) Network analysis of NCI-60 cell lines treated with various drugs suggests the presence of 6 modules of intra-correlated drugs. The correlation of drug-modules to reduced glutathione (GSH), oxidized glutathione (GSSG), UDP-glucuronate and S-adenosylmethionine levels was estimated. Upper numbers in table represent Pearson's correlation coefficient and the lower numbers (in brackets) represent the correlation p-value. B) The blue module. The edges represent the topological overlap of the drugs. The network was automatically generated using Cytoscape [52] with the CoSE algorithm. C) D492 and D492M cells treated with everolimus (0.005 μM), LY-294002 (1 μM), rapamycin (0.1 μM) and paclitaxel (0.005 μM). Results are presented as mean + std (n≥4). D) Mechanism of glutathione synthesis inhibition by buthionine sulphoximine (BSO). GCL Glutamate-cysteine ligase, GSS Glutathione synthetase. E) Effects of 24 hour BSO-treatment on glutathione (GSH) concentration in D492 and D492M cells. F) D492 and D492M cells treated with everolimus (0.005 μM) with and without BSO (50 μM). G) D492 and D492M cells treated with paclitaxel (0.005 μM) with and without BSO (50 μM). For F,G) the Y-axis represents percentage of viability compared to non-treated cells after 72 hours of treatment (mean + std, n≥4). Student's two-tailed t-test and ANOVA were used to measure significance of results in C) and F,G), respectively. ns not significant.

Additional Files

Additional file 1 — Primers used for quantitative Real-Time PCR
A table showing nucleotide sequences of *IDH2* and *IDH1* primers used in this study. 'FWD' and 'REV' are abbreviations for forward and reverse, respectively.

Additional file 2 — An R-script for the weighted correlation network analysis
This file is a .R file which can be opened in RStudio.

Additional file 3 — NMR data from 1H and 13C labelling experiments of D492 and D492M
A) PC 1 scores after Principal Component Analysis of ^{1}H NMR data and B) Corresponding loading plot from PC 1*. C) NMR peak area after incubation with 1,2-^{13}C-glucose for 6 hours. Integrated peak areas of 4-^{13}C glutamate. Results are presented as mean + std (n=3). D) NMR peak area after incubation with 1-^{13}C-glutamine for 6 hours. The metabolites show integrated peak areas of 1-^{13}C glutamate, 1-^{13}C glutathione and 1-13C proline. Results are presented as mean + std (n=2). *: Peak assignment: a: isoleucine, b: leucine, c: valine, d: lactate, e: alanine, f: arginine, g: glutamine, h: glutamate, i: glutathione, j: phosphocholine (10 times higher than shown), k: myo-inositol, l: glycine, m: asparagine, n: proline, o: amp, p: adp, q: atp: r: threonine, s: glucose, t: fumarate, u: tyrosine, v: phenylalanine, w: NAD+, x: NADP, y: NADH.

Additional file 4 — UPLC-MS label enrichment in various metabolites after culturing of D492 and D492M with 1,2-^{13}C-glucose for 6 hours

Additional file 5 — Dependency of D492 and D492M on glucose and glutamine
A) Cell number of D492 and D492M in H14 medium, H14 without glucose and H14 without glutamine. B) Growth rate calculations from numbers used in A. Values are presented as mean + standard error (n=3). Asterisks represent significance of pairwise comparison between full medium and the depleted medium.

Additional file 6 — UPLC-MS label enrichment in various metabolites after culturing of D492 and D492M with 1-^{13}C-glutamine for 6 hours

Additional file 7 — UPLC-MS label enrichment in various metabolites after culturing of D492 and D492M with 5-^{13}C-glutamine for 6 hours

Additional file 8 — Results from ^{13}C-metabolic flux analysis
This file is an Microsoft Excel file (.xlsx) containing information about the reactions, their formulas and the calculated flux values (and errors) in the 13C-MFA of D492 and D492M.

Additional file 9 — UPLC-MS label enrichment in various metabolites after culturing of D492 and D492M with 1,2-^{13}C-glucose for 6 hours, either with or without shRNA-lentiviral silencing of *IDH2* expression

Additional file 10 — UPLC-MS label enrichment in various metabolites after culturing of D492 and D492M with 1-^{13}C-glutamine for 6 hours, either with or without shRNA-lentiviral silencing of *IDH2* expression

Additional file 11 — UPLC-MS label enrichment in various metabolites after culturing of D492 and D492M with 5-^{13}C-glutamine for 6 hours, either with or without shRNA-lentiviral silencing of *IDH2* expression