Published in final edited form as:

Cancer Res. 2011 July 1; 71(13): 4707–4719. doi:10.1158/0008-5472.CAN-10-4554.

TGF β /TNF α -Mediated Epithelial-Mesenchymal Transition Generates Breast Cancer Stem Cells with a Claudin-Low Phenotype

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Abstract

Breast cancer recurrence is believed to be caused by a sub-population of cancer cells that possess the stem cell attribute of treatment resistance. Recently, we and others have reported the generation of breast cancer stem cells (BCSCs) by epithelial to mesenchymal transition (EMT), although the physiological process by which these cells may arise in vivo remains unclear. We show here that exposure of tumor cells to TGF β and TNF α induces EMT and, more importantly, generates cells with a stable BCSC phenotype which is demonstrated by increased self-renewing capacity, greatly increased tumorigenicity, and increased resistance to oxaliplatin, etoposide and paclitaxel. Furthermore, gene expression analyses found that the TGF β /TNF α -derived BCSCs showed down regulated expression of genes encoding Claudin 3, 4 and 7 and the luminal marker, cytokeratin 18. These changes indicate a shift to the claudin low molecular subtype, a recently identified breast cancer subtype characterized by the expression of mesenchymal and stem cell-associated markers and correlated with a poor prognosis. Taken together, the data show that cytokine exposure can be used to generate stable BCSCs ex vivo, and suggest that these cells may provide a valuable tool in the identification of stem cell-directed biomarkers and therapies in breast cancer.

Introduction

Breast cancer recurrence is believed to be caused by a sub-population of cancer cells possessing stem cell attributes of tumor initiation, resistance to chemotherapy, radiation and other forms of treatment (1–3). Breast cancer stem cell (BCSC) characteristics can be induced through genetic and epigenetic mechanisms or extrinsically by micro environmental stimuli, conferring many unique properties such as ability to seed tumors at sites distant from the primary tumor, resistance to apoptosis-inducing drugs and enhanced migratory and metastatic potential (3–6). Current challenges in BCSC research include identifying unique and reliable molecular markers for BCSC isolation and generating stable, homogeneous BCSCs for propagation in culture for drug screening and to identify therapeutic targets. Several techniques have been used to enrich BCSCs including sorting for CD24^{−/low}CD44⁺ cells, selecting for side-population (SP) cells that exclude Hoechst dyes,

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isolating spheroids (mammospheres) from suspension cultures or isolating ALDH1 positive cells (7–10). Recently, we and others (11–13) have demonstrated the generation of CSCs through induction of epithelial-mesenchymal transition (EMT), a biological process involving coordinated molecular, biochemical and cellular changes resulting in the loss of cell-cell adhesion, apical-basolateral polarity, and epithelial markers, but acquisition of motility, spindle-cell shape, and mesenchymal markers (14–16).

Defining how EMT processes contribute to BCSC phenotypic characteristics *in vivo* has been limited by the lack of experimental models that recapitulate the processes known to induce EMT *in vivo*. Prior studies have shown that stable BCSC populations can be produced by forcing expression of key transcription factors (12). By contrast, induction with TGF β , a major inducer of EMT during tumor progression (14–18) has previously led to only transient activation of BCSC characteristics (12). In our previous report, we showed that *in vivo* generation of stable BCSCs required CD8 T cells suggesting multiple pathways must be activated in addition to TGF- β (11). Given that prior studies have shown that TNF α , an inflammatory mediator associated with cell-mediated immunity, results in a stable EMT phenotype when used with TGF β (17), we speculated this combination of cytokines also results in stable generation of cells with the BCSC phenotype. To test this, we exposed breast cancer cells derived from an epithelial breast tumor to the cytokine combination and derived stable cell populations with BCSC characteristics. Furthermore, the *ex vivo* generated BCSCs had characteristics of the claudin-low breast cancer subtype. These findings provide key insight into BCSC development *in vivo* and establish a new *in vitro* experimental model for generating mesenchymal BCSCs for evaluation of characteristics and methods of therapeutic targeting.

Materials and Methods

Cell culture and reagents

Mouse mammary carcinoma cell line (MMC) is an epithelial tumor cell line established from a spontaneous tumor of a neu-tg mouse as previously described (11). Both MMCTT and ETMT were each generated twice by treatment of MMCs with 100 ng/ml TGF β and 50 ng/ml TNF α for 30 and 60 days, respectively. MMCTTE cells were epithelial cells derived from MMCTT cells upon withdrawal of TGF β and TNF α treatment and subsequent culture for 30 days. ANV cells are mesenchymal breast cancer stem like tumor cell lines produced *in vivo* by injection of MMC cells into non-transgenic parental FVB/N mice (11). Derivation of ANV5 was confirmed using fluorescent *in situ* hybridization of the rat neu gene and karyotyping analysis (11). MMC and ANV5 cells were tested for mycoplasma using PCR-based IMPACT Profile III test (RADIL, Colombia, MO) and together with the *in vitro* derived MMCTT and ETMT cell lines maintained in RPMI supplemented with 10% FBS, Glutamate, and antibiotics. MCF10A cells were obtained from ATCC (Manassas, VA) and immediately resuscitated and expanded in DMEM/F12 media containing 5% horse serum, EGF, insulin, hydrocortisone and antibiotics. ATCC performed authentication on MCF10A cell lines through short tandem repeat profiling, karyotyping, and cytochrome c oxidase I testing. Test for bacterial and fungal contamination was performed by ATCC using current USP methods for viral testing adhering to United States Code of Federal Regulation (9 CFR 113.53) guidelines, mycoplasma testing via direct culture and Hoechst DNA staining and Limulus Amoebocyte Lysate (LAL) assay to measure Endotoxin values. The cells were then split when enough cells were obtained and then immediately treated with the cytokines or frozen as seeding stocks. All cell lines were treated with Plasmocin (InvivoGen, San Diego, CA) every two weeks to prevent mycoplasma contamination. MCF10ATT cells were generated in duplicate by treatment of MCF10A cells with same doses of TGF β and TNF α for 40 days. TGF β and TNF α were purchased from R&D System (St Paul, MN).

RNA Isolation, RT-PCR and qPCR

Total RNA was purified from cells using RNeasy Plus kit (Qiagen, Valencia, CA). RNA quantity and purity were determined using a NanoDrop ND-1000, and RNA integrity was assessed by determining the RNA integrity number and 28S/18S ratio using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). PCR primers were designed with Primer Quest (IDT, Inc., Coralville, IA). Reverse transcription-PCR (RT-PCR) was performed using SuperScript One Step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, CA) using 200 ng of RNA in a Bio-Rad MyCycler. PCR samples were analyzed on 1.5% agarose gels and imaged on a Gel Doc XR (Bio-Rad, Hercules, Ca). First strand cDNA for qPCR was synthesized using the RT2 First Strand cDNA Kit (SABiosciences, Frederick, MD). Gene expression and signaling pathway analyses were done using RT2 Profiler PCR Array qPCR kit and detected with the RT2 SYBR Green qPCR Master Mix (SABioscience) according to the manufacturer's protocol and run on ABI 7900HT with standard 96 block (Applied Biosystems, Carlsbad, Ca). Expression analysis was performed using the manufacturer's online analysis tool and gene expression was normalized to housekeeping genes. Differential expression is measured as fold expression relative to the MMC cell line.

Immunoblot analysis

Cell lysates were prepared with standard RIPA buffer and after determining protein concentration, equal protein amounts of samples were resolved by SDS-PAGE gel, transferred onto PVDF membranes, blocked with 5% nonfat milk in TBST and incubated with primary antibody at room temperature for 4hrs or overnight at 4°C. After incubation with appropriate horseradish peroxidase-conjugated secondary antibodies in blocking buffer, protein expression was detected using Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Primary polyclonal antibody to E-cadherin, N-Cadherin and β -actin-HRP, and secondary antibodies were purchased from Santa Cruz (Santa Cruz, CA). Anti- β -actin was used as loading control.

Mammosphere formation assay and cell imaging

For mammosphere formation, MMC, MMCTT, ETTM, MCF10A and MCF10ATT cell lines were grown in 4 mls DMEM/F12 media with 1:50 B27 (Invitrogen), 20 ng/mL EGF, 20 ng/mL, 10 μ g/mL insulin, penicillin, streptomycin, and amphotericin B in 12-well plates at a density of 5,000 cells/mL as described previously (19). Additional 0.5mLs of media were added every 3 days for 15 days. The number of mammospheres formed were observed and counted under a Leica DC 200 microscope (Leica Microsystems, Bannockburn, IL). Images of spheroids and adherent cells were obtained with a Leica DC 200 microscope (Leica Microsystems) and Fujifilm FinePix 6800 Zoom camera (Fujifilm, Valhalla, NY).

Flow cytometry and cell sorting

Cell surface expression of E-Cadherin, N-Cadherin, CD24 and CD44 on MMC, MMCTT, ETTM, MCF10A and MCF10ATT cells was determined by flow cytometry analysis on a FACScan (Becton Dickinson, Franklin Lakes, NJ). Cells were incubated with conjugated or non-conjugated primary antibodies at 4°C for 30 min, washed and then stained with secondary antibody for 30 min. The cells were then fixed with washing buffer containing 0.5% formaldehyde and run on a BD FACScan flow cytometer (Becton Dickinson). Sorting of CD24⁻/CD44⁺ cells was done on a BD FACS Vantage Cell Sorter and data were analyzed using Win MDI ver 2.8 software (<http://en.bio-soft.net/other/WinMDI.html>). Antibodies used included anti-CD24 PE (eBioscience, San Diego CA), anti-E-cadherin PE, anti-E-Cadherin FITC and anti-CD44 FITC (BD Pharmingen, San Diego, CA), and anti-N-Cadherin (Santa Cruz Biotechnology). Secondary antibody FITC goat anti-rabbit IgG were

from Jackson Immuno Research Laboratories. Isotype antibodies were PE Rat IgG2b (eBioscience) and FITC Rat IgG2b (BD Pharmingen).

In vivo tumorigenicity

Female neu-tg mice on the FVB/N background were maintained as a colony and *in vivo* tumorigenicity assays were performed according to institutional animal care and use (IACUC) policy. Appropriate numbers of MMC, MMCTT and ETM cells were injected subcutaneously into the mice and tumor size measured until the mice were sacrificed. Tumors were measured every other day with vernier calipers, and volumes were calculated as the product of length $\times$ width $\times$ height $\times$ 0.5236.

In vitro cell migration and invasion assay

Migration of MMC and ETM cells were assessed using non-coated membrane transwells (24-well inserts; pore size, 8 μ m; BD Biosciences). About 5×10^4 cells suspended in serum free medium were plated in the top chamber and medium supplemented with serum was used as a chemo attractant in the lower chamber. The invasion assay was conducted as described for migration assay using 1.5×10^5 cells and matrigel-coated membrane (24-well insert, pore size, 8 μ m; BD Biosciences). After 24 hrs of incubation, cells remaining on top of the membrane and not migrating were removed using a cotton swab. Migrated cells on the lower surface of the membrane were stained with Hema 3 Stain (Fisher Scientific, Pittsburgh, PA), photographed, and counted.

Chemotherapy and cytotoxicity assays

Etoposide and Paclitaxel were from Selleck Chemical (Houston, TX); Oxaliplatin was from purchased from Sigma Aldrich (St. Louis, MO). Chemoresistance of MMC and ETM cells was determined by measuring cell viability using the Xcelligence system (Roche Applied Science, Indianapolis, IN). The instrument measures cell status (given as cell Index) in a form of electrical impedance which is determined by cell morphology, cell adhesion and cell viability. As cells attach to the bottom of the plate coated with electrodes, a change in local ionic environment occurs resulting in increased impedance. Measurements were performed according to the instructions of the supplier. After seeding 100 μ L of suspension of 20,000 MMC and ETM cells into the 16-well of the E-plates, the cell index was taken every 5min for about 18 hrs for the cells to reach log phase. The cells were then exposed to increasing doses of oxaliplatin, paclitaxel and etoposide and the cell index read again for additional 60 hrs.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA. Two-tailed Student's t test, the Mann-Whitney Test or the Two-way analysis of variance test was performed to determine statistically significant difference. $P < 0.05$ was considered as significant.

Results

TGF β and TNF α generate mesenchymal tumor cells with BCSC characteristics

We previously isolated murine mammary carcinomas cells (MMCs) from tumors developing in neu-transgenic (i.e. neu-Tg)mice (20). We found that MMC cells had epithelial cell characteristics and rapidly formed tumors when reinjected into neu-tgmice, but when injected into syngeneic wild-type mice, showed an immune-mediated initial delay followed by rapid development of tumors which had undergone EMT and had lost expression of the neu antigen, generating antigen-negative variants (ANVs) (11, 20). We sought to define a

culture model of the MMC/ANV conversion process, and focused on TGF β and TNF α as key immunologic agents. When MMC cells were cultured with TGF β and TNF α for extended periods, they were converted to stable mesenchymal cells (named ETTM cells), showing the characteristic change in cell morphology from rounded to elongated, consistent with EMT (Fig. 1A), which was accompanied by loss of expression of the epithelial marker, E-Cadherin, and a gain in expression of mesenchymal markers, N-Cadherin, Snail, Twist and Zeb1 (Figs. 1A-1C). At earlier time points of treatment with TGF β /TNF α , the cells showed an intermediate phenotype (named MMCTT cells) where although the cells had acquired mesenchymal features, gene expression analysis by immunoblot, RT-PCR and flow cytometry showed only a partial loss of E-Cadherin and partial gain of N-Cadherin suggesting incomplete EMT (Figs. 1A and 1B). While removal of TGF β /TNF α from the culture media led to reversion of MMCTT cells to an epithelial phenotype (named MCTTE cells), the ETTM cells showed a complete and stable conversion. To assess the ability of ETTM cells to re-differentiate *in vivo*, they were injected into neu-tg mice and after tumor formation, removed and reassessed for expression of EMT markers. We found that the tumors expressed both E- and N-Cadherin indicating that, despite the stable culture phenotype, they were able to re-establish heterogeneous tumors with epithelial characteristics. Interestingly, the cells isolated from these tumors, when replated in culture, readily reverted to a mesenchymal phenotype (i.e. N-Cadherin positive and E-cadherin negative), similar to the injected ETTM cells suggesting that the cells possess both the ability to differentiate into E-cadherin expressing epithelial cells as well as maintaining strong self-renewal capacity (Fig. 1D).

TGF β /TNF α -mediated EMT was further confirmed by evaluating EMT genes by PCR pathway analysis. The results showed differential expression of EMT genes including higher levels of *Col1a2*, *Col3a1*, *Col5a2*, *MMP9*, and *MMP3* while epithelial genes like *Ocln*, *ErbB3*, *Tspan13* and *Bmp7* were reduced in ETTM cells (Fig. 1E). Overall, these results demonstrate exposure of MMC tumor cells to TGF β and TNF α results in the generation of stable mesenchymal cells capable of reestablishing heterogeneous tumors with epithelial characteristics.

Since EMT induction by over expression of Snail, Twist or K-ras resulted in generation of cell populations with BCSC characteristics in prior studies, we questioned whether TGF β /TNF α -generated mesenchymal cells also have these BCSC characteristics, such as enhanced self-renewal, a CD24^{-/low}CD44⁺ phenotype, ability to potentially establish heterogeneous tumor and resistance to chemotherapy (7, 11, 12). Self-renewal was examined by assessing for an increased ability of the MMCTT (incomplete transition) and ETTM cells to form mammospheres compared to the MMCs. The results showed that ETTM cells were the most efficient in mammosphere formation, producing more compact and rounded spheroids, than MMCTT cells. MMCs, in contrast, were the least efficient in forming mammospheres (Fig. 2A). Representative staining for CD24 and CD44 showed that the CD24^{-/low}CD44⁺ population within cultures increased with combined TGF β and TNF α treatment from 33% in the MMCs, to 77% in the MCTTs and 100% in the ETTMs (Fig. 2A). The observation that 33% of MMCs were CD24^{-/low}CD44⁺ despite a poorer mammosphere forming efficiency suggests that many of the CD24⁻/CD44⁺ cells are not mammosphere-forming cells, which is consistent with Al-Hajj and colleagues work also showing that only a subset of human CD24^{-/low}CD44⁺ cells are BCSCs (7).

Using the mixed epithelial and mesenchymal tumor cell line, MMCTT, we evaluated the morphology and mammosphere formation efficiency of isolated CD24⁺/CD44⁺ and CD24⁻/CD44⁺ subpopulations to confirm that the mesenchymal and self-renewing phenotype is associated with the CD24⁻/CD44⁺ subset. Results in Figure 2B show that only CD24⁻/CD44⁺ cells had a mesenchymal phenotype and effectively formed mammospheres (Fig 2b).

Staining of tumor cells isolated from ETTM-induced tumors showed CD24⁺/CD44⁺ and CD24⁻/CD44⁺ populations. Consistent with Figure 1D showing that cultures of ETTM-generated tumor cell reverted to mesenchymal cells, the cultured tumor cells also reverted entirely to the CD24⁻/CD44⁺ cells phenotype suggesting that the ETTM cells have the ability to differentiate and to self-renew (Fig. 2C).

A recently identified molecular subtype of breast cancer, claudin-low, is associated with increased expression of mesenchymal markers and correlates with poor prognosis (21, 22). Additionally, residual subpopulations of breast cancer cells that evade chemo- and endocrine therapy, in humans, have a claudin-low and BCSC phenotype characterized by reduced levels of claudin 3, 4, and 7, enrichment of CD24⁻/lowCD44⁺ cells, and increased expression of stem cell-related genes (23). Parallel to these studies, we observed that ETTM cells have considerably reduced expression of claudin 3, 4, and 7 and the luminal marker KRT18, relative to the parental MMC tumor cells (Fig. 2D). Furthermore, pathway analysis showed enriched expression of stem cell-associated genes, including *Colla1*, *Fgf2*, *Ccnd2*, *Igf1*, *Notch1*, *ABCG2* in the ETTM cells (Fig. 2E).

TGFβ/TNFα mediated EMT in human MCF10A cells generates mesenchymal cells with a stem cell-like phenotype

To determine whether TGFβ/TNFα induced EMT in human epithelial mammary cells can generate stem cell-like cells, we treated normal human mammary epithelial MCF10A cells with the cytokines for 40 days, giving rise to MCF10ATT cells. As expected, exposure to TGF and TNF led to acquisition of mesenchymal phenotype accompanied by upregulation of mesenchymal genes (such as Vimentin, Sparc, Foxc2, CDH2) and downregulation of epithelial markers (CDH1, Krt14, Ocln) as shown by real-time qPCR analysis (Figs. 3A and B). We then evaluated the breast cancer stem cell properties of the MCF10ATT cells by flow cytometry of CD24 and CD44 expression. We observed that all the MCF10ATT cells were CD24⁻/CD44⁺ compared to the MCF10A cells which comprised mainly CD24⁺/CD44⁺ population (Fig. 3C). Real-time qPCR pathway analysis also showed differential regulation of stem cell gene (Fig. 3D). To assess the self-renewal ability of the EMT generated MCF10ATT cells, we examined their mammosphere-forming capacity compared to the parental MCF10A cells. As shown in Figs. 3E and F, we found that the stem cell-like MCF10ATT cells were more efficient mammosphere forming units generating more than 100 fold mammospheres compared to the MCF10A cells.

Regulation of EMT and stem cell associated genes in ETTM cells

To further assess the BCSC characteristics of the ETTM cells, pathway analysis of transcription factors and signaling pathways associated with cancer stem cells such as Home box genes, TGFβ, Notch and Hedgehog signaling pathways were performed (24–26). These analyses revealed increased expression, in ETTM relative to MMC, of Homeo box-containing genes such as *Dlx1*, *Hoxc8* and *Hoxa9* as well as lower levels of *Hoxd13*, *Pdx1* and *Pitx1* (Fig. 4A). TGFβ/BMP signaling genes including *TGFB1*, *Colla1*, *Serpine1*, *TGFB2* and *Igf1* were differentially expressed (Fig. 4B), whereas analysis of Hedgehog and Notch signaling genes identified differential expression of *Wnt6*, *BMP4*, *Wnt10a*, *Gas1*, *Gli3*, *Ptch2*, *Ptch1*, *Wisp1*, *Notch4*, *Fos1*, *Fzd1*, *Notch3* and *Hey1* (Figs. 4C and D). These observations indicate widespread regulation of stem-associated genes in ETTM BCSCs. Overall, these observations together shows that TGFβ/TNFα mediate EMT leading to the generation CD24⁻/CD44⁺ cells BCSCs with a claudin-low phenotype.

TGFβ/TNFα-mediated EMT results in increased tumor aggressiveness

Mesenchymal BCSCs are known to have increased aggressiveness relative to epithelial tumor cells. Important characteristics include high tumorigenicity nature as well as enhanced

migration, invasion and chemoresistance (12, 13, 27). Consistent with their mesenchymal phenotype, ETTM cells demonstrated enhanced migration and invasion compared to MMCs (Fig 5A). Relative to MMC and MMCTT, ETTM cells were also much more tumorigenic than the MMCTTs (Fig. 5B). Furthermore, serial dilution of the ETTM cells showed ability to form tumors with as low as 100 cells compared to the MMCs which could only form tumors at high cell numbers (11) (Fig. 5C).

Human CD24^{-/low}CD44⁺ BCSCs are known to have a metastatic gene signature and are associated with a poor prognosis (28). Analysis of expression of metastasis-associated genes in ETTM cells revealed higher levels of *Ccl7*, *Mmp13*, *Mmp9*, *Mmp3*, *Cdh11*, *Hgf*, *Mmp10*, *Ctsk* and *Cdh6* as well as lower levels of *Mcam* and *Tnfsf10*, as compared to MMC (Fig 5D). In addition, assessment of oncogenes and tumor suppressor gene expression signatures showed higher expression of *S100a4*, *Tnf*, *kitlg*, *P53*, *Mycn* and *Est1* as well as lower levels of *Myb*, *Serpineb5*, *Cdh1* and *Kit* (Fig. 5E). Overall, these results demonstrated that ETTM cells, like previously reported CSCs, are substantially more aggressive than their epithelial differentiated counterparts.

ETTM BCSCs are chemoresistant

A clinically important attribute of CSCs is their resistance to conventional therapies such as chemotherapy (2, 29). Prior work by us and others has shown that CD24^{-/low}CD44⁺ BCSCs have elevated levels of drug pumps and DNA repair proteins thereby conferring profound resistance to chemo- and radio-therapies (11, 30). To assess the resistance of ETTM and MMC cells, they were treated with increasing doses of Oxaliplatin, Paclitaxel and Etoposide (11) and growth measured in real time (31). As shown in Figs. 6A–C, BCSCs generated with TGFβ/TNFα were more resistant to chemotherapy as compared to epithelial MMC.

Analysis of drug resistance genes showed increased expression of ABC transporter genes, *Abcb1b*, *Abcc3* and *Abcc5* as well as resistance genes *Hif1a*, and *Pparg* but lower levels of *ErbB3* and *Tnfrsf11a* (Fig. 6D). Analysis of apoptotic genes showed higher expression of *Bcl2l2*, *Birc3* and *Traf1* genes but decreased expression of *Bcl2*, *Bcl2l1*, *Mme5* and *Dapk1* (Fig 6E). DNA damage and repair signaling such as *P53*, *Rad9b* and *Rad51L1* were higher whereas *Gadd45a*, *Rad50* and *Rad54l1* were lower (Fig. 6F).

ETTM cells show altered expression of breast cancer and cell survival pathways

A number of signaling pathways including chemokine pathways have a role in breast cancer pathogenesis. Studies have shown correlation between chemokine-secreting tumor-infiltrating macrophages and poor prognosis of breast cancer (32, 33). Assessment of chemokine receptor signaling genes in ETTM showed increased expression of *Ccl13*, *Cxcr7*, *Ccl11*, *Cxcl12* and *Tnf* as well as downregulation of *Cxcr4* relative to MMC (Fig. 7A). We also observed higher amounts of inflammatory cytokines and receptor genes *Ccl2*, *Ccl5*, *Ccl6*, *Ccl7*, *Ccl8*, *Ccl11*, *Ccr1*, *Ccr7* and *Il1r1* but lower *Ccr3*, *Cxcl1*, *Il1f6* and *Ccl20* (Fig. 7B). In breast cancer, expression of estrogen receptors is both a predictive and prognostic marker as well as an effective means of targeting hormone-dependent breast cancers (34). Gene analysis of breast cancer estrogen receptor signaling identified differential expression of *Thbs2*, *Serpine1*, *Serpineb5*, *Cldn7*, *Krt18* and *Kit* genes (Fig. 7C). Cell cycle regulators are implicated in cancer initiation, progression and resistance to therapy, and were also found to be differentially expressed in ETTM (Fig. 7D) (35). Finally, higher levels of angiogenesis genes were observed with generation of ETTM cells (Fig. 7E). The results of these gene expression analyses of breast cancer and cell survival-associated pathways supports the genotype and phenotype of the ETTM cells as BCSCs.

Discussion

The key BCSC characteristics are increased tumorigenicity, ability to reconstitute a heterogeneous tumor, self-renewal and resistance to therapies. We have shown here that treatment of epithelial MMCs with TGF β /TNF α generates stable mesenchymal BCSCs, which have increased expression of mesenchymal markers such as N-Cadherin, Snail, Slug, Twist, Zeb1, Mmp3 and Mmp9. Different cancer cell lines have different degrees of sensitivity and resistance to TGF β -induced EMT (36–40). The incomplete and reversible EMT observed in MMCTT cells, in contrast to complete and stable EMTs, generated from the cell epithelial MMC cells present an intriguing observation, suggesting that although EMT can begin within a few days of exposure to cytokine, complete EMT involves not only early regulation of actin cytoskeleton as evident in cell morphological changes, but is associated with regulation of a plethora of genes and activation of an array of molecular pathways leading to a transformation of epithelial cells to more migratory and invasive mesenchymal cells. Thus, EMT may not be a rapid but rather a long term process involving regulation of many genes which act in concert to produce the profound observed cellular, molecular, phenotypic and functional changes.

The generation of BCSCs by treatment with TGF β /TNF α suggests that factors secreted by the immune system play an important role in breast cancer progression, which is consistent with our prior *in vivo* studies (11). It also implicates inflammation-induced EMT in cancer metastases and recurrence. TGF β is a potent growth inhibitor which normally functions to regulate aberrant growth of epithelial and hematopoietic cells (41). However, TGF β can also induce proliferation and invasiveness in cancer cells that have evaded the inhibitory effect of TGF β signaling through activation of specific biological processes (42). Although the mechanism by which TGF β can alternatively suppress and promote cancer progression at different stages of tumor development has not been completely elucidated, induction of EMT or at least regulation of mesenchymal genes could be one possible scenario. Furthermore, the simultaneous expression of immune factors and receptors may deregulate immunity and allow the CSCs to subvert immune-mediated elimination, thereby permitting long term survival, which would be consistent with long-term recurrence risk among treated breast cancer patients.

The ability of the BCSCs to interconvert between the CD24^{low}CD44⁺ BCSCs and CD24⁺CD44⁺ tumor cells, as shown by de-differentiation of epithelial cells or through selection in culture presents a unique model for understanding how to inhibit the growth and regeneration of BCSCs (43). Identification of cell surface markers of BCSCs for accurate identification and isolation is essential if BCSC research is to make an impact in patient care. In addition, identification of functionally relevant biological targets for development of monoclonal antibodies or small molecule inhibitors to target BCSCs in combination with conventional treatments could provide the breakthrough needed to minimize metastatic recurrence of breast cancer.

We observed upregulation of signaling pathways linked to stem cells including Home box, hedgehog, TGF β /BMP, Notch and chemokine and chemokine receptors. These pathways are subjects of on-going research to identify possible targets. Chemo resistance to cytotoxic drugs such as Oxaliplatin, Etoposide and Paclitaxel is a crucial property of CSCs which is central to their survival and evasion. The robust chemoresistant and regulation of genes and pathways associated with stemness and survival underscores the major problem posed by CSCs, specifically their ability to be eradicated with existing therapeutic modalities.

There is increasing evidence of the importance of EMT in the generation of the claudin-low sub-type (22). The claudin-low subtype is a recent addition to the four major subtypes of

breast cancer, namely luminal A, luminal B, basal-like and ERBB2 positive subtypes (44). This new subtype exhibits stem cell characteristics with increased expression of immune response genes, EMT genes, and lower expression of luminal differentiation genes (45). The claudin-low subtype also constitutes the residual subpopulation of breast cancer cells that survived after chemo-and endocrine therapy and are enriched in CD24^{-/low}/CD44⁺ cells (23). Our results show that TGF β /TNF α treatment of breast cancer cells with a predominant luminal phenotype results in generation of the claudin-low cells and suggests that targeting proteins involved in the induction or survival of this subtype could be a therapeutic strategy for improving survival of breast cancer patients regardless of the primary subtype.

In conclusion, this study provides new information on the role of the cytokines, TGF β and TNF α in EMT. It also provides initial evidence linking immunity (i.e. cytokines) to the generation of the BCSC through EMT. The *ex vivo* generation of BCSCs cells with cytokines may enable a better understanding of the biology of CSC, identification of definitive biomarkers, and the discovery of biological targets and pathways for development of effective therapies. It could also be useful for drug screening to determine the effectiveness and required doses of CSC-targeted therapies.

Acknowledgments

Grant Support

This work was supported by a generous gift from Martha and Bruce Atwater (KLK); Howard Temin Award, K01-CA100764 (KLK); R01-CA122086 (DCR); and the Mayo Clinic Breast Cancer Specialized Program of Research Excellence Award, P50-CA116201 (JI).

The authors acknowledge the strong support of the Mayo Clinic Comprehensive Cancer Center for providing access to core facilities.

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Figure 1. TGFβ with TNFα generates tumor cells with a stable EMT phenotype that form heterogeneous tumors in vivo

A) Photomicrograph (upper) and flow cytometry analysis (bottom) of epithelial MMC and mesenchymal MMCTT and ETTM cells. B) RT-PCR of mRNA isolated from MMC, MMCTT, MMCTTE, ETTM and ANV5 cells using primers specific for E-cadherin, N-Cadherin, Zeb1, Twist, Snail and GAPDH. ANV5 is a stable BCSC cell line derived in our prior studies (11). C) Immunoblot of cells in (B) showing expression of E-Cadherin, N-Cadherin and β-actin. D) Flow cytometry analysis of N-Cadherin and E-Cadherin on ETTM cells, ETTM primary tumor and cultured cells. E) Expression of EMT-associated genes by qPCR analysis.

Figure 2. Generation of breast cancer stem cells by TGF β and TNF α .

A) top left, Photograph of mammospheres formed by MMC, MMCTT and ETTM cells. A) top right, graph of the mean and s.e.m. (n=6) of mammospheres formed by MMC, MMCTT and ETTM cells. *** = $p < 0.05$. A) bottom left, flow cytometry analysis of co-expression of CD24 and CD44 on MMC, MMCTT and ETTM cells. B) Photograph of adherent cells and mammospheres formed by CD24⁺/CD44⁺ and CD24⁻/CD44⁺ of MMCTT cells. C) Flow cytometry analysis showing expression of CD24 and CD44 on ETTM cells, freshly isolated ETTM tumor cells and cultured ETTM tumor cells. D) Real-time PCR using gene-specific primers detect gene expression of Keratin 18, Claudin 3, 4, 7 and 9. Error bars represent SD of two independent experiments. E) Gene expression analysis of stem cells genes using qPCR profiler arrays. Errors bars represent SD of two independent experiments.

Figure 3. Human immortalized breast epithelial cells exposed to TGF β and TNF α undergo EMT and acquire breast cancer stem cell phenotype

A) Photograph of adherent epithelial MCF10A cells and mesenchymal MCF10ATT cells (MCF10A cells grown in TGF β and TNF α for 40 days). B) Fold regulation plot of EMT-associated genes in MCF10ATT cells assessed by qPCR using MCF10A cells as a reference. Errors bars represent SD of two independent experiments. C) Flow cytometry analysis of cell surface expression of CD24 and CD44 on MCF10A and MCF10ATT cells. D) Fold regulation plot of stem cell genes by qPCR using profiler arrays using MCF10A as reference. Errors bars represent SD of two independent experiments. Photograph (E) and quantification (F) of mammospheres formed by MCF10A and MCF10TT cells. Each bar is the mean (s.e.m.) of 6 replicates.

Figure 4. Regulation of EMT and stem cell associated genes in ETTM cells

a) Gene expression analysis of Home box genes showing upregulation of *Dlx1*, *Hoxc9*, *Hoxc8*, and *Hoxa9*. b) qPCR pathway array analysis shows regulation of genes involved in TGF β /BMP signaling pathway. c) Assessment of regulation of Hedgehog signaling genes showing differential regulation of *Wnt6*, *Wnt10A*, *Gas1*, *Wnt5a*, *gli3*, *Ptc1*, *PtcgD2* and *Wnt5b*. d) qPCR gene expression analysis detects upregulation of *Wisp1*, *Fos11*, *Notch4* and downregulation of *Notch3* and *Hey1*. Error bars represent SD of two independent experiments.

Figure 5. ETTM cells are highly migratory, invasive and tumorigenic

A) top, photomicrograph of MMC and ETTM cells migrated and invaded through matrigel coated or uncoated trans well inserts. A) bottom, quantification of migrated and invaded cells showing statistically significant differences between MMC and ETTM cells. B,C) Mean ($\pm$ s.e.m., $n=5$) tumor sizes over time (days) following implantation of 100,000 MMC, MMCTT and ETTM cells as well as serial dilution of ETTM cells showing high tumorigenicity of ETTM cells and the ability to form tumors at 100 cells. The inset fraction represents the number of mice with tumors in this experiment. For both the 10,000 and 1000 cell doses, the variability was so minor that the error bars are obscured by the symbols. D, E) Gene expression analysis of metastases, oncogenes and tumor suppressor genes showing differential regulation of metallopeptidases and adhesion genes such as *Mmp13*, *Mmp9*, *Mmp3*, *Cdh1*, *cdh66* and *Cdh11*. $*=p<0.05$. Error bars represent SD of two independent experiments.

Figure 6. ETTM stem cells are resistant to oxaliplatin, etoposide and paclitaxel

A) Real-time measurement of MMC and ETTM cell growth (Cell index) following treatment with oxaliplatin over a period of 40 hrs. Lines are the means of 3 replicates. B,C) Treatment of MMC and ETTM with different doses of etoposide and paclitaxel for 45 or 60 hrs showing resistance to cell death by ETTM cells as measured by electrical impedance. Bars are the mean (s.e.m.) of 3 replicates. D, E) qPCR pathway array analyses of drug resistance and apoptosis genes, F) qPCR pathway array analyses of DNA damage and repair genes. For panels D-F, error bars represent SD of two independent experiments.

Figure 7. Breast cancer and stem cell associated genes are regulated in ETTM cells qPCR analysis of chemokine receptor genes (A), inflammatory cytokine and receptor genes (B), breast cancer and estrogen signaling genes (C) and cell cycle and angiogenesis genes (D and E). shows differential regulation of these signaling pathways in ETTM BCSCs compared to non-BCSC parental cells. Error bars represent SD of two independent experiments.