Tissue Transglutaminase Promotes Drug Resistance and Invasion by Inducing Mesenchymal Transition in Mammary Epithelial Cells

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Abstract

Recent observations that aberrant expression of tissue transglutaminase (TG2) promotes growth, survival, and metastasis of multiple tumor types is of great significance and could yield novel therapeutic targets for improved patient outcomes. To accomplish this, a clear understanding of how TG2 contributes to these phenotypes is essential. Using mammary epithelial cell lines (MCF10A, MCF12A, MCF7 and MCF7/RT) as a model system, we determined the impact of TG2 expression on cell growth, cell survival, invasion, and differentiation. Our results show that TG2 expression promotes drug resistance and invasive functions by inducing epithelial-mesenchymal transition (EMT). Thus, TG2 expression supported anchorage-independent growth of mammary epithelial cells in soft-agar, disrupted the apical-basal polarity, and resulted in disorganized acini structures when grown in 3D-culture. At molecular level, TG2 expression resulted in loss of E-cadherin and increased the expression of various transcriptional repressors (Snail1, Zeb1, Zeb2 and Twist1). Tumor growth factor-beta (TGF-β) failed to induce EMT in cells lacking TG2 expression, suggesting that TG2 is a downstream effector of TGF-β-induced EMT. Moreover, TG2 expression induced stem cell-like phenotype in mammary epithelial cells as revealed by enrichment of CD44+/CD24−/low cell populations. Overall, our studies show that aberrant expression of TG2 is sufficient for inducing EMT in epithelial cells and establish a strong link between TG2 expression and progression of metastatic breast disease.

Introduction

Tumor cell resistance to systemic therapies and metastasis to distant tissues pose major impediment to successful treatment of breast cancer [1]. Therefore, understanding the molecular mechanisms that contribute to the development of drug resistance and facilitate metastasis could provide valuable targets for effective control and treatment of the disease. Transglutaminase 2 (TG2), a pro-inflammatory protein, has received considerable attention recently for its potential role in cancer cells. There is ample evidence supporting that metastatic and drug-resistant breast cancer cells express high basal levels of TG2 [2] and that its expression is associated with increased cell survival, invasion and motility of cancer cells [3]. However, the knowledge on how TG2 promotes these phenotypes remains elusive.

TG2 is a multifunctional protein implicated in diverse physiological and pathological processes [4]. In addition to transamidation activity, TG2 can catalyze GTPase [5], protein disulfide isomerase [6] and kinase activities [7]. In normal tissues, TG2 expression is upregulated in response to tissue injury and other stressors; there it plays a role in restoring the normal homeostasis [8]. In such pathological conditions as tissue fibrosis and cancer, TG2 expression within the cell or its microenvironment promotes cell adhesion and modulates intracellular signaling [9]. For example, intracellular TG2 is known to activate focal adhesion kinase (FAK), protein kinase B, and Akt [10], cyclic AMP response-element binding protein [11], platelet-derived growth factor [12], and the nuclear factor-kB (NF-kB) [13]. In the extracellular environment, TG2 can modify the extracellular matrix proteins and alter cell-cell (heterotypic) and cell-matrix (heterotypic) interactions [14]. Although, emerging lines of evidence suggest a close link between TG2, drug resistance, and metastasis of cancer cells, the pathways that contribute to these events remain largely unknown.

Recently epithelial-to-mesenchymal transition (EMT), which shares many molecular characteristics with cancer stem cells, has been implicated to play a role in cancer metastasis [15,16]. EMT is a complex dynamic process that occurs during embryonic development for reprogramming of epithelial cells [16]. Its reactivation during adult life has been associated with pathological conditions, such as inflammation, fibrosis, and cancer [16,17]. The EMT promotes the detachment of cancer cells from the primary tumor and facilitates migration via loss of cellular polarity and adhesion [17]. Moreover, emerging evidence suggests a
strong link between EMT and chemoresistance and radioresistance [17,18]. Therefore, understanding of molecular mechanisms that lead to EMT is important for designing novel therapeutic strategies for increasing drug sensitivity and the suppression of metastasis toward better treatment outcomes in cancer patients.

Here we demonstrate that aberrant expression of the pro-inflammatory protein, TG2, in mammary epithelial cells (MECs) is associated with loss of E-cadherin, cellular polarity, upregulation of mesenchymal markers, such as fibronectin, vimentin, N-cadherin and transcriptional repressors, such as Snail1, Zeb1, Zeb2 and Twist1. Moreover, our data suggest that TG2 is a downstream mediator of tumor growth factor-beta (TGF-β)-induced EMT. We conclude that TG2 expression signals the onset of EMT in epithelial cells and contributes to their increased survival and metastatic potential and hence represents a promising therapeutic target for drug-resistant and metastatic breast cancer.

**Results**

**TG2 induces EMT in mammmary epithelial cells**

We previously reported that metastatic breast cancer cells express high basal levels of TG2 [2] and that increased expression of TG2 in breast cancer cells contributes to their increased survival, invasion, motility and drug resistance [2,3]. To understand the role of TG2 in metastatic transformation, we stably transected TG2 into a non-transformed human breast mammary epithelial cell line MCF10A. TG2-overexpressing MCF-10A cells (10A-TG2) showed marked changes in their morphology compared to the vector-transfected (10A-Vec) cells. As shown in Fig. 1A, 10A-Vecl cells appeared rounded with cobblestone epithelial morphology and grew as tightly connected clusters. 10A-TG2 cells, in contrast, displayed spindle-like shape and exhibited scattered distribution of fibroblast-like cells with disrupted cell-to-cell adhesion (Fig. 1A). Expression of TG2 in these cells was confirmed by immunoblotting (Fig. 1B) and immunostaining (Fig. 1C). The mesenchymal nature of 10A-TG2

Figure 1. TG2 induces EMT in mammmary epithelial cells. MCF10A and MCF12A cells were stably transfected with vector alone (10A-Vec and 12A-Vec) or lentiviral-TG2 construct (10A-TG2 and 12A-TG2). (A) Phase-contrast images of 10A-Vec and 10A-TG2 cells after 48 hr culture in medium. Magnification 10X. (B) Immunoblot showing relative expression of TG2 in 10A-Vec and 10A-TG2 cells. (C) Immunofluorescence due to TG2 and EMT markers in 10A-Vec (top panel) and 10A-TG2 (bottom panel) cells. Green fluorescence shows the expression and localization of indicated proteins and DAPI (blue) staining shows the nuclei. (D) Immunoblot analysis of the indicated EMT markers in 10A-Vec and 10A-TG2 cells. Expression of epithelial cell markers (E-cadherin, β-catenin) and mesenchymal cell marker (N-cadherin, fibronectin, and vimentin) was examined by immunoblotting. (E) Morphology (left penal) and immunoblot analysis of TG2, epithelial marker (E-cadherin) and mesenchymal marker (fibronectin) expression in drug-sensitive and drug-resistant (RT) MCF-7 cells. Multiple stable polyclones were established from MCF10A and MCF12A cells and experiments were repeated multiple times with similar results using different clones.

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TG2-induced EMT confers invasiveness, drug resistance and tumorigenic phenotype

Based on the biological contexts, EMT has been classified into three subtypes: Type 1 is associated with embryonic development and neither causes fibrosis nor induces invasive phenotype. Type 2 EMT is associated with wound healing, tissue regeneration, and organ fibrosis and does not induce the invasiveness. Type 3 or oncogenic-EMT is associated with cancer and is characterized by acquisition of invasive and metastatic functions [21]. Accordingly, TG2 expression was associated with increased invasiveness of 10A-TG2 cells compared to the 10A-vec cells (Figs. 3A). These results suggest that TG2-induced EMT is the Type 3 and is associated with the acquisition of invasive and metastatic potential. As a result, we next determined whether TG2-induced EMT would promote the growth of 10A-TG2 cells in soft agar — an in vitro surrogate measure of tumorigenicity [22]. Results shown in Fig. 3B demonstrated that 10A-TG2 cells could grow and form colonies in soft agar whereas, 10A-vec cells failed to survive under these conditions.

MCF10A is a non-transformed human mammary epithelial cell line and is an excellent in vitro model to study mammary gland development and breast cancer progression in 3D cultures. These cells form well-organized acinar structures that mimic the normal mammary end bud in vivo [23]. We used this 3D model to determine whether TG2-induced EMT can disrupt the organization of MCF10A cells. As shown in Fig. 3C, the 10A-vec cells grew into well-organized acinar-like structures with hollow lumens (Fig. 3C and D, top panel). Immunostaining of spheroids with anti-laminin V antibody revealed the presence of continuous and well-defined basement membrane surrounding the acini with apico-basal polarization of cells (Fig. 3D). Similarly, immunostaining of spheroids for E-cadherin further confirmed the apico-basal polarization with cell-to-cell contact in acinar structures (Fig. 3D).

The 10A-TG2 cells, in contrast, demonstrated severe disruption of acinar architecture, characterized by irregular spheroid growth and no lumen formation (Figs. 3C and 3D, lower panels). A distinct feature of 10A-TG2 spheroids was the gain of invasive function; many cells escaped from the acini and invaded the surrounding matrix (Fig. 3C, inset). Similarly, immunostaining for laminin V and E-cadherin revealed diffused basement membrane formation and complete loss of E-cadherin in 10A-TG2 acini (Fig. 3D). Similar changes in growth pattern and organization of acinar structure were observed in TG2-expressing MCF-12A cells, when grown in 3D culture (Supplemental Fig. S2). These results further supported the ability of TG2 to promote invasive potential in MECs. Several lines of evidence suggest that tumor cells undergoing EMT, become resistant to chemotherapy and conversely tumor cells selected for drug resistance exhibit EMT phenotype [17]. Therefore, we next determined whether TG2-induced EMT in MCF-10A cells could confer resistance to chemotherapeutic drug. Results shown in Fig. 3E demonstrated that 10A-TG2 cells were relatively more resistant to doxorubicin-induced cell death compared to the 10A-vec cells. Overall, these results suggest that TG2-induced EMT promotes invasiveness, drug resistance and anchorage-independent growth of MECs.

TG2 is a downstream mediator of TGF-β-induced EMT

Because the EMT gene signature of 10A-TG2 cells (Fig. 2A) closely matched the TGF-β-induced EMT gene signature [24], we next determined whether TG2-induced EMT involved TGF-β signaling. For this purpose, we checked the expression of TGF-β receptor-1 and -2 and phospho-smad 2 and 3 in 10A-vec and 10A-TG2 cells. Interestingly, we did not observe any change in receptors level or phosphorylation status of smad-2 or smad-3 (Supplemental Fig. S5), suggesting that TG2-induced EMT is
independent of TGF-β signaling. The progression of the EMT program is regulated by a series of intracellular signaling molecules, such as NF-κB, MAPK, PI3K, Akt, RhoB, Ras, and c-Fos as well as by cell-surface proteins, such as β4 integrin, α5β1 integrin, and αVβ6 integrin [25]. Previous studies have shown that TG2 expression results in constitutive activation of NF-κB [13,26]. Therefore, next we determined the status of NF-κB activity in 10A-vec and 10A-TG2 cells. Results shown in Fig. 4A revealed almost 4-fold increase in NF-κB activity in 10A-TG2 cells compared with the 10A-vec cells. These results suggest that TG2-induced activation of NF-κB may be responsible for transcriptional regulation of Snail and induction of EMT in 10A-TG2 cells. Indeed, TG2 was recently shown to associate with NF-κB for its recruitment to the promoter sequence of Snail and leading to its transcriptional regulation [27].

We also determined the status of pFAK and pAkt, the other known mediators of EMT [28], in MCF10A sublines. Results shown in Fig. 4B demonstrated the constitutive activation of both FAK and Akt in 10A-TG2 cells. These results suggest that TG2-mediated activation of FAK, Akt and NF-κB may play a role in driving MCF10A cells into EMT by regulating Snail, Zeb1, and Twist genes expression. Because TGF-β can induce TG2 expression [29], we next determined whether TG2 expression is required for TGF-β to induce EMT. MCF10A cells stably transfected with TG2-shRNA and treated with TGF-β showed no or minimal change in their morphology (Fig. 4C). In contrast, MCF10A-control shRNA-transfected cells under similar conditions revealed progressive change in their morphology starting as early as 4 days post-TGFβ treatment. After 12 days of treatment, they appeared elongated and showed mesenchymal appearance (Fig. 4C). Western blot analysis for epithelial markers in TGF-β-treated MCF10A-control-shRNA cells revealed downregulation of E-cadherin on day 4 with complete loss by day 8 of treatment. No such change in E-cadherin level was evident in TG2shRNA-transfected cells even after 12 days of

Figure 2. TG2 expression results in altered expression of Snail1, Twist1, Zeb1, and Zeb2. (A) Real time RT-PCR array showing relative changes in the expression of EMT-related genes in 10A-TG2 cells in comparison to 10A-Vec cells. Y-axis denotes the fold-expression and x-axis denotes the genes. The expression of GAPDH, β-actin and 18S ribosomal RNA was used to normalize variable template loading. (B) RT-PCR analysis for EMT-related transcripts was used to evaluate their expression in 10A-Vec and 10A-TG2 cells. (C) RT-PCR (left panel) and immunoblot (right panel) analysis was performed to validate the expression of transcription factors Snail1, Zeb1 and Twist1 in 10A-Vec and 10A-TG2 cells. Results shown are from a representative experiment repeated at least twice with similar results.
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treatment. Similarly, fibronectin expression in response to TGFβ treatment was found upregulated but only in MCF10A-control shRNA cells (Fig. 4D). Similarly, downregulation of endogenous (in MCF-7/RT cells) or induced TG2 (in MCF-10A/TG2 cells) reversed the EMT process (mesenchymal-to-epithelial) as revealed by increase in E-cadherin and decrease in fibronectin expression (Fig. 4E). These results clearly implied that TG2 is a downstream mediator of TGF-β-induced EMT.

TG2-induced EMT promotes stem cell-like phenotype

Based on recent reports that induction of EMT results in acquisition of stem cell-like characteristics and that EMT and stem
cells share some common molecular links in breast epithelial cells [30], next we determined whether TG2-induced EMT could induce a stem cell state in MCF10A cells. We used flow cytometric analysis to determine the expression of CD44\textsuperscript{high}/CD24\textsuperscript{low} phenotype in 10A-TG2 and 10A-vec cells. The CD44\textsuperscript{high}/CD24\textsuperscript{low} phenotype has been used as a marker to isolate stem cells from normal and cancerous mammary epithelial cells [31]. Results from a representative experiment shown in Fig. 5A revealed that significantly more 10A-TG2 cells expressed CD44\textsuperscript{high}/CD24\textsuperscript{low} stem cell markers. Consistent with these results was the observation that CD326 antigen, an epithelial marker, was significantly downregulated in 10A-TG2 cells. To further validate these results, we next analyzed the CD44\textsuperscript{high}/CD24\textsuperscript{low} expression in doxorubicin-resistant MCF-7/RT breast cancer cells [19]. The constitutive expression of TG2 in MCF-7/RT cells was associated with a similar enrichment of CD44\textsuperscript{high}/CD24\textsuperscript{low} cell population compared to the parental drug-sensitive and TG2-deficient MCF-7 cells (Fig. 5B). However, as the MCF-7/RT subline represents a small subclone of MCF-7 cells, association between TG2 expression and CD44\textsuperscript{high}/CD24\textsuperscript{low} phenotype needs to be further validated. Nevertheless, TG2 expression in yet another mammary epithelial cell line (MCF12A), showed a similar enrichment in CD44\textsuperscript{high}/CD24\textsuperscript{low} expressing cells compared...
to the control vector-infected cells (Supplemental Fig. S4). Taken together, these results suggest a novel TG2-regulated pathway that could confer EMT and stem cell-like phenotype in mammary epithelial cells.

**Discussion**

In this study, we show that aberrant expression of TG2 plays a critical role in promoting the EMT and EMT-dependent processes
in epithelial cells. Thus, stable expression of TG2 in mammary epithelial cells was associated with increased invasion, loss of cell polarity, increased cell survival, anchorage-independent growth and resistance to chemotherapy. Our study provides first evidence on the functional significance of previously observed increased TG2 expression in drug resistant and metastatic tumors [2,3,11,19,26,32].

Many recent reports have demonstrated elevated expression of TG2 in multiple metastatic tumors and those resistant to chemotherapy [2,3,11,33–36] and its expression has been implicated in disease progression [33,35]. Thus, the observation that silencing of TG2 gene expression due to hypermethylation of the CpG island that overlaps transcriptional and translational start site of TG2 may explain relative sensitivity of primary tumors to chemotherapeutic drugs [36]. These findings suggest an interconnecting link between TG2 expression and progression of metastatic and drug-resistant cancer.

TG2 is structurally and functionally a complex protein. Depending on the cellular context, it can promote or suppress tumor growth [33]. Anti-tumorigenic function of TG2 are mainly linked to its ability to irreversibly crosslink proteins, which requires the presence of high calcium (>1 mM) and low GTP (≤9 μM). Therefore, under the physiological conditions intracellular TG2 predominantly exists as a catalytically inactive protein due to low calcium and inhibitory effect of GTP [37]. Recent understanding of the molecular pathways that govern the progression of primary tumors to metastatic disease, points to the EMT as a central stage. The EMT is considered to be an essential process for development of different tissues during embryogenesis [17]. Its reactivation in adults can be considered as a physiological event to control inflammatory response and heal damaged tissues. However, in pathological context, such as in tumors or during organ fibrosis, this healing response may act in a harmful manner and result in metastasis and organ failure. The evidence is accumulating that prolonged induction of EMT plays an important role in cancer progression by converting immobile epithelial cells into fibroblast-like cells with reduced intercellular adhesion, increased motility and invasive behavior of cells [15–18,20,21]. Our current findings that stable expression of TG2 promotes drug resistance and invasiveness in normal and transformed mammary epithelial cells by inducing EMT suggest that TG2 serves as a converging point in conferring drug resistance and metastatic phenotype in cancer cells. A similar correlation between TG2-induced EMT and metastasis was recently observed in ovarian cancer cells. [38]

Metastasis of primary tumors to distant sites involves many steps including, successful invasion, intravasation, survival in circulation, extravasation and colonization by the cancer cells. Many cancer cell types rely on the EMT program for successful execution of these steps [17]. In order for cancer cells to break away from neighboring cells and invade adjacent cell layers, these tumor cells must lose cell-cell adhesion and acquire motility. EMT causes loss of epithelial markers like E-cadherin, desmocollin, desmplakin, and occluding (involved in the formation tight connection between them) which loosens the cell-cell adhesion and helps in the dissemination of cells. It can modulate other adhesion systems and trigger the remodeling of the actin cytoskeleton, leading to the mesenchymal phenotype and increased scattering and motility of carcinoma cells [39]. Acquisition of the mesenchymal phenotype has also been associated with invasive behavior [40]. Indeed we observed that TG2 induced EMT in mammary epithelial cells increases the invasive potential and disrupts the organized growth in 3D culture. Induction of EMT in both normal and pathological conditions is choreographed by a set of EMT inducing transcription repressors, such as Snail, Slug, Twist, Zeb1, and Zeb2. Apart from inducing the EMT program these transcription factors also confer resistance to cell death, including resistance to chemotherapeutic drugs [17]. We found upregulation of some of these transcription factors (Snail1, Twist1, Zeb1, Zeb2) in TG2 expressing cells that was associated with increased resistance to doxorubicin. These observations suggest that TG2-induced EMT promotes drug resistance, an important trait of metastatic cancer cells.

Once detached form the original niche transformed epithelial cells need to acquire autonomy from growth regulatory mechanisms, which could give them the survival advantage. Normal epithelial cells die due to anoikis when detached from the neighboring cells. EMT promotes anchorage-independent growth in transformed mammary epithelial cells [30], a property that generally distinguishes transformed (tumorigenic) from normal (non-tumorigenic) cells [22]. In this study we found that TG2-induced EMT promotes the anchorage-independent growth and protects mammary epithelial cells from death. TG2 is known to rescue cells from anoikis [41], which may explain the ability of MCF-10A-TG2 cells to grow in anchorage-independent manner. After successful invasion, intravasation, survival in circulation, and extravasation, transformed epithelial cells must survive and colonize in the hostile environments of the foreign tissue. This process involves the growth of micrometastases into macroscopic metastases. Recently, it has been proposed that EMT can enable cancer cells not only to disseminate but also to acquire the ability of self-renewal by inducing a stem cell state [30]. In line with these observations, our initial results suggested that TG2-induced EMT in mammary epithelial cells could confer a stem cell-like phenotype (CD44high/CD24low). Currently we are determining the stem cell characteristics of TG2-transfected MCF10A and MCF12A cells by establishing their ability to self-renewal and to differentiate into multiple lineages.

While studies presented here clearly demonstrate the importance of TG2 in promoting the EMT in mammary epithelial cells, we are still working on the molecular intricacies through which TG2 modulates these functions. Previous reports as well as the current study demonstrate that aberrant expression of TG2 in epithelial cells results in constitutive activation of FAK, Akt, and NF-κB [10,13,26]. These pathways are known to be intimately involved in regulation of EMT, conferring drug resistance, and promoting metastasis [18,21,42]. For example, activated NF-κB is considered to be a hallmark of many advanced-stage tumors [43,44]. Thus, constitutively active NF-κB is implicated to play a role in resistance to death-inducing stimuli, including chemotherapeutic agents [45] and to promote metastasis by inducing EMT [42]. The NF-κB-induced EMT has been attributed to the increased stability of Snail due to increased synthesis of ICOP9 signalosome 2, which blocks the ubiquitination and subsequent degradation of Snail [46]. In another report, constitutive activation of NF-κB in MCF10A cells was found to induce the EMT as a result of increased expression of Zeb1 and Zeb2 [47]. Based on these observations it is reasonable to believe that TG2-induced EMT may result from constitutive activation of NF-κB and subsequent increase in Snail, Zeb1, and Zeb2 expression, as observed in this study. Indeed, TG2 was recently shown to associate with NF-κB for its recruitment to the promoter sequence of Snail and leading to its transcriptional regulation [27]. Similarly TGF-β, which is considered to be a potent inducer of EMT both in normal and pathological conditions [17,24], can cross-talk with TG2. Thus, TGF-β can induce TG2 expression [48,49] and TG2 can activate TGF-β [50]. Although, we did not observe any activation of TGF-β signaling in TG2-transfected MCF10A cells, nevertheless, TGF-β failed to induce EMT in absence of TG2, suggesting that TG2 is an important downstream mediator of
TG2-induced EMT. Although additional studies are needed to further validate tumorigenic potential of MCF10A-TG2 cells, our in vitro data clearly support that stable expression of TG2 is sufficient to induce metastatic phenotype. Based on these results, we hypothesize a model that aberrant expression of TG2 contributes to the transformation of primary breast cancer to metastatic capabilities (Fig. 6). These results also support our belief that TG2 is a promising therapeutic target for drug-resistant and metastatic breast cancer.

Methods

Cell lines and vectors

The immortalized human mammary epithelial cells (MCF10A and MCF12A) and breast cancer cell line MCF7 and MCF7-RT were maintained as previously described [23,19]. TG2 gene was subcloned into pCDH lentiviral vector (System Biosciences, Frederick, MD) from pcDNA3.1-TG2 vector (kindly provided by Dr. Gail Johnson, University of Rochester, NY). Control ShRNA and TG2 shRNA lentiviral particles were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 10A-Vec and 10A-TG2 cells were maintained as previously described [23,19]. Soft agar assays were performed as described previously [3,13,19]. Cultures were photographed, and the colonies with diameters larger than 500 mm were counted using Image J software (previously NIH image).

RNA Extraction, RT-PCR, and Quantitative RT-PCR

The detailed procedures for RNA extraction RT-PCR and primers sequences are described in the Supplemental Procedures S1. Quantitative RT-PCR for EMT-associated genes was done using SABiosciences (Frederick, MD) EMT-PCR Array according to the manufacturer’s protocol.

Invasion, Soft Agar Colony Formation, Cell viability assay and NF-κB activity

Invasion assay, cell viability assay to check the drug resistance and NF-κB activity assay were performed as described previously [3,13,19]. Soft agar assays were performed as described previously [22]. Cultures were photographed, and the colonies with diameters larger than 500 mm were counted using Image J software (previously NIH image).

FACS Analysis

The anti-CD44 (clone G44-26), anti-CD24 (clone ML5), and anti-CD45 (clone 2D1) antibodies used for FACS analysis were obtained from BD Biosciences (San Jose, CA). The anti-CD326 (clone HEA-125) was obtained from Miltenyi Biotec (Auburn, CA). Briefly, for each cell line, 5 × 10⁵ cells were aliquoted into 2 tubes; tube 1 was stained with IgG isotype controls for FITC, PE, PerCP, and APC; tube 2 was stained with anti-CD44-FITC, anti-CD24-PE, anti-CD45-PerCP, and anti-CD326-APC. Cells were incubated with appropriate antibodies at room temperature in the dark for 30 minutes, and then washed with PBS. Cells were acquired by 4-color FACS Calibur (BD Biosciences), each sample was acquired 10,000 cells for analysis.

Supporting Information

Procedures S1

Found at: doi:10.1371/journal.pone.0013390.s001 (0.03 MB DOC)

Table S1

Antibodies used.

Found at: doi:10.1371/journal.pone.0013390.s002 (0.05 MB DOC)

Table S2

Primers for RT-PCR.

Found at: doi:10.1371/journal.pone.0013390.s003 (0.04 MB DOC)
Figure S1  Immunofluorescence showing the accumulation of stress fibers in MCF10A-TG2 cells. Found at: doi:10.1371/journal.pone.0013390.s004 (7.7 MB TIF)

Figure S2  Phase-contrast images of acinar structures (4 and 12 days) formed as a result of MCF12A-vec and MCF12A-TG2 cell culture in Matrigel-coated glass-slide chambers for indicated time periods. Found at: doi:10.1371/journal.pone.0013390.s005 (7.7 MB TIF)

Figure S3  Immunoblot showing the expression of TGF-β receptor I & II, pSmad-2, and -3 and total smad-2 and 3 in 10A-vec and 10A-TG2 cells. Found at: doi:10.1371/journal.pone.0013390.s006 (7.7 MB TIF)

Figure S4  Flow cytometric analysis of TG2-transfected (TG2) and vector-alone (-vec) transfected mammary epithelial cells (MCF12A) for CD44+/CD24-/low stem cell marker expression. Found at: doi:10.1371/journal.pone.0013390.s007 (7.7 MB TIF)

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Author Contributions

Conceived and designed the experiments: AK KM. Performed the experiments: AK JX SB HG. Analyzed the data: AK KM. Contributed reagents/materials/analysis tools: JX YD JR. Wrote the paper: AK KM.

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