Breast Cancer Cells Acquire a Stem-Like Phenotype by TGFβ1/EGF Induced Epithelial-Mesenchymal Transition

A thesis presented to
the faculty of
the Russ College of Engineering and Technology of Ohio University

In partial fulfillment
of the requirements for the degree
Master of Science

Chengkai Xiong
May 2013
© 2013 Chengkai Xiong. All Rights Reserved.
This thesis titled
Breast Cancer Cells Acquire a Stem-Like Phenotype by TGFβ1/EGF Induced Epithelial-Mesenchymal Transition

by

CHENGKAI XIONG

has been approved for
the Department of Chemical and Biomolecular Engineering
and the Russ College of Engineering and Technology by

Fabian Benencia
Assistant Professor of Immunology

Dennis Irwin
Dean, Russ College of Engineering and Technology
ABSTRACT

XIONG, CHENGKAI, M.S., May, 2013, Biomedical Engineering

Breast Cancer Cells Acquire a Stem-Like Phenotype by TGFβ1/EGF Induced Epithelial-Mesenchymal Transition

Director of Thesis: Fabian Benencia

Epithelial-Mesenchymal Transition (EMT) is a key developmental program that is often activated during cancer invasion and metastasis. In breast cancer, EMT is associated with cancer stem cell properties including expression of the stem cell antigenic profile, over-expression of transcription factors involved in EMT, adhesion to bone marrow endothelium and increased migratory capabilities. Research suggests that transforming growth factor (TGF)-β and Epidermal Growth Factor (EGF) induces EMT and allows cancer cells to become more stem-like. We have shown that TGF-β1 alone can partly increase expression of EMT-related transcription factors in BT-20 and MCF-7 cells. Furthermore, EGF alone can induce a stem cell-like phenotype in BT-20 cells and promote adhesion on human umbilical vein endothelial cells (HUVECs) but cannot promote expression of EMT-related transcription factors in BT-20 cells. A combination of EGF and TGF-β1 can promote expression of EMT-related transcription factors in BT-20 cells. These results indicate that a combination of EGF and TGF-β1 can induce EMT and cause acquisition of a stem-like phenotype by human breast cancer cell lines.
ACKNOWLEDGMENTS

First and foremost, thank you to my advisor Fabian Benencia, Ph.D. for his excellent guidance he has given me throughout my master’s study. Without his vast knowledge and willingness to help, this project would not have been possible.

To my committee members, Monica Burdick, Douglas Goetz, and Xiaozhuo Chen, thank you for your guidance in assembling this thesis.

Thanks to Michelle Pate for flow cytometry training and my lab mates, Venktesh Shirure, Grady Carlson and Tiantian Liu for their assistance during my experiments. And thanks to Thomas Riggs for his help during these two years.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>3</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>4</td>
</tr>
<tr>
<td>List of Tables</td>
<td>6</td>
</tr>
<tr>
<td>List of Figures</td>
<td>7</td>
</tr>
<tr>
<td>1. Introduction</td>
<td>9</td>
</tr>
<tr>
<td>2. Hypothesis and Special Aims</td>
<td>19</td>
</tr>
<tr>
<td>3. Materials and Methods</td>
<td>21</td>
</tr>
<tr>
<td>4. Results</td>
<td>28</td>
</tr>
<tr>
<td>5. Conclusion and Future Work</td>
<td>47</td>
</tr>
<tr>
<td>References</td>
<td>49</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.</td>
<td>PCR Primer sequence</td>
<td>23</td>
</tr>
<tr>
<td>Table 2.</td>
<td>Real time PCR Primer sequence</td>
<td>25</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td>Major features of epithelial and mesenchymal cells</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>Expression of EMT transcriptional factors</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>Cooperation between the TGF(\beta) and Ras pathways</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>Schematic principle of flow chamber assay</td>
<td>27</td>
</tr>
<tr>
<td>5</td>
<td>Image of morphological changes of BT20 cells treated with and/or EGF</td>
<td>28</td>
</tr>
<tr>
<td>6</td>
<td>CD44/CD24 expression on BT-20 and TGF-(\beta)1 treatment</td>
<td>29</td>
</tr>
<tr>
<td>7</td>
<td>CD44/CD24 expression on BT-20 and EGF treatment</td>
<td>31</td>
</tr>
<tr>
<td>8</td>
<td>CD44/CD24 expression on BT-20 and lower EGF treatment</td>
<td>32</td>
</tr>
<tr>
<td>9</td>
<td>CD44/CD24 expression on BT-20 and TGF-(\beta)1+ EGF treatment</td>
<td>33</td>
</tr>
<tr>
<td>10</td>
<td>CD44/CD24 expression on MCF-7 and TGF-(\beta)1 treatment</td>
<td>34</td>
</tr>
<tr>
<td>11</td>
<td>PCR of the expression of TGF-(\beta) and its receptors</td>
<td>34</td>
</tr>
<tr>
<td>12</td>
<td>CD44/CD24 expression on BT-20 and TGF-(\beta) receptors antagonists treatment</td>
<td>35</td>
</tr>
<tr>
<td>13</td>
<td>CD44/CD24 expression on MCF-7 and TGF-(\beta) receptors antagonists treatment</td>
<td>36</td>
</tr>
<tr>
<td>14</td>
<td>EGFR surface expression on BT-20 cells</td>
<td>37</td>
</tr>
<tr>
<td>15</td>
<td>EMT related molecules in BT-20 cells and TGF(\beta)-1 treatment</td>
<td>38</td>
</tr>
<tr>
<td>16</td>
<td>EMT related molecules in BT-20 cell and TGF-(\beta)1+ EGF treatment</td>
<td>40</td>
</tr>
<tr>
<td>17</td>
<td>EMT related molecules in MCF-7 cells and TGF(\beta)-1 treatment</td>
<td>41</td>
</tr>
<tr>
<td>18</td>
<td>Migration assay of BT-20 cells and TGF-(\beta)1 treatment</td>
<td>42</td>
</tr>
</tbody>
</table>
Figure 19. Migration assay of BT-20 cells and EGF treatment ........................................43
Figure 20. Migration assay of BT-20 cells and TGF-β receptors antagonists’ treatment.44
Figure 21. Migration assay of MCF cells and TGF-β receptors antagonists’ treatment...45
Figure 22. Flow chamber adhesion assay.................................................................46
1. INTRODUCTION

1.1 Breast cancer

Breast cancer is the second most frequently diagnosed cancer in women. It also ranks second among the leading causes of cancer-related deaths in women after lung cancer [1, 2]. The American Cancer Society reports that an estimated 229,060 new cases (226,870 women, 2,190 men) of invasive breast cancer are expected to occur in the US during 2012; an estimated 39,920 breast cancer deaths (39,510 women, 410 men) are expected in 2012 [1].

Most breast cancers begin in the cells that line the ducts (ductal cancer), some begin in the lobules (lobular cancer), and a small number start in other tissues. The cancers originate in the lobules or ducts of the breasts, and then break through the duct or glandular walls to invade the surrounding tissues, lymph nodes and distant organs such as the lungs, liver and bones [2]. There are three stages of breast cancer: non-invasive (in situ) breast cancer, invasive breast cancer and metastatic breast cancer. Non-invasive (in situ) breast cancer means tumors remain within their place of origin; invasive breast cancer means tumors spread outside the membrane and invade the surrounding tissue. Metastatic breast cancer is a stage of breast cancer where the tumors spread into distant organs. The five-year relative survival is highly dependent on the diagnostic stage: 5-year relative survival is 99% for a localized disease, 84% for a regional disease, and 23% for a distant-stage disease [3]. Therefore, breast cancer is a serious human disease for women in US.
The process of tumor metastasis has several steps. First, cancer cells from the primary tumor acquire invasive properties and escape from the original site. Secondly, cancer cells invade the tumor stroma and enter the blood circulation or the lymphatic vascular systems via neo-angiogenesis and basement membrane reconstruction [4]. If circulating tumor cells (CTCs) survive in the blood and lymphatic vascular systems, they may extravasate into the surrounding tissue and eventually reach distant secondary sites such as bone, lungs, brain and liver [5]. Although much research has been conducted on metastatic breast cancer, the specific mechanisms promoting it remain ambiguous.

1.2 Cancer Stem Cells in Breast Cancer

Recent studies have provided evidence of self-renewing, stem-like cells within tumors, which have been called cancer stem cells (CSCs) [6]. CSCs are populations of tumors cells that can undergo self-renewal and differentiation. For this reason, they have also been termed “tumor-initiating cells” [7]. Breast cancer stem cells are considered to be the foundation of breast cancer recurrence and have resistance to both radiation therapy and chemotherapy.

In 2003, an influential study classified a unique identification of human CSC changing the landscape of breast cancer research [8]. They isolated cells from pleural effusions and primary tumors of breast cancer patients. CD44+/CD24−/low/ESA+/lin− cells that were then injected into the mammary fat pad of immune deficiency (NOD/SCID) mice. The fact that as few as 100 CD44+/CD24− cells formed tumors in mice indicated that CD44+/CD24−/low− cells could be recognized as cancer stem cells. On the contrary, as many as 20,000 cells of alternate phenotypes were unable to form tumors.
It is now considered that solid tumors contain a small population of self-renewing tumorigenic cells that lead to and help maintain the tumor mass [9].

CD44 is a multifunctional class I transmembrane glycoprotein that plays a role in both cell–cell and cell–extracellular matrix (ECM) interactions. CD44 can bind to its ligands such as L-selectin, and E-selectin. In many cancers, CD44 plays a major role in initiation and metastasis [6]. CD44 aggregates cells and facilitate cell migration and tumor invasion by inducing cellular growth signals [10]. On the contrary, Lopez et al. [11] proposed that CD44 keeps the tumor within the primary site and suppress tumor metastasis. Similar to CD44, CD24 is a glycosylated cellular adhesion molecule. It is involved in cell adhesion and metastasis [12]. P-selectin, a specific glycoform of CD24, is an adhesion molecule expressed on endothelial cells and platelets. The binding of CD24 and P-selectin may assist circulating tumor cells (CTCs) to pass through blood stream [13]. Schabath et al. [14] suggested that low level of CD24 expression increase the metastatic potential of breast carcinoma cells.

1.3 Epithelial–mesenchymal transition (EMT) occur during cancer metastasis

Epithelial–mesenchymal transition (EMT), a morphogenetic process that allows cells to lose their epithelial phenotype while gaining mesenchymal properties, results in CD44+/CD24− phenotype in breast cancer cells [15]. Mani, et al. investigated that HMLE cells acquired mesenchymal morphology and express CD44+/CD24− phenotype after treatment with recombinant TGF-β1, which indicated that stem cell-like cells were generated by cytokine-induced EMT. It is found that epithelial tumor cells are
predisposed to undergo EMT before entering the peripheral circulation. After EMT, mesenchymal cells leave the primary tumor and migrate separately [15, 16].

1.4 Main features of epithelial and mesenchymal cells

Epithelial tissues constitute the inner surface of the cavities of the human body, and they are also the main constituent of glands. The epithelial tissues function includes protection, secretion, absorption, filtration, and diffusion in the human body. The epithelial cells are adhesive because they are built up by polarized cells connected by strong cell-to-cell and cell-to-substratum adhesions. This adhesive structure keeps the epithelial cells moving from into the stromal compartment and migrating away independently [17].

Mesenchymal tissues, which are composed of mesenchymal cells, are loosely organized. Mesenchymal cells are weakly connected to neighboring cells and have spindle-like morphology. Such properties translate into high cell motility, thereby allowing mesenchymal cells to travel through the basement membrane and the surrounding tissue [18].

There are three major changes that occur during the EMT process. First, trim cobblestone-shaped epithelial cells turn into unconnected spindle-shaped mesenchymal cells. Second, specific cell markers switch from epithelial to mesenchymal subtypes. Finally, stationary cells transform into motile cells, which can invade through the extracellular matrix [19]. Figure 1, summarizes the major features of epithelial and mesenchymal cells.
Figure 1. Major features of epithelial and mesenchymal cells. Epithelial cells are cobblestone-shaped, show epithelial subtypes and have limited mobility. In contrast, mesenchymal cells are separately spindle-shaped, loosely organized and have high migration capacity. During EMT, cancer cells acquire invasive and self-renewal capacity. (Figure adapted from Y. Lie et al, 2010. [20])

1.5 Major criteria to detect EMT

1.5.1 Cadherin switching during Epithelium-Mesenchyme Transition

E-cadherin, a cell-to-cell adhesion protein, is considered to be a hallmark of the epithelial state. The decreased expression of E-cadherin can lead to the dismantling of adherens junctions [21]. E-cadherin expression is decreased at the early stage of tumor development in some types of human cancer, thus leading epithelial cells to exhibit mesenchymal phenotype [21, 22].

N-Cadherin is considered to be a mesenchymal marker because it is expressed on most mesenchymal cells. N-Cadherin can also be detected on some cancer cells, which
promotes cancer cell invasion and metastasis [23]. During EMT, epithelial marker E-cadherin is down-regulated while mesenchymal maker N-cadherin is up-regulated. Maeda et al. [23] observed that the expression of E-Cadherin decreased while N-cadherin increased in the TGF-β1-induced EMT model in mammary epithelial cells.

1.5.2 Transcriptional regulation of EMT

Many EMT-inducing transcription factors, such as TWIST, SNAIL and SLUG, are associated with cancer invasion and metastasis. Yang et al. [24] found that high levels of TWIST expression are essential for breast cancer cells to metastasize. SNAIL, a zinc finger factor, has been recognized as another transcription factor capable of inducing EMTs in epithelial cells. SNAIL can bind to three E-boxes present in the human E-cadherin promoter repressing the transcription of this protein [24, 25]. SLUG is a close relative to Snail, both sharing a high degree of homology at the N-terminal region. Blanco et al. [26] suggested that the expression of E-cadherin was fully repressed by a stable expression of SLUG at transcriptional levels, which induced EMT completely in MDCK cells. Secreted protein acidic and rich in cysteine, also named SPARC, is a secreted protein that belongs to the matricellular family. It is a non-structural protein that can inhibit cell cycle and modulate the activities of cytokine and growth factors [27]. Robert et al. [28] reported that the expression of SPARC can stimulate cell motility and invasive ability. During melanoma development, over expression of SPARC up-regulated Snail while down-regulated E-Cadherin. Other transitional factors include ZEB1, ZEB2, E47 and FOXC2. ZEB1 and ZEB2 are two zinc ginger transcription that repress the expression of E-Cadherin by binding to the promoter regions of E-Cadherin during EMT.
Lee et al. [30] claimed that EMT is promoted by E47 during mammary epithelial branching morphogenesis. The expression levels of the mRNAs encoding Forkhead box C2 (FOXC2) is also upregulated during EMT in human breast cancer [15].

![Figure 2](image)

**Figure 2.** Expression of EMT transcriptional factors in breast stem-like cancer cells. The expression levels of the mRNAs encoding E-cadherin, N-cadherin, vimentin, fibronectin, FOXC2, Slug, SIP1, Twist, and Snail in CD44+/CD24⁻ cells relative to CD44⁻/CD24⁺ as determined by real-time RT-PCR. (Figure adapted from Mani et al., 2007 [15])

1.6 Growth factor receptors and signaling pathways

Since Epithelium-Mesenchyme Transition plays an important role in the progression of cancer, the induction and regulation of EMT process is crucial for scientists to understand the biological mechanism of metastatic cancer. The major inducers of EMT are the binding of growth factors and their receptors.

Transforming growth factor (TGF)-β is a multifunctional cytokine that restrain tumor growth and facilitate tumor metastasis [31]. The ability of TGF-β to inhibit epithelial proliferation as well as enhance tumor metastasis by inducing EMT has been
extensively reported [32]. TGF-β binds to TGF-β type-I/TGF-β type-II serine/threonine kinase receptor complexes and activates Smad2/3, which then interacts with Smad4 and accumulates in the nucleus, where they associate with EMT-inducing transcription factors and regulate gene expression thus promoting the expression of mesenchymal proteins [33]. TGF-β also induces EMT by activating the RTK/Ras/MAPK signaling pathway (short for Receptor Tyrosine Kinase (RTK)/Ras GTPase/MAP kinase (MAPK) signaling pathway) [34].

RTKs are cell-surface transmembrane proteins that transduce extracellular signals. Epidermal growth factor (EGF) and its receptor EGFR induce EMT by two major signal pathways: MAPK signaling pathway and PI3K/AKT signaling pathway. Upon binding of EGF, RTKs are activated and becomes binding sites for GRB, PI3K. Ras is activated which then induces the Ras-Raf-MEK-MAPK signaling cascade. The MAPK signaling pathway can regulate gene expression of transcriptional factors such as Slug [35] and Snail [36]. PI3K/Akt signaling pathway inactivates GSK3b to prevent degradation of Snail and induce EMT [37]. EGF also actives the Rho signaling family and increases migration of cells undergoing the EMT process during metastasis [38].

Researchers found that co-stimulation of TGFβ1 and EGF promote EMT in various cancers. For instance, M Grände, et al. [39] reported that the combination of TGF-β1 and EGF up-regulated N-Cadherin expression and down-regulated E-Cadherin expression in primary cultured pig thyrocytes. They also reported that TGFβ1 and EGF collaborate in degrading tight junctions between epithelial cells and inducing the acquisition the change of a spindle-shaped morphology. Furthermore, it has been shown
that during this process, downstream mediators of RTKs cooperate with TGFβ signaling to affect EMT [40]. Figure 3 highlights the cooperation between TGF-β and Ras pathways.

Figure 3. Cooperation between the TGFβ, Ras pathways. The main pathways involved in TGF-β1 and EGF stimulation include TGFβ pathway, Ras-Raf-MEK-MAPK signaling cascade and Ras-PI3K/Akt signaling cascade.

1.7 Adhesion and EMT

The extravasation of CTCs from blood to tissue leads to cancer metastasis. Such extravasation is closely associated with three members of the selectin family of carbohydrate-binding proteins: E-selectin, L-selectin and P-selectin. The selectin family
medicates cell adhesion and rolling to endothelium by recognizing E-selectin ligands such as Sialyl Lewis X (sLex) and sialyl Lewis a (sLea) expressed on CTCs.

In particular, it has been reported that the adhesion of breast cancer cells to endothelium was mediated by E-selectin and sLeXa [41]. Furthermore, previous studies also demonstrated that human breast cancer cell lines interacted with human umbilical vein endothelial cells (HUVEC), allowing a rolling and adhesion behavior under physiological flow [42]. Therefore E-selectin may play an important role in breast cancer metastasis. Recently, S. Keiichiro et al. [43] indicated that sLeX/a were highly expressed on colon cancer cells undergoing EMT under EGF and or FGFb stimulation. However, K.A. Henson [44] reported that EMT may not be necessary for E-selectin mediated breast cancer homing to the metastatic site. Shirure et al. [45, 46] also showed that stem-like phenotype CD44+/CD24- cells expressed less E-selectin ligands than non-stem cells in breast cancer cell lines. Therefore, it is a matter of current debate whether the induction of EMT affects the expression of E-selectin ligands or not.

A summary of previous studies indicates that stem-like phenotype CD44+/CD24- are highly associated with EMT during breast cancer metastasis. Furthermore, TGFβ-1 and EGF could induce EMT in various cancers. To figure out if TGFβ-1 and/or EGF can induce EMT and allow cancer cells to acquire stem cell features is important for clinical studies. Strategies to block TGFβ-1 and/or EGF can be used in cancer therapeutics.
2. HYPOTHESIS AND SPECIFIC AIMS

2.1 Hypothesis

Breast cancer cells acquire a stem-like phenotype by TGFβ1/EGF induced EMT. After EMT, the migratory and adhesive capability increase in breast cancer cells.

2.2 Objective

This study thus aimed to investigate the capability of TGF-β and/or EGF to induce a stem-like phenotype on human breast cancer cell lines.

2.3 Specific aims

Aim 1: Determine the capability of TGF-β and/or EGF to induce a CD44+/CD24- breast cancer stem cell phenotype in vitro.

Herewith, flow cytometry analysis was used to determine the expression of stem cell surface markers CD44 and CD24 on human breast cancer cells treated with TGF-β and/or EGF and untreated controls.

Aim 2: Identify the gene expression levels of EMT-related molecules in human breast cancer cell lines upon treatment with TGF-β and/or EGF.

RNA was extracted from both normal cells and transformed cells and then reverse transcribed into cDNA. Expression of different molecules associated with EMT was then evaluated by Real-time quantitative PCR.

Aim 3: Define the influence of TGF-β and/or EGF on the invasive and migratory capability of human breast cancer cell lines.
Migration assays were performed to evaluate the migratory capability of human breast cancer cells treated with TGF-β and/or EGF and cells treated with TGF-β receptors antagonists, or untreated cells.

Aim 4: Study the adhesion of human breast cancer cell to activated endothelium under flow.

A flow chamber assay was performed to determine the effects EGF treatment on human breast cancer cells binding to HUVECs.
3. MATERIALS AND METHODS

3.1 Cell culture

BT-20 and MCF-7 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) to use in our experiments. BT-20 cells were maintained in Minimum Essential Medium (MEM, Hyclone, Logan, UT) supplemented with 10% heat inactivated fetal bovine serum (hiFBS) and 1x penicillin–streptomycin (Invitrogen, Carlsbad, CA) [46]. MCF-7 cells were cultured in Dulbecco’s Minimum Essential Medium (DMEM, Hyclone) with 15% heat-inactivated fetal bovine serum (hiFBS, Hyclone) and 1X antibiotic–antimycotic (Gibco, Invitrogen, Carlsbad, CA) [44]. BT-20 cells were harvested for experiments using 0.25% Trypsin, 0.1% EDTA in HBSS with Calcium and Magnesium (Hyclone) as previously described [47]. MCF-7 cells were harvested for experiments using 0.05% trypsin-0.53 mM EDTA in HBSS without calcium or magnesium (Cellgro, Herndon, VA) [44]. All cells were cultured at 37°C and 5% CO₂.

3.2 Epithelium-Mesenchyme Transition induction

The cell culture substrates were constructed in duplicate on six well plates. Each well was seeded with 1 ml of cell suspension containing 1x10⁵ cells/ml. Two ml medium (MEM for BT-20 cells, DMEM for MCF-7 cells) was used to supplement each well during incubation. One day later, cells were treated with growth factors (TGF-β1, R&D Systems; Recombinant human EGF, PeproTech, Rocky Hill, NJ) in normal growth medium (2ml medium/well as previously described) [48]. The concentration of cytokines varied in different experiments. The medium containing the growth factors was replaced
every two days and cells were harvested on day 7 and day 12. Furthermore, Cells were also treated with TGF-β1 receptors inhibitors. SB431542 (sigma Saint Louis, Missouri) is a selective TGFBR1 and TGFBR2 receptor inhibitor. LY364947 (sigma Saint Louis, Missouri) is a relatively selective inhibitor for the TGFBR1 receptor CD44/24 expression was measured via flow cytometry while cell morphology changes were visualized using phase contrast microscopy on cell cultures before detachment. In some experiments RNA was extracted from the recovered cells to test the mRNA expression level of EMT related transcription factors.

3.3 Morphology changes

Morphology changes signifying EMT in cells treated with TGF-β1 were recorded via microscopy using a Motic AE31 microscope and Motic Imagines plus 2.0 ML software [49].

3.4 Flow cytometry

Antibodies included FITC-Mouse anti-human CD44 and PE-mouse anti-human CD24 (BD Biosciences, San Jose, CA), and the isotype controls were mouse IgG2a for CD24 and mouse IgG2b for CD44 (BD Biosciences, San Jose, CA). Cells were washed twice with blocking buffer (DPBS+, 1% BSA) and incubated with CD44/24 antibodies (10 μg/ml CD44 FITC and CD24 PE antibodies) or isotype antibodies (10 μg/ml mouse IgG2a for CD24 and mouse IgG2b for CD44) for 30 minutes at room temperature in the dark as described previously [47]. After incubation cells were washed twice in DPBS+, 1% BSA and fixed in 100ul Cytofix fixation buffer (BD Bioscience) and kept at 4°C. Finally, cells were analyzed using a FACSaria Special Order Research Product flow
cytometer/sorter (BD Biosciences) [49]. Cells with or without TGF-β1 treatment were also stained with APC anti-human EGFR (BioLegend, San Diego, CA) and analyzed by flow cytometry to determine the expression of EGF receptor on BT-20 cells.

3.5 RNA Extraction and cDNA Preparation

RNA was isolated using the RNeasy® Mini Kit. (Qiagen, Valencia, CA). Then RNA was reverse transcribed by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) as previously described [50].

3.6 Qualitative PCR reaction

For qualitative PCR analysis, the PCR cycling was conducted with Taq polymerase at 94°C (30 s), 57°C (30 s), and 72°C (20 s) for 40 cycles [50].

<table>
<thead>
<tr>
<th>Table 1. PCR Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFB1 Forward 5’- CTC TTC GCG CTC TCG GCA GT-3’</td>
</tr>
<tr>
<td>Reverse 5’- CTC TTC GCG CTC TCG GCA GT -3’</td>
</tr>
<tr>
<td>TGFB1R Forward 5’- TCT GCC ACA ACC GCA CTG TCA -3’</td>
</tr>
<tr>
<td>Reverse 5’- AAG ATG GGC AAG ACG GCT CGC-3’</td>
</tr>
<tr>
<td>TGFB2R Forward 5’- ACC GCA CGT TCA GAA GTC GGA TG-3’</td>
</tr>
<tr>
<td>Reverse 5’- GCT TCC GCG TCT TGC CGG TT-3’</td>
</tr>
<tr>
<td>TGFB3R Forward 5’- GGC CAC TTT CCC TGC GCG AT-3’</td>
</tr>
<tr>
<td>Reverse 5’- GTG GCA GCC CAG TTG TGC CT-3’</td>
</tr>
<tr>
<td>GAPDH Forward 5’- CCC CTT CAT TGA CCT CAA CTA CA-3’</td>
</tr>
<tr>
<td>Reverse 5’- CGC TCC TGG AAG ATG GTG AT-3’</td>
</tr>
</tbody>
</table>
3.7 Gel Electrophoresis

cDNA that was amplified in the PCR reaction was analyzed by gel electrophoresis. PCR samples were mixed with DNA Gel loading buffer (5 PRIME) and seeded into the wells of an agarose gel upon applying an electric current.

DNA fragments migrated from negative charge to the positive pole, while large DNA fragments moving slower than small ones. Therefore, separation of DNA molecules was done based on their size. The gel was mixed with ethidium bromide, which produced orange color bands under UV light upon interaction with the DNA. The gel was analyzed in ChemiDoc™ XRS+ with Image Lab™ Software (Bio-Rad Laboratories, Hercules, CA).

3.8 Real-Time quantitative PCR

The expression of EMT-related molecules was analyzed at the RNA level by quantitative real time RT-PCR analysis. For RT-PCR, SYBR Green (FastMix, Quantas Biosciences) was used to detect the PCR reaction. We normalized the cDNA load to the housekeeping gene GAPDH as previously described [26].

Data were expressed as relative expression to GAPDH mRNA molecules. The mathematical assumptions for this approach are based on the Livak Method as follows:

Expression of any particular molecule = $2^{(\text{ct(GAPDH)} - \text{ct(Sample)})}$.

Each amplification experiment was performed in 96-well optical grade PCR plates covered with an optical film) in an iCycler iQ5 real-time PCR instrument (Bio-Rad).
Table 2. Real time PCR Primer sequence

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5’-</th>
<th>Reverse 5’-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snail</td>
<td>CCT TCT CTA GGC CCT GGC TGC T-3’</td>
<td>GTT GGA GCG GTC AGC GAA GG -3’</td>
</tr>
<tr>
<td>Slug</td>
<td>GCG TTT TCC AGA CCC TGG TTG C -3’</td>
<td>TGC TCT GTT GCA GTG AGG GCA AG -3</td>
</tr>
<tr>
<td>Twist</td>
<td>CGC CGC TCG AGA GAT GAT GC -3’</td>
<td>GCT GCC GGT CTG GCT TTT CC -3</td>
</tr>
<tr>
<td>hE-Cadherin</td>
<td>TGA CAC CCG GGA CGT TTA CTA CT -3’</td>
<td>CAC TTC AGG CCG AGC GTC CA -3</td>
</tr>
<tr>
<td>hN-Cadherin</td>
<td>CCT GCT CTG CAT CAT CCT GCT TTT -3’</td>
<td>TGT TTG GCC TGG CGT TCT TTA TCC -3</td>
</tr>
<tr>
<td>SPARC</td>
<td>GCA CGG ACT GTC AGT TCT CT -3’</td>
<td>AAG AAC AAC CGA TTC ACC AA -3</td>
</tr>
<tr>
<td>TGFB1</td>
<td>CGT GGA GCT GTA CCA GAA ATA -3’</td>
<td>TCC GGT GAC ATC AAA AGA TAA -3</td>
</tr>
<tr>
<td>TGFB2</td>
<td>AAC AAG AGC AGA AGG CGA AT -3’</td>
<td>TGC CAT CAA TAC CTG CAA AT -3</td>
</tr>
<tr>
<td>TGFB3</td>
<td>TGG TTT TCC TCC CTC CTG CT -3’</td>
<td>TGG TGC AAG TGG ACA GAG AG -3</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCC CTT CAT TGA CTA CAA CTA CA -3’</td>
<td>CGC TCC TGG AAG ATG GTG AT -3’</td>
</tr>
</tbody>
</table>
3.9 Migration assay

Cell migration was analyzed using an Oris™ cell migration assay kit (Platypus Technologies, LLC., Madison, WI) following the manufacturer’s instructions. Briefly, cells were trypsinized and resuspended in culture medium at 5X10⁵ cells/ml. One-hundred μl of the cell suspension was added to each well of 96-well plate containing the Oris cylindrical stoppers. After overnight incubation at 37 °C in a 5% CO₂ incubator, the stoppers were removed from the 96-well plate and each well washed with PBS to remove any unattached cells. By removing the stoppers a circular surface without cells is created. Advancement of cells towards this region is evidence of migration. Cells were incubated with complete culture medium for several days (migration time varied depending upon cell type and experimental design) and fixed with formalin. Cells were then stained with Crystal violet dye for 30 min and images were captured by Motic AE31 microscope and Motic Imaginex plus 2.0 ML software. Data was analyzed by NIH image J freeware.

3.10 Flow Chamber Adhesion Assays

BT-20 cells were treated with EGF at 10 ng/ml for 7 days. Cells were then harvested by using 0.25% trypsin EDTA in DPBS without calcium or magnesium as above. Cells were washed three times and then resuspended in 0.1% BSA in DPBS with calcium and magnesium at 5X10⁵ cells/ml. In the flow chamber assay, a flow chamber was placed inside a petri dish and a syringe pump created a shear flow (shear rate was 91.6 s⁻¹) by pulling the cell suspension over a HUVEC monolayer in order to observe the adhesive behavior of the tumor cells. HUVEC were stimulated with 1 ng/ml IL-1β for 4-6 hours prior to performing the flow assay in order to stimulate the expression of E-selectin.
The adhesion ability was monitored by an inverted microscope connected to a monitor [51].

Figure 4: Schematic principle of flow chamber assay. Figure adapted from K.A. Henson [44].

3.11 Statistics

The one-way ANOVA method was used to determine the difference of gene expression between treated group and control group. Statistical significance was defined as a p value < 0.05. Error bars represent mean ± SD. Data was analyzed by using the Graph Pad InStat software (GraphPad Software, Inc., San Diego, CA).
4. RESULTS

4.1 Morphological changes on BT20 cells upon TGF-β1/EGF treatment.

Figure 5 shows the morphology of both normal BT-20 cells and BT-20 cells with TGFβ-1, EGF, and TGFβ-1+EGF treatment for 7 days. A shift in cellular morphology from an epithelial shape to a spindle-cell like, fibroblastic shape can be observed after TGF-β1 and TGF-β1+FFEGF treatments. Little morphological changes occurred after EGF treatments.

![Image of morphological changes of BT20 cells treated with TGFβ-1, EGF, and TGFβ-1+EGF treatment for 7 days and control (no cytokine added). The experiment was repeated three times.](image)

*Figure 5:* Image of morphological changes of BT20 cells treated with TGFβ-1, EGF, and TGFβ-1+EGF treatment for 7 days and control (no cytokine added). The experiment was repeated three times.
4.2 Flow cytometry analysis of BT20 cells and TGF-β1 treatment

CD44 and CD24 surface expression on BT20 cells treated with TGF-β1 for 7 or 12 days and non-treated controls were analyzed by flow cytometry. Figure 6 shows the flow cytometry results. In figure 6A, all three groups show CD44+/CD24+ phenotype, and no significant differences are found between various concentrations of TGF-β1 treatment in 7 days. Figures 6B shows both CD44 and CD24 signals became lower than control group after 12 days treatment of 20ng/ml TGF-β1. The experiment shows that stem-like cells may not be obtained in this inductive condition.

Figure 6: CD44/CD24 surface expression on BT20 and TGF-β1 treatment.
Flow cytometry analysis of CD44 and CD24 surface expression on BT20 cells treated with TGF-β1 (5ng/ml and 20ng/ml) and controls (no cytokine added). 6A shows the cells and treatment for 7 days; 6B shows the cells and TGF-β1 treatment (5ng/ml and 20ng/ml) for 12 days. TGF concentration is expressed in ng/ml. The experiment was repeated three times.
4.3 Flow cytometry analysis of BT20 cells upon EGF treatment

CD44 and CD24 surface expression on BT20 cells treated with different concentration of EGF for 7 days and controls (no cytokine added) was analyzed by flow cytometry. Figure 7 shows the flow cytometry results. Untreated BT-20 cells with CD44^+/CD24^+ phenotype are shown in figure 7A. After EGF treatment (5, 10, 20, 30, and 40 ng/ml), BT-20 cells showed a CD44^+/CD24^- phenotype. Therefore, in terms of surface marker expression, stem-like cells can be obtained in BT-20 cells upon EGF treatment for 7 days.
Figure 7: CD44/CD24 surface expression on BT20 and EGF treatment.
Flow cytometry analysis of CD44 and CD24 surface expression on BT20 cells treated with TGF-β1 (5ng/ml and 20ng/ml) and controls (no cytokine added). 7A shows the cells without treatment for 7 days; 7B-7F shows the cells and EGF treatment (7B:5ng/ml, 7C:10 ng/ml, 7D: 20 ng/ml, 7E:30 ng/ml, 7F: 40 ng/ml) for 7 days. EGF concentration is expressed in ng/ml. The experiment was repeated three times.

In order to show this is a dynamic transformation, we treated BT-20 cells with a lower concentration of EGF for 3 days. As shown in figure 8, we were able to obtain a “middle stage” phenotype, a phenotype that between normal and stem cells. The expression of CD24 went down in BT-20 upon 2.5ng/ml or 5ng/ml EGF stimulation.
4.4 Flow cytometry analysis of BT20 cells and TGF-β1 plus EGF treatment

CD44 and CD24 surface expression on BT-20 cells treated with TGF-β1 plus EGF (10ng/ml for each) for 7 days and controls (no growth factors added) was analyzed by flow cytometry. Figure 9 shows the flow cytometry results. After treatment, transformed BT-20 cells showed CD44+/CD24- phenotype. However, when we repeated this experiment, the stem-like phenotype could not be obtained again.
Figure 9 CD44/CD24 surface expressions on BT-20 cells and TGF-β1 + EGF treatment. Flow cytometry analysis of CD44 and CD24 surface expression on BT-20 cells treated with TGF-β1 and EGF for 7 days and controls (no cytokine added). Treatment concentration is expressed in ng/ml. The experiment was repeated three times, only once did we observe this result.

4.5 Flow cytometry analysis of MCF-7 cells and TGF-β1 treatment

CD44 and CD24 surface expression on MCF-7 cells treated with TGF-β1 for 7 days and controls (no cytokine added) was analyzed by flow cytometry. Figure 10 shows the flow cytometry results. The expression of both CD44 and CD24 decrease after TGF-β1 treatment. Cells under treatment had higher autofluorescence so then the isotype controls for treated and untreated are different.
4.6 PCR analysis of TGF-β family molecules

As shown in Figure 11, we were able to detect expression of TGF-β family molecules on breast cancer cell lines BT-20 and MCF-7. Both BT-20 and MCF-7 cells can express TGF-β1 and its receptors

Figure 10. CD44/CD24 surface expressions on MCF-7 cells and TGF-β1 treatment
Flow cytometry analysis of CD44 and CD24 surface expression on MCF-7 cells treated with 10 ng/ml TGF-β1 for 7 days and controls (no cytokine added). TGF concentration is expressed in ng/ml. The experiment was repeated three times

Figure 11. PCR result of the expression of TGF-β and its receptors at the level of RNA in BT20 cells (14A) and MCF-7 cells (14B). GAPDH is the housekeeping gene used as a control for cDNA load.
4.7 Flow cytometry analysis of BT-20 cells and TGF-β receptors antagonists treatment

As shown in the PCR, these cells produce TGF-β by themselves. Then, the idea would be to see what happens when these cells are totally deprived of TGF-β (both external and self-produced). LY364947 is a relatively selective inhibitor for the TGFBR1 receptor. SB431542 is a selective TGFBR1 and TGFBR2 receptor inhibitor. To investigate the effect of autologous TGF-β produced BT-20 cells, we treated them with TGF-β receptors antagonists LY-364947 or SB-431542 (1.5ng/ml) and controls (DMSO, solvent for the inhibitors) for 3 days. Then, CD44 and CD24 surface expression on BT-20 cells was analyzed by flow cytometry. Figure 12 shows the flow cytometry results. All three groups show CD44⁺/CD24⁺ phenotype. No significant differences were found in BT-20 cells with or without TGF-β receptors antagonists treatment for 3 days.

![Figure 12. CD44/CD24 surface expression on BT-20 and TGF-β receptors antagonists treatment](image)

4.8 Flow cytometry analysis of MCF-7 cells and TGF-β receptors antagonists’ treatment

In a series of complementary studies, MCF-7 cells were treated with TGF-β receptors antagonists LY-364947 or SB-431542 (1.5ng/ml) and control solvent (DMSO)
for 3 days. Then, CD44 and CD24 surface expression on MCF-7 cells was analyzed by flow cytometry. Figure 13 shows the flow cytometry results. In figure 13, control group and LY-364947 group shows CD44+/CD24+, while SB-431542 shows CD44+/CD24+. LY-S364947 group showed CD44 signal higher than control group. Therefore, the expression of CD44 increase after TGF-β receptors inhibitor LY-364947 treatment, and expression of CD24 increase while CD44 decrease after TGF-β receptors inhibitor SB-431542 treatment.

Figure 13. CD44/CD24 surface expression on MCF-7 and TGF-β receptors antagonists treatment. Flow cytometry analysis of CD44 and CD24 surface expression on MCF-7 cells treated with TGF-β1, LY-364947 or SB-431542 (1.5ng/ml) and vehicle (DMSO) for 3 days. The experiment was repeated three times.

4.9 Flow cytometry analysis of EGFR expression on BT-20 cells and TGF-β1 treatment

EGFR is the cell-surface receptor for EGF. In order to identify the expression of this receptor in our cells and also determine if TGF-β1 could boost its expression, EGFR surface expression on BT-20 treated with TGF-β1 for 7 days and control no growth factor added) was analyzed by flow cytometry. In figure 14, the expression of EGFR was
extremely high with or without treatment of TGF-β1. No significant differences are found among these groups.

Figure 14, EGFR surface expression on BT-20 cells. Flow cytometry analysis of EGFR surface expression on BT-20 cells treated with TGF-β1 (2.5ng/ml, 5 ng/ml and 10ng/ml) and controls (no cytokine added). 13A shows the cells without treatment for 7 days; 13B,13C and 13D show the cells and TGF-β1 treatment (13B: 5ng/ml, 13C:10 ng/ml, 13D: 2.5ng/ml) for 7 days. TGF concentration is expressed in ng/ml.

4.10 RT-qPCR analysis of EMT-related molecules in BT-20 cells upon TGFβ-1 treatment

To investigate the effect of autologous TGF-β produced BT-20 cells, we treated them with TGFβ-1 for 7 or 12 days (5 ng/ml). After treatment, relative gene expression of
Snail, Slug, Twist, E-Cadherin, N-Cadherin, SPARC, TGFB1, TGFB2, TGFB3 were tested by Real-time qPCR. Figure 15 shows the gene expression of transcription factors involved in EMT after treatment with TGF-β1. We were able to see upregulation of N-Cadherin, TGFB2 and TGFB3 (treated for 7 days) in BT-20 cells with respect to control group (not TGF-β1 treated) at the level of RNA.

*ANOVA* variance analysis was conducted to determine the significance of EMT related molecules expressed in transformed BT-20 cells vs. Untreated BT-20 cells, p<0.05. The experiment was repeated three times.

**Figure 15.** Expression of EMT related molecules mRNA in BT-20 cells is shown in relation to the housekeeping gene GAPDH. In this Real-Time PCR analysis, samples were run in duplicate and represent 3 independent experiments. Errors are reported as mean ± SD.
4.11 RT-PCR analysis of EMT related molecules in BT-20 upon EGF and/or TGFβ-1 treatment

BT-20 cells were treated with EGF alone or EGF plus TGFβ-1 for 7 days (10 ng/ml). After treatment, relative gene expression of Snail, Slug, Twist, E-Cadherin, N-Cadherin were tested by Real-time qPCR. Figure 16 shows the gene expression of transcription factors involved in EMT after treatment of EGF and EGF plus TGF-β1. In figure 16, the expression of Slug, twist and N-Cadherin increase in BT-20 cells and EGF plus TGF-β1 treatment with respect to control group (not TGF-β1 treated) at the level of RNA.
Figure 16. Expression of EMT related molecules mRNA in BT-20 cells is shown in relation to the housekeeping gene GAPDH. In this Real-Time PCR analysis, samples were run in duplicate and represent 3 independent experiments. Errors are reported as mean ± SD. *ANOVA variance analysis was conducted to determine the significance of EMT related molecules expressed in transformed BT-20 cells vs. Untreated BT-20 cells, p<0.05. The experiment was repeated three times.

4.10 RT-PCR analysis of relative gene expression of EMT related molecules in MCF-7

MCF-7 cells were treated with TGF-β-1 for 7 days (5 ng/ml). After treatment, relative gene expression of Snail, Slug, Twist, E-Cadherin, N-Cadherin, SPARC, TGFβ1,
TGFB2, TGFB3 were tested by Real-time qPCR. Figure 17 shows the gene expression of transcription factors involved in EMT after treatment of TGF-β1. In figure 17, we were able to see upregulation of SLUG, TWIST, N-Cadherin and TGFB2 in MCF-7 cells with respect to control group (not TGF-β1 treated) at the level of RNA.

**Figure 17.** Expression of EMT related molecules mRNA in MCF-7 cells is shown in relation to the housekeeping gene GAPDH. In this Real-Time PCR analysis, samples were run in duplicate and represent 3 independent experiments. Errors are reported as mean ± SD. *ANOVA variance analysis was conducted to determine the significance of EMT related molecules expressed in transformed MCF-7 cells vs. untreated MCF-7 cells, p<0.05. The experiment was repeated three times.
4.11. Migration assay

Migration assay for BT20 and MCF-7 cells upon different growth factor treatment was performed. Pictures were taken every 24 hours. Migration assay was performed after stimulation for 3 days. After 7 days’ migration assay, both untreated and treated cells were fixed and stained with a blue dye and photographed.

4.11.1. BT-20 cells and TGF-β1 treatment

After TGF-β1 treatment, the cell-free areas within BT-20 cells cultures shrunk faster than control group (Figure 18). Therefore we concluded that TGF-β1 treatment increased the migration ability of BT-20.

*Figure 18.* Figure 18A1 shows images of changing area of BT20 cells without treatment; Figure 10A2 shows images of changing area of BT20 cells treated with TGF β-1 for 7 days and control (no cytokine added). 18 B shows the quantitative changing areas for 7 days and control (no growth factor added). The experiment was repeated three times.
4.11.2. BT-20 cells and EGF treatment

After EGF treatment, the areas without cells within BT-20 cells decreased slightly faster than in the control group (Figure 19). Therefore we concluded that EGF treatment slightly increased the migration ability of BT-20.

*Figure 19. 19A shows the quantitative changing areas for 7 days and control (no growth factor added). Figure 19B1 shows images of changing area of BT20 cells without treatment; Figure 19B2 shows images of changing area of BT20 cells treated with EGF for 7 days and control (no cytokine added). The experiment was repeated three times.*
4.11.3. BT-20 cells and TGF-β receptors antagonists’ treatment

After TGF-β receptors antagonists’ treatment, the cell-free areas within BT-20 cells cultures decreased at slower rate than control group (Figure 20). Therefore TGF-β1 receptors antagonists’ treatment decreased the migration ability of BT-20.

*Figure 20. Figure 20A shows images of changing area of BT20 cells with TGF-β receptors antagonists and control. 20B shows the quantitative changing areas for 7 days and control (no growth factor added). (no cytokine added). The experiment was repeated three times.*
4.11.4. MCF-7 cells and TGF-β receptors antagonists’ treatment

After TGF-β receptors antagonists’ treatment, the areas within MCF-7 cells decreased slower than control group (figure 20). Therefore TGF-β1 receptors antagonists SB-431542 treatment decrease the migration ability of MCF-7.

Figure 21. 21A shows images of changing area of MCF-7 cells with TGF-β receptors antagonists and control (no cytokine added). Figure 21 B shows the quantitative changing areas for 7 days and control (no cytokine added). The experiment was repeated three times.
4.12 Flow chamber analysis of adhesion of treated BT-20 to endothelium

BT-20 cells were treated with EGF for 7 days (10 ng/ml). After treatment, the adhesion behavior was tested by a flow chamber assay. BT-20 cells treated EGF showed more adhesion interaction with HUVEC cells. Figure 22 shows the binding interactions in untreated and EGF treated groups.

*Figure 22. Flow chamber adhesion assay. Binding interactions means the number of BT-20 cells/minute mm² that bound to HUVEC. The shear rate was 91.6 s⁻¹. The experiment was repeated one time.*
5. CONCLUSION AND FUTURE WORK

Both BT-20 and MCF-7 cells can express TGFβ-1 receptor and EGF receptor on their surface, so they might be prone to modulation by these growth factors. When BT-20 cells underwent EMT, a shift of morphology from epithelial shape to a spindle-cell like shape was observed. Furthermore, the gene expression of some EMT related molecules such as N-Cadherin, TGFB2 and TGFB3 were up-regulated after TGFβ-1 stimulation. TGF-β1 treatment also increased the migration ability of BT20 cells while TGF-β1 receptors antagonist treatment reduced this migration capability. Unexpectedly, there were no changes in CD24 expression in these conditions, which indicates that TGF-β1 can partly induce a fully stem-like phenotype on BT-20 cells.

We then switched to another breast cancer cell line MCF-7. Herewith, upregulation of SLUG, TWIST, N-Cadherin, and TGFB2 in MCF-7 cells and TGFβ-1 treatment at the level of RNA was observed. Similar to BT-20 cells, TGF-β1 treatment increased while TGF-β1 receptors antagonist treatment decreased this migration capability of MCF-7 cells. In FACS test, the expression of both CD44 and CD24 are decreased after TGF-β1 treatment. Thus, TGF-β1 seems to partially induce a stem-like phenotype on MCF-7 cells.

Studies on TGFβ-1 induced EMT on breast cancer cells demonstrated that TGF-β1 can only partly induce a fully stem-like phenotype. Therefore, we began to investigate the capability of EGF to induce a stem-like phenotype on human breast cancer cell lines. BT-20 cells showed CD44+/CD24- phenotype after treatment with EGF, which indicated EGF alone can induce a surface-stem like phenotype. However, EGF alone could not
affect the expression of EMT related molecules on BT-20 cells as determined by RT-qPCR. A single flow chamber assay implied that EGF treatment increased the adhesion of BT-20 cells to endothelium. However, repeated experiments need to be done to make a conclusion. The expression of E-selectin ligands on the surface of EGF or EGF+ TGF-β stimulated cells also need to be test at the RNA and protein level in future studies.

Additionally, when BT-20 cells were stimulated by both TGF-β1 and EGF, EMT related molecules SLUG, TWIST and N-Cadherin were up-regulated after TGFβ-1+EGF stimulation. Meanwhile, a single experiment showed that transformed BT-20 cells obtained CD44+/CD24- phonotype. Experiments using various EGF and TGF- β1 concentrations are needed to test our suspicion that co-stimulation of TGF-β1 and EGF can induce BT-20 cells to undergo EMT and acquire a stem-like phenotype.

According to the fact that EGF alone cannot induce breast cancer cells to undergo EMT in these cells,, the Ras/MAPK pathway may not be sufficient to induce EMT taking into account the literature review depicted in the introduction section. But when TGF-β1 was added, EMT signaling molecules could be induced on BT-20 cells. Taking into account that TGF-β1 and EGF signaling pathways might work together to induce EMT signaling molecules in breast cancer cells. Further study should focus on finding an ideal combined concentration of TGF-β1 and EGF to obtain a full EMT phenotype.
REFERENCES


