TGF-β (BETA) AND PERIOSTIN MODULATE EACH OTHER’S EXPRESSION IN BOTH BREAST STROMA AND TUMOR CELLS

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Abstract

Breast cancer is the most common cancer in female population worldwide. In addition to mutations, the breast tumor microenvironment especially the tumor cell - stroma interactions through extracellular matrix components and multiple growth factors have been shown to promote tumor progression. Among those, increases in both TGF-β (transforming growth factor beta) activities and periostin expression were associated with tumor cell survival, proliferation and metastasis. TGF-β role in breast cancer progression including its ability to promote periostin expression has been extensively studied. In contrast, the role of periostin in cancer progression remains to be fully understood. Thus, the present study aimed to determine whether TGF-β and periostin have effect on each other’s expressions in breast tumor and stroma cells using in vitro cell models. Through Western blot analyses and ELISAs, the periostin and TGF-β expressions of both stroma and tumor cells were analyzed following TGF-β and periostin treatments, respectively. The results indicate that TGF-β treatments led to significant increase in periostin expression in fibroblasts (p<0.05). In addition, periostin was differentially expressed by human breast cancer cells following TGF-β1 treatment. The TGF-β activities involved activation of pSMAD2 in both L929 fibroblasts and MCF10A mammary cells. Taken together, all experimental data indicate that within the breast tumor TGF-β and periostin likely participate in a regulation loop. Whether this putative regulation loop is critical to metastasis remains to be determined. Should periostin play a critical role in breast cancer progression, it could become a specific target in the preventive and/or therapeutic development of breast cancer patients.
List of Abbreviations

Akt/PKB Protein Kinase B
BMP Bone Morphogenic Protein
3D Three Dimensional
ECM Extracellular Matrix
EGFR Endothelial Growth Factor Receptor
EMT Epithelial Mesenchymal Transition
ELISA Enzyme Linked Immunosorbent Assay
FAK Focal Adhesion Kinase
FAS I Fasciclin I
HGF Hepatocyte Growth Factor
IL Interleukin
JNK c-Jun N terminal Protein Kinases
LAP Latency Associated Protein
LTBP Latent TGF-β Binding Protein
mRNA Messenger Ribonucleic Acid
MAP kinase Mitogen Activated Protein Kinase
MMP Matrix Metalloproteinase
OSF-2 Osteoblast Specific Factor 2
PP2a Protein Phosphatase 2a
RGD Arginine-Glycine-aspartic Acid
SDS-PAGE Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis
SP Signal Peptide
TGF-β Transforming Growth Factor Beta
<table>
<thead>
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<th>Abbreviation</th>
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<tr>
<td>TβRI</td>
<td>Transforming Growth Factor Beta Receptor Type-1</td>
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<tr>
<td>TβRII</td>
<td>Transforming Growth Factor Beta Receptor Type-2</td>
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<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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1. Introduction

Breast cancer is the most common cancer for women in the Western world, and the incidence has been increasing since 1940. The highest incidence rates are observed in North America, Europe whereas lower risks for breast cancer are found in Asia and Africa (Fredslund and Bonefeld-Jorgensen, 2012). Breast cancer progression is affected by the clinico-pathological characteristics of the tumors and by extrinsic factors that also influence the biologic behavior of breast tumor (Zhang et al., 2010). The cancer microenvironment includes in addition to tumor cells a wide variety of stromal and vascular cells (e.g., fibroblasts, myo-fibroblasts, leukocytes, myo-epithelial, endothelial and immune cells) (Wels et al., 2008). These cells are not themselves malignant; however, through their interactions with the cancer cells and their surroundings, they actively participate in the cellular and molecular events associated with cancer progression including cell invasion and metastasis (Li et al., 2007; Bacac and Stamenkovic, 2008). For example, fibroblasts which produce most of the connective tissue present within the tumor mass including different collagens, proteolytic enzymes and their inhibitors, growth factors and determinants of intercellular adhesions (Sappino et al., 1990; Tlsty, 2001), can influence epithelial tumor cells and those stromal–epithelial interactions have been shown to promote tumor progression (Schor et al., 1988). Monocytes/macrophages, neutrophils, and lymphocytes are also recruited and reside within the tumor stroma. Macrophages and neutrophils release many factors, such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), matrix metalloproteinase 2 (MMP2), and interleukin-8 (IL-8), that can influence angiogenesis (Li et al., 2007). Myeloid suppressor cells are also present within the tumor microenvironment at different time during the cancer progression. Myeloid suppressor cells are phenotypically similar to macrophages and granulocytes and produces both matrix metalloproteinase 9 (MMP9) and VEGF critical to the extracellular matrix (ECM) remodeling and angiogenesis, respectively (Serafini et al., 2004; Yang et al., 2004).

The ECM is the product of the secretions of multiple cell types, mainly stromal fibroblasts and epithelial cells and provides structural scaffolding for cells (Bissell and Radisky, 2001). As in normal tissue homeostasis, the secretion and remodeling of the ECM within the tumor microenvironment are regulated by complex interactions among tumor cells, fibroblasts and macrophages (Howlett and Bissell, 1993; Elenbaas and Weinberg, 2001; Hu and Polyak, 2008; Curran and Keely, 2013). However, ECM alterations generated by tumor epithelial cells and stroma cells play a critical role in the initiation and progression of neoplasms. Indeed, both the composition and the density of the ECM have been shown to modulate the breast cancer progression in part through three dimensional (3D) culture experiments (Petersen et al., 1992; Weaver et al., 1997; Swamydas et al., 2010; Lance et al., 2013). The ECM is composed of approximately 300 proteins (Tlsty and Coussens, 2006; Hynes and Naba, 2012) including periostin a potential marker for tumor aggressiveness with clinical implications in multiple human cancers, including the breast cancer (Puglisi et al., 2007; Zhang et al., 2010).
Periostin (about 90 kilo Dalton (kDa), also termed osteoblast-specific factor 2) is a disulfide-linked bone adhesive secretory protein initially identified in mice (Tilman et al., 2007) and a member of the fasciclin I family with homology with the insect cell adhesion molecule FAS I (Ruan et al., 2009). In mammals, four fasciclin-related genes have been identified: periostin, βIG-H3, stabilin-1, and stabilin-2 (Norris et al., 2007). Recently, five additional isoforms differing by their alternative splicing in the C-terminal region of human periostin have been identified (Takeshita et al., 1993; Ruan et al., 2009). Periostin is also a member of the superfamily of transforming growth factor beta (TGF-β)-inducible proteins (Takeshita et al., 1993; Horiuchi et al., 1999). Both the murine and human periostin genes are highly conserved and located on chromosome 3 and 13q, respectively (Litvin et al., 2005). The periostin N terminal region is highly conserved and contains a signal peptide (SP) and a cysteine-rich region (EMI domain). The signal peptide is necessary for periostin secretion, and the cysteine-rich region (EMI domain) promotes the formation of periostin multimers in non-reducing conditions. In addition to these two domains, periostin has four internal FAS domains homologous to the insect cell adhesion protein fasciclin I that serve as ligands for integrins. Through its FAS domains, the periostin N-terminal region regulates cell functions through integrin binding (Takeshita et al., 1993; Horiuchi et al., 1999). The periostin C-terminal region consists of one hydrophilic domain, which regulates the cell–matrix organization and interactions through binding of other ECM proteins including collagen IV, fibronectin, tenascin C and periostin itself (Norris et al., 2007).

In multicellular organisms, cell-cell and cell–ECM adhesive interactions are essential for cell proliferation, survival and functions (Kumar, 1998). Periostin promotes integrin-dependent cell adhesion and motility. Periostin is expressed in a wide variety of normal adult tissues and fetal tissues (Tai et al., 2005). However, an excessive production of periostin contributes to the generation of a tumor-supportive microenvironment (Morra and Moch, 2011). Indeed, periostin is frequently overexpressed in various tumors including ovarian (Gillan et al., 2002), colon (Bao et al., 2004), thyroid (Fluge et al., 2006), lung (Sasaki et al., 2001) and breast (Shao et al., 2004) cancers. Periostin increased expression was associated with advanced cancer stages and increased tumor cell proliferation, adhesion, and migration (Bao et al., 2004; Kudo et al., 2006; Siriwardena et al., 2006; Erkan et al., 2007). In breast tumors, periostin expression was increased compared to normal breast tissue and the periostin expression was highest in advanced stages (Zhang et al., 2010) suggesting that periostin may play an important role in the progression of breast cancer.

Phenotype and activity of cancer cells are strongly regulated by their binding to ECM proteins (Egeblad and Werb, 2002). Integrins, which expressions are frequently altered in cancer cells (Varner and Cheresh, 1996), mediate cell-ECM interactions through the activation of multiple intracellular pathways which in turn alter cell behaviors promoting conditions associated with disease formation (Luo et al., 2007). Through the secretion of ECM proteins, cancer cells modify the composition of their adjacent stroma toward a supportive environment for their growth (Bissell and Radisky, 2001). Periostin binding typically to the integrins αVβ3, αVβ5 and α6β4 promotes the recruitment of the epidermal growth factor receptor (EGFR) and activates the protein kinase B (Akt/PKB) and focal
adhesion kinase (FAK)-mediated signaling pathways in tumor cells (Li et al., 2010). These periostrin-activated signaling pathways promote cellular survival, angiogenesis (Gillan et al., 2002; Bao et al., 2004; Tilman et al., 2007) and resistance to hypoxia induced cell death (Ouyang et al., 2009). Thus periostrin - integrin interactions modulate the ECM integrin interactions, alter the microenvironment and the activities of stromal cells triggering different intracellular signaling including the activation of genes involved in tumor progression (Shao et al., 2004; Kudo et al., 2007). Indeed, overexpression of stroma derived periostrin played a key role in breast cancer metastasis (Contie et al., 2011). Further, periostrin transfection of epithelial cells promoted the epithelial mesenchymal transition, an important step in cancer progression (Yan and Shao, 2006).

Periostin is highly expressed in bone tissue which is one of the preferential breast cancer metastasis sites mostly by osteoblast like cells (Coleman and Rubens, 1987). The key regulator of periostrin expression, transforming growth factor-β (TGF-β) is a critical growth factor involved in the vicious cycle that promotes bone destruction once metastasis occurred. TGF-β has been shown to stimulate the expression of periostrin messenger RNA (mRNA) (Takeshita et al., 1993) and protein expression (Horiuchi et al., 1999) in mice. Taken together, these observations suggest that TGF-β and periostrin interacts in the bone microenvironment to promote bone metastasis from breast cancer (Sasaki et al., 2003). Whether similar regulation loops between TGF-β and periostrin in the primary tumor also play a role in early phases of cancer progression remain to be determined.

Originally isolated from platelets, concentrations of TGF-β were 100-fold greater in bone. Osteoblasts (i.e., bone forming cells). Osteoblasts were found to express large amounts of TGF-β which through autocrine binding to TGF-β receptors amplify TGF-β concentrations in bone (Patil et al., 2011). TGF-β1 is the prototypic family member of 33 secreted structurally related cytokines including TGF-β, activin, and bone morphogenetic proteins (BMPs) (Imamura et al., 2012). The mature TGF-β1 is enzymatically cleaved from the C terminal of a large propeptide (Annes et al., 2003). The homodimerization of two 12.5-kd polypeptides joined by one disulfide bond form the mature TGF-β. An inactive complex of TGF-β combining propeptide and TGF-β, the latent TGF-β binding protein (LTBP) is present within the ECM (Feng and Derynck, 2005). In ECM, TGF-β is activated by local proteolytic cleavage, interactions with integrins or pH changes (Buck and Knabbe, 2006).

TGF-β is a multifunctional cytokine that in its active form regulates mammalian development, differentiation and homeostasis (Tian et al., 2011). TGF-β is also a potent anticancer agent that prevents the uncontrolled proliferation of epithelial, endothelial, and hematopoietic cells. Aberrations in the TGF-β signaling pathway altered TGF-β-mediated growth arrest and promoted human malignances (Blobe et al., 2000; Shi and Massague, 2003). TGF-β signals through the binding of the transmembrane TGF-β Receptor type II (TβRII) and subsequent phosphorylation of TGF-β Receptor type I (TβRI). In turn, phosphorylation of TβRI activates downstream signaling cascades (Caestecker, 2004) especially the SMAD pathway, but can also activate mitogen activated protein kinase (MAP kinase), phosphoinositols-3-kinase (PI3K), and protein phosphatase (PP2A) signaling
pathways (Feng and Derynck, 2005). Receptor-activated SMAD2 or SMAD3 proteins form complexes with SMAD4, and those heteromeric SMAD complexes following nuclear translocation bind to specific promoter elements and regulate transcription of TGF-β-responsive genes (Buck and Knabbe, 2006).

In normal cells, TGF-β signaling inhibits cell growth and inactivation of the SMAD pathway can lead to tumorigenesis. Mutations in both TGF-β family receptors and the SMAD proteins have been found in some tumor cells. For example, TßRII is inactivated in most human gastrointestinal cancers and SMAD4 in almost half pancreatic carcinomas. Many other somatic and hereditary disorders are a result of mutations or malfunctions in the TGF-β/SMAD pathway (Shi and Massague, 2003; Savary and Moustakas, 2011).

TGF-β has been studied in both breast normal development and breast cancer using multiple mouse models (Bierie and Moses, 2006). The importance of the TGF-β switch with suppressive activities in normal and early tumors and stimulative activities in more advanced tumors promoting metastasis has been detailed (Bierie and Moses, 2006). TGF-β controls many important stromal cells - tumor cell interactions including those involved in immune evasion and angiogenesis during tumor progression. In addition, TGF-β signaling mediated by stromal fibroblasts suppressed tumor formation and progression. Overall, the suppressor or promoter TGF-β roles are dependent on the tumor microenvironment (Hanahan and Weinberg, 2000; Vogelstein and Kinzler, 2004).

In tumors, TGF-β is a potent inducer of the epithelial–mesenchymal transition (EMT), a phenotypic conversion of epithelial cells to mesenchymal cells through loss of polarity and cohesiveness and acquisition of the migratory features (Bissell and Radisky, 2001; Heldin et al., 2009). In addition to occurring in various morphogenetic processes during normal embryonic development, EMTs are involved in pathological situations including fibrosis, tumor invasion and metastasis (Thiery, 2003). TGF-β also binds to integrins during EMT. Indeed, TGF-β1 LAPs (latency associated protein) has an integrin binding motif, arginine–glycine–aspartic acid (RGD). Multiple αV integrins including αVβ1, αVβ3, αVβ5, αVβ6 and αVβ8 interact with this RGD motif. The TGF-β-integrin binding activates TGF-β1 (Munger et al., 1999; Annes et al., 2004). Interestingly, antibody-mediated blockade of αV integrin, particularly αVβ6, down-regulates the TGF-β induced EMT and inflammation associated with cancer progression and metastasis (Bates et al., 2005; Aarsen et al., 2008). Overall, αV integrins induce EMT through the activation of TGF-β and unregulated TGF-β can lead to pathological EMT (Mamuya and Duncan, 2012).

TGF-β and periostin both individually and together plays significant roles in the tumor microenvironment influencing tumor cell survival, migration and metastasis (Pasche, 2001; Ruan et al., 2009; Bernabeu et al., 2009). Although periostin and TGF-β interrelationships (Conway et al., 2011) have already been shown in vulvogenesis and their simultaneous expression detected in colorectal carcinoma (Wooi and Hang, 2009), interrelations between TGF-β and periostin in breast tumor microenvironment have yet to be determined.
1.1 Aim of the Study

The overall aim of this study is to better understand the role of the interrelations between periostin and TGF-β in breast tumor microenvironment. Specifically, the present work aims to determine whether TGF-β promotes periostin expression and/or periostin promotes TGF-β by tumor and/or stroma cells within the breast tumor microenvironment using in vitro cell models. In this study, the expression of TGF-β and periostin in total cell lysates and supernatants will be determined by Western blot analyses and ELISAs. The proposed experiments will provide evidence on the effects of periostin on TGF-β protein expression, the effects of TGF-β in production of periostin in both breast tumor cells, fibroblasts and macrophages. Taken together, these studies are expected to provide compelling evidence that periostin and TGF-β forms a regulation loop in breast tumor microenvironment.
2. Materials and Method

The overall research approach is shown in figure 1. After preparing appropriate number of cells (fibroblast, macrophages, human breast cells and murine mammary cells), assigned cells were treated with TGF-β1 and periostin. Following treatment cells were harvested where cellular lysates and supernatants were collected for western blotting and ELISA respectively. After Densitometric analysis of expression of TGF-β1 and periostin in immunoblots and results generated from ELISA, statistical significance was assessed. Then effects of TGF-β1 on periostin expression and effects of periostin on TGF-β1 expression were evaluated and final results were obtained.

Fig. 1: Schematic showing research approach.

2.1 Cells and Cultures

All cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured according to ATCC’s recommendations. Used cells included mammary/breast cells, fibroblasts and macrophages derived from human and mouse tissues (see Table 1).
Table 1: Cells analyzed for periostin and TGF-β expression.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cell Type</th>
<th>Species</th>
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<tbody>
<tr>
<td>MCF 10A</td>
<td>Breast epithelial cells</td>
<td><em>Homo sapiens</em></td>
</tr>
<tr>
<td>MCF 7</td>
<td>Locally invasive breast cancer cells</td>
<td><em>Homo sapiens</em></td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Highly metastatic breast cancer cells</td>
<td><em>Homo sapiens</em></td>
</tr>
<tr>
<td>4T1</td>
<td>Highly metastatic mammary cancer</td>
<td><em>Mus musculus</em></td>
</tr>
<tr>
<td>J774</td>
<td>Macrophage</td>
<td><em>Mus musculus</em></td>
</tr>
<tr>
<td>RAW</td>
<td>Macrophage</td>
<td><em>Mus musculus</em></td>
</tr>
<tr>
<td>L929</td>
<td>Fibroblast</td>
<td><em>Mus musculus</em></td>
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Briefly, cells of human origin were cultured in DMEM/F12 (Dulbecco’s Modified Eagle Medium) media (Cellgro, Manassas, VA) supplemented with bovine serum (10%; Lonza, Allendale, NJ) amphotericin B (Cellgro, Manassas, VA), and gentamycin (Cellgro). MCF10A cells were cultured in the same base media where bovine serum is replaced with horse serum (5%, Lonza, Allendale, NJ) supplemented with insulin (10 μg/ml, Sigma-Aldrich, St. Louis, MO), epidermal growth factor (20 ng/ml, Sigma-Aldrich) and hydrocortisone (0.5 μg/ml, Sigma). Cells of murine origin were cultured in DMEM based media supplemented with gentamycin and amphotericin and bovine serum (10%). Cell viability and counts were assessed using Trypan blue dye (Cellgro). All cell cultures were conducted at 37°C, 5% CO2 and >90% humidity conditions. After preparing the cells, treatments were conducted following seeding cells to confluence and 24-hr incubation in serum-free media.

L929 mouse fibroblasts, 4T1 mouse mammary tumor cells, and RAW and J774 mouse macrophages were incubated with either 0, 0.1, 1.0 or 10 μg/ml of periostin (R&D Systems, Minneapolis, MN) for 24 hours.

Human breast cells (MCF10A, MCF7, MDA-MB-231) and RAW and J774 mouse macrophages were incubated for 24 hours with 20 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN). L929 mouse fibroblasts were treated with the same concentration of TGF-β1 for 6 hours, 24 hours, 48 hours and 72 hours.

### 2.2 Secreted/Shed Proteins and Protein Cell Lysates

Following incubation with cell treatments, supernatants were collected, centrifuged to remove debris (including dead cells) and stored at -20°C until use. Cell lysates were obtained following scraping of cells and multiple washes with PBS (phosphate buffered saline), centrifugation and resuspension of the pellets in 50uL of the protein lysate solution (iNtRON Biotech Pro-prep). The cell lysis was promoted with a freezing cycle and sonication. The cell lysates were stored at -20°C until use.
2.3 Western Blotting

Protein lysate immunoblottings were conducted as described earlier (Swamydas et al., 2011). 25 µg of total protein per well were loaded on 8% (for periostin, SMAD detection), 12% and 15% (for TGF β detection) poly-acrylamide gels and ran in SDS-PAGE under denaturing conditions and the proteins were then transferred onto nitrocellulose membranes. Loading of equal protein amounts was confirmed by staining membrane with 0.1% Ponceau S (Sigma) and further assessed by evaluating the even expression of β-actin by immunoblots. Briefly, after a 1-hr incubation with TBS-T (Tris buffered saline with 0.1% Tween 20) containing 5% nonfat milk to block non-specific binding, membranes were incubated with antibodies specific for periostin and TGF-β1, 2, or 3 (Santa Cruz biotechnology), pSMAD2 and pSMAD3 (Cell signaling, Danvers, MA) or β-actin (Sigma Aldrich, St Louis, MO) for one hour. Then the membranes were washed with TBS-T (0.1% Tween 20) 4 times for 5 minutes each. Then Following one-hour incubation with the appropriate HRP-conjugated secondary antibody and washing with TBS-T (0.1% Tween 20) for 20 minutes, a chemiluminescent substrate (H₂O₂) (Propietary Luminol and peroxide solution) (Pierce, Rockford, IL), applied over the membrane by which the presence of protein was detected on the membrane using a biochemiluminescent imaging system and the VisionWork software (UVP, Upland, CA). Differences in protein expression were determined by densitometry using Quantity One software (Bio-Rad, Hercules, and CA) following normalization to β-actin expression.

2.4 Periostin and TGF-β ELISAs

After treatment with periostin and TGF-β (for different time periods) supernatants were collected and centrifuged to remove the dead cells (2.5k for six minutes at 4°C). These supernatants were assessed by Enzyme Linked Immunosorbent Assay (ELISA) for periostin and TGF-β concentrations following the manufacturer’s recommendations (R&D Systems, Minneapolis, IN). Briefly, 96-well plates were coated with the capture antibody and incubated overnight. Following blocking with 1% bovine serum albumin for 1 hour, supernatant samples were added and the plates were incubated for 2 hours. In the subsequent incubations, a biotin conjugated detection antibody and streptavidin-horseradish peroxidase (HRP) were added for 120 and 20 minutes, respectively. The presence of HRP-conjugated complexes was determined following the addition of the substrate solution (TMB, Pierce Inc. Rockford, IL) and the enzymatic reaction stopped by the addition of 2N H₂SO₄. (Sulphuric Acid) Optical densities (450 nm) resulting from HRP activities were measured using a micro plate reader (Biotek, Winooski, VT) and concentrations (pg/ml) derived from standards ran along with the samples.

2.5 Statistical Analyses

Statistical analyses were conducted using one-way and two-way ANOVAs and Newmann-Keul post-hoc tests (Prism, Graphpad Software, Inc., La Jolla, CA). Significance was set a priori to p value below 0.05.
3. Results and Discussion

Solid tumor development and progression involve cellular abnormal functions mostly associated with mutations and gene expression deregulation that also lead to alterations in the extracellular matrix composition (Ruan et al., 2009; Nuzzo et al., 2012). Tumor cells and the surrounding stroma cells within the tumor evolve together but their respective roles in the tumor initiation and progression is still discussed (Li et al., 2007).

In the tumor microenvironment periostin, an ECM protein (Morra and Moch, 2011) especially along with the multifunctional cytokine TGF-β (Ikushima and Miyazono, 2010) has emerged as a potential role player in carcinogenesis. Several studies (Kudo et al., 2006; Puppin et al., 2008; Bernabeu et al., 2009; Imamura et al., 2012) have suggested a combined impact of these two proteins on cell proliferation, survival and metastasis. In the present study, as expected TGF-β1 stimulated periostin expression especially in fibroblasts, but also in tumor cells. In addition, data from present study indicate that periostin mediates TGF-β1 expression by tumor and stroma cells within the breast tumor microenvironment.

3.1 TGF-β1 promotes the Expression of Periostin in Both Stroma and Tumor Cells

3.1.1 TGF-β1 promotes the Expression of Periostin in Stroma Cells

The effect of TGF-β1 on the production of periostin by mouse fibroblast cells (L929) and macrophages (RAW) were determined. L929 fibroblasts incubated for 24hrs with TGF-β1 secreted significantly more periostin compared to samples incubated for 6 hours (p <0.01, Fig. 2).

![Graph showing periostin expression](image)

**Fig. 2:** TGF-β1 promoted the expression of periostin by L929 fibroblasts. The concentrations of periostin (pg/ml) measured by ELISA in the supernatant of L929 fibroblast cultures were significantly higher following 24-hr incubation with TGF-β1 (20 ng/ml). (n=3), ( **p <0.01)**
In contrast to most organs normal tissue stroma, which contains few fibroblasts and a normal ECM, tumor stroma typically contains a large number of fibroblasts (Ronnov-Jessen et al., 1996). The importance of reactive stroma in the development of tumorigenesis has been demonstrated (Dolberg et al., 1985). Here, the stimulation of the L929 normal subcutaneous areolar adipose tissue derived fibroblasts with TGF-β1 led to highly significant level of periostin secretion. (Fig 1) This result confirms previous observations in normal fibroblasts (Borg and Markwald, 2007) and in PDL fibroblasts (Wen et al., 2010).

The effect of TGF-β1 on periostin expression by mouse macrophages (RAW) was evaluated by Western blots. Treatment of RAW macrophages with TGF-β1 for 24 hours led to a significant increase in the expression of periostin in the RAW cell lysate (Fig. 3AB).

![Western Blot Image]

Fig. 3: TGF-β1 promotes the expression of periostin in RAW macrophages. (A) Two representative Immunoblots of the expression of periostin in RAW macrophage lysates following a 24hr treatment with TGF-β1. (B) Periostin expression normalized to β-actin expression determined by densitometry. (n=2) (*p<0.05).

Macrophages play a key role in tumor progression (Sica et al., 2008) and a macrophage rich tumor microenvironment has high metastatic potential (Nardin and Abastado, 2008). Indeed, data obtained from this study (Fig 2) indicate that TGF-β1 stimulated expression of periostin in RAW macrophages.
3.1.2 Periostin is differentially expressed by Human Breast Cancer Cells following TGF-β1 Treatment

The effect of TGF-β1 on the expression of periostin by human breast cancer cells was determined. By immunoblots (Fig 4A), periostin expression appeared to increase, regardless of the cells tested. Moreover, the periostin expression was much higher (p< 0.001, p<0.0001) in MCF7 cells than in MCF10A and MDA-MB-231 cells (Fig. 4B). Furthermore, the expression of periostin was significantly (p<0.001) increased following TGF-β1 treatment in MCF7 cells but the expression of periostin was not significant in MCF10A and in MDA-MB-231 cells following TGF-β1 treatment (Fig. 4B).

Fig. 4: TGF-β1 treatment significantly increased the periostin expression in MCF7 human breast cancer cells. (A) Representative immunoblots of periostin in expression in the lysate of MCF10A, MCF7 and MDA-MB 231 cells. β-actin expression was used as a loading control. (B) The periostin expression significantly higher in MCF7 cells than in MCF10A and MDA-MB-231 cells was promoted by TGF-β1 treatment (**p<0.001,****p<0.0001). Data are representative of at least three independent experiments.
The concentrations of periostin secreted by MCF10A, MCF7 and MDA-MB-231 cells in the presence or not of TGF-β1 were determined by ELISA. As shown Fig. 5, periostin concentrations following TGF-β1 treatment were significantly lower in MCF7 (B) cells (p <0.05) but not in MCF10A (A) and MDA-MB-231 (C) cells.

![Graph A](image1.png) ![Graph B](image2.png) ![Graph C](image3.png)

**Fig. 5: TGF-β1 treatment reduced the secretion of periostin.** Periostin concentrations (pg/ml) were determined by ELISA in (A) MCF-10A, (B) MCF7 and (C) MDA-MB-231 cell supernatants collected following treatment without or with TGF-β1. Periostin concentrations following TGF-β1 treatment were significantly lower in MCF7 cells but not in MCF10A and MDA-MB-231 cells (p <0.05). Each set of point represents one independent experiment.

Here, TGF-β1 tended to promote periostin expression in the human breast cell tested (Fig. 4, Fig. 5). This observation is in line with data demonstrating higher periostin expression in malignant breast tissue compared to normal breast tissue (Zhang *et al.*, 2010). Furthermore, higher tumor periostin expression was correlated with increased aggressiveness and progression of breast tumors (Siriwardena *et al.*, 2006; Kudo *et al.*, 2006; Erkan *et al.*, 2007). Following secretion, the ECM protein periostin likely binds to integrins promoting the survival, proliferation and metastasis of tumor cells (Curran and Kelly, 2013). In the presence of TGF-β, normal epithelial breast cells secreted comparable periostin concentrations than in control conditions. However, TGF-β tended to increase the periostin secretion by the aggressive MDA-MB-231 breast cancer cells supporting the observation that metastasis is closely related to periostin expression (Zhang *et al.*, 2010). Of note, periostin expression in breast tumor cells was highly variable (Tilman *et al.*, 2007). Nevertheless, TGF-β1 has previously demonstrated to promote increased periostin expression by different cells (Horiuchi *et al.*, 1999). As in the tumor microenvironment, TGF-β is an EMT inducer (Erkan *et al.*, 2007), so TGF-β1-stimulated increased periostin expression may have a key role in the metastatic process.
3.2 TGF-β1 and the SMAD2 Pathway: Role in Stromal Cells and Breast Cells

3.2.1 SMAD2 Pathway is activated in L929 Cells following Incubation with TGF-β1

To determine whether TGF-β promotion of periostin expression in L929 fibroblast was associated with the canonical TGF signaling through the SMAD cascade, the expression of these proteins was assessed by Western blots. The expressions of total SMADs or of pSMAD1, pSMAD3 and SMAD4 by L929 fibroblasts were not altered following TGF-β1 treatment (not shown). However, pSMAD2 expression in L929 fibroblasts was significantly increased following TGF-β1 treatment (Fig. 6, p<0.01).

![Figure 6: Following incubation with TGF-β1 L929 fibroblasts expressed significantly higher levels of pSMAD2. (A) Representative Immunoblots of the phosphorylated SMAD2 expression in L929 fibroblast lysates; (B) Densitometric quantification of L929 fibroblast expression of pSMAD2 following normalization to β-actin expression indicates that TGF-β1 stimulated a significantly higher expression of Phosphorylated SMAD2 compared to control conditions (**p<0.01). Data are representative of at least three independent experiments.

The periostin secreted through TGF-β1- stimulation involving the pSMAD2 pathway (Fig. 6) in fibroblasts likely participates to the generation of a tumor supportive microenvironment in part through the activation of the Akt/PKB pathway following integrin binding (Morra and Moch, 2011).
3.2.2 TGF-β1 increases SMAD2 Phosphorylation in Normal Breast Cells

To evaluate whether TGF-β1 promotion of periostin expression in Human breast cancer cell line was associated with the canonical TGF-β signaling through the SMAD cascade, the expression of these proteins was assessed by Western blots. The expression of total pSMAD2 was significantly increased in Normal Breast cell line (MCF10A) but did not show any significant change in Breast cancer cell line MCF7 which is locally invasive and MDA-MB-231 which is highly metastatic. In contrast, pSMAD2 expression in normal breast cell was significantly increased following TGF-β1 treatment (Fig. 7, p<0.01).

![Western blots showing pSMAD2 expression in MCF10A, MCF7, and MDA-MB-231 cells with and without TGF-β1 treatment](image)

![Graph showing the densitometric quantification of pSMAD2 expression](image)

**Fig. 7:** TGF-β1 promoted the expression of pSMAD2 was significantly higher in normal breast MCF10A cells (A) Representative Immunoblots of the phosphorylated SMAD2 expression in lysate of MCF10A, MCF7 and MDA-MB-231 cells (B) Densitometric quantification of expression of pSMAD2 following normalization to β-actin expression indicates that TGF-β1 stimulated a significantly higher expression of PhosphoSMAD2 in MCF10A compared to control conditions and MCF7 cell line (**p<0.01, *p<0.05). Data are representative of at least three independent experiments.
Here alterations in the TGF-β signaling pathway regardless of the stroma or tumor cells tested lead to the stimulation of the SMAD pathway as shown previously (Xie et al., 2002). The phosphorylation of SMAD pathway is essential during normal cellular activities (Imamura et al., 2012). In early stage mammary gland tumor TGF-β acts via this SMAD pathway (Koumoundourou et al., 2007). In normal breast cells (MCF10A) specifically, pSMAD2 expression increased following TGF-β1 stimulation (Fig. 7) but peristin expression did not increase (Fig. 4) and in locally invasive breast cancer cells (MCF7) peristin expression was increased significantly (Fig. 4) following TGF-β1 stimulation but pSMAD2 expression was not significant (Fig. 7). This may be due to modulations by other pathways than the SMAD pathway including the c-Jun N-terminal protein kinases (JNK) or p38/MAPK pathways or the results of mutation(s) related to the TGF-β pathway (Levy and Hill, 2006). Indeed, TGF-β also induces other breast tumor cell pathways which can act independently as well as together with the SMAD pathway (Imamichi et al., 2006). Effect of TGF-β1 on the expression of peristin is shown in the following figure (Fig. 8).

**Fig. 8: Summary of effect of TGF-β1 on peristin expression.**

Human Breast cells (MCF10A, MCF7 and MDA-MB-231) and stromal cells (macrophages, fibroblasts) were stimulated with TGF-β1 (20 ng/ml) for 24 hours. Following stimulation human breast cancer cells (MCF7 which is locally invasive) and stromal showed significantly increased peristin expression where stromal fibroblasts also showed involvement of pSMAD2 pathway. This expressed peristin plays significant role in generation of a tumor supportive microenvironment by increasing cell proliferation and cell survival.
3.3 Periostin stimulates and decreases the TGF-β1 Expression in Tumor Cells and in L929 Fibroblasts, Respectively

3.3.1 Periostin promotes TGF-β1 Expression in Tumor Cells

The expression of TGF-β1 in 4T1 cancer cell incubated with increasing periostin concentrations significantly increased in a dose-dependent manner (Fig. 9A, 9B; p<0.0001). Furthermore, the concentrations of active TGF-β1 secreted increased following treatments of 4T1 cells with increasing concentrations of periostin (Fig. 10A, p<0.05). The ratio between active and total TGF-β1 also indicated that as increasing periostin concentrations were used to treat 4T1 cells, these cells secreted higher doses of active TGF-β1 (Fig. 10B, p<0.05).

![Image of immunoblots and densitometric quantification]

**Fig. 9**: Periostin promotes the expression of TGF-β1 in 4T1 mammary tumor cells in a dose-dependent manner. (A) Representative Immunoblots of 4T1 cell lysates for the expression of TGF-β1 following 24-hr incubation with increasing periostin concentrations. (B) Densitometric quantification following normalization to the β-actin expression demonstrated that both doses of periostin (1 and 0.1 µg/ml) promoted significant increases in 4T1 cell TGF-β1 expression compared to control conditions (****p<0.0001, **p<0.01). Data are representative of at least three independent experiments.
Periostin stimulates the secretion of active TGF-β1 in 4T1 mammary cancer cells. (A) The secretion of active TGF-β1 secretion by 4T1 cells was stimulated by treatment with both 0.1 and 1.0 µg/ml of periostin, with the higher concentration leading to a significant increase in active TGF-β1 (*p<0.05). (B) Furthermore, the increase of ratio active TGF-β1 to total TGF-β1, as the dose of periostin used to treat 4T1 cells increased, was significant at both periostin concentrations tested (**p<0.01; *p<0.05).

Here periostin induced increased TGF-β1 expression in 4T1 mammary cancer cells (Fig. 9, Fig. 10) confirming previous observations that periostin induced TGF-β1 in epithelial cells (Sidhu et al., 2010).

### 3.3.2 Periostin promoted Differential Expression of TGF-β1 in Stromal Cells

Following incubation with increasing concentrations of periostin L929 fibroblasts expressed lower TGF-β1 levels as measured by immunoblots on cell lysates (Fig. 11AB, p<0.01). L929 secreted TGF-β1 tended to increase as the periostin concentration increased (Fig. 11C, p=0.54).
Fig. 11: Expression of TGF-β1 in Mouse L929 fibroblast cells following periostin stimulation. (A) Representative immunoblots of the expression of TGF-β1 by L929 fibroblast following treatment with increasing concentrations of periostin. (B) The densitometric quantification of the TGF-β1 expression following normalization to β-actin indicates that L929 cells treated with higher dose of periostin (1.0 µg/ml) and (0.1 µg/ml) had significantly lower TGF-β1 compared to L929 cells under control conditions (**p<0.01). (C) The concentrations of TGF-β1 secreted and present in the supernatants of L929 cells tended to increase following incubation with higher concentrations of periostin which was not significant. Data are representative of at least three independent experiments.

All experimental data from this present study indicate that following stimulation with increasing concentrations of periostin, L929 fibroblasts TGF-β1 expression was variable (Fig. 11). During tumor progression, TGF-β and SDF-1 (stromal cell derived factor 1) converted normal fibroblast into carcinoma-associated fibroblasts (Kojima et al., 2010). Moreover, in breast carcinoma 80 percent of stroma fibroblasts were converted into carcinoma-associated fibroblasts and those cells plays critical roles in cancer progression (Sappino et al., 1988; Kalluri and Zeisberg, 2006). Whether the secreted TGF-β participates to the phenotypical conversion to carcinoma-associated fibroblasts, possibly through autocrine signaling as shown here in L929 fibroblasts, remains to be determined.
Incubation of RAW or J774 macrophages with increasing concentrations of periostin had no significant effects on the expression of TGF-β1 (Fig. 12).

**Fig. 12: Effects of periostin on RAW, J774 macrophages TGF-β1 expressions.** Representative Immunoblots of the expression of TGF-β1 by RAW (A) and J774 (B) macrophages. The expression of TGF-β1 in RAW (C) and J774 (D) cells was quantified by densitometry analyses and normalized to β-actin expression. Moreover, the ratios of active TGF-β1 / total (active + latent) TGF-β1 following periostin treatment of RAW (E) and J774 (F) cells are presented after analyzing ELISA. Regardless of the macrophages tested, the expression of TGF-β1 was not altered by incubation with increasing periostin concentrations (not significant). Data are representative of at least three independent experiments.
At the concentrations tested, periostin was associated with a trend toward an increased TGF-β1 expression (Fig. 12A, 12C, 12E) in RAW macrophages possibly participating in the phenotype conversion to tumor associated macrophages which promotes tumor progression (Curran and Keely, 2013). As TGF-β1 can modulate the macrophage phenotype (i.e., from anti-tumor to pro-tumor), the alterations in TGF-β1 secretion promoted by periostin may refine the local macrophage phenotypes (Sica et al., 2008).

Effect of periostin on the expression of TGF-β1 is shown in the following figure (Fig. 13).

Murine mammary cancer cell line (4T1) and stromal cells (macrophages, fibroblasts) were stimulated with periostin at a dose of (0, 0.1, 1.0 µg/ml) for 24 hours. Following stimulation Murine mammary cancer cell line (4T1) showed significantly increased TGF-β1 expression with increase dose of periostin. But in stromal fibroblast expression of TGF-β1 decreased with increased periostin dose and probably this TGF-β1 plays role in converting the normal fibroblast in cancer associated fibroblast (CAF) which play significant role in tumor progression in a microenvironment. In macrophages increased TGF-β1 expression was evident in a dose dependent manner and its role in probable conversion of macrophage into tumor associated macrophage (TAM) was speculated. Altogether this periostin influenced expression of TGF-β1 can act in both autocrine and paracrine manner and influence the generation of a tumor supportive microenvironment.
Taken together, these results suggest that TGF-β and periostin appear to regulate each other’s expression in both normal epithelial and epithelial tumor cells, and stromal cells. Furthermore, the data may support a possible loop present between TGF-β and periostin in a tumor microenvironment. Both autocrine and paracrine signaling loops are likely to be involved in the signaling between stroma and tumor cells during breast cancer progression and it is shown in the following figure (Fig. 14).

![Possible regulation loop between TGF-β and periostin in a tumor microenvironment.](image)

**Fig. 14:** Possible regulation loop between TGF-β and periostin in a tumor microenvironment.

Breast tumor and stromal cells upon TGF-β stimulation expresses periostin which in turn binds to cell surface integrin receptor, activates Akt/PKB pathway and increases cell survival and cell proliferation leading to progression of tumorigenic process. This expressed periostin can again act on the stromal cells and breast tumor cells and modulate TGF-β expression. In breast tumor cells, periostin stimulates TGF-β expression which can influence the tumor microenvironment significantly by acting (autocrine and paracrine manner) on the same or other tumor cells, fibroblast and macrophages leading to more periostin expression. However this TGF-β can also modify normal macrophage to tumor associated macrophage, can convert normal fibroblast into carcinoma associated fibroblast and plays profound role in epithelial mesenchymal transition and metastasis. As a whole once a TGF-β-periostin regulation loop starts in a tumor microenvironment, it continues in a cyclical manner and helps the progression of overall tumorigenic process.
4. Conclusion and Future Prospects

Breast cancer is a heterogeneous disease involving different environmental and molecular factors responsible for its initiation and progression. Beside intrinsic tumor factors including mutations, extrinsic factors also play critical roles in breast tumor progression. Both the density and composition of the ECM have been demonstrated to promote breast cancer progression and metastasis. Among the numerous molecules involved in breast cancer progression both periostin and TGF-β present within the breast tumor microenvironment have been associated with worst clinical prognosis. Data indicate that not only does TGF-β promote the expression of periostin by fibroblasts as already demonstrated; TGF-β also promotes periostin expression by tumor cells. Furthermore, periostin also differentially modulated TGF-β expression in stroma and tumor cells. Together the independent regulations of TGF-β expression by periostin and of periostin expression in both stroma and tumor cells by TGF-β suggest a role for periostin in breast cancer progression. Moreover, the data indicate that within the breast tumor TGF-β and periostin may likely participate into a regulation loop. Whether this putative regulation loop is critical to metastasis remains to be determined. Should periostin play a critical role in breast cancer progression, it could become a specific target in the preventive and/or therapeutic development of breast cancer patients.
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References


