Uncovering the Role of TGFβ and BMP in Triple Negative Breast Cancer Stem Cells

Busra Gungor, Philip Owens, and Harold L. Moses

KEYWORDS. Cancer stem cells, TGFβ, breast cancer, DMH1

ABSTRACT. Triple negative breast cancer is considered to be the most difficult breast cancer subtype to treat due to lack of targeted therapy and is diagnosed annually in approximately 400,000 women worldwide. Increasing evidence has shown that cancer stem cells (CSC) are responsible for tumor re-initiation and metastasis. It was hypothesized that inhibiting the CSC will prevent tumor self-renewal and growth. Mammospheres from different human breast cancer cell lines were grown and quantified to determine the effects of transforming growth factor b (TGFβ) and bone morphogenetic protein (BMP) stimulation. Cancer stem, differentiated progenitor, and epithelial-mesenchymal-transition (EMT) markers were evaluated for differential gene expression. Both TGFβ and BMP stimulation were sufficient to enhance the stem cell phenotype. Interestingly, one cell line (HCC1937) had diminished response to TGFβ/BMP stimulation. HCC1937 cells were observed to be highly enriched for stem cell/EMT gene expression, which indicated that lack of response may be due to saturation in TGFβ/BMP signaling. Inhibition of BMP signaling with a kinase inhibitor DMH1 reduced tumor volume in a dose-dependent response in three human stem-like cell lines. These results reveal that targeting the CSC via TGFβ/BMP inhibition may be a promising therapeutic approach for breast cancer.

INTRODUCTION.

Among the different types of cancer, breast cancer has become the second leading cause of death of women around the world. Despite recent advances in treatment, nearly 1.3 million women worldwide are diagnosed with breast cancer and more than 450,000 die each year [1]. Breast cancer generally begins with the transformation of a normal cell to a malignant tumor through genetic mutations. Enhanced cell growth results in the buildup of large, irregular cancer cells that eventually block the mammary ducts [2]. By growing beyond their normal size and disrupting the basement membrane, cancerous cells become invasive and have the ability to travel through the blood stream and colonize other parts of the body such as bone, brain, and lung [2].

Receptor proteins allow molecules to bind to them and send messages to the cell. In breast cancer cells, the most commonly known receptors are estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Triple negative breast cancer (TNBC) lacks all three receptors. While TNBC accounts for only 20% of breast cancer cases, it comprises the majority of breast cancer deaths [1]. Research has shown that not only do victims of triple negative breast cancer have low survival rates, but they also experience higher recurrence (in 3-5 years after treatment).

Cancer stem cells (CSC) are predicted to drive tumor growth through unlimited self-renewal. Self-renewal allows cancer stem cells to repopulate and maintain specific capabilities [4] perpetuating the stem cell pool throughout life. Self-renewal is division with maintenance of the undifferentiated state. This requires cell cycle control and often maintenance of multipotency or pluripotency, depending on the stem cell. Self-renewal programs involve networks that balance proto-oncogenes (promoting self-renewal), TNBC patients resort to non-specific chemotherapy, a treatment with the mechanism to kill rapidly dividing cells. CSCs appear difficult to treat because they grow at a slower rate than cancerous cells. Drug resistance in cancer stem cells is considered to be a main reason for cancer relapse [5]. While pathways involved in cancer stem cell determination have been explored, an effective approach has been targeting these pathways with specific drugs to eliminate CSCs.

The transforming growth factor (TGFβ) signaling pathway has been linked to the regulation of various cellular processes, including stem cell activity [6]. Bone morphogenetic proteins (BMP) are members of the TGFβ super family and also demonstrate unique functions regulating cell behavior [7]. The role of BMP and TGFβ becomes essential in determining stem cell identity. The ID1 protein plays a central role in the creation of tumors [8]. Furthermore, it represents a strong candidate for driving breast cancer progression and development. Since ID1 is increased by bone morphogenetic protein 4 (BMP4), levels of expression of ID1 can be associated with increased stem-cell activity [9]. Thus, ID1 is used to detect levels of stem-cell activity in the current research. This study tests the effects of stimulation with BMP and TGFβ on the self-renewal capacity of human triple negative breast cancer cell lines. It is hypothesized that stimulation via TGFβ and BMP will increase the growth and differentiation of stem cells. Inhibition of the BMP signaling pathway with a drug (DMH1) will limit self-renewal ability of the cancer stem cell and prevent tumor progression. The research presented here sought to shed light on future drugs to eliminate cancer stem cells.

MATERIALS AND METHODS.

Cell Culture.

Breast cancer human cell lines, reflecting primary breast tumors, were chosen based on stem-high and stem-low properties as classified by their level of expression of ID1 RNA expression: BT20-stem low; HCC70, HCC1937, HCC1143, HCC1187-stem high. Cells were grown in suspension in 6-well plates (10,000 cells/well) to form mammospheres. Once established (3 days), each well was treated with one of the following conditions: untreated, BMP4 at 100 ng/mL, or TGFβ at 10 ng/mL. Cells were grown for 10 days in DMEM medium supplemented with EGF (10ng/ml) and triple antibiotic.

Mammosphere Quantification with GelCount Software.

After letting mammospheres grow for 10 days, the 6-well plates were scanned using the GelCount™ plate scanner and analyzed using the GelCount Version 1.4 software (Oxford Optronics, Oxford, UK). Plates were scanned at 600 dpi and mammospheres between 80-1200 μm were examined. To optimize the number of cells counted, the colony detection algorithm was customized and universally applied to all experiments. Total tumor volume was calculated by multiplying the number of spheres with average volume of mammospheres per plate.

Immunofluorescence Staining.

Chamber slides were cultured with HCC1937 and BT20 cells, and treated with either BMP4 or TGFβ1. Cells were washed with 200 μl of phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (Sigma, St. Louis, MO), and permeabilized 0.1% Triton 100X (Sigma) in PBS buffer for 15 minutes. Primary antibodies were diluted in 12% BSA and then incubated overnight at 4°C. Secondary antibodies were diluted 1:200 in 12% BSA and incubated for 30 minutes at room temperature. Slides were mounted with Slow-Fade Light Anti fade Kit (Invitrogen, Carlsbad, CA) containing DAPI to stain the nucleus of all cells blue.

Reverse Transcriptase PCR Analysis (RT-PCR).

Total RNA was purified from cells using Rneasy Qiagen kit (Qiagen, Valencia, CA), converted to cDNA with the VILO cDNA synthesis kit (Invitrogen), and
combined with Lumino CT SYBR Green qPCR Ready Mix (Sigma). Primer sequences were constructed with Primer BLAST (NCBI). Genes were chosen based on previous studies related to the TGFβ/BMP signaling pathway, stem cells, differentiated cells, or epithelial mesenchymal transition (EMT). RT-PCR was performed using the Bio-Rad CFX instrument. Gene expression was normalized to GAPDH and differential expression was measured as a fold expression.

**ApoTox-Glo ™ Triplex Assay.**

DMH1 drug was added to a 96-well plate at different concentrations (5 μM -160μM). ApoTox-Glo ™ Triplex Assay (Promega, Madison, WI) was used to assess viability, cytotoxicity and caspase activation events within 4 stem-high cell lines (HCC70, HCC1143, HCC1187, HCC1937).

**RESULTS.**

The stem-like characteristics of human triple negative breast cancer cells were examined by immunofluorescent staining of cells with E-cadherin/Vimentin and CK5/CK8. Studies have shown that epithelial-mesenchymal transition (EMT) is often activated during cancer invasion and metastasis [10]. In BT20 cells, or “stem-low” cells, Vimentin is not expressed, and there is a lack of staining (green) regardless of BMP4 or TGFβ stimulation (Figure 1A-C). However, the HCC1937 show a noticeable increase in Vimentin expression cells, suggesting that the HCC1937 cells exhibit “stem-high” qualities since EMT is induced through both stimulation of TGFβ or BMP4 (Figure 1D-F). Cells were also stained with CK5, a basal marker, and CK8, a luminal marker. Luminal refers to more differentiated cells, thus making them easier to target; basal characteristics indicate that cells are less differentiated. The HCC1937 cells indicate the induction of a more basal-like morphology with TGFβ and BMP4 treatment. (Figure 1J-L). Again, no significant difference is observed in BT20 cells with both treatments (Figure 1G-I). The results illustrate how TGFβ and BMP4 induce less differentiation and self-renewal, suggesting that there is an increase in stem cells due to the increase in basal cells and subsequent decrease in luminal cells.

**Figure 1.** Induction of EMT and Luminal/Basal morphology via TGFβ and BMP4 stimulation. (A)-(C) Stem-low cells (BT20) do not express Vimentin. (D)-(F) Stem-high cells (HCC1937) show more mesenchymal morphology with treatment due to increased expression of Vimentin. (G)-(I) Stem-low cells (BT20) do not show significant change with TGFβ or BMP4 treatment. (J)-(L) Stem-high cells (HCC1937) show more basal-like morphology.

Stem cell, differentiated cell, EMT, and TGFβ/BMP signaling markers were analyzed (Figure 2). ID1 and p21 were significantly induced with stimulation, indicating that the cells were competent to respond to TGFβ/BMP (Figure 2A). Stem-low cells (BT20) showed that BMP and TGFβ induce both the expression of FN1 and ALDH1A1A genes. Stem-high cells (HCC1937) induced FN1 only with TGFβ stimulation, demonstrating a unique response to TGFβ in differentiated cell lines. The stem cell markers reveal that HCC1937 cells are in fact stem-high since they have a greater fold change relative to the untreated BT20 cells (Figure 2B). The HCC1937 cells reveal a significant fold change in both TGFβ and BMP4 stimulation. On the other hand, CD24 was only induced in BT20 cells (Figure 2C). TGFβ induces CDH2 (also known as N-cadherin, a common mesenchymal marker) in both cell lines. However, for SNAIL2, BMP inhibits the gene, while TGFβ induces it (Figure 2D). Several other genes indicative of stem cell and EMT function were also screened to determine change in gene expression relative to BT20 untreated cells (Table 1).

**Figure 2.** Transcriptional response of TGFβ/BMP4 stimulation in mammospheres reveals distinct roles in cancer stem cell differentiation and EMT. RT-PCR performed to measure transcriptional response after 10 days of mammosphere growth. Fold change is relative to untreated BT20 cDNA. (A) TGFβ/BMP Signaling Markers (B) Stem Cell Markers (C) Differentiated Cell Markers (D) EMT Markers * p<0.05 ** p<0.01
DMH1, a specific kinase inhibitor, was used to inhibit BMP signaling by blocking BMP Smad phosphorylation. Both the cytotoxicity and apoptosis graphs show no significant difference from the untreated tumors (Figure S1). To test the efficacy of DMH1 and its ability to reduce tumor growth, stem-high cell lines (HCC70, HCC1143, HCC1187, HCC1937) were treated with the drug and observed over 10 days. The results showed a statistically significant decrease in tumor volume of mammospheres with increased doses of DMH1 (Figure 3A-B). Continuing treatment for a longer time also revealed a greater reduction in mammospheres. Figure 3C shows the representative images of the effects of DMH1 on the tumors. The pictures indicate the ability of DMH1 to sufficiently reducing mammosphere size and volume.

DISCUSSION.

Both TGFβ and BMP stimulation confirmed that HCC1937 cells were stem-high and BT20 cells were stem-low. An induction of epithelial-mesenchymal transition (EMT) was observed, revealing that HCC1937 cells exhibit stem cell characteristics (Figure 1). Furthermore, it was shown that stimulation of TGFβ and BMP may induce a more basal-like morphology in HCC1937 cells (Figure 1). Thus, these results demonstrate that TGFβ and BMP stimulation induce less differentiation and self-renewal capabilities and that BMP does not necessarily antagonize TGFβ in TNBC. Also, the decrease in luminal cells and increase in basal cells suggests that stimulation via TGFβ and BMP results in an increasing number of stem cells.

Examining gene markers indicated that HCC1937 cells had diminished response to TGFβ/BMP stimulation (Figure 2). Additionally, the stem-high cells (HCC1937) were observed to be highly enriched in stem cell and EMT gene expression. This indicates that the lack of response by these cells may be due to saturation in TGFβ/BMP signaling.

These results have demonstrated the induction of epithelial mesenchymal transition is possible via TGFβ and BMP4 stimulation. It can be speculated that the difference in EMT induction may be Snail2 (SLUG) dependent. EMT is not only known for its promotion of metastases, but also its acquisition of stem cell properties [10]. Studies have shown that under conditions of chemotherapy, the breast cancer cells are the most difficult to eliminate [5]. This study utilizes a unique approach to explore the effects of stimulation of TGFβ and BMP on stem-high and stem-low TNBC human cell lines. The results demonstrate that epithelial-mesenchymal transition is induced through stimulation via TGFβ and BMP4. Inhibiting the BMP pathway by directly targeting the “source,” or cause, of CSCs could be the beginning of developing a highly effective treatment. The mammospheres treated with the DMH1 drug indicate that it has great potential to reduce tumor volume and size without poisoning the cells. The dose-dependent response observed in three human stem-like cells indicates that DMH1 may be a promising cancer therapeutic drug. The data obtained from the research is widely applicable to today’s development of medicine.

More research can be done to determine whether a similar inhibition of TGFβ and BMP can be used to make effective therapies. Future experiments should also be performed to determine the response and toxicity of DMH1 in normal
cells, providing a base line for a more appropriate comparison. In the future, DMH1 could also be used as part of adjuvant chemotherapy and combined with other cancer treatment drugs, like the standard chemotherapy treatment paclitaxol, to examine the effects of combined drug-therapy on tumor volume and self-renewal capabilities of cancer stem cells.

ACKNOWLEDGMENTS. I would like to thank Dr. Moses and Dr. Philip Owens for providing support and guidance throughout this project as well as everyone in the Moses Laboratory for their kindness and expertise. I would like to acknowledge Joseph Roland and the Epithelial Biology Core (EBC) for use of the GelCount plate reader and Dr. Charles Hong for use of the DMH1 drug. Finally, I would like to thank Dr. Mary Loveless for her constant guidance and the School for Science and Math at Vanderbilt for giving me the opportunity to conduct this research. The project described was supported by Award Number R25RR024261 from the National Center For Research Resources. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center For Research Resources or the National Institutes of Health.

SUPPORTING INFORMATION.

Supplemental Methods.

Figure S1. Inhibition of BMP with DMH1 shows low toxicity and apoptosis.

REFERENCES.

Busra Gungor is a student at Martin Luther King Jr. Magnet High School in Nashville, Tennessee, and enrolled in the School for Science and Math at Vanderbilt.