Role of the Type III TGF-beta Receptor Cytoplasmic Domain in Breast Cancer Progression

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

2009
ABSTRACT

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Abstract

Breast cancer remains among the most common cancers of the developed world. Despite advances in treatment modalities, deaths due to breast cancer are the second leading cause of cancer death among women. The transforming growth factor-beta (TGF-β) pathway is an important modulator of breast cancer progression, acting in a tumor suppressing fashion in early carcinogenesis but switching in a poorly understood fashion to a promoter of cancer progression in later stages. Mutations and loss of function of TGF-β components are common across a variety of cancers. In particular, the expression of the type III TGF-β receptor (TβRIII) is decreased with cancer grade and clinical progression in prostate, lung, ovarian, and pancreatic cancers. In an effort to enhance our understanding of the biology of TGF-β on carcinogenesis, this dissertation looks at the role of TβRIII in breast cancer progression.

Through an examination of clinical specimens, loss of TβRIII was seen at both the message and protein levels with increasing tumor grade. Analysis of correlated patient outcomes showed that low TβRIII expression was predictive of a shorter time to recurrence, demonstrating clinical relevance for TβRIII expression. The contribution of TβRIII to tumor progression was further examined by examining known TGF-β functions, including proliferation, apoptosis, migration, and invasion. TβRIII had no effect on proliferation or apoptosis, but had a suppressive effect on metastasis in vivo, as mammary cancer cells stably expressing TβRIII that were orthotopically injected exhibited lower metastatic burden and local invasion. In vitro, breast cancer cells exhibited suppression of migration and invasion in transwell assays. Finally, soluble TβRIII (sTβRIII) was shown to recapitulate the suppressive effects on invasion.
To further explore other potential mechanisms by which T\(\beta\)RIII may be mediating its tumor suppressive effects, I examined the contribution of the cytoplasmic domain of T\(\beta\)RIII, which is known to be critical in the regulation of T\(\beta\)RIII cell surface expression and downstream signaling. \textit{In vitro}, I demonstrated that abrogation of the cytoplasmic domain attenuates the T\(\beta\)RIII-mediated suppression of migration and invasion. T\(\beta\)RIII’s suppressive effects are also concomitant with loss of TGF-\(\beta\) signaling, as abrogation of the cytoplasmic domain failed to attenuate TGF-\(\beta\) signaling while the full length receptor was able to do so. \textit{In vivo}, I also showed that in the absence of the cytoplasmic domain, T\(\beta\)RIII is unable to suppress metastasis and local invasion. Finally, a closer dissection of the cytoplasmic domain revealed that abolishing the interaction of T\(\beta\)RIII with the scaffolding protein GIPC also attenuated T\(\beta\)RIII’s ability to dampen TGF-\(\beta\) signaling and invasion.

In sum, T\(\beta\)RIII was established as a prognostic marker for recurrence-free survival of breast cancer patients and as a suppressor of metastasis, migration, and invasion. Furthermore, several mechanisms contribute to T\(\beta\)RIII’s tumor suppressive effects, namely the generation of sT\(\beta\)RIII and the interaction of T\(\beta\)RIII with GIPC. Taken together, these studies further demonstrate the importance of TGF-\(\beta\) signaling in cancer biology, elucidate mechanisms by which T\(\beta\)RIII suppresses breast carcinogenesis, and expand upon our understanding of the emerging roles of T\(\beta\)RIII in regulating tumor biology in general.
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<td>ALK</td>
<td>Activin receptor-like kinase</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-Dimethylbenz(α)anthracene</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular domain</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>Erk</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GIPC</td>
<td>GAIP-interacting protein C-terminus</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>H &amp; E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HER-2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HHT-2</td>
<td>Hereditary hemorrhagic telangiectasia type 2</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
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<tr>
<td>IL-1R</td>
<td>Interleukin-1 receptor</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>Message ribonucleic acid</td>
</tr>
<tr>
<td>MSP</td>
<td>Macrophage stimulating protein</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plaminogen activator inhibitor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIN</td>
<td>Prostatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>POD</td>
<td>Postoperative day</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>SERM</td>
<td>Selective estrogen receptor modulator</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2-containing inositol phosphatase</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>shGIPC</td>
<td>Short hairpin RNA to GIPC</td>
</tr>
<tr>
<td>SMURF</td>
<td>Smad ubiquitination regulatory factor</td>
</tr>
<tr>
<td>sTβRIII</td>
<td>Soluble type III transforming growth factor-beta receptor</td>
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<td>TβRIII</td>
<td>Type III transforming growth factor-beta receptor</td>
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<td>TβRII</td>
<td>Type II transforming growth factor-beta receptor</td>
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<tr>
<td>TβRI</td>
<td>Type II transforming growth factor-beta receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
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1. Introduction


Targeted therapies for breast cancer rely on an understanding of cellular signaling in both normal and neoplastic tissue. One signaling pathway, the transforming growth factor-beta (TGF-β), is an important regulator of both normal mammary gland development as well as mammary carcinogenesis. It is involved in a wide variety of cellular processes, including proliferation, apoptosis, migration, and invasion, in addition to contributing to angiogenesis and modulation of the immune system. With the wide variety of roles it plays, TGF-β has sometimes diametrically opposed effects that are cell and context specific. Moreover, TGF-β possesses a unique dichotomy of function in cancer progression. Generally acting as a tumor suppressor early in breast cancer progression, TGF-β signaling undergoes a still unrevealed switch that leads to tumor promotion in the later stages of cancer. Highlighting the complexities inherent in TGF-β signaling, along with our current efforts to better our understanding of it, several strategies are outlined that may enable the creation of focused therapies in the prevention and treatment of breast cancer.
1.1 Overview

Worldwide, breast cancer is one of the most significant causes of cancer morbidity and mortality with over one million new cases and 400,000 deaths every year globally [1]. Through advances in detection and treatment modalities, the number of women with poor outcomes after diagnosis has steadily decreased. The prevalence of breast cancer remains high, however, justifying continued studies into the etiology and biology of breast cancer with the aim of further reducing morbidity and mortality.

Chemotherapeutic and targeted agents are used in adjuvant and neoadjuvant settings in combination with surgical resection and radiation therapy for the treatment of breast cancer. Until recently, chemotherapeutic drugs were blunt instruments lacking specificity for breast cancer cells. The relatively recent introduction of targeted therapies heralded a new paradigm of breast cancer treatment, promising greater specificity and thus a lower attendant potential for toxicity. The selective estrogen receptor modulator (SERM) tamoxifen and the HER-2/neu receptor antagonist trastuzumab, targeting ER-positive and HER-2-overexpressing breast cancers respectively, have revolutionized the treatment of breast cancer patients. The demonstrated efficacy of these targeted drugs provides proof of principle for developing rational, targeted strategies for breast cancer therapy based on studies of both normal and tumor biology.

The transforming growth factor-beta (TGF-β) signaling pathway, an important regulator of normal mammary gland development and homeostasis whose disruption is a common event during mammary carcinogenesis, is a pathway with promise for potential chemotherapeutic targeting. As such, what follows is background of TGF-β biology at the molecular, cellular, and systemic levels in normal and tumor states and the
potential for and challenges posed by targeting the TGF-β pathway in the prevention and treatment of breast cancer.

1.2 The TGF-β Signaling Pathway

1.2.1 Canonical TGF-β signaling

The canonical signaling cascade for TGF-β involves the binding of TGF-β ligand dimer to its cognate cell surface receptors. TGF-β binds either to the type III TGF-β receptor (TβRIII, or betaglycan) dimer, which in turn presents TGF-β to the dimeric type II TGF-β receptor (TβRII), or binds directly to TβRII. Once either of these occurs, ligand binding to TβRII favors recruitment and interaction with the type I receptor (TβRI) dimer, forming a multimeric ligand-receptor complex. In this complex, TβRII transphosphorylates serine residues on TβRI in the cytoplasmic domain, thereby activating TβRI serine/threonine kinase activity. Activated TβRI then recruits and phosphorylates receptor Smads. Depending on the ligand, members of the Smad2/3 axis or the Smad 1/5/8 axis are activated. Once phosphorylated, these receptor Smads bind to Smad4, a co-Smad. This complex then translocates to the nucleus and directly interacts with other transcription factors to regulate expression of TGF-β-responsive genes in a cell- and context-specific manner [2, 3]. Concomitant with activation of TGF-β responsive genes, a negative feedback loop attenuates activation of the pathway with the upregulation of the inhibitory Smad Smad7. Smad7 competes with the receptors Smads for binding to the activated receptors and recruits an E3 ubiquitin ligase, the Smad ubiquitination regulatory factor (SMURF), which consequently leads to internalization of the receptor/Smad7/SMURF complex for proteasomal degradation [4].
TGF-β binds to either TβRIII or TβRII which leads to complex formation with TβRI. Activated TβRI phosphorylates Smad2 or Smad3. The Smad2/3 complex with Smad4 translocates to the nucleus and regulates target gene transcription.

1.2.2 Non-canonical TGF-β signaling

In addition to the canonical signaling pathway, TGF-β signaling has also been demonstrated to elicit downstream effects through crosstalk with other signaling pathways, including the NFκB, p38, and JNK MAPK pathways. TGF-β and NFκB signaling have been shown to exhibit crosstalk at several points along their respective pathways. At the receptor level, interleukin-1 (IL-1) and its cognate receptor interleukin-1 receptor (IL-1R) are able to stimulate Smad signaling; likewise, TGF-β2 and the TGF-β receptor complex can stimulate NFκB. This signaling crosstalk requires the interaction of the TGF-β receptor complex with the IL-1 receptor complex [5]. In a neuronal model system, TGF-
β1 activation of NFκB could be recapitulated by the addition of a constitutively active type I receptor, ALK1 [6]. Another important modulator of NFκB signaling is transforming growth factor beta activated kinase-1 (TAK1), a MAPKKK member that is itself activated by TGF-β stimulation. This particular kinase has been shown in various model systems to activate NFκB, in addition to having effects on downstream MAPK effectors such as p38 and JNK MAPK [7-9].

In addition, effects on non-canonical signaling are seen by the ability of TGF-β pathway components to activate p38 mitogen-activated protein kinases (MAPK). In an NMuMg mouse mammary model, a mutant form of TβRII unable to phosphorylate downstream Smads demonstrated that TGF-β activation of p38 could occur independent of Smad signaling [10]. Phosphorylated p38 MAPK also has been shown to elicit increased activation of Smad2 [11]. In addition, TβRIII has been shown to activate p38 MAPK activation in a ligand independent manner, and this activation is dependent on an intact cytoplasmic domain [12].

There is also crosstalk between the TGF-β and JNK MAPK pathways. JNK can phosphorylate Smad3, which consequently leads to the potentiation of TGF-β dependent Smad3 activation and enhancement of Smad3 translocation to the nucleus. In addition, stimulation of Mv1Lu cells with TGF-β leads to a biphasic activation of JNK; the peak of the first phase of activation occurs within a relatively rapid 10 minutes [13, 14]. JNK can also enhance TGF-β mediated Smad3 signaling through the phosphorylation of the linker region, further evidence that there is crosstalk between these pathways [15]. Thus, JNK MAPK can impinge on the TGF-β pathway to modify TGF-β gene responses, while the rapid activation of JNK by TGF-β suggests a complementary Smad-independent mechanism to elicit JNK-mediated downstream effects.
In addition to these effects, TGF-β can effect changes on Rho GTPases. In particular, TGF-β stimulation can regulate the activation of GTPases involved in cytoskeletal organization, such as Cdc42 and RhoA. In a prostate cancer cell line, TGF-β stimulation induced a rapid 15-minute peak of activation of Cdc42 and a slightly longer time to peak activation of RhoA [16]. Similar results were found in NMuMg cells in which TGF-β stimulation led to a rapid induction of RhoA activation in as little as 5 minutes, with peak induction occurring at 15 minutes [17]. Effects on these GTPases have also been shown to occur in the absence of ligand stimulation. In an ovarian cancer cell system, overexpression of TβRIII led to constitutive activation of both Cdc42 and Rac1 in the absence of exogenous ligand [18]. Coupled with the fact that these GTPases can regulate NFκβ and MAPK activity themselves [19], the complexity of the crosstalk among the above described pathways becomes clear, and likely accounts for the context-specific and highly variegated signaling and cellular functional effects seen across different biological systems.

1.3 Effects of TGF-β on Cancer Biology

The relative simplicity of the canonical TGF-β signaling pathway as outlined above belies the complexities of TGF-β-mediated biology. The TGF-β superfamily of growth factors influences a wide variety of biological processes in both normal and disease states. Physiologically, TGF-β plays prominent roles in development, wound healing, and modulating immune system responses. Subversion of these normal processes by TGF-β misregulation can contribute, for example, to abnormal fibrotic changes, immunosuppression, and carcinogenesis. Underlying these systemic manifestations, TGF-β signaling is involved at the cellular level in the intricate regulation of proliferation, apoptosis, differentiation, extracellular matrix deposition, migration, and
invasion. The following discussion examines the role of TGF-β signaling in cellular and systemic processes relevant to breast cancer development and progression.

1.3.1 Regulation of Proliferation

Proliferation is perhaps the most well-characterized role for TGF-β in carcinogenesis. Normally, TGF-β acts as a potent inhibitor of proliferation. TGF-β activation leads to cell cycle arrest at the G1/S phase. TGF-β signaling primarily impinges on proliferation through the upregulation of cyclins and the cyclin-dependent kinase inhibitors, including p15 [20] and p21 [21]. Furthermore, activation of TGF-β signaling represses c-myc [22], which itself has also been shown to be involved in the repression of p15 and p21 in a feedback loop [23].

During cancer progression, however, cells become resistant to TGF-β-mediated inhibition of proliferation and instead TGF-β can promote a malignant phenotype. In the MDA-MB231 breast cancer cell line, as well as the Ras-transformed MCF-10A breast line, c-myc repression was found to be lost, in contrast to non-cancer breast cells. Importantly, inhibition of proliferation was found to be dependent on the binding of TGF-β activated Smad complexes to the c-myc promoter to repress transcription. In these breast cancer models where c-myc repression was lost, this Smad complex binding was attenuated [24]. Resistance to TGF-β mediated inhibition of proliferation is seen in a gastric cancer model as well. A panel of micro RNAs inappropriately overexpressed in gastric cancer were identified, and two of these were shown to inhibit the TGF-β mediated expression of p21 [25]. Thus, the loss of TGF-β mediated inhibition of proliferation can contribute to the progression of cancer.
1.3.2 Regulation of Apoptosis

In addition to proliferation, another mechanism by which cell number can be controlled is apoptosis, or programmed cell death. TGF-β mediates the induction of apoptosis in normal epithelia as well as in hematopoietic cells. TGF-β and its family members influence prototypical regulators of apoptosis, including upregulation of the pro-apoptotic factors Bax and caspases [26, 27], and downregulation of the anti-apoptotic Bcl-2 family members [28]. In hematopoietic cells, TGF-β activation of Smads leads to the transcriptional activation of the inositol phosphate SHIP, which itself acts to inhibit Akt-mediated cell survival [29]. DAP-kinase, a protein responsible for triggering downstream mitochondria-associated apoptotic factors, has also been shown to play a critical role in apoptosis triggered by TGF-β. TGF-β stimulation leads to a Smad-dependent transcriptional activation of DAP-kinase, which consequently leads to increased apoptosis [30]. In addition, in an example of a TGF-β receptor interaction that is involved in the regulation of apoptosis, TβRII has been shown to interact with the pro-apoptotic protein Daxx, a protein that is associated with the Fas-receptor and JNK-mediated programmed cell death. Furthermore, the interaction of TβRII and Daxx is critical in mediating TGF-β induced apoptosis in hematopoietic cells [31].

Similar to inhibition of proliferation, TGF-β mediated regulation of apoptosis also undergoes a switch in cancer progression. While TGF-β can modulate the tumor suppressor p53 by inducing p53 activity and consequent apoptosis in a Smad-dependent manner [32], there is evidence in certain systems that TGF-β signaling in fact suppresses apoptosis [33]. In MCF-7 breast cancer cells, over-expressing dominant negative TβRII or treating with a TβRI inhibitor increased apoptosis compared to normal breast epithelia [34]. Furthermore, micro RNAs overexpressed in gastric cancer were
found to have inhibitory effects on TGF-β mediated activation of the pro-apoptotic mediater BIM, in addition to their negative effects on p21-mediate cell cycle arrest [25].

1.3.3 Regulation of Angiogenesis

Tumorigenesis eventually reaches a point where the volume of the tumor begins to outstrip the ability of blood vessels supplying oxygen and nutrients to adequately supply the tumor’s increasing metabolic needs [35]. Angiogenesis is the process by which tumors recruit vasculature to meet these needs, and thus plays an important role in cancer progression. This makes targeting angiogenesis an attractive strategy for pharmacotherapy; for example, the efficacy of bevacizumab, a monoclonal antibody directed against the angiogenic activator vascular endothelial growth factor (VEGF), in treating metastatic colon and non-small cell lung cancers validates the clinical utility of anti-angiogenic agents. In concert with VEGF and other growth factors critical in angiogenesis, TGF-β plays an important role by upregulating their secretion into the cellular milieu. TGF-β induces VEGF expression in a smooth muscle cell model [36]. In addition, TGF-β stimulation under hypoxic conditions was able to induce the expression of VEGF in a hepatoma line [37]. Similarly, a gene signature describing breast cancer cell lines whose metastases target bone revealed the overexpression of the pro-angiogenic factor connective tissue growth factor (CTGF), and importantly this growth factor was expressed to a greater degree with TGF-β stimulation [38].

Along with upregulation of these growth factors, TGF-β also regulates the activity of endothelial cells responsible for the composition of the local vascular milieu. Recruitment of these vascular endothelial cells requires the breakdown of mature vessels and the basement membrane. This process is mediated in part by the activation of matrix metalloproteinases (MMP’s), which enhance the migratory and invasive
abilities of endothelial cells necessary for angiogenesis. TGF-β has been shown to be sufficient for the upregulation of TGF-β; in a breast cancer line, for example, TGF-β upregulates MMP-9 to induce angiogenesis [39]. Vascular endothelial cells also exhibit a reduction in cell adhesion in response to TGF-β, contributing in yet another fashion to the pro-angiogenic function of TGF-β [40, 41]. Thus, TGF-β signaling triggers enhanced growth factor and endothelial cell activity in vitro.

Additionally, TGF-β signaling is not only sufficient but critical in inducing angiogenesis in vivo in animal models and humans. Overexpressing TβRIII, in part through an inhibition of TGF-β signaling through the sequestering of ligand, decreases angiogenesis in both the primary and metastatic tumor of an orthotopic breast cancer model [42]. Downregulation of TGF-β signaling as a result of the deletion of TGF-β signaling pathway components such as TβRI, TβRII, and TβRIII in mice leads to the appearance of vascular defects, particularly in embryonic development [43-46]. Genetic manifestations of aberrant TGF-β signaling can result in defects of angiogenesis. Mutations of the type III TGF-β receptor endoglin and type I receptor ALK-1 in humans are linked to defective vascular formation in hereditary hemorrhagic telangiectasia type 2 (HHT-2) [47, 48]. In breast and lung cancer patients, elevated levels of circulating TGF-β correlate with increased microvessel density and poor prognosis [49, 50]. Taken together, the above findings demonstrate that TGF-β signaling has a positive effect on angiogenesis.

1.3.4 Regulation of the Immune System

Another facet that affects cancer progression is the systemic surveillance of cancer cells, for which the immune system is responsible. One of the various roles that TGF-β plays is its modulation of the immune system. TGF-β signaling has an overall
suppressive effect on the immune system, specifically on cellular immunity mediated by T-cells and antigen presenting cells (APCs). TGF-β influences T-cell development at the level of both proliferation and differentiation. TGF-β suppresses T-cell proliferation, as seen in CD4+ cells [51] and CD8+ cells [52], and promotes T-cell differentiation, as exemplified by the conversion of naïve CD4+CD25- T-cells to regulatory CD4+CD25+ T-cells [53] and by the conversion of naïve T-cells to helper T-cells [54]. Given the intimate interaction between T-cells and APCs required for cell-mediated immunity, it is not surprising that TGF-β also regulates APCs themselves. Mice with conditional dendritic cell knockout of a TGF-β-activating integrin lack regulatory T-cell activity and display an autoimmune phenotype in the colon [55]. TGF-β also inhibits macrophage activation [56] and Langerhans cell development [57].

Enhanced TGF-β activity, as seen for example with higher TGF-β levels associated with increased tumor burden, would therefore be predicted to promote immunosuppression. This would create an environment whereby cancer cells could more effectively evade immune system surveillance. Findings from mouse models are consistent with this notion. Abrogation of TGF-β signaling through the use of transgenic dominant negative TβRII in either CD4+ or CD8+ T-cells are able to more effectively eliminate tumors derived from thymoma or melanoma cell lines [58]. In addition, deletion of Smad4 in T-cells of mice leads to an enhanced formation of gastrointestinal carcinomas [59]. In both breast cancer and colon cancer models, IL-17 secreted by CD8+ cells promotes tumor growth. This effect is enhanced with TGF-β as TGF-β signaling results in increased IL-17, and blockade of TGF-β signaling with neutralizing antibody in vivo reduces IL-17 expression and consequently increases apoptosis [60]. Taken together, TGF-β signaling has a suppressive effect on the immune system; given
the role of the immune system in scavenging cancer cells and preventing tumorigenesis, enhanced local and systemic TGF-β levels associated with cancer progression serve to help tumors evade the body's defenses.

1.3.5 Regulation of Migration and Invasion

The TGF-β signaling pathway is a key regulator of cellular migration and invasion through effects on cellular adhesion, motility, and the extracellular matrix. In normal epithelia, TGF-β facilitates extracellular matrix deposition by increasing the biosynthesis of extracellular matrix proteins and by inhibiting the degradation of extracellular matrix proteins via upregulation of PAI-1 and TIMP-1 [61]. In the later stages of carcinogenesis, however, TGF-β fosters a permissive environment for migration and invasion by upregulating matrix metalloproteases (MMPs) and other enzymes that degrade extracellular matrix [62]. In an immunohistochemical analysis of breast tumors, TGF-β staining was concentrated at the tumor periphery as compared to the central tumor bulk [63], suggesting that TGF-β may play an important function in the invasive front of the tumor. Furthermore, in a pre-neoplastic breast epithelial line, TGF-β triggered the transcriptional induction of MMP-2 [64] and MMP-9 [65]. Interestingly, while TGF-β modulates p53-mediated apoptosis as previously discussed, p53 affects TGF-β-mediated migration. A p53 mutant was shown to downregulate TβRII and attenuate Smad2/3 phosphorylation, thereby decreasing migration in vitro [66].

TGF-β is an important regulator of epithelial-mesenchymal transition (EMT), whereby epithelial cells undergo a morphological and phenotypical change. This occurs in both normal and pathological processes, including embryonic differentiation, tissue development, and carcinogenesis. Important in this process is the cells assuming a more motile and invasive characteristic [67]. There are numerous cell types in which
TGF-β alone can enhance motility and invasion in this manner, including normal mammary epithelia [68, 69], breast cancer [70], pancreatic cancer [71], endothelial cells [72], and heart tissue [73]. The changes that take place after TGF-β activation differ by cellular context, but common traits shared among these tissues is a loss of cell-cell junctions, as well as a reorganization of the actin cytoskeleton to elicit a more motile phenotype. That TGF-β stimulation alone can induce the motility phenotype begs the question of whether downstream Smad activation is itself sufficient to propagate this signal. Indeed, in some contexts, this is the case. Activated Smad4 is essential for increasing migration associated with TGF-β induced EMT in pancreatic cancer [74], and the same requirement of Smad4 is similarly seen in a breast cancer model [75]. However, given the significant crosstalk with other pathways, it is not surprising to note that in certain other cellular contexts, non-Smad pathways are often required for this to occur. The p38 MAPK pathway has been shown to be required for EMT in the NMuMg breast cancer model [76, 77] with pharmacological inhibition of p38 MAPK abrogating TGF-β mediated EMT. In another breast cancer model system, NF-κB activation was required for TGF-β induced EMT [78]. ROCK/Rho signaling, an important mediator of cytoskeletal reorganization in its own right, has also been shown to be critical for the increased motility seen with TGF-β induced EMT, as inhibition of this pathway through dominant negative RhoA expression blocked TGF-β induced EMT [17].

What has become increasingly clear is that TGF-β signaling within the epithelia is not the only factor at play in the physiological context of cell migration and invasion. Indeed, in addition to the EMT that occurs with the cells themselves, contributions of the surrounding stroma have received increasing attention and study. From the deposition and breakdown of the extracellular matrix in which the epithelia reside, to the release of
paracrine cytokines and growth factors, fibroblasts take part in a complex interplay with the parenchyma. Generally, fibroblasts tend to keep their associated epithelia in check, with increased TGF-β signaling to the fibroblasts leading to the regulation of growth factor release and extracellular matrix reorganization hostile to tumorigenesis and local invasiveness. In a prostate cancer model, abrogation of TGF-β signaling through the use of dominant negative TβRII leads to prostatic intraepithelial neoplasia (PIN) [79]. Similar use of dominant negative TβRII in a breast cancer model led to a hyperproliferative and more invasive mammary carcinoma, with concomitant enhancement of secretion of growth factors TGF-α, MSP, and HGF and consequent activation of their cognate receptor tyrosine kinases [80].

As a cancer develops, fibroblasts can undergo a transdifferentiation into myofibroblasts in a TGF-β dependent manner [81]. Myofibroblasts facilitate tumor development and can play a significant role in stromal-epithelial interactions not only through the enhanced generation of matrix metalloproteinases and growth factors, but also in their ability to guide epithelia motility, thus enhancing migration and invasion of tumor cells. Conditioned media from myofibroblasts of colon tissue enhanced the migratory ability of epithelial cells in vitro, and increased TGF-β3 secretion by the myofibroblasts induced this effect [82]. Myofibroblasts themselves are more motile than fibroblasts, and this presumably facilitates myofibroblast invasion to localize with the burgeoning tumor to facilitate tumor escape. TGF-β signaling also plays a role in potentiating this myofibroblast motility, as co-culture of colon cancer cells secreting TGF-β enhances myofibroblast migration and invasion in Matrigel [83]. Further examination of the parenchymal-stromal will potentially yield a more intricate understanding of the enhancement of migration and invasion in tumorigenesis and metastasis.
Table 1: Summary of TGF-β functions

<table>
<thead>
<tr>
<th>Function</th>
<th>General effect of TGF-β signaling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation</td>
<td>↓</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>↑</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>↑</td>
</tr>
<tr>
<td>Immune surveillance</td>
<td>↓</td>
</tr>
<tr>
<td>Migration</td>
<td>↑</td>
</tr>
<tr>
<td>Invasion</td>
<td>↑</td>
</tr>
</tbody>
</table>

1.4 TGF-β in Cancer Progression

1.4.1 TGF-β pathway alterations in cancer

The cellular and systemic activities of TGF-β take on great clinical and therapeutic significance in the face of evidence that most human cancers exhibit alterations in the TGF-β signaling pathway. All three TGF-β receptors display evidence of mutation, deletion, or reduced protein expression with corresponding implications for tumor biology and clinical prognosis. Inactivating TβRI mutations are associated with advanced stages of breast cancer [84, 85], and are seen with frequency of up to one-third in ovarian cancers [86]. Loss of TβRII expression in prostate cancer is associated with tumor stage, Gleason score, and decreased 4 year survival rate [87]. Reduced expression or inactivating mutation of TβRII receptor has been observed in some breast
and colon cancers [88, 89]. Loss of TβRII expression in hyperplastic breast lesions is associated with an increased risk of invasive breast cancer [90], while reduced TβRII expression is associated with higher tumor grades [91]. TβRIII displays reduced expression in breast, prostate, and ovarian cancers, as well as in kidney, pancreatic, and lung cancers [42, 92-96].

In addition to mutations or changes in expression of receptors, changes in Smad levels have also been seen across different cancers. Germline mutations of Smad4 are seen in up to 50% of patients with the hereditary disorder juvenile polyposis, a condition predisposing patients to colon cancer [97]; mouse models consistent with this link are seen in heterozygous Smad4 knockout mice demonstrating increased gastrointestinal polyp formation [98]. Examination of a panel of pancreatic adenocarcinomas similarly revealed allelic loss of Smad4 [99]. Mutations or loss of the receptor Smads, Smad2 and Smad3, have also been reported in certain colon, lung, and gastric tumors, though the clinical significance of these mutations has not been as well-established as Smad4 [100-102]. Finally, alterations in Smad signaling components that negatively regulate TGF-β signaling reveal further clinical findings consistent with those of loss of function of Smad4, Smad2 and Smad3. Lower levels of the inhibitory Smad7 predicted better disease-free survival in colon cancer patients, while Smad7 duplication showed a decrease in disease-free survival [103]. Similarly, lower levels of Smurf2 predicted better survival rates, and high levels of Smurf2 expression were predictive of local invasiveness and lymph node metastasis in esophageal cancers [104].

TGF-β ligand expression is elevated in the late stages of a wide variety of cancers, including breast, colon, lung, gastric, pancreatic, and hepatic carcinomas [105]. With regards to downstream mediators, microarray analysis of human breast carcinomas
reveals that decreased levels of phosphorylated Smad2 are associated with decreased survival times in patients with stage II breast cancer [106]. Notably, a plethora of evidence points to TGF-β as a major player in breast cancer progression. Increasing circulating TGF-β levels are correlated with advancing stages of breast cancer [107], and elevated TGF-β levels, as well as those of the family member BMP-7, are prognostic of a poorer outcome for breast cancer patients [108, 109]. After surgical removal of cancerous breast tissue, circulating TGF-β levels decline, suggesting that reduced tumor burden leads to a decrease in TGF-β secretion [110]. In addition, an aggressive subpopulation of breast cancers featuring increased invasion and poorer outcome was associated with increased TβRII expression and TGF-β activation [111]. These findings demonstrate that increased TGF-β levels or activity are associated with later stages of breast cancer and less favorable prognoses.
<table>
<thead>
<tr>
<th>TGF-β pathway component</th>
<th>Tumor type</th>
<th>Alteration</th>
<th>Clinical outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>breast, colon, lung, gastric, pancreatic, hepatic</td>
<td>↑ expression</td>
<td>metastasis, lymph node metastasis, ↓ survival</td>
</tr>
<tr>
<td>TβRI</td>
<td>breast, prostate</td>
<td>↓ expression, inactivating mutation</td>
<td>↑ tumor grade, ↓ survival</td>
</tr>
<tr>
<td>TβRII</td>
<td>breast, colon, lung</td>
<td>↓ expression, inactivating mutation</td>
<td>↑ tumor grade, ↓ survival</td>
</tr>
<tr>
<td>TβRIII</td>
<td>breast, prostate, ovarian, lung pancreatic, kidney</td>
<td>↓ expression</td>
<td>↑ tumor grade, ↓ survival</td>
</tr>
<tr>
<td>Smad2</td>
<td>colon, lung</td>
<td>inactivating mutation</td>
<td>none observed</td>
</tr>
<tr>
<td>Smad3</td>
<td>gastric</td>
<td>↓ expression</td>
<td>none observed</td>
</tr>
<tr>
<td>Smad4</td>
<td>pancreatic, colon</td>
<td>↓ expression, inactivating mutation</td>
<td>↑ tumor grade</td>
</tr>
<tr>
<td>Smad7</td>
<td>colon</td>
<td>↑ expression</td>
<td>↓ survival</td>
</tr>
<tr>
<td>Smurf2</td>
<td>esophogeal</td>
<td>↑ expression</td>
<td>↓ survival</td>
</tr>
</tbody>
</table>
Figure 2: Alterations in the TGF-β signaling pathway in breast cancer.

The progression of human breast cancer is linked to changes at different levels of the TGF-β signaling cascade, from the levels of TGF-β ligand and its cognate receptors to the expression and activation of the downstream signaling mediator Smads.

Taken together with our current understanding of the biology of TGF-β signaling, these findings are seemingly paradoxical. Losses or deficiencies in TGF-β signaling are associated with increasingly malignant tumors, suggesting that decreased TGF-β signaling output is associated with advancing stages of cancer and that TGF-β acts as a tumor suppressor. Increased TGF-β levels, however, are also associated with increased tumor progression and poorer survival, suggesting that TGF-β promotes tumorigenesis. This dichotomy of TGF-β activity remains a central and fundamental focus of investigation. Delving into the intricacies of the complex interplay of tumor-suppressing and tumor-promoting activities is critical to our eventual understanding of TGF-β in
cancer and, importantly, in developing sound therapeutic strategies in breast cancer treatment.

1.4.2 Changes in TGF-β responsiveness – dichotomy of TGF-β signaling

The dichotomy of TGF-β signaling, whereby TGF-β has opposing effects in early versus late carcinogenesis, remains one of the mostly critically examined questions in studies of the multifaceted cytokine. As described above, alterations in specific components of the TGF-β signaling pathway can contribute to changes in TGF-β signaling during carcinogenesis. In addition to this, the dichotomy of TGF-β-mediated effects is explained by the observation that cells develop altered responsiveness to TGF-β or sensitivity to TGF-β signaling. Indeed, though the previously mentioned alterations in TGF-β signaling pathway components are found in various types of cancer, breast cancer is actually more likely to be associated with subtle alterations in cellular responses downstream of the receptors and effectors of TGF-β signaling. The majority of breast cancers is not associated with overt loss of function or expression of TβRII or TβRI, while TβRIII is lost at both the mRNA and protein levels in the progression from early to late stages of breast cancer in a majority of cancers [42]. Smads, including Smad2 and Smad4, are also not frequently mutated in human breast cancer [106, 112].

Indeed, the scaffolding of the TGF-β signaling pathway remains largely intact in human breast cancers. What ultimately determines TGF-β’s role during cancer progression—tumor suppression or tumor promotion—is the milieu and time point in which cells are exposed. Generally, TGF-β tends to act in a tumor suppressor role in normal tissue and in early carcinogenesis but as a tumor promoter in the later stages of carcinogenesis. Studies in animal models support this dual, context-dependent role for
TGF-β in various cancer models. In a skin cancer model, chemical promotion and initiation of tumor was induced with DMBA/TPA treatment on the skin of transgenic TGF-β mice. These mice exhibited a decrease in the number of benign skin papilloma formation, as would be expected in TGF-β’s tumor suppressive role as an inhibitor of proliferation. However, these mice went on to sustain a higher degree of conversion to malignant lesions, along with a great incidence of spindle cell carcinomas, demonstrating a switch in TGF-β’s behavior to that of a tumor promoter [113]. Targeted mammary expression of dominant-negative TβRII in the context of chemically-induced breast cancer enhanced the tumorigenicity of both mammary and pulmonary tumors [114]. In contrast, expression of dominant-negative TβRII in a mouse model of oncogenic Neu-driven breast cancer decreased tumor but decreased lung metastatic potential [115]. Targeted expression of activated TβRI in the mammary glands of oncogenic Neu mice elicited yet another response: delayed initial tumor onset but increased lung metastasis [115].

The precise mechanisms underlying the switch in the functional effects of TGF-β signaling remain unclear. An attractive possibility is that early tumor epithelia become gradually more resistant to the anti-proliferative effects of TGF-β, effectively driving an anomalous feedback cycle that increases TGF-β signaling. This TGF-β signaling eventually leads to autocrine and paracrine effects, both on the adjacent epithelia and the extracellular environment. In an increasingly unregulated fashion, TGF-β signaling ramps up to a critical point at which it becomes pro-oncogenic.

In this context, it should be noted that TGF-β effects depend not only on the epithelia *in situ* but also the surrounding extracellular matrix. The differential effects of TGF-β in epithelial versus mesenchymal cells is long-established, but more intense
study of the interplay between the tumor and its microenvironment has been undertaken only recently [116]. Stromal secretion of TGF-β contributes to the inhibition of proliferation and tumor progression in the epithelia [79]. Changes in stromal TGF-β signaling can also dictate cancer progression and the balance of oncogenic potential. One study demonstrated, for example, that fibroblasts lacking TβRII are able to induce an invasive phenotype in adjacent carcinomas, featuring increased proliferation rate, greater angiogenesis, and reduced apoptosis [80]. Furthermore, TβRII-null fibroblasts grafted into the mammary fat pads of wild-type mice induced epithelial changes in the new local microenvironment [80].

This interplay between the stromal and epithelial compartments lends credence to another explanation for the functional dichotomy of TGF-β in cancer progression. In this model, the carcinoma in situ derived from an epithelial cell type acquires traits of a mesenchymal cell in the process of EMT. Epithelial cells are characterized by a low level of motility, enhanced cell-cell contact, and flattened morphology. In contrast, mesenchymal cells exhibit relatively higher levels of motility and invasiveness, decreased cell-cell contact, and a spindle-like morphology. A subset of cancer cells at the primary site switch to a more mesenchymal phenotype. The more invasive mesenchymal cells can then break through the immediate extracellular matrix, including the basement membrane, greatly facilitating metastasis to secondary sites [67].

Interestingly, differences in TGF-β signaling in epithelial cells and mesenchymal cells parallel those of early and late carcinogenesis; that is, TGF-β in epithelial cells inhibits proliferation, migration, and invasion, whereas TGF-β tends to promote these processes in mesenchymal cells. We have found that TβRIII modulates the EMT-associated migration and invasion of pancreatic carcinomas. Specifically, during EMT TβRIII
expression decreases, and either membrane-bound or soluble TβRIII can inhibit this EMT-associated migration and invasion [95]. Evidence also implicates TGF-β and EMT in breast cancer progression. In one model, TGF-β induced EMT and consequent migration in a PI3K-dependent manner [117]. In another cell culture model of breast cancer, dominant negative TβRII was associated with a decrease in tumor progression with concomitant inhibition of EMT [118]. Thus, the EMT model presents an attractive explanation for the dichotomy of TGF-β signaling.

Importantly, alterations in cells’ responsiveness to TGF-β do not occur in a vacuum. As described by Hanahan and Weinberg, there are multiple hallmarks of cell behavior that distinguish normal from cancer cells, including the evasion of apoptosis, growth signal self-sufficiency, insensitivity to anti-growth signals, sustained angiogenesis, limitless replicative potential, and tissue invasion and metastasis [119]. Mindful of these changes during carcinogenesis, one must consider that alterations in other key mediators of oncogenesis impact TGF-β signaling. For instance, TGF-β counteracts mitogenic signals of epithelial cells through inhibition of proliferation under wild-type conditions, and by abrogation of TβRII alone, a hyperproliferative phenotype is observed. However, in cells made premalignant with KRAS oncogene expression, TGF-β’s role as a mediator of apoptosis becomes dominant, and abrogation of TβRII leads to a decrease in apoptosis and consequently enhances squamous cell carcinoma development [120]. In addition, an examination of metastatic breast cancer cells demonstrate intact TGF-β signaling components, while half of these samples demonstrate insensitivity to TGF-β mediated inhibition of proliferation through the induction of p15 and repression of c-myc. This deficiency in this subset of cells was found to be caused by an increase in a dominant negative isoform of the transcription
factor C/EBPβ, the aberrant expression of which causes a coordinate dysregulation of proliferation regulation [121]. Thus, differential effects of TGF-β in neoplastic disease versus normal cellular contexts must take into account the changes in the intracellular makeup of the cell that occur during cancer progression.
In early carcinogenesis, TGF-β plays a tumor suppressive role, affecting among other processes proliferation, and extracellular matrix deposition. TGF-β becomes tumor promoting, and can do so through two generalized alternatives. Components of the TGF-β signaling pathway can be lost or mutated (e.g., loss of Smad expression or cell surface receptor mutation), or cancer cells can subvert the functional effects of TGF-β tumor suppression and create an environment whereby TGF-β becomes tumor promoting. Adapted from Massague, 2008 [122].

1.4.3 TGF-β as a target for breast cancer therapy

Efforts are underway to target the TGF-β pathway for the prevention or treatment of breast cancer. Unlike other targeted therapies, however, the therapeutic strategy for TGF-β is not straightforward. The complex and sometimes diametrically opposed aspects of TGF-β signaling render the outcome of TGF-β inhibition difficult to predict.
Nevertheless, the varying degrees of success in initial forays into this field of TGF-β targeted therapy have laid the groundwork for more promising approaches.

Given the observed anti-proliferative effect of TGF-β in early carcinogenesis, one potential strategy for preventing the initiation or progression of early stage breast cancer is to functionally increase local TGF-β signaling. TGF-β seems to play an inhibitory role in the early stages of hormone-dependent breast cancers in particular [123]. In fact, tamoxifen use in ER-positive breast cancers augments TGF-β secretion and consequent growth inhibition [124]. In addition, a human breast cancer line treated with a histone deacetylase inhibitor displayed increased expression of TβRII, consequent downstream TGF-β signaling, and inhibition of cancer cell proliferation [125].

Despite these telling findings, to date no definitive evidence demonstrates high efficacy in directly exploiting the early anti-proliferative effects of TGF-β. The difficulties in employing a strategy of functionally increasing TGF-β signaling for prevention and treatment of early breast cancer are compounded by the contrasting role of TGF-β in later stages of cancer. Given that several models suggest that primary tumors initially inhibited by TGF-β signaling eventually give rise to more aggressive and malignant tumors, some serious considerations must be made. First, what constitutes an “early stage” of breast cancer in the spectrum of TGF-β function must be defined so as to avoid a scenario in which artificially enhanced TGF-β signaling unintentionally becomes tumor-promoting. The association of stage or grade with TGF-β functionality has not yet been rigorously established. Second, the present benefit of TGF-β-mediated delay in tumor onset must be weighed against the future risk a more aggressive tumor phenotype. The risk-benefit ratio for each individual patient would need to account for improvements in
morbidity and quality of life as well as the availability and efficacy of treatment strategies for the contingency of an increasingly aggressive tumor.

In comparison to the complications inherent to exploiting TGF-β’s tumor suppressing role in early carcinogenesis, the approach of targeting increased TGF-β signaling in later stages of cancer is much more straightforward. A number of therapeutic strategies designed to inhibit TGF-β signaling locally or systemically are currently being evaluated.

One attractive strategy for targeting breast carcinomas directly, one exploits the fact that both TβRI and TβRII are receptor kinases whose downstream effects are mediated by the phosphorylation of their target residues. High-throughput screening for specific activity against the TβRI and TβRII kinase domains have identified many promising small molecule inhibitors. A few compounds, including SB-431542, SB-505124, and SB-203580, have been shown to specifically inhibit TβRI with consequent inhibition of proliferation, angiogenesis, motility, and TGF-β-dependent transcription [126-128]. Another small molecule inhibitor, Ki26894, has been shown to selectively and effectively inhibit TGF-β responsiveness in a breast cancer-specific assay. In a xenograft mouse model of a highly metastatic human breast cancer cell line, application of Ki26894 significantly inhibited metastatic potential and prolonged survival time [129]. Continued small molecule discovery combined with testing in preclinical trials promises to expand the possibilities of carcinoma-directed TGF-β therapy.

A complementary approach for TGF-β inhibition is based on limiting the bioavailability of the TGF-β ligand itself. Increased circulating serum TGF-β levels are correlated with increasing tumor grade [105], making the attenuation of TGF-β ligand another avenue of intense investigation. At the cellular level, increased availability of
TGF-β for autocrine and paracrine signaling is thought to contribute to tumor-promoting activity, and TGF-β mediates important interplay between the epithelial and stromal compartments in the tumor microenvironment [116]. The use of neutralizing antibodies to sequester TGF-β ligand has yielded encouraging results. One such antibody has been used in a mouse xenograft model to abrogate TGF-β-mediated tumor progression and metastasis of the human breast cancer line MCF-7 [130], with similar results reported using another human cancer line MDA-MB231 [131]. Another way to sequester TGF-β ligand is to utilize soluble forms of the cognate TGF-β receptors which, when circulated, bind to free serum TGF-β and prevent signaling to cell surface receptors. A soluble form of recombinant TβRII has been used to inhibit breast cancer progression in a mouse model, both increasing apoptosis in the primary tumor and decreasing pulmonary metastatic potential [132]. Similarly, introduction of a soluble recombinant TβRIII reduced metastasis and angiogenesis in a xenograft of a human breast cancer line [133]. Importantly, soluble TβRIII affects other important mediators of breast cancer progression, including apoptosis [134], migration, and invasion [42].

In addition to carcinoma-directed strategies, therapies are now increasingly geared towards modulation of TGF-β specifically in the immune system. As TGF-β signaling suppresses the immune system in general and cell-mediated immunity in particular, an approach inhibiting TGF-β signaling in the immune compartment would be predicted to bolster immune surveillance of rogue cancer cells. In experiments demonstrating proof-of-principle using a syngeneic mouse model, expression of dominant negative TβRII in T-cells led to a marked decrease in metastases of murine melanoma and lymphoma lines [58]. In another experiment, CD8+ T-cells were sensitized in a nude mouse xenograft model of prostate cancer, manipulated to express
dominant negative T\(\beta\)RII, and then re-introduced into the mice. Remarkably, introduction of modified T-cells reduced metastasis compared to introduction of unmodified control T-cells [135]. These results illustrate the significant role of TGF-\(\beta\) in the interplay between tumor and cell-mediated immunity. Further, continued research into immune-targeted approaches for breast cancer therapy is justified, with simultaneous consideration for the undesired side effects of immunosuppression.

Because TGF-\(\beta\)’s sometimes antithetical effects depend on temporal and spatial context, a straightforward approach to utilizing TGF-\(\beta\)-modulating chemotherapeutic or biologic agents in the treatment of breast cancer is unlikely. However, initial preclinical investigations reveal opportunities which may be exploited to enhance the mode and timing of treatment. As our understanding of TGF-\(\beta\) signaling in proliferation, apoptosis, migration, invasion, angiogenesis, and immune system modulation continues to advance, so will our knowledge of how these diverse biological processes are implicated in breast cancer progression. As further efforts reveal the nuances of the TGF-\(\beta\) pathway, a wider range of successful therapeutic strategies will be developed. TGF-\(\beta\)-targeted therapies that minimize serious side effects while selectively and potently targeting breast cancer cells will add alternatives to traditional therapies to the clinician’s armamentarium.
1.5 The Type III TGF-β Receptor

The type III TGF-β receptor (TβRIII, also known as betaglycan) is a member of the family of TGF-β signaling receptors. It is an 851 amino acid cell surface receptor characterized by chondroitin sulfate and heparan sulfate proteoglycan chains found on the extracellular portion of the protein [136, 137]. TβRIII also has a short cytoplasmic tail (42 amino acids) that contains no known intrinsic kinase activity, suggesting that it cannot directly phosphorylate downstream mediators of signaling like TβRI or TβRII. However, the cytoplasmic domain does contain residues that are important for its interaction with scaffolding proteins, including β-arrestin2 as well as GAIP-interacting protein C-terminus (GIPC) [138, 139]. TβRIII is found ubiquitously in epithelial cells and is the most abundant and ubiquitously expressed TGF-β superfamily coreceptor. TβRIII
binds to a variety of ligands, including all three TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3), along with FGF, inhibin, activin, BMP-2, BMP-4, and BMP-7 [140-142]. TGF-β has classically been regarded as a co-receptor which presents ligand to the TβRI/TβRII receptor complex [143]. However, evidence points increasingly to a role for TβRIII in TGF-β signaling that is more complex than might appear at first glance. TβRIII is shed into the extracellular matrix as a soluble form cleaved from its short cytoplasmic domain [137], and this process has been shown to be mediated in part by MMP-1, a matrix metalloprotease [144]. This shed form of TβRIII has been shown across different models, including in breast and prostate xenograft models, to reduce downstream TGF-β signaling through the sequestration of ligand [133, 145]. Precisely how TGF-β signaling is modulated by TβRIII has yet to be fully defined.

Even though TβRIII has no signaling motif itself, recent studies suggest that TβRIII has essential, nonredundant roles for in regulating signaling through and independent of TβRI [141]. Knockout mouse models of TβRIII demonstrate TβRIII-null embryos that die on embryonic day 16.5, exhibiting defects in hepatic and cardiovascular development [146]. TβRIII has also been demonstrated to have an essential role in EMT during the development of endocardial cushion in chicks [46]. Similarly, TβRIII is critical in mediating differential TGF-β responses in gastrointestinal cells [147].

Mutations of TβRIII arising in patient tumor samples have not been reported, but altered expression of TβRIII has been well documented. In a minority of tumor types, increased TβRIII is associated with higher tumor grade, typically in liquid tumors examined. Non-Hodgkin’s lymphomas with high grade are associated with increased expression of TβRIII at the message level [148]. Gene expression arrays also revealed
upregulation of TβRIII in B-cell chronic lymphocytic leukemias (B-CLL) compared to normal B-cells [149]. However, the overwhelming evidence demonstrates loss of TβRIII with cancer progression across different tumor types. A decrease in TβRIII levels is seen in prostate cancer and is correlated with increasing tumor stage [92], while patients with non-small cell lung carcinomas having low TβRIII expression exhibit increasing tumor grade and disease progression [96]. Similarly, ovarian and pancreatic cancers with decreased TβRIII expression correlate with increasing tumor grade [93, 95]. In addition, staining of patient biopsies of endometrial adenocarcinomas demonstrated progressive loss of message and protein levels of TβRIII as neoplasias became more poorly differentiated [150]. Finally, renal cell carcinomas (RCC) exhibited loss of TβRIII at the message level compared to patient-matched normal renal tissue [94].

In sum, TβRIII plays a role in the modulation of TGF-β signaling through an incompletely understood process. This modulation of TGF-β signaling is critical and non-redundant. Furthermore, changes in TβRIII expression are seen in a wide variety of cancers, with loss of TβRIII expression being the overwhelming alteration observed particularly in solid tumors. Taken together, given that modulation of TGF-β signaling and changes in responsiveness to TGF-β in target cells underlie many of the phenotypes observed in breast cancer progression, the question of TβRIII’s functional role in breast cancer progression naturally follows. In this dissertation, I examine the role of TβRIII in breast cancer progression, particularly as it relates to mediating migration, invasion, and metastatic potential.
2. TβRIII as a suppressor of breast cancer progression

This is a modified version of a paper published in the JCI, which I contributed to by assisting with experimental design, as well as technical assistance and data analysis.


2.1 Introduction

Transforming growth factor-β (TGF-β) is a member of a superfamily of cytokines involved in regulating and mediating a variety of normal and pathological processes, including wound healing, fibrosis, and cancer progression [61, 151]. In the context of breast cancer, TGF-β plays important roles in both normal and pathological processes. In normal mammary epithelial cells, TGF-β functions in ductal and glandular development as an inhibitor of proliferation [152]. In mammary carcinogenesis, TGF-β initially acts as a tumor suppressor, as supported by murine models in which overexpressing TGF-β suppresses tumor formation [153] and a chemically-induced breast cancer mouse model in which overexpressing a dominant negative TβRII enhances tumor progression [114]. Throughout breast cancer development, however, cells become increasingly resistant to the antiproliferative effects of TGF-β, and TGF-β then functions as a tumor promoter. Indeed, dominant-negative TβRII decreased lung metastatic potential in oncogenic Neu mice, while targeted expression of activated TβRI in the mammary glands increased lung metastases [115].

Changes in expression of specific TGF-β pathway components can contribute to breast cancer progression. For example, loss of TβRII expression correlates with high tumor grade and tumor invasiveness in early cancer progression [90, 91], while in later
stages, increased TGF-β levels in breast cancer patients correlate with poor prognosis [108]. We have shown that TβRIII specifically is downregulated in a variety of cancers, including non-small cell lung, ovarian, pancreatic and prostate cancer models [92, 93, 95, 96]. Concomitant with these changes in TβRIII expression, enhancement of migration, invasion, and tumor progression have also been demonstrated. Given the wide variety of cancers in which loss of TβRIII expression is seen in cancer progression, we sought to define the role of TβRIII in breast cancer progression.

2.2 Materials and Methods

TβRIII gene expression analysis on cDNA filter array. A filter array containing normalized cDNA from 50 breast cancers and corresponding normal tissues (Cancer Profiling Array; Clontech; Takara Bio Co.) was probed with 32P-labeled cDNA probes for TβRIII following methods recommended by the manufacturer. In the 50 breast cancer samples, 33 were ductal carcinoma, 10 were lobular carcinoma, and 2 were tubular carcinoma; the remaining samples consisted of 1 each of mixed lobular/ductal carcinoma, medullary carcinoma, mucinous adenocarcinoma, fibrosarcoma, and DCIS. The TβRIII cDNA probe was PCR amplified using the forward primer GTAGTGGGTTGGCCAGATGGT and reverse primer CTGCTGTCTCCCCTGTGTG. Purified PCR products (25 ng) were labeled by random primed DNA labeling using α-32P-dCTP following the manufacturer’s protocol (Roche Diagnostics). Labeled cDNA probe was purified on a BD CHROMA SPIN+STE-100 column (BD Biosciences – Clontech). Images were acquired using a phosphorimager, and subsequent data analysis was performed using NIH ImageJ software (http://rsb.info.nih.gov/ij/). A normal/tumor ratio of 2 or higher was considered to be significant.
Breast cancer tissue array. A polyclonal antibody recognizing TβRIII protein was custom made by immunizing rabbits with a GST-fusion protein of the entire cytoplasmic domain of human TβRIII. The IgG fraction of pre-immune and immune rabbit serum was collected using Immuno-Pure IgG Purification kit (Pierce Biotechnology), and the specificity of the antibody was established by comparing staining of the breast cancer tissue arrays (Cooperative Breast Cancer Tissue Resource; National Cancer Institute) with preimmune serum and with the immune serum under identical conditions, by the specific pattern of staining of cells known to express TβRIII (breast epithelial cells) and lack of staining of cells known not to express TβRIII (lymphocytes), and by Western blot deparaffinized, rehydrated, treated with 3% hydrogen peroxide, and then blocked with 10% goat serum. The arrays were incubated with anti-TβRIII antibody overnight at 4°C, washed in PBS, and further incubated with HRP-conjugated anti-rabbit IgG (Vector Laboratory). Counterstaining was performed using hematoxylin. As a negative control, duplicate sections were immunostained with IgG purified from prebleed rabbit serum. The immunoreactivity for TβRIII in breast epithelial and breast cancer cells was relatively uniform within a specimen and was thus semiquantitatively scored by staining intensity in a blinded manner with 0-1 defined as no or weak staining, 2-3 as moderate staining, and 4-5 as intense staining. Standards for each staining score were used to maintain consistent scoring across specimens.

Cell culture and reagents: 4T1 cells stably transfected with the luciferase gene under puromycin selection were generously provided by MW Dewhirst (Duke University). MDA-MB231 cells were obtained from ATCC.
**Fibronectin migration and Matrigel invasion assays.** Fibronectin (Calbiochem) was coated on transwells (Costar) at a concentration of 50μg/mL in serum-free DMEM, while Matrigel transwells (BD Biosciences) were preincubated with serum-free DMEM for 2 hours at 37°C. For migration assays, 50,000 cells were plated in 200μL of serum-free DMEM in the upper chamber; for invasion assays, 200,000 cells were plated in 200μL of and allowed to migrate for 24 hours towards 600μL of 10% FBS in DMEM. Cells on the upper surface were scraped with a cotton swab, and wells were stained with 3 Step Stain Kit (Richard-Allen Scientific). Filters were then mounted on microscope slides with Vectamount (Vector Laboratories). Three random fields on each filter were counted. Cells were plated in duplicate, and each experiment was conducted at least three times.

**TGF-β binding and crosslinking assay.** For cell media, we incubated 100 pM 125I-TGF-β1 with 500 uL of the cell medium in the presence of protease inhibitors for 3 hours at 4°C. The 125I-TGF-β1-sTβRIII complex was then crosslinked with 0.5 mg/mL disuccinimidyl suberate and immunoprecipitated with a polyclonal antibody recognizing the extracellular domain of TβRIII (R&D Systems). The final complex was visualized after SDS-PAGE and autoradiography.

**In vivo metastasis.** Animal procedures were approved by the Institutional Animal Care and Use Committee of Duke University. Empty vector pcDNA3.1-neo and TβRIII lines were generated by transfecting 4T1 cells with Lipofectamine 2000 (Invitrogen), further selected with G418, and confirmed by 125I-TGFβ binding and crosslinking as previously described [42]. 25,000 cells were injected into the right axillary mammary fat pad of 6-week old virgin female Balb/c mice. The resulting primary tumors were excised, weighed, and measured with calipers (volume calculated by 0.52 x length x width²).
Bioluminescent imaging with intraperitoneal injections of 150 μg/g luciferin (Xenogen) was conducted with an IVIS camera (Xenogen) every three days. Regions of interest were defined automatically, and luciferase units expressed as photons/s/cm²/steradian. Background signal was defined by imaging a mouse without luciferin. Mice were sacrificed at the end of the study, organs visually inspected, and samples of interest preserved for immunohistochemical analysis.

**Immunohistochemistry.** Tissue samples were formalin fixed, paraffin embedded, and cut onto microscope slides. Slides were then either stained for hematoxylin and eosin, or prepared for immunohistochemical analysis. Staining for TUNEL (Roche Diagnostics) and PCNA (Santa Cruz) was performed according to manufacturers’ instructions.

**Statistical analysis.** All data are presented as mean +/- SEM. 2-tailed Student’s t-test was performed. p-values of less than 0.05 were considered significant.

### 2.3 Results

#### 2.3.1 Loss of TβRIII expression in breast carcinogenesis

As TβRIII is downregulated in cancers including non-small cell lung, ovarian, pancreatic and prostate [92, 93, 95, 96], the expression status of TβRIII in human breast cancer was investigated. A cDNA array of 50 human breast cancer samples with matched normal controls (Figure 5) was analyzed. In a vast majority of the samples examined (greater than 80% of the matched samples), TβRIII mRNA levels in tumor samples were lower than their patient-matched normal tissue samples.
Figure 5: TβRIII mRNA levels in normal versus patient-matched tumors

TβRIII mRNA levels were detected by hybridizing $^{32}$P-labeled human TβRIII cDNA probe to the Clontech Cancer Profiling Array I. The portion of the array containing breast samples is shown, with tumor specimens (T) and matched normal breast tissue (N). Data courtesy of Dr. Mei Dong, a post-doctoral fellow, whom I aided with analysis.

In addition to message levels of TβRIII, immunohistochemical analysis of patient tumors was conducted to confirm loss of TβRIII in breast cancer progression. A breast cancer tissue array was stained which contained 252 breast cancers of different stages (20 DCIS, 64 lymph node-negative, 64 lymph node-positive, and 64 distant metastases) and 40 normal breast specimens. These samples also had available clinical data including tumor size, TNM stage, number of nodes positive, invasive grade, ER status, and progesterone receptor (PR) status. TβRIII expression progressively decreased from normal to DCIS to invasive tumor, and in certain DCIS samples, TβRIII expression in normal glands served as a useful internal control (Figure 6). In addition, to directly assess the role of TβRIII loss of expression in breast cancer progression, matched tissue sets of normal breast and invasive breast cancer specimens, or matched DCIS and invasive breast cancer specimens were analyzed. Samples on the tissue array were scored for expression levels of TβRIII. When examining TβRIII expression in matched normal breast and invasive breast cancer specimens, TβRIII expression decreased in every case (10 of 10), with 6 cases decreasing from high expression (IHC score of 5) in normal breast tissue to low expression (IHC score of 0-1) in the matching
invasive breast cancer tissue (Figure 7). When examining TβRIII expression in matched DCIS and invasive breast cancer specimens, TβRIII expression decreased in 63% of the case (5 of 8), with 1 additional case where expression was already absent at the DCIS stage (Figure 8). These data indicate that TβRIII expression is significantly decreased in human breast cancer, with loss of TβRIII expression correlating with breast cancer progression.

![Image: Representative IHC analysis of TβRIII expression](normal, DCIS-1, DCIS-2, invasive cancer)

**Figure 6: Representative IHC analysis of TβRIII expression**

TβRIII expression in normal breast ductal cells, low grade DCIS (DCIS-1), high grade DCIS (DCIS-2), and invasive cancer. Magnification 10x. Data courtesy of Dr. Mei Dong, whom I aided with experimental design.
Figure 7: TβRIII expression in matched normal breast vs. invasive cancer

Immunoreactivity was scored on a scale of 0-5 and characterized as low staining (0-1), medium staining (2-3), or high staining (4-5). M. Dong and I scored the samples, while I conducted the data analysis.

Figure 8: TβRIII expression in matched DCIS vs. invasive cancer

Immunoreactivity was scored on a scale of 0-5 and characterized as low staining (0-1), medium staining (2-3), or high staining (4-5). Blue lines indicate a decrease of TβRIII in matched samples. Red lines indicate an increase of TβRIII in matched samples. M. Dong and I scored the samples, while I conducted the data analysis.
2.3.2 TβRIII expression attenuates metastatic potential in vivo

The frequent loss of TβRIII expression observed during progression to invasive disease suggested that TβRIII loss during mammary carcinogenesis may specifically promote tumor invasion and metastasis in vivo. To investigate a causal role for decreased TβRIII expression in breast cancer progression, the effect of TβRIII on in vivo tumor growth and metastasis was examined using a murine model for mammary carcinogenesis. Murine 4T1 mammary cancer cells, which are derived from a BALB/c murine mammary tumor, share many characteristics with human mammary cancers including spontaneous lung metastasis in immunocompetent mice and have been widely used as a model of breast cancer [154, 155]. The 4T1 cells were genetically engineered to express the firefly luciferase gene so that by periodically injecting the substrate luciferin into mice carrying these cells and taking bioluminescent images, in vivo growth and metastatic potential could be followed closely. The 4T1 cells were stably transfected with TβRIII (4T1-TβRIII) or control plasmid pcDNA-Neo (4T1-neo). The cells were then injected into the axillary mammary fat pads of BALB/c mice. The primary tumor was measured every 2 days starting from day 10 after injection and removed on day 20. Tumor metastases were then followed by bioluminescent imaging every 3 days over a period of 19 days. No significant difference was observed in the growth of the primary tumors from 4T1-TβRIII or 4T1-Neo cells as shown by tumor growth curve (Figure 9) and tumor mass at the time of resection (Figure 10), establishing that TβRIII had no effect on tumorigenicity in vivo. However, mice injected with 4T1-TβRIII cells demonstrated a significantly delayed onset of tumor metastasis as well as a significant reduction in both the size and number of lung metastases compared with the mice injected with control 4T1-Neo cells (Figure 11, Figure 12). Proliferation and apoptosis of
the primary tumor and metastatic lesions was also assessed with staining for the proliferation marker PCNA and apoptotic marker TUNEL. Consistent with the lack of significant difference seen in primary tumor volume or mass, there was no difference in PCNA staining or TUNEL staining in the primary tumors (Figure 13). In addition, metastatic lesions to the lung also exhibited no significant difference in proliferation or apoptosis (Figure 13). These data support a specific suppressor effect of TβRIII on metastasis, but not on primary tumorigenesis.

Figure 9: Primary tumor growth in vivo

Primary tumor growth was recorded by measuring tumor size every 2 days beginning at 10 days after injection and presented as mean +/- SEM. Data courtesy of Dr. Mei Dong, whom I aided with technical assistance conducting the experiment.
Figure 10: Primary tumor mass

Weight of the primary tumors upon surgical removal on day 20 after injection. Data are mean +/- SEM (n=16). Data courtesy of Dr. Mei Dong, whom I aided with technical assistance conducting the experiment.

Figure 11: Bioluminescent imaging of 4T1-Neo, 4T1-TβRIII

Bioluminescent imaging was performed every 3 postoperative days (POD). Representative images are shown. Red and violet signals correspond to the maximum and minimum intensity values, respectively, with other colors representing values in between. Data courtesy of Dr. Mei Dong, whom I aided with technical assistance conducting the experiment.
Figure 12: Average luminescent signal in each group

Average luminescent signal in each group at the indicated time points. ** p<0.01. Data courtesy of Dr. Mei Dong, whom I aided with technical assistance conducting the experiment.

Figure 13: Proliferation and apoptosis of primary tumor and metastatic lesions

Tissue sections of primary tumors and lung metastases from mice implanted with 4T1-Neo and 4T1-TRIII cells were immunostained for PCNA and TUNEL to evaluate cell proliferation and apoptosis, respectively. Representative staining frequency and intensity is shown (original magnification x40). Data courtesy of Dr. Mei Dong, whom I aided with histopathological analysis.
2.3.3 TβRIII expression attenuates invasion

Further pathologic examination of the primary tumors demonstrated that the 4T1-Neo tumors exhibited increased invasion of the surrounding normal mammary tissue (Figure 14A) and skin (Figure 14B), while the 4T1-TβRIII tumors exhibited little to no invasion and instead maintained a distinct margin with the adjacent normal tissue (Figure 14C). In addition, primary recurrences in the 4T1-Neo mice exhibited invasion of tumor cells into the blood vessels, resulting in internal hemorrhage (Figure 14D). Pathologic examination of tumor metastasis revealed distant metastasis to the mesentery (Figure 14E), the paratracheal lymph nodes (Figure 14F), and the cecum in addition to the lung in control 4T1-Neo mice, while 4T1-TβRIII exhibited only lung metastases. In addition, when lung metastases were observed in 4T1-TβRIII mice, these metastatic lesions were always small, well circumscribed, and isolated (Figure 14H, Figure 14I) compared to the large, locally invasive lung metastases observed in 4T1-Neo mice (Figure 14G).
Figure 14: Local invasiveness in 4T1-Neo, 4T1-TβRIII tumors

Representative H & E staining (original magnification, x10) of (A and B) primary tumors from mice implanted with 4T1-Neo cells exhibiting local invasion (red arrows) of tumor cells into the adjacent normal mammary tissue (A) and skin (B). C, a representative primary tumor from mice implanted with 4T1-TβRIII cells demonstrated the absence of local invasion, as indicated by the clear margin between the tumor and the adjacent normal mammary tissue (yellow arrow). D, a recurring tumor in a mouse at the primary injection site of 4T1-Neo cells exhibiting internal bleeding due to invasion of tumor cells into the blood vessels. E, A metastatic tumor (black arrow) adjacent to the pancreas (Green arrowhead) found on the mesentery of a mouse implanted with 4T1-Neo cells. F, a significantly enlarged paratracheal lymph node adjacent to the trachea (blue arrowhead) containing metastatic tumor cells (black arrow) in a mouse with 4T1-Neo cells, indicating the presentce of lymphatic metastasis. G, multiple large metastatic tumor nodules (black arrows) in the lung of a mouse implanted with 4T1-Neo cells. H and I, representative lung metastases in mice implanted with 4T1-TβRIII cells (black arrows). Data courtesy of Dr. Mei Dong, whom I aided with histopathological analysis.

To further define the mechanisms by which TβRIII regulated breast cancer invasiveness and metastasis in vivo, the effect of expressing TβRIII in breast cancer cell
lines in vitro was examined. Using the tumorigenic, invasive, and metastatic human breast cancer cell line MDA-MB231, TβRIII was overexpressed. Overexpression of TβRIII dramatically repressed the ability of MDA-MB231 cells to invade through Matrigel and significantly attenuated the responsiveness of MDA-MB231 cells to TGF-β induced invasion (Figure 15). This demonstrates a direct effect of TβRIII on inhibiting breast cancer cell invasion.

Figure 15: TβRIII-mediated suppression of invasion in vitro

MDA-MB231 cells were infected with equivalent amounts of adenoviral constructs carrying GFP or HA-tagged TβRIII. 75,000 cells were then seeded in a Matrigel-coated upper chamber and treated with TGF-β1 (15pM) 2 hours later. Cell invasion through the Matrigel after 24 hours incubation was detected by H & E staining and quantitated. Data courtesy of Dr. Mei Dong, whom I aided with experimental design.

2.3.4 Soluble TβRIII attenuates invasion

The extracellular domain of TβRIII can be proteolytically cleaved in the juxtamembrane region [156], and the resulting soluble TβRIII (sTβRIII) has been demonstrated to suppress tumor growth and angiogenesis, potentially through binding and sequestering TGF-β and preventing signaling through the membrane-bound receptor [157]. To assess whether the effects of TβRIII could be mediated by the
production of sTβRIII, media from 4T1 and MDA-MB231 cells were examined to
determine whether they produced sTβRIII. Conditioned media from each cell line was
collected, crosslinked with iodinated TGF-β1, and specifically immunoprecipitated
sTβRIII with an antibody to the extracellular domain. These studies confirmed that both
the 4T1-TβRIII and MDA-MB231-TβRIII cell lines produce a significant amount of
sTβRIII (Figure 16). Accordingly, the effect of sTβRIII on MDA-MB231 breast cancer cell
invasion in vitro was examined. Conditioned media collected from COS-7 cells
transiently transfected with full-length TβRIII or sTβRIII potently decreased TGF-β
induced invasion of MDA-MB231 breast cancer cells through Matrigel (Figure 17).
Detection of sTβRIII in media of MDA-MB231-TβRIII and 4T1-TβRIII cells. Cells were plated to confluence, media collected and debris removed. Media was then subjected to $^{125}$I-TGF-β1 binding and crosslinking followed by immunoprecipitation. Data courtesy of Dr. Mei Dong, whom I aided with optimization of the experiment.

Matrigel invasion assay was performed after resuspending MDA-Mb231 cells in the conditioned media collected from pcDNA3.1-Neo-, TβRIII-, and sTβRIII-transfected COS-7 cells. Data are mean +/- SEM, n=3 in triplicate. Data courtesy of Dr. Mei Dong, whom I aided with experimental design.
2.3.5 Decreased TβRIII expression predicts decreased recurrence-free survival

As decreased TβRIII expression is frequently observed in human breast cancers and restoring TβRIII expression decreased invasiveness and metastasis *in vivo*, there was the possibility that TβRIII expression could be a useful prognostic marker for breast cancer patients. This was done by examining publicly available microarray data sets in which both TβRIII expression and recurrence-free survival data were available [158-161]. TβRIII expression was set as a dichotomous variable, with high expression as above the mean and low expression as below the mean. In the largest data set (that of Wang et. al., [161]), composed of 286 patients with lymph node-negative breast cancers, low expression of TβRIII was significantly associated with a decrease in recurrence free survival (Figure 18, *p*=0.043), with recurrence defined as a distant metastatic event. The hazard ratio (HR) for recurrence based on TβRIII expression (HR, 1.569) was higher than that for ER status (HR, 1.18) or for Her2/Neu status (HR, 1.06) [162].
Figure 18: TβRIII and recurrence-free survival

Low levels of TβRIII predict decreased recurrence-free survival in women with breast cancer. Five-year recurrence-free survival for breast cancer with high or low TβRIII expression was analyzed based on a microarray data set containing 286 patients. Kaplan-Meier survival analysis was utilized, with log-rank test used to determine statistical significant. *p<0.05.

In addition, the predictive value of TβRIII was assessed to determine whether it was independent of other known prognostic factors. As all samples in the Wang et. al. data set [161] came from lymph node-negative patients, ER status was analyzed, which was the only other available prognostic factor available within the data set. A Pearson correlation coefficient of -0.08 (95% confidence interval, -0.19 to 0.036) supported little correlation between TβRIII expression and ER status, although the data set was not large enough to power the analysis (p=0.177). In 3 other completely independent data sets (Sorlie et. al., [158], containing 74 locally advanced ER-positive and –negative primary breast cancers; van’t Veer et. al. [159], containing 97 ER-positive and –negative lymph node-negative breast cancers; and Ma et. al., [160], containing 60 hormone receptor-positive breast cancers), there was a trend toward decreased recurrence-free survival associated with low TβRIII expression, although in each case the number of
patients was not large enough to reach statistical significance. Taken together, these
data suggest that TβRIII expression is predictive of recurrence-free survival in breast
cancer patients.

2.4 Discussion

In the present study, we demonstrate that expression of the TGF-β coreceptor
TβRIII was frequently decreased at the mRNA level and approximately 70%
demonstrating decrease or loss at the protein level. As previously discussed, loss of
TGF-β signaling components in breast cancer is a relatively rare event [106, 163]. Given
this, loss of TβRIII expression we see in breast cancer tissue samples makes it the most
common TGF-β signaling pathway alteration described in human breast cancer to date.
Loss of TβRIII expression was an early event, occurring initially in the preinvasive state,
DCIS, with degree of loss correlating with breast cancer progression. In addition, loss of
TβRIII has a functional effect in vivo and in vitro, as restoring TβRIII expression
dramatically inhibited tumor invasiveness in Matrigel and metastasis in the 4T1
orthotopic mouse model. Furthermore, decreased TβRIII expression is predictive of
recurrence free survival in patients, highlighting the significance of TβRIII expression on
clinical outcome in breast cancer patients. Taken together, these results support loss of
TβRIII expression as a frequent and important step in breast cancer progression, directly
promoting breast cancer invasion and metastasis.

How might loss of TβRIII expression contribute to breast cancer invasion and
metastasis? The answer may lie in TβRIII’s role as a modulator of TGF-β signaling.
Canonically thought of as a coreceptor, TβRIII initially was shown to enhance TGF-β
signaling by presenting ligand to the TβRII-TβRI complex [143]. However, TβRIII’s
effects on TGF-β signaling are undoubtedly more complex, as there is evidence that
TβRIII can also act to dampen TGF-β signaling through sequestration of ligand [133, 145], particularly in the setting of systemic administration of a soluble form of TβRIII.

Our data are consistent with the latter observation of TβRIII functioning as a dampener of TGF-β signaling, as cells expressing TβRIII additionally elicited sTβRIII generation (Figure 16), and conditioned media containing sTβRIII was sufficient to inhibit migration in Matrigel (Figure 17). This suggests that the invasive properties in vitro may in part be due to downregulation of TGF-β signaling, which is consistent with the overwhelming evidence in the literature defining TGF-β as a promoter of migration and invasion [68-73].

In addition, TGF-β signaling has effects on non-canonical signaling pathways. In particular, the TGF-β pathway has effects on other signaling pathways, including p38, JNK, and NF-κB as described in the Introduction. Given their roles in migration, attenuation of these pathways by decreased TGF-β signaling could contribute to the decrease in invasion and metastasis observed. In fact, TβRIII has been observed to inhibit breast cancer cell migration through the dampening of NF-κB signaling [164]. In addition, TβRIII may suppress invasion and metastasis independent of TGF-β signaling. TβRIII can suppress migration of breast cancer cells in a TGF-β independent, cytoplasmic domain-dependent manner [18], which opens the possibility that there may be similar effects on invasion and metastasis as well. Expression of TβRIII then may have an effect on migration, invasion, and metastasis not only via TGF-β signaling, but through TGFβ-signaling independent pathways as well.

The contribution of the full length TβRIII at the cell surface and its canonical function as a presenter of ligand cannot be discounted. Reconciling the complex, opposing effects of TβRIII function requires a closer examination of TβRIII itself. In
addition to its long extracellular domain, TβRIII contains a short cytoplasmic domain with no known intrinsic kinase activity. However, the cytoplasmic domain does contain specific sites that are required for TβRIII to interact with other proteins. Particularly, the terminal three amino acids contain a PDZ-binding domain regulating TβRIII interaction with GIPC, a scaffolding protein which in various cellular contexts can either upregulate or downregulate TGF-β signaling [139]. In addition, the phosphorylation of threonine residue 841 allows for the interaction of TβRIII with β-arrestin2, another scaffolding protein which can enhance internalization of TβRIII [138]. In light of these observations, cell surface TβRIII in the cellular context of our experiments may also in fact be contributing to a downregulation of TGF-β signaling and could consequently lead to suppression of migration, invasion, and metastasis. Thus, a closer examination of the cytoplasmic domain may yield a more refined understanding of the mechanism by which TβRIII leads to the observed inhibition of invasion and metastasis and is explored in further detail below.
3. The cytoplasmic domain of TβRIII plays a role in breast cancer progression

This is a modified version of a paper submitted to Carcinogenesis.

3.1 Introduction

Mechanistically, TβRIII appears to function as a suppressor of cancer progression through several discrete mechanisms. We have demonstrated that cell surface TβRIII undergoes ectodomain shedding, releasing the soluble extracellular domain, which can sequester TGF-β ligand to inhibit TGF-β signaling and inhibit invasion in breast cancer models in vitro and lung metastasis in vivo [42]. TβRIII also inhibits cancer cell migration through interacting with the scaffolding protein β-arrestin2 to activate Cdc42 and reduce directional persistence of cancer cells [18].

The cytoplasmic domain of TβRIII is a relatively short region (42 amino acids) with no known intrinsic kinase activity but functional importance in TβRIII expression and in mediating downstream signaling. TβRII phosphorylates TβRIII’s cytoplasmic domain at Thr841, resulting in β-arrestin2 binding [138]. This interaction is important for TβRIII internalization and ultimately leads to downregulation of TGF-β signaling. In addition, the adaptor protein GIPC binds to the PDZ-binding domain located at the terminal three amino acids of TβRIII. Depending on cellular context, the interaction of GIPC with TβRIII can either upregulate or downregulate cell surface expression [139]. In terms of signaling, evidence suggests that the cytoplasmic domain is critical in mediating downstream Smad signaling of TGF-β2 [165]. In addition, modulation of p38 MAP kinase signaling by TβRIII has been shown to depend on an intact cytoplasmic domain, independent of Smad signaling [12, 166]. These findings suggest that the cytoplasmic tail is important in the regulation of the expression of TβRIII at the cell surface and plays
a role in mediating both Smad-dependent and Smad-independent signaling. Accordingly, the contribution of the cytoplasmic domain of TβRIII in mediating the suppressor of cancer progression role of TβRIII in vitro and in vivo is examined here.

3.2 Materials and Methods

Cell culture and reagents: 4T1 cells stably transfected with the luciferase gene under puromycin selection were generously provided by MW Dewhirst (Duke University). Antibodies used were phospho-p65 (Cell Signaling), IκB (Santa Cruz), pSmad2 (Cell Signaling), total Smad2 (Cell Signaling), TβRIII (R&D Systems) and GIPC (Santa Cruz). shGIPC plasmid was generously provided by JC Rathmell (Duke University) [167]. SB431542 was obtained from Sigma.

Immunoblotting. Cells were plated overnight, serum starved for four hours, and stimulated for 20 minutes at varying doses of TGF-β. Lysates were harvested with hot sample buffer and boiled for 5 minutes. Lysates were separated using SDS-PAGE gels, transferred onto PVDF membranes, blocked in 5% milk in PBS/Tween, and incubated overnight at 4°C with gentle agitation.

Fibronectin migration and Matrigel invasion assays. Fibronectin (Calbiochem) was coated on transwells (Costar) at a concentration of 50μg/mL in serum-free DMEM, while Matrigel transwells (BD Biosciences) were preincubated with serum-free DMEM for 2 hours at 37°C. For migration assays, 50,000 cells were plated in 200μL of serum-free DMEM in the upper chamber; for invasion assays, 200,000 cells were plated in 200μL of and allowed to migrate for 24 hours towards 600μL of 10% FBS in DMEM. Cells on the upper surface were scraped with a cotton swab, and wells were stained with 3 Step...
Stain Kit (Richard-Allen Scientific). Filters were then mounted on microscope slides with Vectamount (Vector Laboratories). Three random fields on each filter were counted. Cells were plated in duplicate, and each experiment was conducted at least three times.

**TGF-β binding and crosslinking assay.** For cell media, we incubated 100 pM $^{125}$I-TGF-β1 with 500 uL of the cell medium in the presence of protease inhibitors for 3 hours at 4°C. The $^{125}$I-TGF-β1-sTβRIII complex was then crosslinked with 0.5 mg/mL disuccinimidyl suberate and immunoprecipitated with a polyclonal antibody recognizing the extracellular domain of TβRIII (R&D Systems). The final complex was visualized after SDS-PAGE and autoradiography.

**In vivo metastasis.** Animal procedures were approved by the Institutional Animal Care and Use Committee of Duke University. Empty vector pcDNA3.1-neo, TβRIII, and TβRIII-cyto lines were generated by transfecting 4T1 cells with Lipofectamine 2000 (Invitrogen), further selected with G418, and confirmed by $^{125}$I-TGFβ binding and crosslinking as previously described [42]. 25,000 cells were injected into the right axillary mammary fat pad of 6-week old virgin female Balb/c mice, using 15 mice in each group. After twelve days, the resulting primary tumors were excised, weighed, and measured with calipers (volume calculated by $0.52 \times \text{length} \times \text{width}^2$). Bioluminescent imaging with intraperitoneal injections of 150μg/g luciferin (Xenogen) was conducted with an IVIS camera (Xenogen) every three days for 24 days. Regions of interest were defined automatically, and luciferase units expressed as photons/s/cm²/steradian. Background signal was defined by imaging a mouse without luciferin. Mice were
sacrificed at the end of the study, organs visually inspected, and samples of interest preserved for immunohistochemical analysis.

*Immunohistochemistry.* Tissue samples were formalin fixed, paraffin embedded, and cut onto microscope slides. Slides were then either stained for hematoxylin and eosin, or prepared for immunohistochemical analysis. Staining for TUNEL (Roche Diagnostics) and PCNA (Santa Cruz) was performed according to manufacturers’ instructions. Phospho-Smad2 (Cell Signaling) was performed following antigen retrieval with boiling Tris-EDTA (10mM Tris, 1mM EDTA, 0.05% Tween, pH 9.0) for 5 minutes.

*Statistical analysis.* All data are presented as mean +/- SEM. 2-tailed Student’s t-test was performed. p-values of less than 0.05 were considered significant.
3.3 Results

3.3.1 TβRIII’s cytoplasmic domain has a role in TβRIII-mediated inhibition of breast cancer cell migration and invasion

We have previously demonstrated a role for TβRIII in inhibiting breast cancer metastases in vivo and cancer cell migration and invasion in vitro [42]. The cytoplasmic domain of TβRIII has a critical role in regulating TβRIII cell surface expression and endocytosis through its interaction with GIPC [139] and β-arrestin2 [138]. The cytoplasmic domain is also important in mediating TβRIII-dependent downstream signaling to both Smad-dependent [2, 165, 168] and Smad-independent signaling pathways [12, 164, 169]. Accordingly, to further define the mechanism by which TβRIII suppresses breast cancer progression, we investigated the specific contribution of the cytoplasmic domain of TβRIII in the 4T1 syngeneic murine model of mammary carcinogenesis.

Full length TβRIII (TβRIII), TβRIII lacking the cytoplasmic domain (TβRIII-cyto) or empty vector (Neo) was stably expressed in the 4T1 cell line (Figure 19). While TβRIII inhibited migration by approximately 50% (Figure 20), consistent with our prior results [42], TβRIII-cyto failed to inhibit the migration of 4T1 cells (Figure 20). In addition, while TβRIII expression inhibited invasion by approximately 40%, TβRIII-cyto expression failed to inhibit invasion (Figure 21). Interestingly, TβRIII-cyto slightly enhanced the invasive potential of 4T1 cells in comparison to the negative control (Figure 21), although these results were not significantly different. These results suggest an important role for the cytoplasmic domain of TβRIII in TβRIII-mediated inhibition of breast cancer cell migration and invasion.
Figure 19: TβRIII expression in 4T1 stable cell lines

4T1 cells were transfected with empty vector control (Neo), full length TβRIII (TβRIII), or TβRIII lacking the cytoplasmic domain (TβRIII-cyto), selected by growth in G418 (600μg/mL) and pools of resistant colonies selected. Cells were subjected to $^{125}$I-TGF-beta binding and crosslinking to examine TβRIII expression, with β-actin as loading control.

Figure 20: Migration of Neo, TβRIII, and TβRIII-cyto cells

4T1-Neo, TβRIII, or TβRIII-cyto were plated on fibronectin transwells and allowed to migrate for 24 hours towards media with 10% FBS. Counts of three random fields were averaged and representative data of three independent experiments are shown. *p<0.05.
4T1 cells were infected with adenoviral GFP, full length TβRIII, or TβRIII-cyto for 48 hours, washed, and plated on Matrigel inserts. Cells were allowed to invade for 24 hours towards media with 10% FBS. Counts of three random fields were averaged and representative data of three independent experiments are shown. *p<0.05.

3.3.2 TβRIII's cytoplasmic domain contributes to TβRIII-mediated inhibition of migration and invasion by inhibiting TGF-β signaling

Given TβRIII's known role in regulating TGF-β signaling, the contribution of the cytoplasmic domain of TβRIII to TGF-β signaling was examined. 4T1 cells stably expressing TβRIII, TβRIII-cyto or empty vector were stimulated with TGF-β and analyzed for activation of pathways known to be downstream of TGF-β and TβRIII, including the Smad2 [2], p38 [12, 166] and NFκB [164, 169, 170] pathways. While TβRIII expression decreased TGF-β-mediated Smad2 phosphorylation, TβRIII-cyto expression had no effect (Figure 22). In contrast, activation of the NFκB signaling pathway, as assessed by phosphorylation of p65 or IκB levels, or p38 signaling, as assessed by phosphorylation of p38, were not differentially regulated by TβRIII and TβRIII-cyto (Figure 22), suggesting a specific role for cytoplasmic domain of TβRIII in negatively regulating TGF-β signaling.
Figure 22: Signaling in Neo, TβRIII, and TβRIII-cyto cells

4T1 cells stably transfected with TβRIII, TβRIII-cyto or empty vector (Neo) were serum starved for four hours, treated with the indicated doses of TGFβ for 20 minutes, and subjected to Western blot analysis with the indicated antibodies.

To investigate whether the effects of TβRIII in inhibiting breast cancer cell migration and invasion might be through TβRIII-mediated attenuation of TGF-β signaling, we directly assessed the effect of altering autocrine TGF-β signaling on breast cancer cell migration and invasion. Consistent with a role for inhibition of TGF-β signaling, directly inhibiting TGF-β signaling with the ALK5 pharmacological inhibitor SB431542 inhibited the migration of Neo and TβRIII-cyto to the same extent as TβRIII (Figure 23). If TβRIII inhibits migration and invasion by inhibiting TGF-β signaling, we hypothesized that activating TGF-β signaling downstream of TβRIII utilizing constitutively active type I TGF-β receptor (ALK5QD) might bypass this inhibition. Consistent with this hypothesis, while ALK5QD expression had minimal effect on the migration or invasion of 4T1-Neo and 4T1-TβRIII-cyto cells (Figure 24, Figure 25), ALK5QD expression significantly attenuated TβRIII-mediated inhibition of migration (Figure 24) and invasion.
(Figure 25). Taken together, these data support a role for TβRIII-mediated inhibition of autocrine TGF-β signaling in TβRIII-mediated inhibition of migration and invasion.

**Figure 23: Invasion of Neo, TβRIII, and TβIII-cyto cells with TGF-β blockade**

4T1-Neo, TβRIII, or TβIII-cyto cells were preincubated with either vehicle (DMSO) or the ALK5 inhibitor SB431542 (20 μM) for 20 minutes prior to plating on Matrigel transwells, and allowed to invade for 24 hours. Counts of three random fields were averaged and representative data of three independent experiments are shown. Counts of three random fields were averaged and representative data of three independent experiments are shown are shown. *p<0.05.
Figure 24: Migration of Neo, TβRIII, and TβRIII-cyto cells with constitutive TGF-β signaling

4T1-Neo, TβRIII, or TβRIII-cyto cells were infected with either adenoviral GFP or a constitutively active ALK5 mutant (ALK5QD) for 48 hours, plated on transwells, and incubated for 24 hours. Counts of three random fields were averaged and representative data of three independent experiments are shown. *p<0.05.
4T1-Neo, TβRIII, or TβRIII-cyto cells were infected with either adenoviral GFP or a constitutively active ALK5 mutant (ALK5QD) for 48 hours, plated on transwells, and incubated for 24 hours. Counts of three random fields were averaged and representative data of three independent experiments are shown. *p<0.05.

3.3.3 The interaction of TβRIII with GIPC is required for TβRIII-mediated suppression of TGF-β signaling and invasion

As TβRIII-mediated inhibition of TGF-β signaling appears to have a predominant role in TβRIII-mediated inhibition of migration and invasion, we further investigated the mechanism of TβRIII action. TβRIII undergoes ectodomain shedding to release the soluble extracellular domain, soluble TβRIII (sTβRIII), which can function to bind and sequester ligand and can also inhibit breast cancer invasion [42, 133]. We initially investigated whether TβRIII and TβRIII-cyto differed in their ability to produce sTβRIII. Examining the conditioned media from 4T1-TβRIII and 4T1-TβRIII-cyto revealed that both TβRIII and TβRIII-cyto were shed to a similar extent (Figure 26). As the sTβRIII produced by TβRIII and TβRIII-cyto might function to differentially inhibit breast cancer
migration or invasion, we examined the ability of conditioned media from 4T1-TβRIII and 4T1-TβRIII-cyto cells to inhibit 4T1 cell migration. Consistent with our prior results [42], conditioned media from TβRIII and TβRIII-cyto both inhibited 4T1 cell migration to a similar extent (Figure 27), further supporting that differential shedding was not responsible for differences in TβRIII-mediated inhibition of TGF-β signaling, migration and invasion between TβRIII and TβRIII-cyto.

**Figure 26: Expression of soluble TβRIII in media of Neo, TβRIII, and TβRIII-cyto cells**

Conditioned media from 4T1 cells stably transfected with TβRIII, TβRIII-cyto, or empty vector (Neo) were subjected to 125I-TGF-beta binding and crosslinking, with β-actin from plated cells used as loading control.
Figure 27: Migration of Neo cells with conditioned media from TβRIII and TβRIII-cyto cells

4T1-Neo cells were resuspended in the indicated conditioned media, plated on fibronectin transwells and allowed to migrate for 24 hours towards media with 10% FBS. Counts of three random fields were averaged and representative data of three independent experiments are shown. *p<0.05.

We next investigated the contribution of specific previously defined TβRIII cytoplasmic domain functions, namely the interactions with scaffolding proteins GIPC and β-arrestin2. TβRIII mutants unable to bind β-arrestin2 (TβRIII-T841A) or GIPC (TβRIII-del, lacking three C-terminal amino acids) were expressed in 4T1 cells alongside with TβRIII and TβRIII-cyto (Figure 28). Consistent with our prior results (Figure 22), TβRIII inhibited TGF-β signaling while TβRIII-cyto did not (Figure 29). Interestingly, while TβRIII-T841A also inhibited TGF-β signaling (Figure 29), TβRIII-del was completely unable to inhibit TGF-β signaling (Figure 29), suggesting a specific role for this region of the cytoplasmic domain of TβRIII in inhibiting TGF-β signaling. To investigate the contribution of interactions with GIPC and β-arrestin2 on breast cancer
invasion, we expressed TβRIII, TβRIII-cyto, TβRIII-T841A or TβRIII-del in 4T1 cells. Again, TβRIII inhibited invasion, while TβRIII-cyto did not (Figure 30). Consistent with the TGF-β signaling data, TβRIII-T841A also inhibited invasion (Figure 30), while TβRIII-del was completely unable to inhibit invasion (Figure 30). The tight correlation between the ability of TβRIII mutants to inhibit TGF-β signaling (Figure 29) and inhibit breast cancer invasion (Figure 30) supports a functional relationship between these effects. This functional relationship is further supported by the ability of pharmacological inhibition of ALK5 to inhibit invasion in the context of TβRIII-del expression (Figure 31).

**Figure 28: Expression of TβRIII, TβRIII-cyto, TβRIII-T841A, and TβRIII-del**

4T1 cells were infected with adenovirus for GFP, TβRIII, TβRIII-cyto, TβRIII-T841A, and TβRIII-del for 48 hours. Cells were then harvested by hot sample buffer lysis and subjected to western blot analysis with the TβRIII-ECD antibody. β-actin was used as a loading control.
Figure 29: TGF-β dose response in Neo, TβRIII, TβRIII-cyto, TβRIII-T841A, and TβRIII-del cells

4T1 cells were infected with adenoviral constructs of GFP, TβRIII, TβRIII-cyto, a mutant unable to bind β-arrestin2 (TβRIII-T841A), or a mutant unable to bind GIPC (TβRIII-del) for 48 hours. Cells were then plated and serum starved for four hours, stimulated with the indicated doses of TGFβ for 20 minutes, and subjected to Western blot analysis with the indicated antibodies.

Figure 30: Invasion of TβRIII, TβRIII-cyto, TβRIII-T841A, TβRIII-del cells

4T1 cells were infected with adenoviral constructs of GFP, TβRIII, TβRIII-cyto, TβRIII-T841A, or TβRIII-del for 48 hours, plated on Matrigel transwells, and allowed to invade for 24 hours. Counts of three random fields were averaged and representative data of three independent experiments are shown. Counts of three random fields were averaged and representative data of three independent experiments are shown. *p<0.05.
4T1 cells were infected with GFP, TβRIII, or TβRIII-del constructs for 48 hours, preincubated with either vehicle (DMSO) or the ALK5 inhibitor SB431542 (20 μM) for 20 minutes prior to plating on Matrigel transwells, and allowed to invade for 24 hours. Counts of three random fields were averaged and representative data of three independent experiments are shown. Counts of three random fields were averaged and representative data of three independent experiments are shown. *p<0.05.

While deletion of the C-terminal three amino acids in TβRIII-del is known to abrogate the interaction of TβRIII with GIPC [139], this region could also mediate other TβRIII functions. To investigate the specific function of GIPC, we utilized shRNA-mediated silencing of GIPC expression, which efficiently decreased GIPC expression (Figure 32). Consistent with a role for GIPC in TβRIII-mediated inhibition of autocrine TGF-β signaling and breast cancer invasion, shRNA-mediated silencing of GIPC expression attenuated both TβRIII-mediated inhibition of TGF-β signaling (Figure 32) and TβRIII-mediated inhibition of invasion (Figure 33). Taken together, these data support a model in which TβRIII through interacting with GIPC inhibits TGF-β signaling to inhibit breast cancer invasion.
Figure 32: TGF-β signaling and GIPC knockdown

4T1 cells stably transfected with TβRIII or empty vector (Neo) were cotransfected with either GFP or shGIPC plasmid for 48 hours. Cells were then serum starved for four hours, stimulated with the indicated doses of TGFβ for 20 minutes, and subjected to western blot analysis with the indicated antibodies.

Figure 33: Invasion and GIPC knockdown

4T1 cells stably transfected with TβRIII or empty vector (Neo) were cotransfected with either GFP vector or shGIPC plasmid for 48 hours. Cells were then plated on Matrigel transwells, and allowed to invade for 24 hours. Counts of three random fields were averaged and representative data of three independent experiments are shown. *p<0.05.
3.3.4 The cytoplasmic domain of TβRIII has a role in TβRIII-mediated suppression of breast cancer progression

Having established that the cytoplasmic domain of TβRIII has an important role in TβRIII-mediated inhibition of migration and invasion, coupled with our previous findings that TβRIII suppresses breast cancer metastases in vivo, we examined the role of the cytoplasmic domain of TβRIII in TβRIII-mediated suppression of breast cancer progression in vivo in the 4T1 syngeneic murine model of breast cancer. The 4T1 cells were stably cotransfected with a constitutively active luciferase reporter, which allowed the non-invasive in vivo tracking of cells during tumor development, along with either Neo, TβRIII, or TβRIII-cyto. After cells were injected, primary tumors were allowed to develop for twelve days, after which they were surgically excised and assessed. After excision of the primary tumor, the formation of metastases was followed by bioluminescent imaging every three days over a period of 24 days. Consistent with our previous studies [42], there were no significant differences in primary tumor growth among the 4T1-Neo, -TβRIII, or -TβRIII-cyto groups, as measured by both primary tumor mass and volume (Figure 34, Figure 35). However, consistent with our previous studies [42], mice with the 4T1-TβRIII cells had a delay tumor metastasis onset and decreased metastatic tumor burden as compared to mice injected with the control 4T1-Neo cells (Figure 36, Figure 38). In contrast to the results with mice with the 4T1-TβRIII cells, mice with the 4T1-TβRIII-cyto cells had no delay in onset of tumor metastases and no decrease in metastatic tumor burden as compared to mice with the control 4T1-Neo cells (Figure 36, Figure 38). Upon necropsy, the 4T1-Neo, 4T1-TβRIII and 4T1-TβRIII-cyto mice were examined for gross metastatic lesions. Consistent with bioluminescent imaging, significantly more lesions were found in the mice with 4T1-Neo cells and 4T1-TβRIII-cyto as compared to mice with 4T1-TβRIII cells (Figure 37, Table 3).
Figure 34: Primary tumor mass of Neo, TβRIII, and TβRILL-cyto groups

After 12 days, tumors were excised and weighed. Bars above are presented as mean ± s.e.m. N=15.

Figure 35: Primary tumor size of Neo, TβRIII, and TβRIII-cyto groups

Tumors were measured with calipers and volume calculated based on the formula 0.52 x length x width² and presented as mean ± s.e.m., N=15.
Figure 36: Time series of tumor burden in Neo, TβRIII, and TβRIII-cyto groups

4T1 cells stably transfected with TβRIII, TβRIII-cyto or empty vector (Neo) were injected into the axillary mammary fat pad of groups of 15 Balb/c mice, with excision of the primary tumors after 12 days. Bioluminescence imaging was conducted every 3 postoperative days (POD) and representative images are shown.

Figure 37: Metastatic events in Neo, TβRIII, and TβRIII-cyto groups

The number of grossly observed metastatic lesions upon necropsy in each group of mice is presented. *p<0.05.
Table 3: Summary of metastases

<table>
<thead>
<tr>
<th></th>
<th>Number of mice with metastases</th>
<th>Number of total metastatic events</th>
<th>Sites of metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neo</td>
<td>8 of 11 (73%)</td>
<td>10</td>
<td>lungs, liver</td>
</tr>
<tr>
<td>TβRIII</td>
<td>5 of 12 (42%)</td>
<td>7</td>
<td>lungs</td>
</tr>
<tr>
<td>TβRIII-cyto</td>
<td>9 of 11 (82%)</td>
<td>13</td>
<td>lungs, liver, kidney</td>
</tr>
</tbody>
</table>

Figure 38: Scatterplot of bioluminescent intensities of tumors

Signal intensities for each mouse are plotted, with open symbols indicating a mouse that died before POD 24, and horizontal lines indicating mean intensities. *p<0.05.

Further histological examination of the primary tumors demonstrated local invasiveness of the primary tumor into the stroma in the 4T1-Neo (Figure 39A) and 4T1-TβRIII-cyto (Figure 39C) tumors, while the 4T1-TβRIII tumors instead displayed a well-demarcated border with the surrounding stroma (Figure 39B). Furthermore, lung metastases from 4T1-Neo (Figure 39D) and 4T1-TβRIII-cyto (Figure 39F) tumors...
revealed invasion into the lung parenchyma, while lung metastases from 4T1-TβRIII tumors (Figure 39E) were smaller and well-circumscribed. In addition, while 4T1-Neo and 4T1-TβRIII-cyto tumors both metastasized to the liver (Figure 39G, Figure 39I), and a 4T1-TβRIII-cyto tumor metastasized to the kidney (Figure 39J), none of the 4T1-TβRIII tumors metastasized to these sites (Figure 39H). These studies support a specific suppressor effect of TβRIII on breast cancer invasiveness and metastasis mediated through its cytoplasmic domain.
Figure 39: Histology of primary tumors and metastatic lesions

Representative H&E stains (at 10x) of primary tumors (A, B, C) demonstrate local invasion of tumor cells into the surrounding stroma in Neo and TβRIII-cyto mice (A,C), while the TβRIII tumors exhibit a clearly demarcated tumor border (B). Lung metastases (D,E,F) with a more invasive phenotype in the Neo and TβRIII-cyto groups (D,F); note that the TβRIII-cyto tumor impinges upon the airway (F), while the tumor adjacent to the airway in the TβRIII tumor retains its architecture (E). Liver metastases were also noted in the Neo and TβRIII-cyto groups (G,I), while none of the TβRIII liver tissues examined revealed metastatic lesions (H). A metastatic tumor in the kidney was noted in one of the TβRIII-cyto mice (J). Scale bar=100µm.
To investigate the mechanism of the cytoplasmic domain of TβRIII on decreasing metastasis in vivo, we performed immunohistochemistry for PCNA as a marker of proliferation in the primary tumor and lung metastases. There were no significant differences in PCNA staining between 4T1-Neo, 4T1-TβRIII and 4T1-TβRIII-cyto tumors in either the primary or metastatic lesions (Figure 40, Figure 41). We also performed TUNEL staining as a marker of apoptosis in the primary tumor and lung metastases. Similarly, there were no significant differences observed between 4T1-Neo, 4T1-TβRIII and 4T1-TβRIII-cyto tumors for TUNEL staining (Figure 42, Figure 43). These results suggested that differences in proliferation or apoptosis do not account for the differences in invasion and metastases among 4T1-Neo, 4T1-TβRIII and 4T1-TβRIII-cyto tumors.

![Figure 40: Proliferation in primary tumors](image)

Tissue sections of primary tumors from mice implanted with 4T1-Neo, 4T1-TβRIII and 4T1-TβRIII-cyto cells were immunostained for PCNA to evaluate cell proliferation. Three random fields were counted with ImageJ software used to quantify positively staining cells and total number of cells. Indices were calculated for each count by normalizing the number of positively staining cells to the total number of cells.
Tissue sections of lung metastases from mice implanted with 4T1-Neo, 4T1-TβRIII and 4T1-TβRIII-cyto cells were immunostained for PCNA to evaluate cell proliferation. Three random fields were counted with ImageJ software used to quantify positively staining cells and total number of cells. Indices were calculated for each count by normalizing the number of positively staining cells to the total number of cells.

**Figure 41: Proliferation in lung metastases**

Tissue sections of primary tumors from mice implanted with 4T1-Neo, 4T1-TβRIII and 4T1-TβRIII-cyto cells were immunostained for TUNEL to evaluate apoptosis. Three random fields were counted with ImageJ software used to quantify positively staining cells and total number of cells. Indices were calculated for each count by normalizing the number of positively staining cells to the total number of cells.

**Figure 42: Apoptosis in primary tumors**
Tissue sections of lung metastasis from mice implanted with 4T1-Neo, 4T1-TβRIII and 4T1-TβRIII-cyto cells were immunostained for TUNEL to evaluate apoptosis. Three random fields were counted with ImageJ software used to quantify positively staining cells and total number of cells. Indices were calculated for each count by normalizing the number of positively staining cells to the total number of cells.

As we had observed differences in TGF-β signaling between 4T1-Neo, 4T1-TβRIII and 4T1-TβRIII-cyto which potentially accounted for differences in cell migration and invasion in vitro, we explored whether differences in TGF-β signaling could account for differences in cancer progression in vivo. Tissues from 4T1-Neo, 4T1-TβRIII and 4T1-TβRIII-cyto primary tumors and their lung metastases were analyzed for phospho-Smad2 staining. Although there was no difference in phospho-Smad2 staining in the 4T1-Neo, 4T1-TβRIII and 4T1-TβRIII-cyto primary tumors (Figure 44A-C), we did observe a dramatic decrease in phospho-Smad2 staining in pulmonary metastases from 4T1-TβRIII tumors relative to pulmonary metastases from 4T1-TβRIII-cyto and 4T1-Neo tumors (Figure 44D-F). These results suggest that TβRIII specifically suppresses TGF-β signaling in the metastatic tumor microenvironment specifically through functions mediated through its cytoplasmic domain.
Figure 44: TGF-β signaling of primary tumors and lung metastases

Tissues sections of primary tumors and lung metastasis from mice implanted with 4T1-Neo, 4T1-TβRIII and 4T1-TβRIII-cyto cells were stained with phospho-Smad2 antibody. Representative results are shown (at 40X). Scale bar=100µm.
3.4 Discussion

Breast cancer remains a leading cause of morbidity and mortality worldwide, with most of this morbidity and mortality resulting from recurrent and metastatic disease [1]. Previous studies have amply demonstrated an important role for TGF-β signaling during mammary carcinogenesis. In early breast cancer progression, TGF-β generally acts as a tumor suppressor, inhibiting proliferation and promoting apoptosis. However, breast cancer cells in an established tumor become resistant to TGF-β-mediated effects on proliferation and apoptosis through an incompletely understood process and instead respond with increased migration and invasion. We have previously reported that the TGF-β superfamily co-receptor TβRIII is a suppressor of breast cancer progression, with frequent loss during breast cancer progression corresponding with decreased patient survival [42]. Functionally, TβRIII inhibited tumor invasiveness in vitro and tumor invasion, angiogenesis and metastasis in vivo [42]. Mechanistically, TβRIII appeared to function, at least in part, by undergoing ectodomain shedding, with the resulting sTβRIII antagonizing TGF-β signaling to reduce invasiveness and angiogenesis in vivo [42]. In the present study, we further investigate the mechanism by which TβRIII mediates its suppressor of cancer progression function. We demonstrate that the cytoplasmic domain of TβRIII has an important role in TβRIII-mediated suppression of cancer progression, as deletion of the cytoplasmic domain abolishes the ability of TβRIII to inhibit migration or invasion in vitro and invasion and cancer progression in vivo.

Mechanistically, TβRIII-mediated inhibition of migration and invasion appears to be modulated by TβRIII-mediated inhibition of TGF-β signaling, as supported by (1) the tight correlation between the ability of TβRIII mutants to inhibit TGF-β signaling and to inhibit migration and invasion in vitro, (2) the tight correlation between diminished TGF-β
signaling and decreased invasion and metastasis mediated by TβRIII in vivo, (3) the ability of directly inhibiting TGF-β signaling to mimic the effects of TβRIII on inhibiting migration in vitro and (4) the ability of bypassing TβRIII-mediated inhibition of TGF-β signaling to abrogate TβRIII-mediated inhibition of migration and invasion in vitro. 

Finally, we demonstrate that a discrete function of the cytoplasmic domain of TβRIII, namely binding to the PDZ domain containing protein, GIPC, is largely responsible for mediating the effects of TβRIII on inhibiting TGF-β signaling and inhibiting cell migration and invasion in vitro, as either inhibiting the ability of TβRIII to bind GIPC or silencing GIPC expression is sufficient to abolish these effects. These results provide another mechanism by which TβRIII mediates its suppressor of cancer progression effects and emphasize the importance of the conserved TβRIII cytoplasmic domain in mediating TβRIII functions.

How does TβRIII inhibit TGF-β signaling through its cytoplasmic domain and GIPC binding function? While we have previously demonstrated that TβRIII can inhibit TGF-β signaling through generation of sTβRIII, which sequesters TGF-β to inhibit TGF-β signaling [42], here we show that TβRIII and TβRIII-cyto do not differ in their ability to produce or to inhibit cell migration through sTβRIII. Thus, differences in generation of sTβRIII likely do not account for cytoplasmic domain-mediated inhibition of TGF-β signaling. We have also previously demonstrated that GIPC binding to TβRIII stabilized TβRIII on the cell surface and increased TGF-β signaling in epithelial cells and myoblasts [139]. However, these studies utilized GIPC overexpression as opposed to the loss of function studies performed here. In addition, breast epithelial and/or breast cancer cells could have altered responsiveness to GIPC. In any case, the ability of GIPC to regulate cell surface stability of TβRIII suggests that GIPC might serve to
regulate TGF-β signaling by regulating its internalization and trafficking. Consistent with this hypothesis, the other scaffolding protein that interacts with the cytoplasmic domain of TβRIII, β-arrestin2, functions to regulate TβRIII endocytosis and TGF-β signaling [138]. In addition, we have demonstrated that internalization of TβRIII through both clathrin-dependent and clathrin-independent signaling pathways is important for TβRIII-mediated signaling through both Smad-dependent and Smad-independent signaling pathways [168]. Alternatively, GIPC could be functioning as a scaffolding protein to link TβRIII to other pathways that either directly or indirectly inhibit TGF-β signaling. Current studies are aimed at defining the precise mechanism by which TβRIII and GIPC function to inhibit TGF-β signaling, including the connection between receptor trafficking and signaling.

Here we present data supporting a model for TβRIII in inhibiting breast cancer progression through inhibition of TGF-β signaling, not through generation of sTβRIII, but through its cytoplasmic domain. In separate studies we have also demonstrated that TβRIII might inhibit breast cancer progression through TGF-β signaling independent and β-arrestin2-dependent activation of Cdc42 to inhibit breast cancer cell migration [18] as well as through β-arrestin2-dependent inhibition of NF-κB signaling to inhibit breast cancer cell migration [164]. Taken together, these results suggest several mechanisms that could either act in concert or in isolation to mediate the suppressor of cancer progression function of TβRIII. These multiple mechanisms by which TβRIII functions also provide an explanation for the selective pressure which likely results in the frequent loss of TβRIII expression during breast cancer progression, as we have already reported [42]. While these mechanisms may function in isolation, as TβRIII-cyto also produces sTβRIII (Figure 26), why does it not have any effect in inhibiting cell migration and
invasion \textit{in vitro} and cancer progression \textit{in vivo}? While this paradox remains to be fully explored, it is possible that the different mechanisms defined operate in different contexts or that T\(\beta\)RIII-cyto, which cannot be internalized and downregulated \cite{168}, functions as a constitutively active ligand presenter and functionally competes with sT\(\beta\)RIII as a suppressor of signaling. These possibilities are currently being explored.

In summary, our work has demonstrated that the cytoplasmic domain of T\(\beta\)RIII is critical for its function in inhibiting migration and invasion, as well as T\(\beta\)RIII’s role in attenuating TGF-\(\beta\) signaling. \textit{In vivo}, the cytoplasmic domain is important for the ability of T\(\beta\)RIII to inhibit metastatic potential. Furthermore, we have identified the interaction with GIPC as a critical mediator of T\(\beta\)RIII’s effects \textit{in vitro} on signaling and invasion. Coupled with further work to identify the contribution of GIPC / T\(\beta\)RIII interactions \textit{in vivo}, these studies have the potential to open a path towards a novel target for the treatment of breast cancer patients.
4. Summary and Future Directions

4.1 Summary

TGF-β plays a significant role in cancer progression, having effects on proliferation, apoptosis, differentiation, migration, and invasion. Though TGF-β generally acts in a tumor suppressive fashion, a switch occurs sometime during carcinogenesis whereby TGF-β can act paradoxically to promote tumor progression. This dichotomy of functional effects in cancer progression remains one of the central mysteries of TGF-β signaling. TGF-β signaling has been shown to be a significant modulator of breast cancer progression specifically. Evidence to date suggests that loss of expression of TGF-β signaling components is a relatively uncommon event in patients with breast cancer, and that contributions of TGF-β to cancer progression are due to changes in how cells respond to signals. My studies have focused on the contribution of the type III TGF-β receptor (TβRIII). First thought of as a co-receptor for ligand presentation to the TGF-β signaling apparatus, TβRIII is emerging as a more complex regulator of TGF-β signaling and function. Furthermore, TβRIII loss has been seen in cancer progression in other cancers [92, 93, 95, 96], lending support to the notion that TβRIII loss may be a significant event in breast cancer progression.

In contrast to other signaling components, TβRIII is significantly downregulated both at the message and protein levels with breast cancer progression. Samples from a variety of patients were compared and analyzed. Loss of TβRIII was demonstrated to occur not just with increasing grade, but was seen across several different subtypes of breast cancer. We had also demonstrated that there is loss of heterozygosity at the TβRIII locus in breast cancer patients, and that this was correlated with a decrease in TβRIII expression. Furthermore, TGF-β stimulation led to the downregulation of TβRIII
transcription [42]. These data suggest multiple potential mechanisms of TβRIII loss in breast cancer, and that the loss of TβRIII expression may be a relatively ubiquitous phenomenon that, in conjunction with other data, has implications for a variety of breast cancers in terms of understanding underlying biology, clinical outcome, and therapeutic approach.

Having established that TβRIII loss of expression is a significant event in breast cancer progression, we sought to further establish whether this loss functionally impacts the biology of the tumor. We used an orthotopic mammary cancer model to address this question. Given TGF-β’s role in tumor cell growth, we first examined whether there were differences in tumor growth. Gross examination of the tumor revealed no differences in growth. Tumor growth was further dissected with a closer look at the underlying processes of proliferation and apoptosis. With immunohistochemical analysis, TβRIII demonstrated no significant effect on the proliferative or the apoptotic behavior of tumor cells in vivo. Thus, TβRIII in this model reveals no effects on tumor growth, proliferation, or apoptosis.

Cancer progression is also characterized by the metastasis of primary tumor. Thus, the next question addressed was whether TβRIII had any effect on metastatic potential. In the orthotopic mouse model, TβRIII did have a significant effect on metastatic tumor formation, both delaying the onset of metastatic lesions as well as decreasing the overall metastatic tumor burden. Furthermore, this was accompanied by a decrease in local invasiveness, as histopathological analysis of both primary tumor and metastatic lesions revealed that TβRIII tumors tended to have well-demarcated borders and less involvement with the surrounding parenchyma and stroma. This difference in invasiveness was confirmed in vitro, with a Matrigel transwell assay used to
demonstrate that overexpression of TβRIII results in the attenuation of TGF-β stimulated invasion. We have also seen that primary and metastatic tumors expressing TβRIII exhibit decreases in angiogenesis as seen by vessel density and number [42]. Together with the decrease in invasiveness, TβRIII-mediated decreases in angiogenesis reveals another potential mechanism by which TβRIII suppresses metastasis.

Given that invasion of cells with TGF-β stimulation was significantly altered in the presence of TβRIII, coupled with the fact that soluble TβRIII (sTβRIII) can dampen TGF-β signaling through the sequestration of ligand, we next determined whether sTβRIII may be contributing to TβRIII’s suppression of invasion. Having established that TβRIII overexpression in our *in vitro* system does in fact yield sTβRIII species that bind TGF-β, we used conditioned media containing sTβRIII to show that sTβRIII is sufficient to induce the suppression of invasion *in vitro*. This suggests a potential mechanism *in vivo* by which TβRIII may suppress metastasis; the generation of sTβRIII leads to an overall decrease in TGF-β signaling through the sequestration of ligand, and this decrease in TGF-β signaling contributes to attenuation of local invasion at both the primary and metastatic sites, leading to an end result of a suppression of metastatic disease.

The clinical relevance of TβRIII on the suppression of metastatic potential was further examined by a retrospective analysis of previously published microarray data containing gene transcripts of breast cancer patients. Complementing the gene arrays were the accompanying data of clinical outcomes and characterization of tumor samples. The significance of TβRIII expression in breast cancer patients was further amplified with the finding that TβRIII expression levels correlate with patient prognosis; patients with low TβRIII levels have a shorter time to recurrence compared to those with
high levels of TβRIII. Thus, while TβRIII expression has a measurable biological effect, it may also be a useful prognostic marker for predicting patient survival.

Examination of the structure of TβRIII reveals that, in addition to the extracellular domain that is important for sTβRIII sequestration of ligand, TβRIII contains a short cytoplasmic domain that contributes to its regulation of cell surface expression as well as non-canonical TGF-β signaling. Thus, I sought to determine the contribution, if any, of the cytoplasmic domain in mediating TβRIII’s effects on breast cancer metastasis. My initial approach was to use a deletion mutant abolishing the entire cytoplasmic domain and determining whether this mutant had any differential effects compared to the full length receptor.

In transwell assays, the role of the cytoplasmic domain of TβRIII on migration and invasion in vitro was assessed. Abrogation of the cytoplasmic domain failed to suppress both migration through fibronectin and invasion through Matrigel, suggesting an important role for the cytoplasmic domain in regulating TβRIII’s function. Furthermore, this effect was associated with changes in TGF-β signaling; while full length TβRIII suppressed TGF-β signaling, deletion of the cytoplasmic domain failed to suppress Smad activation. TβRIII might also be affecting migration and associated breast cancer progression through other mechanisms. Specifically, as mentioned previously, TβRIII might inhibit migration through TGF-β signaling independent and β-arrestin2-dependent activation of Cdc42, as seen in ovarian cancer cells [18]. TβRIII can also mediate its effects on migration through β-arrestin2-dependent inhibition of NF-κB signaling to inhibit cell migration in another breast cancer cell line [164]. Given that TβRIII is likely to act through multiple mechanisms to suppress cancer in a context-specific manner, I examined other signaling pathways to determine their potential
contribution to breast cancer progression. I examined MAPK and NF-κB signaling. Changes in both MAPK and NF-κB signaling were not detected with either the full length or cytoplasmic domain deletion mutant. These findings suggest that non-TGF-β pathways are less likely in this system to contribute to TβRIII’s effects on migration and invasion, and suggest that full length TβRIII may be modulating its effects in part through attenuation of TGF-β signaling.

TβRIII’s effects on TGF-β signaling and consequent migration and invasion were further examined. Abrogation of TGF-β signaling through the use of a pharmacological inhibitor of TβRI revealed abrogation of invasion. Furthermore, a constitutively active mutant of TβRI demonstrated that migration and invasion suppressed by TβRIII could be rescued. These data taken together demonstrate that TβRIII mediates invasion and migration through modulation of the TGF-β signaling pathway.

As previously mentioned, the cytoplasmic domain of TβRIII contains sites important for interaction with scaffolding proteins, namely β-arrestin2 and GIPC [138, 139]. Given their roles in regulating cell surface expression and consequent modulation of downstream TGF-β signaling, I sought to address the question of whether the effects seen with abrogation of the cytoplasmic domain were due to the inability of TβRIII to interact with these proteins. Using mutants specific to the interactions with each of these proteins, I demonstrated that dampening of TGF-β signaling by TβRIII could be abrogated by the deletion of the GIPC binding domain alone. The importance of the interaction of GIPC with TβRIII in modulating TGF-β signaling was confirmed with the shRNA knockdown of GIPC yielding similar results. Finally, the functional effect of abolishing GIPC and TβRIII interaction was assessed by looking at Matrigel transwell invasion. By using both the GIPC-binding mutant as well as GIPC knockdown, I showed
that inhibition of invasion by TβRIII could be abrogated by eliminating the TβRIII interaction. Furthermore, TGF-β signaling modulated this GIPC-dependent effect on invasion, as the use of pharmacological inhibition of TβRI led to abrogation of invasion.

Having demonstrated the importance of the cytoplasmic domain in TGF-β signaling as well as its contribution to mediating migration and invasion in vitro, I next determined whether the cytoplasmic domain has biological effects in vivo. As expected, there were no differences in tumor growth, proliferation, or apoptosis with the deletion of the cytoplasmic domain. However, deletion of the cytoplasmic domain did affect metastatic growth. While TβRIII suppressed metastatic tumor formation, deletion of the cytoplasmic domain abrogated TβRIII-mediated suppression of metastasis, as assessed by overall metastatic burden, time to onset, and sites of metastases. Furthermore, decreases in local invasiveness of primary tumor and metastases in tumor cells with TβRIII were not seen in those tumors with the deletion mutant. Finally, these effects were associated with changes in TGF-β signaling in the metastatic lesions, as TβRIII decreased Smad2 phosphorylation while the deletion mutant failed to have the same effects.

In conclusion, this work in aggregate amplifies the existing body of knowledge defining TGF-β signaling as an important mediator of breast cancer progression. TβRIII is identified as a commonly downregulated TGF-β signaling component with prognostic implications for breast cancer patients. It is shown to be important in breast cancer progression through its suppressive effects on migration, invasion, and metastasis. Additionally, TGF-β signaling is critical to TβRIII’s tumor suppressive effects. These effects are also modulated by the cytoplasmic domain, and in particular TβRIII’s interaction with GIPC contributes to the invasive properties suppressed by TβRIII.
4.2 Future directions

I have demonstrated the importance of the cytoplasmic domain of TβRIII in suppressing migration, invasion, and metastasis, as well as associated dampening of TGF-β signaling. Furthermore, I have also shown that the interaction of TβRIII with GIPC is important in modulating TGF-β signaling as well as in the suppression of invasion *in vitro*. Determining whether this interaction has biological relevance would significantly add to our understanding of the GIPC-TβRIII interaction. The experiments with GIPC interaction with TβRIII utilized transient overexpression, and constructs were thus not stably expressed as they were in the *in vivo* experiments utilizing the complete cytoplasmic deletion. Thus, a logical next step would be to develop a 4T1 cell line with the same constitutively active luciferase reporter and stably express the GIPC deletion mutant. After verifying expression of this GIPC deletion mutant, as well as *in vitro* functional effects on invasion as seen in my work, these could then be orthotopically injected into mice and compared with an empty vector control group and a TβRIII group. Given the observations we have seen thus far, the expectation would be for the GIPC deletion mutant group to phenocopy the cytoplasmic deletion mutant seen in my experiments. That is, while full length TβRIII would lead to a decrease in metastatic burden, number of metastatic sites, and local invasiveness, deletion of the GIPC binding domain would abrogate these effects.

My dissertation demonstrated the importance of GIPC in mediating TβRIII’s suppressive effects *in vitro*. Beyond looking at a mouse model, a more clinically relevant question would be to determine whether GIPC expression itself is correlated with or predictive of disease progression. GIPC in cancer has been examined in a relatively small number of studies in which GIPC expression is measured in a panel of cancer
tissue samples or cell lines. The data do not demonstrate a clear trend of changes in expression in cancer progression. In an array of pancreatic cancer tumor tissues, GIPC protein was largely overexpressed compared to normal pancreatic tissue [171]. Furthermore, GIPC expression of human breast cancer cell lines revealed a majority that overexpressed GIPC compared to a normal breast epithelial cell line [172]. However, in another study examining a panel of tissue samples by immunohistochemical staining, GIPC was overexpressed in gastric and pancreatic cancers, while kidney, colorectal, and prostate cancers had decreased expression of GIPC [173]. These disparate results are not altogether unsurprising, given the multifaceted and complex role of GIPC as a mediator of internalization, cell surface receptor expression, and downstream signaling. Overall expression levels of GIPC may be important in its effects on T\(\beta\)RIII and other cell surface receptors, but subcellular localization of GIPC also likely plays a role in mediating GIPC’s effects. It may be that because of the wide variety of proteins with which GIPC interacts, most studies have focused on disrupting the GIPC interaction with a protein of interest. There are few studies that directly knockdown GIPC to determine GIPC’s biological effects; however, one study did demonstrate that tumor growth of a pancreatic cell line was decreased with knockdown of GIPC and subsequent orthotopic injection [174]. Beyond this example of GIPC’s effects on tumor growth, GIPC’s generalized role in invasiveness and metastasis remains to be explored, along with GIPC’s association with patient survival and outcome.

Another key issue that begs further consideration is reconciling the functions of the soluble form of T\(\beta\)RIII and the cytoplasmic deletion mutant. As previously discussed, sT\(\beta\)RIII sequesters ligand to inhibit TGF-\(\beta\) signaling [133], and T\(\beta\)RIII is cleaved to yield sT\(\beta\)RIII species [144]. In our hands, expression of full length receptor yields sT\(\beta\)RIII in
conditioned media, and reduces migration and invasion in vitro, consistent with the existing literature [133]. Overexpressing the cytoplasmic deletion, furthermore, also yields sTβRIII in the conditioned media, and this species functions similarly to the sTβRIII species arising from full length TβRIII expression in terms of signaling and in migration and invasion in vitro. This leads to an apparent paradox: if sTβRIII is sufficient to dampen TGF-β signaling and consequently have tumor suppressive effects, how does the cytoplasmic deletion mutant, which also gives rise to a sTβRIII that similarly suppresses migration and invasion, fail to inhibit migration, invasion, and metastasis? It may be that the cytoplasmic domain also contributes to tumor suppressive activity itself independent of sTβRIII generated, and that deletion of the cytoplasmic domain has a dominant negative effect on sTβRIII’s suppressive functions on TGF-β signaling, migration and invasion. One way to address this would be to create a full length receptor unable to generate sTβRIII by identifying the cleavage site of TβRIII and mutating it, and in fact this avenue is being explored currently. One would expect then that this cleavage mutant could either mimic the full length receptor or have no suppressive effect. If it were to mimic the full length receptor in vitro and in vivo, we could conclude that sTβRIII is sufficient but not required for TβRIII-mediated suppression of migration and invasion. If the cleavage mutant had no suppressive effects, then sTβRIII would be shown to be absolutely essential in mediating TβRIII’s effects, and that the observed effects of the cytoplasmic deletion mutant and the GIPC-binding mutant are due to a dominant negative effect that overrides the contribution of sTβRIII.

As described in the Introduction, TβRIII binds a wide variety of other TGF-β superfamily ligands, including BMP’s, activin, and inhibin [140-142]. These ligands operate through different type II and type I receptors, and can modulate Smads 1/5/8 to
mediate their downstream effects [140-142]. My studies focused on the specific effects of TGF-β on the ALK5/Smad2 axis of signaling. It is possible that the effects on migration, invasion, and metastasis observed for TβRIII-mediated dampening of TGF-β signaling may have concurrent mechanisms of action mediated by these other superfamily members. For example, in the MDA-MB231-D mammary cancer cell line, BMP-2 has been shown to potentiate invasion in vitro and enhance mammary cancer metastasis in a xenograft model [175]. Furthermore, in an ovarian cancer cell model, a mutant TβRIII that was deficient in its ability to bind inhibin exhibited a decrease in invasion in vitro [176]. Smads1/5/8 have been shown to mediate effects in endothelial cells, particularly on the stimulation of endothelial cell proliferation and migration [177]. In addition, Smads1/5/8 are integral to BMP effects on tumor growth suppression in bone [178]. Disruptions to these processes lead to alterations in angiogenic and tumor growth potential, and such alterations would be interesting to examine in the context of TβRIII-mediated alterations in signaling. Studies to determine whether sTβRIII and GIPC interaction are important in mediating these effects with other superfamily ligands would elucidate yet another mechanism by which metastasis is inhibited by TβRIII in vivo. Also interesting would be an examination of whether these pathways converge and synergize or compete for TβRIII in these and other functional effects.

The wide variety of mechanisms mentioned, including the effects of sTβRIII, contribution of TGF-β independent pathways like Cdc42 and NF-κB, and the role of GIPC and the cytoplasmic domain of TβRIII, may be further explored by looking at other types of cancer. As TGF-β signaling is context-dependent, and as one or more of these mechanisms in concert may contribute to the observed differences in migration, invasion, and metastasis, examining other systems may yield more insight. Generalizing
these insights to a wider variety of cancers would enhance the relevance of our biological understanding of TβRIII’s tumor suppressor effects. In addition, TGF-β signaling has a significant impact on other processes, both normal and pathological [2]. As described before, beyond cancer progression, TGF-β has demonstrated roles in processes as varied as embryonic development, vascular remodeling, immune responses, and wound healing [2]. These functions are intimately involved with cellular migration and invasion. In embryonic development and vascular remodeling, motility and local invasiveness of tissues is critical with EMT driving the process of tissue reorganization [67]. Immune responses and wound healing require the localization of cells to target regions of interest, whether to mount an attack against a foreign pathogen or to bring cells into an area to initiate a cascade of repair [179]. My findings with regards to migration and invasion may have broader applicability beyond cancer disease. Further examination of processes such as these may not only generalize these insights, but may also better define how these multiple mechanisms of TβRIII’s potentially work together to mediate migration and invasion specifically. Ultimately, the goal of examining the effects of TβRIII would ideally lead to better outcomes for breast cancer patients. As I have demonstrated, TβRIII may be used as a prognostic indicator for recurrence free survival. Whether high levels of TβRIII actually lead to better overall survival remains to be seen, and as more clinical specimens and survival data become available, this is an important question which can be readdressed. TβRIII as a marker for recurrence-free survival may also prove to be useful in terms of planning treatments for patients. Again, as more data become available, it may be possible to determine gene profiles in conjunction with other predictive markers that predict patient response to chemotherapy.
Further defining how TβRIII acts to suppress metastasis may lead to a more targeted prevention or treatment approach that enhances the quality of life and survivability of breast cancer patients. Our work has shown that sTβRIII is sufficient to inhibit migration and invasion \textit{in vitro}. In addition, our work has shown that in the 4T1 mouse model, there is a tight link between TGF-β signaling and invasiveness. Previous studies utilizing systemic soluble TβRIII in mice show some promise \cite{133, 145} in inhibiting metastatic growth. This modality hinges upon repression of TGF-β signaling through the sequestration of ligand. As described in the Introduction, it is evident from human trials that using a strategy of TGF-β signaling repression alone is fraught with complications due to TGF-β’s multifaceted roles on various tissues, tumor type, and tumor stage. There are other factors that beg consideration with regards to therapeautic strategies. In particular, TβRIII exhibits loss of heterozygosity in breast cancer patients; as a tumor suppressor, targeting TβRIII might be of therapeautic value through the inhibition of epigenetic loss of TβRIII expression. In addition, TβRIII –mediated effects on migration and invasion are modulated in part through its cytoplasmic domain, in particular through its interactions with GIPC, β-arrestin2, and Cdc42. A closer examination of these components in other cellular contexts and tumor types would reveal the extent of how generalizable these effects might be in various cancer types, as well as any potential side effects which could obviate such therapy.
References


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