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Original Articles

Epithelial–mesenchymal transition induces similar metabolic alterations in two independent breast cancer cell lines

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ABSTRACT

Epithelial–mesenchymal transition (EMT) induces invasive properties in epithelial tumors and promotes metastasis. Although EMT-mediated cellular and molecular changes are well understood, very little is known about EMT-induced metabolic changes. HER2-positive BT-474 breast cancer cells were induced to undergo a stable EMT using mammosphere culture, as previously described by us for the ERα-positive MCF-7 breast cancer cells. Two epithelial breast cancer cell lines (BT-474 and MCF-7) were compared to their respective EMT-derived mesenchymal progeny (BT-474$_{EMT}$ and MCF-7$_{EMT}$) for changes in metabolic pathways including glycolysis, glycogen metabolism, anabolic pathways and gluconeogenesis. Both EMT-derived cells displayed enhanced aerobic glycolysis along with the overexpression of specific glucose transporters, lactate dehydrogenase isoforms, monocarboxylate transporters and glycogen phosphorylase isoform. In contrast, both EMT-derived cells suppressed the expression of crucial enzymes in anabolic pathways and gluconeogenesis. STAT3, a transcription factor involved in tumor initiation and progression, plays a role in the EMT-related changes in the expression of specific enzymes and transporters. This study provides a broad overview of similar metabolic changes induced by EMT in two independent breast cancer cell lines. These metabolic changes may provide novel therapeutic targets for metastatic breast cancer.

Introduction

Worldwide, breast cancer accounted for 521,000 global deaths in 2012, according to the World Health Organization. In the US, the National Cancer Institute predicts 232,670 new cases along with 40,000 deaths from breast cancer in 2014. Breast cancer is composed of several subtypes with distinct molecular characteristics, clinical behaviors and treatment options. The most common subtypes of invasive breast carcinoma are estrogen receptor/progesterone receptor (ER/PR)-positive and human epidermal growth factor receptor 2 (HER2)-positive [1]. These subtypes are composed of cells that although neoplastically transformed, still display a significant degree of epithelial organization including basal–apical polarity and E-cadherin-mediated adherent junctions. These ER/PR-positive and HER2-positive subtypes are less invasive and confer a more favorable diagnosis compared to triple-negative (ER-, PR- and HER2-) subtype [2]. Nevertheless, these subtypes can eventually give rise to distant metastatic lesions [3].

Metastasis is a complex process that involves a series of events including localized stromal invasion, intravasation, transport through circulation, extravasation and colonization. The early phase of

Abbreviations: EMT, epithelial–mesenchymal transition; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; GATA3, GATA binding protein 3; ZEB1, zinc finger E-box binding homeobox 1; ZEB2, zinc finger E-box binding homeobox 2; SLUG, zinc-finger binding transcription factor sanil2; ER, estrogen receptor; PR, progesterone receptor; HER2, amplified ERBB2 oncogene; GLUT1, glucose transporter 1; GLUT3, glucose transporter 3; GLUT12, glucose transporter 12; HK1, hexokinase 1; HK2, hexokinase 2; HK3, hexokinase 3; GPI, glucose-6-phosphate isomerase; PFKM, phosphofructokinase, muscle; PFKL, phosphofructokinase, liver; PFKP, phosphofructokinase, platelet; ALDOA, aldolase A; ALDOB, aldolase B; ALDOC, aldolase C; TPI1, triosephosphate isomerase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK1, phosphoglycerate kinase 1; PGK2, phosphoglycerate kinase 2; PGAM1, phosphoglycerate mutase 1; PGAM2, phosphoglycerate mutase 2; ENO1, enolase 1; ENO2, enolase 2; PKM1, pyruvate kinase, muscle 1; PKM2, pyruvate kinase, muscle 2; LDHA, lactate dehydrogenase A; LDHB, lactate dehydrogenase B; LDHC, lactate dehydrogenase C; MCT1, monocarboxylate transporter 1; MCT2, monocarboxylate transporter 2; MCT4, monocarboxylate transporter 4; GYS1, glycogen synthase, muscle; GYS2, glycogen synthase, liver; PYGL, glycogen phosphorylase, liver; PYGB, glycogen phosphorylase, brain; PYGM, glycogen phosphorylase, muscle; G6PD, glucose-6-phosphate dehydrogenase; TKT, transketolase; TALDO1, transaldolase 1; GFPT1, glutamine–fructose-6-phosphate transaminase 1; PHGDH, phosphoglycerate dehydrogenase; PC, pyruvate carboxylase; PCK1, phosphoenolpyruvate carboxykinase 1; PCK2, phosphoenolpyruvate carboxykinase 2; FBP1, fructose-1,6-bisphosphatase 1; FBP2, fructose-1,6-bisphosphatase 2; G6PC, glucose-6-phosphatase, catalytic subunit; IL6, interleukin 6; JAK2, janus kinase 2; STAT3, signal transducer and activator of transcription 3.

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localized stromal invasion by epithelial tumor cells involves the downregulation of intercellular adhesions, loss of apicobasal polarity, and cytoskeletal reorganization that results in a mesenchymal phenotype, coincident with enhanced motility and invasiveness, and the acquisition of cancer stem cell properties [4]. These changes constitute the epithelial–mesenchymal transition (EMT), a process that is orchestrated by the re-expression of embryonic transcription factors, including ZEB1, ZEB2, SLUG and SNAIL, in response to genetic damage coupled to multiple signals within the microenvironment, including the hormone/growth factors, degree of oxygenation, inflammation and extracellular matrix components [5,6]. As EMT is an early step in the metastatic process, it represents an important target in the development of anti-metastatic adjuvant therapies. However, the fact that EMT is induced by multiple factors predicts that complete prevention of EMT is probably not possible. Thus, it is important to better define the characteristics of EMT-derived cancer cells that may provide therapeutic targets to inhibit metastasis. Here, we examined metabolic pathways in EMT-derived breast cancer cells.

Normal cells with healthy mitochondria metabolize glucose into lactate only under hypoxic conditions. In contrast, tumor cells typically import more glucose and metabolize it into lactate even in the presence of oxygen. This process is referred to as 'aerobic glycolysis' or the 'Warburg effect' [7]. A high glycolytic rate confers the following growth advantages for tumor cells: faster synthesis of ATP as compared to mitochondrial oxidative phosphorylation (OXPHOS); synthesis of ATP independently of oxygen; generation of fewer reactive oxygen species (ROS); and support of cell proliferation by providing increased quantities of glycolytic intermediates, several of which are precursors for biosynthetic pathways [8,9]. It is important to note that the TCA cycle and OXPHOS still contribute to ATP production and TCA cycle-related anabolic pathways to some extent in neoplastic cancer cells. An increasing body of research suggests that aerobic glycolysis is just one component of the global changes in the metabolism of tumor cells compared to normal cells, referred to as 'metabolic reprogramming' [10].

The vast majority of studies on metabolic reprogramming have been performed in the setting of neoplastic transformation. Considerably less is known about metabolic reprogramming in the context of metastatic transformation. Since EMT promotes metastatic transformation of epithelial tumor cells, we examined whether EMT induces additional metabolic changes in breast cancer cell lines. We previously reported that culturing ERα-positive, epithelial MCF-7 breast cancer cells in prolonged mammosphere culture induced EMT and generated a stable population of mesenchymal cancer cells (termed MCF-7_{EMT} cells herein) [11]. Compared to parental MCF-7 cells, MCF-7_{EMT} cells are highly motile, generate larger tumors *in vivo* and display cancer stem cell phenotype characterized by $CD44^{hi}$/$CD24^{lo}$ expression. In the current study, we similarly induced EMT through mammosphere culture in HER2-positive, epithelial BT-474 breast cancer cell line and generated stable mesenchymal BT-474_{EMT} cells with $CD44^{hi}$/$CD24^{low}$ expression. We compared the metabolic pathways of EMT-derived mesenchymal BT-474_{EMT} and MCF-7_{EMT} cells with their parental epithelial BT-474 and MCF-7 cells in order to determine EMT-induced metabolic reprogramming. Here we show for the first time that EMT induces multiple metabolic changes including enhanced aerobic glycolysis with increased expression of specific transporters and enzymes related to glycolysis. Surprisingly, EMT was also associated with a significant suppression of crucial enzymes within some anabolic side pathways and gluconeogenesis that would otherwise extract carbons from glycolysis or promote the flux of carbons against the flow of the glycolytic pathway. These novel findings identify several metabolic components as potential therapeutic targets for metastatic breast cancer.

Materials and methods

Cell culture

BT-474 and MCF-7 cell lines were obtained from the American Type Culture Collection (Manassas, VA). EMT in BT-747 cells was induced using prolonged mammosphere culture method as described earlier [11]. BT-474 and MCF-7 and their corresponding EMT-derived BT-474_{EMT} and MCF-7_{EMT} cells were all cultured in DMEM/F-12 supplemented with 10% heat inactivated FBS (Gibco, Grand Island, NY) and 1× MycoZap™ Plus-CL antibiotic (Lonza, Walkersville, MD). BT-474 and MCF-7 cells were additionally supplemented with 1× Insulin–Transferrin–Selenium solution (Gibco, Grand Island, NY). All cells were cultured in a humidified incubator maintained at 5% CO_2 and 37 °C.

Flow cytometry

BT-474 and BT-474_{EMT} cells were trypsinized and 1 million cells were plated in FACS tubes. Cells were washed with FACS buffer (5% FBS in 1× PBS). Cells were labeled with CD24-PE/Cy7 and CD44-Alexa Fluor 647 conjugated antibodies (BioLegend, San Diego, CA) in FACS buffer by incubating at 4 °C for 30 minutes in the dark. Cells were washed twice with FACS buffer and then labeled with viability dye eFluor® 506 (eBioscience, San Diego, CA) for 20 minutes. Cells were washed again and analyzed on BD LSR II flow cytometer (BD Biosciences, San Jose, CA). Unstained, isotype and single antibody controls were also performed for each cell line. Only live cells were included in the data analysis using FlowJo software (Ashland, OR). Flow cytometry analysis was performed twice for each cell type.

Glucose uptake and lactate production

BT-474 and MCF-7 cells (200,000 cells/well) and BT-474_{EMT} and MCF-7_{EMT} cells (50,000 cells/well) were plated in regular growth media. Cells were allowed to attach for 24-hours and washed with PBS. 5 mM glucose medium was added and cells were cultured for another 24 hours. Glucose and lactate concentrations in the culture medium were determined by fluorometric based Glucose Assay Kit and Lactate Assay Kit (BioVision, Inc., Milpitas, CA) according to the vendor's instructions using Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT). The amount consumed or produced by cells was determined by comparing the concentration in the medium incubated without cells and then normalized to cell number. These assays were performed twice with three experimental replicates for each cell type.

Conditional media and drugs

RPMI 1640 Medium Modified without L-Glutamine, without Amino acids and Glucose (US Biological, Swampscott, MA) was supplemented with 2.5 mM L-glutamine, 1× MEM essential and non-essential amino acid mixtures, 10% heat inactivated FBS, 15 mM HEPES (Gibco, Grand Island, NY); 0.5 mM sodium pyruvate, chemically defined lipid mixture 1, 15 mM sodium bicarbonate (Sigma, St. Louis, MO) and 1× MycoZap™ Plus-CL antibiotic (Lonza, Walkersville, MD). To this base medium, 20 mM D-galactose or 5 mM D-glucose (Sigma, St. Louis, MO) was added to make galactose or 5 mM glucose media, respectively. 2-Deoxy-D-glucose (Sigma, St. Louis, MO) and metformin (Tocris Bioscience, Bristol, UK) solutions were made using sterile distilled water and oligomycin A (Tocris Bioscience, Bristol, UK) solution was made using ethanol. 2-Deoxy-D-glucose, metformin and oligomycin A were used at concentrations of 5 mM, 10 mM and 5 μM, respectively.

Growth and viability assays

For a 6-day growth curve, all four cell lines were plated at a density of 10,000 cells/well in a 6-well plate in regular growth media. Cells were counted on days 2, 4 and 6 using TC20 Automated Cell Counter (Bio-Rad, Hercules, CA). For growth analysis (in 20 mM galactose, 2-deoxy-D-glucose, metformin and oligomycin A), BT-474 and MCF-7 cells were plated at a density of 300,000 cells/well and BT-474_{EMT} and MCF-7_{EMT} cells were plated at a density of 50,000 cells/well in a 6-well plate in the regular growth media. Cells were allowed to attach for 24 hrs, washed with PBS and medium of interest was added. Cells were cultured for 3 days and then live cells were counted using TC20 Automated Cell Counter (Bio-Rad, Hercules, CA) by trypan blue exclusion method. Viability assays were performed three times for each cell type.

Real-time qPCR and primers

In all assays, three separate RNA samples for each cell type extracted on different days or independent experiments were analyzed. Total RNA was isolated using TRIzol reagent (Ambion RNA, Carlsbad, CA). DNase treatment was performed using TURBO DNA-free kit (Ambion RNA, Carlsbad, CA). cDNA was made from 1 μg of total RNA using iSCRIPT cDNA synthesis kit (Bio-Rad, Hercules, CA). 50 ng cDNA was used to perform real-time PCR using SYBR green based SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA). Gene expression was normalized to TATA-box binding protein (TBP) using $2^{-\Delta Ct}$ method. Relative expression ($2^{-\Delta Ct}$) values less than 0.001 were considered extremely low/undetectable; between 0.001 and 0.01 were considered very