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Transforming growth factor-beta signaling in breast cancer

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TABLE OF CONTENTS

1. ABSTRACT

Transforming growth factor-β (TGF-β) is a multifunctional polypeptide that regulates cell growth, differentiation, and extracellular matrix formation. Studies on genetically engineered animal models have demonstrated that TGF-β-mediated signaling pathway plays a critical role in both normal development and tumorigenesis of the breast. In pathogenesis of breast cancer, the role of TGF-β appears featured with growth-inhibitory effects at early stages of carcinogenesis, but aggressive oncogenesis with transition to more advanced malignant states. The TGF-β signaling pathway is also tissue-context and ligand content-dependent. Therein, therapeutic modulation of TGF-β signaling may be a multifactorial event.

2. INTRODUCTION

The transforming growth factor β (TGF-β) ligand family is composed of several multifunctional growth factors, including TGF-β (TGF-β1/2/3 isoforms), activins, and bone morphogenetic proteins (BMPs) (1). The TGF- β was first discovered in relation to its capability of inducing a transformed morphology and clonogenic growth in soft agar of non-transformed, anchorage-dependent normal rat kidney cells and fibroblasts (2). However, subsequent studies demonstrated that TGF-β inhibits the growth of normal epithelial cells, demonstrating the complexity of the function (3). TGF-β ligands and receptors are expressed in nearly all types of cells, including epithelial, stromal, immune, lymphoid, and endothelial cells. In these cells, the TGF-β signaling regulates development, differentiation,

Table 1. TGF-β signaling molecules

Signaling Molecules	Function
Type I receptor (TβRI)	Bind to TGF-β/TβRII complex and phosphorylates Smad2/3
Type II receptor (TβRII)	Bind to TGF-β and activate TβRI
Type III receptor (TβRIII)	Unclear
Receptor-activated Smad (Smad2, Smad3)	Signal transmission/DNA binding
Common mediator Smad (Smad4)	Smad2/3 mediator
Inhibitory Smad (Smad6, Smad7)	TGF-β signaling inhibitors

References: 4, 5, 26, 55

extracellular matrix formation, cell cycle, angiogenesis, hematopoiesis, chemotaxis, and immune functions. Recent studies indicate that the TGF- β signaling demonstrates dual functions in mammary tumor development. TGF- β appears inhibitory at early stage of tumorigenesis, whereas tumor cells at advanced stages can evade antiproliferative control and undergo tumorigenic progression in response to TGF-β. In this review, discussion will focus on recent progress on the role of the TGF-β in mammary gland development and tumorigenesis, and its potential avenues toward cancer therapies.

3. TGF-β LIGANDS AND THEIR RECEPTORS

The TGF-β ligands consist of three isoforms, TGF-β1/2/3 (1, 4). In the TGF-β signaling pathway, biological signals are transmitted via binding of the TGF-β ligands to two types of receptors, TGF-β type I (TβRI) and TGF-β type II (TβRII). The three TGF-β isoforms often elicit similar responses (5). Table 1 summarizes the key signaling molecules and their functions in this pathway.

Active TGF-β is produced through the maturation of a propeptide (6). For instance, TGF-β1 gene encodes a 390 amino acid polypeptide. At the dibasic cleavage site (residue 278), this polypeptide is cleaved into a latency-associated peptide (LAP) and TGF-β1 during post-translational modification (6, 7). LAP and TGF-β1 form an inactive, noncovalently associated small latent TGF-β complex (SLC), which is secreted. Alternatively, this complex can be linked via a disulfide bond to a latent TGF-β binding protein (LTBP) for storage. This trimolecular aggregate is called large latent complex (LLC). LTBP secures the ligands in extracellular matrix (ECM) via a cross-link at the N-terminus catalyzed by transglutaminase (8). This latent complex primarily mediates the biological activity of TGF-β, and therefore, TGF-β message RNA levels do not usually reflect the protein production or activity (9-11). Latent TGF-β can be activated by heat, chaotropic agents, pH, proteases, urokinase plasminogen activator, integrins, fibronectin fibrils, and thrombospondin-1 (12-16). Thrombospondin-1 stimulates TGF-β release by the interaction of a motif (K412RFK415) in thrombospondin-1 with the LSKL motif in LAP at the amino terminus (15).

There are three TGF-β receptors identified thus far. TβRI and TβRII are transmembrane serine-threonine kinase receptors (5). TGF-β binds to TβRII and then recruits TβRI, forming the heterodimeric TβRII/TβRI complex with bound TGF-β (Figure 1). Formation of the heterodimer allows the transphosphorylation of specific serine and threonine residues in juxtamembrane segment of TβRI by TβRII kinase, thereby activating TβRI kinase and initiating the signaling process (17). TGF-β receptor III (TβRIII), also known as betaglycan (18), is not signaling. The biological function of the TβRIII is unclear, but its short cytoplasmic domain and subsequent loss of intrinsic kinase activity may indicate its role as a co-receptor, enhancing ligand binding to TβRII (19).

Activated TβRI kinase phosphorylates C-terminal serine residues of Smad2 and Smad3, two distinct proteins in a subclass of R-Smads (receptor-activated Smads) (20). Phosphorylated R-Smads bind to a common mediator Smad4, also referred to as Co-Smad, forming a functional trimeric protein complex. This complex is translocated into the nucleus, binds to the Smad-binding element in promoter of the target genes, recruits transcription factors, and controls the transcription of these genes (20, 21).

The activity of the TGF-β signaling pathway is regulated by a negative regulatory feedback loop mediated by inhibitory Smads (I-Smad), Smad6 and Smad7. I-Smads competitively bind to TGF-β/receptor complex and inhibit the phosphorylation of R-Smad (22). In addition, Smad7 can recruit phosphatases to dephosphorylate and thus inactivate the receptor complex.

Recent studies revealed that TGF-β mediates cell cycle through the RhoA/$p160^{ROCK}$ signaling pathway (23). In mammary gland epithelial cells, by activating RhoA, TGF-β stimulates $p160^{ROCK}$ translocation to the nucleus, alters the phosphorylation of the linker region of Smad2/3 at Ser^{203} and Ser^{207} residues, and triggers downstream gene expression, such as $p15^{INK4B}$ and $p21^{cip1/waf1}$. This results in inhibition of pRb phosphorylation and cell cycle arrest or triggers apoptosis through the regulation of various pro-apoptotic and anti-apoptotic molecules: p53, Bad, Bax, Bik, Bcl-2, and Bcl-XL (24, 25).

4. TGF-β SIGNALING IN MAMMARY GLAND DEVELOPMENT

The role of the TGF-β signaling pathway in cell proliferation and tissue formation is complicated. In mammalian breast development, the TGF-β signaling pathway is involved in establishing proper gland structures, maintaining epithelium in functionally undifferentiated status, and inducing apoptosis in the involuting gland (26).

In mammary glands, the expression of TGF-β isoforms is strictly regulated with the development of mammary glands. In mice, all three TGF-β isoforms are expressed in epithelium in all developmental stages of the breast, where TGF-β acts as an inhibitor of ductal elongation and branch formation. During puberty, the TGF-β activity is decreased in mammary ducts in response to proliferation signals, preparing for proliferation (27). In transgenic mice expressing TGF-β1 s223/225 (a constitutively active TGF-β) specifically in mammary epithelial cells, ductal tree hypoplasia occurred (28). TGF-β also regulates development of the alveolar epithelium. Transgenic mice harboring active TGF-β were unable to lactate because of the inhibition of secretory epithelium

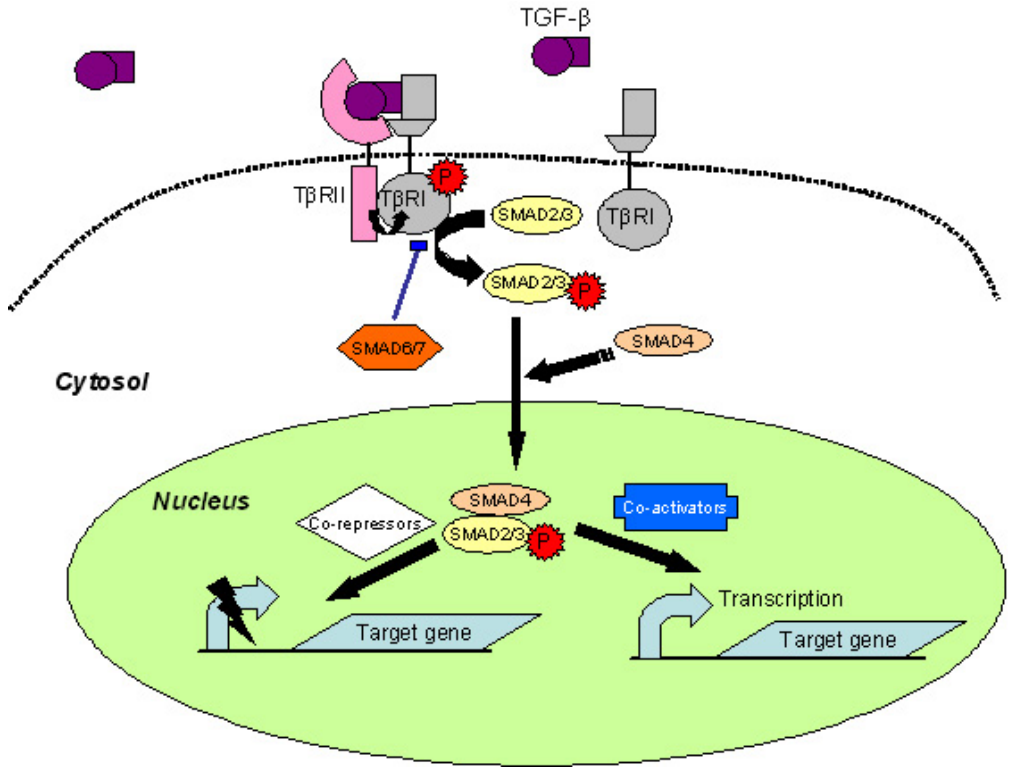

Figure 1. TGF-β/Smad signaling transduction pathway. TGF-β binds to TGF-β receptor II (TβRII) on cell surface, which recruits and activates TGF-β receptor I (TβRI), a serine-threonine kinase. Activated TGF-β/TβRII/TβRI complex phosphorylates Smad2/3 that in turn associates with Smad4 and moves to nucleus. In the nucleus, Smad complex binds to DNA and regulates targeting gene expression through recruitment of transcriptional co-repressors or co-activators. Smad6 or Smad7 are the inhibitory Smads which can competitively bind to TGF-β/TβRII/TβRI complex, inhibiting Smad2/3 phosphorylation.

development stemming from early apoptosis in differentiating alveolar cells (29). On the contrary, disrupting TGF-β signaling also causes inappropriate alveolar development. Mice carrying TβRII antisense RNA controlled by the MMTV promoter displayed precocious lobuloalveolar development, indicating a critical role of TβRII in maintaining non-differentiated status of virgin mammary gland epithelium (30). Furthermore, treating mice with slow-release plastic pellets containing TGF-β resulted in reversible regression of the end-buds in developing mammary gland during puberty, but not in the alveolar buds in pregnancy (31). This selective regression action indicates that TGF- β functions in cell type and/or tissue context-dependent manners and that TGF-β activity is differentially regulated during distinct stages of mammary gland development (32).

During pregnancy, TGF-β1 expression is decreased while TGF-β2 and TGF-β3 are elevated until the onset of lactation (33). Studies using specific antibodies that recognize latent and active TGF-β indicated that TGF-β1 activation was primarily localized in luminal epithelial cells, not in cap and myoepithelial cells (27). TGF-β1 promotes apoptosis during involution. In the involuting gland, TGF-β1 arises from days 1 to 10 after weaning, with an expression peak at day 6. This expression profile is consistent with bulk mammary epithelial cell death during post-lactational mammary gland involution (34). Finally, the response of the cultured TAC-2.1 epithelial cells varies with the concentrations of TGF-β. At picomolar levels, the TGF-β inhibited branching morphogenesis, whereas it stimulated at fentomolar levels (35). Therefore, the TGF-β signaling not only holds a pleiotropic role in mammary gland development, but is also tissue context- and ligand dose-dependent.

5. TGF-β SIGNALING IN MAMMARY GLAND TUMORIGENESIS

TGF-β promotes cell growth inhibition, apoptosis, and differentiation, and therefore, is considered as a potent tumor suppressor (36). However, recent studies indicate that TGF-β plays a dual role in mammary tumorigenesis. During advanced stages, TGF-β factually stimulates cancer cell invasion and metastasis (37, 38).

The involvement of TGF-β signaling pathway in tumorigenesis was documented first by functional changes of the signaling molecules in this pathway, such as TGF-β receptor and Smad mutations (37, 39-41). In human breast cancer, however, the alterations of TGF-β signaling molecules are relatively rare, except for TβRII downregulation (42, 43). Pathological studies of archived breast samples, including benign lesions, ductal carcinoma

in situ (DCIS), and invasive mammary carcinomas (IMC), indicated that TβRII downregulation correlated with progression and aggression of both in-situ and invasive breast carcinomas (44, 45). In mice expressing dominant-negative TβRII in mammary epithelium, spontaneous epithelial tumor occurrence was significantly increased (46). TβRI may also prevent mammary gland tumor formation. In mice carrying the Neu oncogene, active TβRI expressed specifically in the mammary epithelium diminished epithelial tumor appearance (47). In TGF-β1 and TGF-α double-transgenic mice, the frequency of tumors was significantly reduced compared to that in TGF-α transgene alone. In addition, mammary gland tumorigenesis induced by 7,12-dimethylbenz[a]anthracene was prevented by TGF-β expression (48).

Although its biological function remains unclear, TGF-β receptor III (TβRIII) may act as a suppressor of breast cancer. TβRIII has a short cytoplasmic domain, and therefore, its intrinsic kinase activity and role in TGF-β signaling need to be defined. However, the decrease or loss of TβRIII expression occurred in approximately 90% of breast cancer at mRNA levels and 70% at protein levels. In addition, TβRIII loss occurred at substantially high levels in advanced, invasive breast carcinomas. Therefore, TβRIII loss may be a negative prognostic factor for patients with invasive breast cancer (49).

Premature stem cells are involved in pathogenesis of mammary gland cancer (50). TGF-β induces premature senescence of mammary stem cells and thus may suppress tumorigenesis (50-52). This hypothesis was confirmed by a telomerase study. Telomerase underpins stem cell renewal and proliferation and thus enhances the occurrence of breast cancer. Li's report indicated that TGF-β can repress telomerase reverse transcriptase (TERT) expression by stimulating rapid entrance of Smad3 into nucleus (53). In the nucleus, Smad3 associates with c-myc, binds to the promoter region of TERT gene, and suppresses its expression. This negative regulation of telomerase activity can be interrupted by the Smad3 antagonist, Smad7 (53). Currently, TERT is proposed as a diagnostic and prognostic biomarker of breast cancer, as well as a potential therapeutic target of this disease (54).

On the contrary, considerable evidence indicates that TGF-β functions as a tumor promoter through the autocrine and paracrine actions, favoring tumor cell growth, invasion, and metastasis (55). In Neu transgenic mice, TβRI kinase activated c-myc and ligand-independent phosphatidylinositol-3 kinase (PI3K)/Akt in mammary cells, rendering cellular resistance to TGF-β-mediated growth arrest (56). In this model, TGF-β may synergize with the oncogene Neu to enhance survival and transformation of mammary epithelial cells.

A reduced response of tumor cells to TGF-β signaling often accompanies an increase in secretion of this ligand (57, 58). In breast cancer patients with poor prognosis, TGF-β1 levels were often elevated in plasma, tumor cells, and associated stroma (59-61). Factually, in transgenic mice expressing either activated or dominant-negative TGF-β ligands or receptors, a biphasic role of TGF-β signaling appeared in mammary tumor progression. In Neu transgenic mice, constitutively active TβRI increased the latency of mammary tumor formation, but enhanced the frequency of extravascular lung metastasis (47). On the contrary, the dominant-negative form of TβRII reduced tumor metastasis in Neu mice, but shortened the median latency of tumors induced by polyomavirus middle-T (47, 59). Constitutive expression of active TGF-β1 did not affect tumor latency in transgenic mice but enhanced the tumor invasiveness and metastasis to lungs (62, 63). All these findings indicate a tumor stage-related dual function of TGF-β signaling in mammary tumorigenesis.

The dual role of TGF- β in tumorigenesis also appears in human breast cancer. TβRII inactivation enhances the invasiveness of premalignant or low-grade breast tumor cells, but reduces the metastasis of high-grade tumors (64). As for TGF-β1, this ligand induces mammary epithelial-to-mesenchymal transition (EMT), resulting in the loss of epithelial polarity, disruption of cellular adhesion, and tumor cell invasion (65-67). TGF-β1 also upregulates integrin-linked kinase, increasing cellular motility (68). In addition, high levels of TGF-β1 mRNA correlated with enhanced angiogenesis and poor prognosis of breast cancer (69). Therefore, TGF-β may be an important regulating factor of tumor invasion and metastasis at later stages (63).

Tumor metastasis is a multifactorial event, including tumor cell invasion into stroma and formation of blood and lymphatic vessels (43). Investigations in the past decades have demonstrated the importance of peritumoral stroma in the development and/or evolution of tumors (70). Stromal cells support and facilitate tumor growth by secreting growth factors and/or proteases, such as vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), and TGF-β. These factors constitute a microenvironment that benefits tumor cell growth and progression (71, 72). Cheng's work (73) demonstrated that co-transplanting TβRII knock-out mammary fibroblasts with carcinoma cells promoted growth and invasion of tumor cells, associated with an increase in activity of several tyrosine kinase receptors: erbB1, erbB2, RON, and c-Met. In an *in vitro* assay, cancer cells cultured under fibroblast-conditioned medium showed increased proliferation and motility (73), indicating the role of stromal TGF-β signaling in neoplastic progression.

6. TGF-β PATHWAY AS A TARGET FOR CANCER THERAPY

Therapeutic potential of the TGF-β signaling pathway is derived from its supportive function in late-stage tumors, enhancing tumor invasion, neoangiogenesis, metastasis, and the escape of immunosurveillance (74). In addition, TGF-β signaling is also involved in anti-tumor drug resistance in *in vivo* and *in vitro* studies (75, 76). Current cancer therapeutic approaches that target the TGF-

β pathway include antagonism of TGF-β ligand binding to heteromeric receptor complex, intracellular inhibition of TβRI kinase, and sequence-specific degradation of TGF-β mRNA. Among them, the most extensively investigated intervention agents are sorted into small-molecule and large-molecule inhibitors (77, 78).

Ki26894, a TβRI kinase inhibitor, is a representative of small-molecule inhibitors. Systemic administration of Ki26894 via intraperitoneal injection effectively reduces the number and size of lung metastasis in both orthotropic xenografts and experimental metastasis models of human breast carcinomas (79, 80). Other small compounds such as SB-203580, an inhibitor of TβRI kinase (81), and SD-093 and LY580276, inhibitors of epithelial-to-mesenchymal transition (82, 83), all showed promising potency in suppression of tumor cell invasion and metastasis. In addition, TGF-β signaling often promotes metastasis by activating survival signals, such as epidermal growth factor receptors (84, 85); therefore, Gleevec, a specific tyrosine kinase receptor inhibitor, effectively blocks TGF-β-induced proliferation of human osteosarcoma cells (86).

Large-molecule inhibitors of the TGF-β signaling include peptides, monoclonal antibodies, and antisense oligonucleotides/antisense RNA (78). Short phospho-Smad peptide [pSmad3(-3), KVLTQMGSPSIRCSS(PO4)VS] is a specific substrate of active TβRI, inhibiting TGF-β-induced Smad2 phosphorylation in mouse mammary epithelial cells (82). Bioengineered protein composed of extracellular domain of TβRII and Fc domain of murine IgG1 heavy chain (Fc:TβRII) demonstrated capability of enhancing the apoptosis of primary tumors and inhibiting tumor cell motility, intravasation, and lung metastases (87). In Fc:TβRII transgenic mice, tumor metastasis to distant organs was significantly less than in wild type animals (87). In addition, a monoclonal anti-TGF-β antibody (1D11) significantly suppressed metastasis of highly metastatic 4T1 murine breast cancer cells to the lungs in animal studies (88).

DNA vaccine is another immunological approach in cancer therapy. TGF-β is known as an important factor regulating tumor cell migration toward blood vessels, the first step of metastasis (89). Tumor-associated macrophages (TAMs) are key players in this process through the production of a variety of factors, including TGF-β (89). Legumain is a protein specifically expressed in TAMs (90). An ongoing approach immunologically targets legumain to induce the destruction of TAMs, remodeling tumor microenvironments and inhibiting tumor growth and metastasis (91). In the 4T1 breast carcinoma metastatic model, a legumain-based DNA vaccine administered after surgical resection of primary tumors significantly increased the lifespan in 75% (6/8) of the experimental mice up to 3 months, and 62% of mice were completely free from metastases, demonstrating the effectiveness of TAM-targeted tumor growth and metastasis inhibition (92).

TGF-β antisense RNA is a novel strategy targeting TGF-β signaling pathway by triggering mRNA degradation (93). AP12009 is a complementary antisense RNA of TGF-β2 mRNA and showed promising therapeutic efficacy in animal tumor models with TGF-β2 overexpression, such as malignant glioma and pancreatic cancer (94). A recent study further demonstrated that combining the TGF-β antibody and antisense RNA completely regressed 4T1 tumors in 40% of the mice tested (95).

Compared to small-molecule inhibitors, these large-molecules are characterized with specificity and prolonged duration. However, their limited tissue penetration is a major concern for clinical applications. Small-molecule inhibitors penetrate tissue better, but their tumor-selectivity is usually low. Circumventing the shortcomings of large- and small-molecules and choosing appropriate treatment modalities for patients with different TGF-β signaling responses is a major challenge that needs to be more extensively investigated in the future.

7. CONCLUSION

In this article, we update the current understanding of TGF-β signaling, with focus on mammary gland development and tumorigenesis, and discuss the therapeutic perspectives of this pathway. TGF-β signaling pathway is complicated and functions in mammary gland in ligand content- and tissue context-dependent manners. TGF-β signaling also functions biphasically in tumor suppression and progression. TGF-β normally prevents uncontrolled cell proliferation; however, once aberrant genetic or epigenetic events abolish the cytostatic function of TGF-β, tumor cells evade TGF-β control and acquire the ability to proliferate, invade, and metastasize. Therefore, understanding the bifunctional features of TGF-β signaling in tumorigenesis is important to the development and the clinical practice of antitumor agents targeting this pathway. In addition, development of more specific agents with better tissue penetration would be the effort of the investigators in cancer therapeutic studies.

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Abbreviations: TGF: Transforming growth factor; TβRII: TGF-β type II receptor; TβRI: TGF-β type I receptor; TβRIII: TGF-β type III receptor; MMTV: mouse mammary tumor virus; TERT: telomerase reverse transcriptase; and TAMs, Tumor-associated macrophages

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