Tumor and Stem Cell Biology

TGFβ/TNFα-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 subpopulation 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 (BCSC) by epithelial–mesenchymal transition (EMT), although the physiologic process by which these cells may arise in vivo remains unclear. We show here that exposure of tumor cells to TGFβ and TNFα induces EMT and, more importantly, generates cells with a stable BCSC phenotype which is shown by increased self-renewing capacity, greatly increased tumorigenicity, and increased resistance to oxaliplatin, etoposide, and paclitaxel. Furthermore, gene expression analyses found that the TGFβ/TNFα-derived BCSCs showed downregulated 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.

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Introduction

Breast cancer recurrence is believed to be caused by a subpopulation 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 microenvironmental 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 identifying therapeutic targets. Several techniques have been used to enrich BCSCs, including sorting for CD24+/lowCD44+ cells, selecting for side population cells that exclude Hoechst dyes, isolating spheroids (mammospheres) from suspension cultures, or isolating ALDH1 positive cells (7–10). Recently, we and others (11–13) have showed the generation of cancer stem cells 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 TGFβ, a major inducer of EMT during tumor progression (14–18) had 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 that multiple pathways must be activated in addition to TGFβ (11). Given that prior studies have shown that TNFα, an inflammatory mediator associated with cell-mediated immunity, results in a stable EMT phenotype when used with TGFβ (17), we speculated that 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-transgenic (neu-tg) mouse as previously described (11). Both MMCTT and ETTM were generated twice by treatment of MMCs with 100 ng/mL TGFβ and 50 ng/mL TNFα for 30 and 60 days, respectively. MMCTT cells were epithelial cells derived from MMCTT cells upon withdrawal of TGFβ and TNFα treatment and subsequent culture for 30 days. Antigen-negative variants (ANV) cells are mesenchymal breast cancer stem-like tumor cell lines produced in vivo by injection of MMC cells into nontransgenic parental FVB/N mice (11). Derivation of ANV5 was confirmed by using fluorescent in situ hybridization of the rat neu gene and karyotyping analysis (11). MMC and ANV5 cells were tested for mycoplasma by using PCR-based IMPACT Profile III test (RADIL) and together with the in vitro derived MMCTT and ETTM cell lines maintained in Roswell Park Memorial Institute supplemented with 10% FBS, glutamate, and antibiotics. MCF10A cells were obtained from American Type Culture Collection (ATCC; Manassas) and immediately treated with the cytokines or frozen as seeding stocks. All cell lines were treated with Placmocin (InvivoGen) every 3 days for 15 days. The number of mammospheres was determined by flow cytometry analysis and adherent cells were obtained with a Leica DC 200 microscope (Leica Microsystems). Images of spheroids were analyzed on 1.5% agarose gels and imaged on a Gel Doc XR (Bio-Rad). First strand cDNA for quantitative PCR (qPCR) was synthesized by using the RT2 First Strand cDNA Kit (SA Biosciences). Gene expression and signaling pathway analyses were done by using RT2 Profiler PCR Array kit and detected with the RT2 SYBR Green qPCR Master Mix (SA Biosciences) according to the manufacturer’s protocol and run on ABI 7900HT with standard 96 block (Applied Biosystems). Expression analysis was conducted by 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 radioimmunoprecipitation assay buffer and after determining protein concentration, equal protein amounts of samples were resolved by SDS-PAGE gel, transferred onto polyvinylidene difluoride membranes, blocked with 5% nonfat milk in tris-buffered saline and Tween 20 and incubated with primary antibody at room temperature for 4 hours or overnight at 4°C. After incubation with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies in blocking buffer, protein expression was detected by using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Primary polyclonal antibody to E-cadherin, N-adherin and β-actin-HP, and secondary antibodies were purchased from Santa Cruz (Santa Cruz Biotechnology). Anti-β-actin was used as a loading control.

Mammosphere formation assay and cell imaging

For mammosphere formation, MMC, MMCTT, ETTM, MCF10A, and MCF10ATT cell lines were grown in 4 mL DMEM/F12 media and adherent cells were obtained on a BD FACSVantage Cell Sorter, and data were analyzed by using WinMDI version 2.8 software (http://en.bio-soft.net/other/)
WinMDI.html). Antibodies used included anti-CD24 PE (eBioscience), anti-E-cadherin phycoerythrin (PE), anti-E-cadherin fluorescein isothiocyanate (FITC) and anti-CD44 FITC (BD Pharmingen), and anti-N-cadherin (Santa Cruz Biotechnology). Secondary antibody FITC goat anti-rabbit IgG were from Jackson ImmunoResearch 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 conducted according to Institutional Animal Care and Use Committee (IACUC) policy. Appropriate numbers of MMC, MMCTT, and ETTM 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 × width × height × 0.5236.

In vitro cell migration and invasion assay
Migration of MMC and ETTM cells were assessed by using noncoated membrane transwells (24-well insert; pore size, 8 μm; BD Biosciences). About 5 × 10⁴ cells suspended in serum-free medium were plated in the top chamber, and medium supplemented with serum was used as a chemoattractant in the lower chamber. The invasion assay was conducted as described for migration assay by using 1.5 × 10⁵ cells and Matrigel-coated membrane (24-well insert; pore size, 8 μm; BD Biosciences). After 24 hours of incubation, cells remaining on top of the membrane and not migrating were removed by using a cotton swab. Migrated cells on the lower surface of the membrane were stained with Hema 3 Stain (Fisher Scientific), photographed, and counted.

Chemotherapy and cytotoxicity assays
Etoposide and paclitaxel were from Selleck Chemical; oxaliplatin was purchased from Sigma Aldrich). Chemoresistance of MMC and ETTM cells was determined by measuring cell viability by using the Xcelligence system (Roche Applied Science). 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 carried out according to the instructions of the supplier. After seeding 100 μL of suspension of 20,000 MMC and ETTM cells into the 16-well of the E-plates, the cell index was taken every 5 minutes for about 18 hours 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 hours.

Statistical analysis
Statistical analysis was conducted by using GraphPad Prism version 4.00 for Windows (GraphPad Software). Two-tailed Student’s t-test, the Mann–Whitney U test, or the 2-way ANOVA was carried out to determine statistically significant difference. P < 0.05 was considered as significant.

Results
TGFβ and TNFα generate mesenchymal tumor cells with BCSC characteristics
We previously isolated MMCs from tumors developing in neu-tg mice (20). We found that MMC cells had epithelial cell characteristics and rapidly formed tumors when re injected into neu-tg mice, 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 ANVs (11, 20). We sought to define a culture model of the MMC/ANV conversion process, and focused on TGFβ and TNFα as key immunologic agents. When MMC cells were cultured with TGFβ and TNFα 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–C). At earlier time points of treatment with TGFβ/TNFα, 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 B). Although removal of TGFβ/TNFα 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 redifferentiate 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 reestablish 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).

TGFβ/TNFα-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, whereas epithelial genes like Ocln, Erbb3, Tspan13, and Bmp7 were reduced in ETTM cells (Fig. 1E). Overall, these results show exposure of MMC tumor cells to TGFβ, and TNFα results in the generation of stable mesenchymal cells capable of reestablishing heterogeneous tumors with epithelial characteristics.

Because EMT induction by overexpression of Snail, Twist, or K-ras resulted in generation of cell populations with BCSC...
characteristics in prior studies, we questioned whether TGFβ/TNFα-generated mesenchymal cells also have these BCSC characteristics, such as enhanced self-renewal, a CD24⁻/CD44⁺ phenotype, ability to potently 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 with 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 least efficient in forming mammospheres (Fig. 2A). Representative staining for CD24 and CD44 showed that the CD24⁻/CD44⁺ population within cultures increased with combined TGFβ and TNFα 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⁻/CD44⁺ despite a poorer mammosphere forming efficiency suggests that many of the CD24⁻/CD44⁺ cells are not mammosphere-forming cells, which is consistent with the work of Al-Hajj and colleagues also showing that only a subset of human CD24⁻/CD44⁺ cells are BCSCs (7).

By 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 showing that cultures of ETTM-generated tumor cell reverted to mesenchymal cells as shown in Figure 1D, 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 Col1a1, 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, and CDH2) and downregulation of epithelial markers (CDH1, Krt14, and Ocln) as shown by real-time qPCR analysis (Fig. 3A and B). We then evaluated the BCSC properties of the MCF10ATT cells by flow cytometry of CD24 and CD44 expression. We observed that all the MCF10ATT cells were CD24+/CD44+ compared with the MCF10A cells which were mainly CD24+/CD44+ (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 with the parental MCF10A cells. As shown in Figure 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 with 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 Homeobox genes, TGFβ, Notch, and Hedgehog signaling pathways were carried out (24–26). These analyses revealed increased expression, in ETTM relative to MMC, of Homeobox-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 Igfbp3 were...
differentially expressed (Fig. 4B), whereas analysis of Hedgehog and Notch signaling genes identified differential expression of Wnt6, BMP4, Wnt10a, Gli1, Gli3, Ptc1, Ptc1, Wisp1, Notch4, Fosl1, Fzd1, Notch3, and Hey1 (Fig. 4C and D). These observations indicate widespread regulation of stem-associated genes in ETTM BCSCs. Overall, these observations together show that TGFβ/TNFα mediate EMT leading to the generation of 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 showed enhanced migration and invasion compared with 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 with the MMCs which could only form tumors at high cell numbers (ref. 11; Fig. 5C).

Human CD24−/lowCD44+ 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 Ccld, Mmp13, Mmp9, Mmp3, Cdhh11, Hgf, Mmp10, Csk, and Cdh6 as well as lower levels of Mcam and Tnfsf10, as compared with MMC (Fig. 5D). In addition, addition 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, Serpinb5, Cdh1, and Kit (Fig. 5E). Overall, these results showed that ETTM cells, like previously reported CSCs, are substantially more aggressive than their epithelial differentiated counterparts.

**ETTM BSCSs are chemoresistant**

A clinically important attribute of CSCs is their resistance to conventional therapies such as chemotherapy (2, 29). Prior works by us and others have shown that CD24−/lowCD44+ BCSCs have elevated levels of drug pumps and DNA repair proteins, thereby conferring profound resistance to chemotherapies and radiotherapies (11, 30). To assess the resistance of ETTM and MMC cells, they were treated with increasing doses of oxaliplatin, acitaxel, and etoposide (11) and growth measured in real-time (31). As shown in Fig. 6A–C, BCSCs generated with TGFβ/TNFα were more resistant to chemotherapy as compared with epithelial MMC.

Analysis of drug resistance genes showed increased expression of ABC transporter genes, Abcb1b, Abcc3, and Abcc5, as well as resistance genes Hif1α, and Pparg but lower levels of Erbb3 and Tnfrsf1a (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 Gad45a, 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 progression. Studies have shown a 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, Cc11, 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 Cc22, Cc15, Cc67, Cc7, Cc82, Cc11, Ccr1, Ccr7, and Ilr1 but lower Ccr3, Cxcl1, Hif6, and Cc20 (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, Serpine5b, 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; ref. 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 support 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 TGFβ/TNFα 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 TGFβ-induced EMT (36–40). The incomplete and reversible EMT observed in MMCTTs cells, in contrast to complete and stable ETTMs, 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 morphologic 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 TGFβ/TNFα 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 implicate inflammation-induced EMT in cancer metastases and recurrence. TGFβ is a potent growth inhibitor which normally functions to regulate aberrant growth of epithelial and hematopoietic cells (41). However, TGFβ can also induce
proliferation and invasiveness in cancer cells that have evaded the inhibitory effect of TGFβ signaling through activation of specific biological processes (42). Although the mechanism by which TGFβ 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 1 possible scenario. Furthermore, the simultaneous expression of immune factors...
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 hours. Lines are the means of 3 replicates. B and C, treatment of MMC and ETTM with different doses of etoposide and paclitaxel for 45 or 60 hours showing resistance to cell death by ETTM cells as measured by electrical impedance. Bars are the mean (SEM) of 3 replicates. D and E, qPCR pathway array analyses of drug resistance and apoptosis genes. F, qPCR pathway array analyses of DNA damage and repair genes. D–F, error bars represent SD of 2 independent experiments.
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<sup>−/low</sup>/CD44<sup>+</sup> BCSCs and CD24<sup>+</sup>/CD44<sup>+</sup> tumor cells, as shown by dedifferentiation 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 Homeobox, hedgehog, TGFβ/BMP, Notch, and chemokine and chemokine receptors. These pathways are subjects of ongoing research to identify possible targets. Chemoresistance 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 subtype (22). The claudin-low subtype is a recent addition to the 4 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 chemos- and endocrine therapy and are enriched in CD24−/low/CD44+ cells (23). Our results show that TGFβ/TNFα 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, TGFβ and TNFα 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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In this article (Cancer Res 2011;71:4707–19), which was published in the July 1, 2011, issue of Cancer Research (1), the labels for panels B and C in Fig. 4 are transposed. The data within the panels, however, are correct.

Reference


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