EFFECTS OF ALTERATIONS TO THE TUMOR MICROENVIRONMENT DRIVEN BY
TRANSFORMING GROWTH FACTOR BETA ON TUMOR PROGRESSION

By

Michael William Pickup

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Approved:
Harold Moses, M.D.
Barbara Fingleton, Ph.D.
Andries Zijlstra, Ph.D.
Ambra Pozzi, Ph.D.
Timothy Blackwell, M.D.
To my parents, Cheryl and Timothy, for being infinitely supportive and inspirational.
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<table>
<thead>
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<th>Full Form</th>
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<tbody>
<tr>
<td>A.U.</td>
<td>Arbitrary Unit</td>
</tr>
<tr>
<td>AKT</td>
<td>RAC-Alpha serine/Threonine-protein Kinase</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>Angiopoetin like 4</td>
</tr>
<tr>
<td>aSMA</td>
<td>Alpha Smooth Muscle Actin</td>
</tr>
<tr>
<td>BAMBI</td>
<td>BMP and Activin Membrane-Bound Inhibitor homolog</td>
</tr>
<tr>
<td>BAPN</td>
<td>Beta-Aminopropionitrile</td>
</tr>
<tr>
<td>BIM</td>
<td>BCL-2 Like 11</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenic Protein</td>
</tr>
<tr>
<td>BST2</td>
<td>Bone Marrow Stromal Cell Antigen 2</td>
</tr>
<tr>
<td>CAF</td>
<td>Carcinoma Associated Fibroblast</td>
</tr>
<tr>
<td>CCL5</td>
<td>Chemokine (C-C Motif) Ligand 5</td>
</tr>
<tr>
<td>CDK4</td>
<td>Cyclin-dependent Kinase 4</td>
</tr>
<tr>
<td>COL1A2</td>
<td>Collagen 1 alpha 2</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective Tissue Growth Factor</td>
</tr>
<tr>
<td>DAPK</td>
<td>Death Associated Protein Kinase 1</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal Carcinoma In Situ</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA Methyltransferase</td>
</tr>
<tr>
<td>DPC4</td>
<td>Deleted in Pancreatic Cancer 4</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to Mesenchymal Transition</td>
</tr>
<tr>
<td>FSP</td>
<td>Fibroblast Specific Protein</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>HGF</td>
<td>Human Growth Factor</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard Ratio</td>
</tr>
<tr>
<td>ID1</td>
<td>Inducer of Differentiation 1</td>
</tr>
<tr>
<td>IDC</td>
<td>Invasive Ductal Carcinoma</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL-11</td>
<td>Interleukin 11</td>
</tr>
<tr>
<td>INK4B</td>
<td>Cyclin-dependent Kinase Inhibitor 2B</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>K14</td>
<td>Keratin 14</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>LOX</td>
<td>Lysyl oxidase</td>
</tr>
<tr>
<td>LOXL2</td>
<td>Lysyl oxidase like 2</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase 1</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid Derived Suppressor Cell</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse Mammary Tumor Virus</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
</tr>
<tr>
<td>MYC</td>
<td>V-Myc Myelocytomatosis Viral Oncogene Homolog</td>
</tr>
</tbody>
</table>
NAF - Normal Associated Fibroblasts
NIH - National Institutes of Health
NK - Natural Killer
PDGFb - Platelet derived growth factor beta
PI3K - Phosphotidyl Inositide 3 Kinase
PyMT - Polyoma Middle T Virus
qPCR - quantitative polymerase chain reaction
RHOA - Ras Homolog Family Member A
SMAD - Mothers against decapentaplegic homolog
SNAI1 - Snail Homolog 1
TGF-β - Transforming Growth Factor Beta
TGFBR2 - Type II Transforming Growth Factor Beta Receptor
THBS1 - Thrombospondin 1
TIEG - Kruppel-Like Factor 10
VEGFa - Vascular endothelial growth factor alpha
ZO1 - Zonal Occludins 1
CHAPTER I

INTRODUCTION

Overview

The transforming growth factor β (TGF-β) pathway has been established as essential for cancer progression due to its prominent role in the regulation of cell growth, differentiation and migration. Through the canonical and non-canonical arms of the signaling pathway, TGF-β instigates cellular phenotypic changes and mediates its role as both a tumor suppressor and a tumor promoter. Indeed, the first described phenotypic effect of TGF-β signaling was the induction of a cellular cytostatic program (Tucker et al. 1984) and provided the first evidence for the pathway being tumor suppressive (Massague 2004). However, there was also evidence to the contrary, such as carcinomas that overexpressed TGFβ1 ligand having increased tumor progression (Gorsch et al. 1992; Hasegawa et al. 2001). The initial in vitro evidence for pro-tumorigenic effects of TGF-β consisted of the induction of a mesenchymal phenotype in epithelial tumor cells [commonly known as an epithelial to mesenchymal transition (EMT)] after prolonged exposure to TGF-β (Caulin et al. 1995; Miettinen et al. 1994). These early studies into the functional outcome of active TGF-β signaling underlie the difficulties in implementing clinically efficacious treatment regimens targeting the TGF-β pathway. The contextual cues driving the tumor suppressor and tumor promoter roles of TGF-β, as well as the switch between these two phenotypes are not fully understood.
As the understanding of tumor progression has expanded, the importance of the tumor microenvironment has been clearly demonstrated. It is interesting to note that TGF-β signaling both endogenously in human disease as well as in genetically engineered mouse models of cancer is associated with characteristic epithelial changes as well as significant changes in the stromal tumor microenvironment (Hazelbag et al. 2002; Walker et al. 1994; Wikstrom et al. 1998). TGFβ1 expression in invasive breast cancer correlates with progression of the cancer towards metastasis, extracellular matrix deposition, and the infiltration of immune cells (Walker et al. 1994). These findings have laid the ground work for recent studies which have established TGF-β signaling not only as an important mediator of epithelial phenotypic changes but also of changes in the stromal environment that are essential for tumor progression. Such findings lead us to hypothesize that microenvironmental TGF-β signaling, particularly in stromal fibroblasts, could regulate the progression of breast carcinomas. As such, we sought to address the functional contribution of stromal TGF-β signaling to phenotypic epithelial cell changes as well as the ultimate goal of understanding its role in metastasis.

**The TGF-β Signaling Pathway**

TGFβ1, β2 and β3 ligands act as the primary mediators of TGF-β signaling (Bierie and Moses 2006b; Massague 2012; Siegel and Massague 2003) and are secreted as inactive homodimeric polypeptides that can bind to latent TGF-β binding proteins, which promote extracellular sequestration (Rifkin 2005). Upon activation, the ligands bind to the type II TGF-β receptor causing recruitment and phosphorylation of the type I
TGF-β receptor resulting in downstream signaling activation (Shi and Massague 2003) (Fig.1). The strength of this signal depends on which ligand is bound, as the ligands vary in their binding affinity for this receptor. This variation in ligand binding promotes differential ligand presentation to the type II TGF-β receptor (Stenvers et al. 2003). The type III TGF-β receptor can augment the initialization of the signaling cascade through promotion of differential ligand binding (Moustakas et al. 1993). The final heterotetrametic form of the active receptors initiates downstream signaling through either SMAD-mediated canonical signaling or SMAD-independent non-canonical signaling (Shi and Massague 2003). Canonical signaling involves phosphorylation of the carboxy-terminal serine residue of the internal modulator smad proteins, SMAD2 or SMAD3, by the activated receptors (Derynck and Zhang 2003; Feng and Derynck 2005). To facilitate this interaction, adaptor proteins such as Smad anchor for receptor activation (SARA) are necessary (Tsukazaki et al. 1998). This phosphorylation induces oligomerization of SMAD2 or SMAD3 with SMAD4, which is necessary for nuclear translocation (Schmierer and Hill 2005). Through interactions with a variety of transcription co-factors, the nuclear localized SMAD complex initiates transcriptional activation or repression of a number of genes (Figure 1). A major target of TGF-β initiated transcription is SMAD7, known as an inhibitory Smad due to its ability to competitively inhibit the phosphorylation of SMAD2 or SMAD3 by TGFβR1 (Hayashi et al. 1997). In this way, canonical TGF-β signaling regulates the activity of the pathway preventing the potentially harmful effects of overactivation of the TGF-β pathway. TGF-β signaling has
also been linked to the activation of other signaling pathways, which comprises the non-canonical, Smad independent arm of the signaling pathway (Moustakas and Heldin 2005). The active hetero-tetrameric receptor instigates activation of the PI3K/Akt, RhoA, and MAPK pathways among others (Massague and Gomis 2006).

The outcome of these signaling pathways can either suppress cell proliferation or induce cellular migration and invasion. Studies into the cytostatic phenotype induced by TGF-β have established numerous intermediaries including repression of MYC and cyclin dependent kinase 4 (CDK4), as well as the induced expression of CDK inhibitors p21 (also known as CIP1) and INK4B (also known as p15) (Ewen et al. 1995; Hannon and Beach 1994; Polyak et al. 1994). Furthermore, SMAD dependent activation of TIEG1, DAPK and BIM, among others, results in the triggering of programmed cell death (Pardali and Moustakas 2007). Studies elucidating the tumor suppressor role of TGF-β corroborated evidence that loss of TGF-β signaling components was associated with carcinoma progression (Amendt et al. 1998; Bottinger et al. 1997). The signaling pathway downstream of EMT induction by TGF-β has also been partly mapped. Inhibitor of DNA binding 1 (ID1), a transcriptional regulator, is inhibited by TGF-β, which results in decreased expression of E cadherin and ZO1, two factors known to help maintain an epithelial phenotype. TGF-β signaling also induces the expression of EMT associated transcription factors, such as snail 1 (SNAI1), SNAI2 and lymphoid enhancer-binding factor 1 (LEF1), which help to promote loss of cellular adhesions and cytoskeletal rearrangement (Huber et al. 2005). Non-canonical signaling pathways activated by TGF-β, particular the RHO–ROCK and AKT pathways, were also shown to be essential in the
Figure 1. TGF-β Signaling During Tumor Progression. Early in tumorigenesis, TGF-β acts as a tumor suppressor in part through the SMAD dependent induction of cell cycle arrest. Thus, one can hypothesize that selective pressure leads to the expansion of tumor cells harboring inactivating mutations in the TGF-β pathway, thus allowing them to overcome the growth inhibitory effects of active TGF-β signaling (Green = TGF-β responsive, Red = TGF-β non-responsive). Normal TGF-β signaling in TGF-β responsive cells feeds through the type II TGF-β receptor to activate downstream signaling targets. Canonical signaling is activated through phosphorylation of the type I TGF-β receptor to induce nuclear localization and transcriptional activity of SMADs. Non-canonical signaling occurs independently of SMAD proteins and includes activation of RHOA, AKT and MAPK pathways. As outlined by Levy and Hill (2006), loss of TGF-β responsiveness can occur through loss of function mutations, loss of expression, or promoter methylation of receptors or SMADs.
Figure 1. TGF-β Signaling During Tumor Progression

Progression

**TGF-β Responsive Tumor Cell**
- Non-Smad Signaling:
  - MAPK
  - Akt
  - Par6

**TGF-β Non-Responsive Tumor Cell**
- Non-Smad Signaling:
  - MAPK
  - Akt
  - Par6

Transforming Growth Factor Beta Ligand
Transforming Growth Factor Beta Receptor I
Transforming Growth Factor Beta Receptor II

Receptor SMAD: SMAD2, SMAD3
Nuclear SMAD: SMAD4
Methyl
promotion of cellular migratory and invasive phenotypes observed on treatment with TGF-β (Dumont et al. 2003). TGF-β’s induction of Rho-ROCK signaling has been linked not only with cell migration, but with specifically single cell migration. Interestingly, abrogation of TGF-β signaling did not completely shut down epithelial migration but rather switched the cells towards a cohesive migratory phenotype (Giampieri et al. 2009). However, it is noted in this study that only single cells escaped into the vasculature which allowed for the establishment of metastasis. Thus, the dichotomous effects of TGF-β signaling was established in which active signaling is associated with tumor suppression early in tumor development through initiation of growth arrest and with tumor promotion through the induction of EMT and cellular migration and invasion in late stage tumors.

Regulation of TGF-β Signaling in Human Cancer

As pro- and anti-tumorigenic functions for TGF-β have been established, a key to understanding and effectively targeting the TGF-β pathway will involve delineating alterations to the pathway in human disease. As with most signaling pathways, deregulation leading to altered pathway activation can occur at several levels. Mutation or altered expression of ligands, receptors, or intercellular signaling mediators can affect proper cellular response to pathway activation.

In terms of progression of human cancer, there is little evidence to support mutations in TGF-β ligands (Levy and Hill 2006). Most alterations to this level of the signaling pathway occur through the induced overexpression and increased secretion of
the ligand (cBioPortal). Numerous studies across cancer types has associated increased expression of the TGF-β ligands with increased progression and metastasis of the disease as well as being indicative of poor patient survival (Decensi et al. 1998; Gorsch et al. 1992). This overexpression induces increased activation of the TGF-β pathway. However, as will be mentioned, later this does not necessitate induced epithelial changes and has been linked with increased stromal activation which also effects tumor progression.

Acting as a bottleneck for signaling activation, alterations in receptor expression and function are an essential component to abrogating or enhancing downstream signaling activation. Some of the first identified drivers of receptor inactivation in human cancers were associated with microsatellite instability in colon cancers (Markowitz et al. 1995). Loss of proper DNA mismatch repair machinery can lead to amplification of microsatellite regions. Sporadic generation of these microsatellite regions in promoter and coding regions of genes can lead to abrogation of their expression or expression of non-functioning proteins (Brentnall 1995). Such is the case for TGFBR2, which has been shown to have its expression abrogated due to such mutations in colon, lung, gastric, pancreatic cancer as well as gliomas (Goggins et al. 1998; Myeroff et al. 1995). More recently, work into the epigenetic regulation of gene expression has shown this to be an important factor in regulating TGF-β signaling in tumor cells. Epigenetic modulation of gene expression primarily occurs through modification of histones to promote nucleotide region availability and DNA methylation of CpG islands in gene promoter regions to modulate transcription factor binding and
promoter activity (Azad et al. 2013). Histone deacetylation is essential to mediating loss of TGF-β responsiveness in lung cancer as altered chromatin structure correlated with loss of TGFBR2 expression (Osada et al. 2001). TGFBR2 expression could be rescued through the treatment of cells endogenously with an inhibitor of histone deacetylase (Ammanamanchi and Brattain 2004). Similarly, promoter regions of these receptors have been shown to be methylated leading to suppression of their expression in numerous cancers (Chou et al. 2010; Hinshelwood et al. 2007; Pinto et al. 2003). Such mutations and epigenetic modifications promote loss of TGF-β responsiveness and underlie a cause for the loss of TGF-β’s cytostatic effects on cellular proliferation.

Upon ligand binding to TGFBR2, downstream signaling is initiated in cells through either canonical or non-canonical intercellular mediators. As SMADs represent the only TGF-β specific downstream signaling target, these proteins have been focused on for alterations leading to differential TGF-β response in tumor cells. Chromosomal loss of 18q21 has been found in nearly 90% of pancreatic cancer patients (Hahn et al. 1996). This chromosomal region contains many genes, but importantly this is where Deleted in Pancreatic Cancer 4 (DPC4), also known as SMAD4, is found. These findings have been extended to other cancer such as colon cancers. As a central hub for TGF-β signaling mediating nuclear shuttling of SMAD2 and SMAD3, loss of this chromosomal region is essential to mediating loss of TGF-β signaling in cancer cells. It is interesting to note that all mediators of TGF-β signal transduction specific to the tumor epithelium are associated with a loss of expression supporting the role of TGF-β as a tumor suppressor in tumor development. However, it should be noted that TGF-β ligands are typically
found to be overexpressed which potentially indicates that the pro-tumorigenic effects of TGF-β signaling lies outside of the epithelium in the tumor microenvironment.

As mentioned above, histone modification and promoter methylation play a role in the expression of TGF-β signaling components. However, these processes also play a role in determining the outcome of TGF-β signal activation. Actions of TGF-β directly, as well as known functions of the pathway, have been linked with altered histone and promoter modifications (Sann Sanda Khin 2011). At the histone level, transcriptional activation of SMAD2 targets requires histone acetylation by p300 prior to assembly of transcriptional machinery to drive gene expression (Ross et al. 2006). At the promoter level, numerous cancer types show hypermethylation of RunX3 to attenuate TGF-β’s growth suppressive functions (Yeh et al. 2011). Active TGF-β signaling is required for the maintenance of this methylation as tumors overexpress SMAD7, which inhibits TGF-β signaling, lose methylation of promoters and reverse the effects of gene silencing (Papageorgis et al. 2010). Such data lends credence to the push for use of histone deacetylase (HDAC) and dna methyltransferase (DNMT) therapeutics which could act to reactivate silenced tumor suppressor functions of the TGF-β pathway.

Epithelial TGF-β Signaling Effects on the Tumor Microenvironment

Even without the introduction of oncogenic changes, normal epithelium exposed to TGF-β results in phenotypic changes in the stroma. For example, expression of a constitutively active TβRII receptor in mammary epithelium results in mammary glands with greater collagen deposition surrounding the ductal epithelium (Muraoka-Cook et
al. 2006). Notably, mammary and pancreatic carcinoma cells harboring activated ALK5 induce a significant increase in angiogenesis on implantation into mice (Safina et al. 2007; Schniewind et al. 2007) (Figure 2). Active TGF-β signaling in mammary tumor cells also promotes tumor progression through SMAD independent induction of matrix metalloproteinase (MMP) expression resulting in enhanced angiogenesis and tumor cell invasion (Safina et al. 2007). One of the more interesting effects of TGF-β signaling in malignant epithelium on the tumor microenvironment stems from its recently discovered association with microRNA (miRNA) regulation. These potent modulators of gene expression are aberrantly expressed in numerous cancer types and linked with numerous pro- and anti-tumorigenic functions (Esquela-Kerscher and Slack 2006). TGF-β has recently been shown to promote the expression of DROSHA, which drives pre-miRNA accumulation through cleavage of pri-miRNA sequences (Davis et al. 2008), thus increasing the maturation of miRNAs. However, TGF-β signaling can also inhibit the function of specific miRNAs. TGF-β expression in hepatocellular carcinoma (HCC) cells induces the expression of CC motif chemokine ligand 22 (CCL22), through inhibition of miR-34a expression, and promotes recruitment of regulatory T cells (P. Yang et al. 2012). TGF-β signaling also reduces the expression of miR-29a thereby regulating the induction of angiogenesis in endothelial cells (J. Wang et al. 2013). TGF-β also induces the expression of miR-494 to regulate recruitment of myeloid derived suppressor cells (MDSCs) to tumor tissue. TGF-β induced miR-494 also modulates MDSC function through promoting expression of pro-tumorigenic Arg1 and MMP2, 13, and 14 (Y. Liu et al. 2012b).
Figure 2. Microenvironmental Changes Mediated by Tumor Cell TGF-β Signaling. TGF-β signaling in tumor cells induces the expression of numerous mediators of extracellular change. Tumors showing increased activity of TGF-β in the tumor cells are characterized by increased extracellular matrix (ECM) deposition through increased secretion of matrix proteins and maturation through ECM modifying enzymes such as LOXL4. Additionally, TGF-β signaling in tumor cells drives the induction of endothelial cell recruitment and proliferation driving increased angiogenesis. Conversely, TGF-β suppresses the expression of numerous cytokines and chemokines such as CXCL1 and CXCL5. Loss of TGF-β responsiveness relieves this suppression and results in enhanced immune cell infiltration. These microenvironmental changes promote epithelial cell and stromal cell phenotypic responses, which significantly affect tumor progression. Shown are the phenotypic changes resulting specifically from epithelial derived factors listed.
Figure 2. Microenvironmental Changes Mediated by Tumor Cell TGF-β Signaling

<table>
<thead>
<tr>
<th>TGF-β Responsive Tumor Cells (single cell invasion)</th>
<th>TGF-β Non-Responsive Tumor Cells (cohesive invasion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>• CTGF</td>
<td>• CXCL1</td>
</tr>
<tr>
<td>• PDGFβ</td>
<td>• CXCL2</td>
</tr>
<tr>
<td>• HGF</td>
<td>• CXCL5</td>
</tr>
<tr>
<td>• THBS1</td>
<td>• ADAMTS9</td>
</tr>
<tr>
<td>• IL-8</td>
<td>• GCSF</td>
</tr>
<tr>
<td>• VEGFa</td>
<td></td>
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<tr>
<td>• MMP9</td>
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<td>• CXCL12</td>
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<td>• LOXL4</td>
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- Tumor Cell Undergone EMT
- Macrophage
- Immature Collagen
- Polymorphonuclear Myeloid Cell
- Mature/Crosslinked Collagen
- Dendritic Cells
Induction of TGF-β signaling through genetic alterations or through the treatment of carcinoma cells with TGF-β has identified numerous gene targets, many of which are conserved in various cancer types (Hills et al. 2010; Maupin et al. 2010; Sartor et al. 2010; S. E. Wang et al. 2008). These experiments have begun to address the functional significance of gene expression induced by TGF-β by correlating these changes with patient data that predicts poor patient outcome (Coulouarn et al. 2008; S. E. Wang et al. 2008). Importantly, many of these gene expression targets have well characterized functions in modification of the tumor microenvironment and link with phenotypic changes seen on alteration of TGF-β signaling in mouse models of cancer (Fig. 2). TGF-β signaling in epithelial cells induces the expression of numerous extracellular matrix genes, including collagen 1 α1 (COL1α1) and COL4α1, as well as matrix modifying enzymes MMP2, MMP9 and lysyl oxidase-like 4 (LOXL4). These same studies also provide supporting evidence for the observed angiogenic phenotypes on TGF-β activation in that gene expression analysis showed a significant increase in vascular endothelial growth factor A (VEGFA) and Thrombospondin 1 (THBS1) (Hills et al. 2010; Maupin et al. 2010; Sartor et al. 2010; S. E. Wang et al. 2008). Given the previously mentioned EMT and migratory phenotypic changes induced on activation of TGF-β signaling, as well as the TGF-β epithelial gene signature associated with tumor recurrence, the identification of stromal changes solidifies epithelial TGF-β signaling as a pro-tumorigenic signaling pathway. However, such investigations do not segregate the epithelial and stromal effects of epithelial TGF-β signaling on the promotion of tumor
cell metastasis and thus do not address the specific contribution of each to the correlation of TGF-β signaling with poor patient outcome.

As tumors progress, the growth inhibitory effects of TGF-β are overcome through the loss of TGF-β pathway elements or downstream signaling targets (Bierie and Moses 2006b; Massague 2008). Loss of TGF-β responsiveness in tumor cells has significant effects on tumor progression not only through altered epithelial characteristics, but also through gene expression changes affecting the tumor microenvironment. The primary association of activated TGF-β signaling has been with increased metastasis and poor patient prognosis primarily through induction of EMT (Giampieri et al. 2009; Mima et al. 2013). However, abrogation of TGF-β signaling in carcinoma cells can also result in increased metastasis (Bierie et al. 2008; Forrester et al. 2005). Loss of TGFβ signaling components in both mouse models of cancer and human cancer has been associated with poor prognosis through increased progression and metastasis (Bottinger et al. 1997; Levy and Hill 2006; S. L. Lu et al. 2006; Malkoski et al. 2012; Paiva et al. 2012). Similar to receptor activation in normal mammary epithelium, pancreatic epithelium expressing a dominant negative type II TGF-β receptor results in increased desmoplasia and angiogenesis in adult mice (Bottinger et al. 1997). Abrogation or attenuation of TGF-β signaling either at the receptor or SMAD level in mouse models of cancer has various expected epithelial effects such as loss of growth inhibition, but also induces a number of stromal changes. Such changes include activation of stromal fibroblasts, deposition of collagenous extracellular matrix, infiltration of a variety of immune cells, and increased angiogenesis (Bierie and Moses 2006a). Recent work has shown that the
infiltration of MDSCs is increased in tumors in which epithelial TGF-β signaling is abrogated (L. Yang et al. 2008) (Fig.2). The increased recruitment of these cells is primarily associated with the increased expression of the chemokines CXCL1 and CXCL5, the expression of which is normally inhibited by TGF-β. Although this altered chemokine expression pattern is associated with increased myeloid cell infiltration into the tumor microenvironment, these chemokines can also drive the activation of stromal fibroblasts through the induction of connective tissue growth factor (CTGF) expression (Ijichi et al. 2011). Perhaps the most interesting induced gene expression change to result from abrogation of TGF-β signaling in epithelial cells is TGF-β ligand itself (Gewin et al. 2010; Lin et al. 2012). Given that the epithelium expressing this cytokine can no longer respond to it, any effects derived from its expression would either be on a separate population of epithelial cells retaining their ability to respond to TGF-β or cells found in the stromal microenvironment. This intriguing aspect of altered gene expression on epithelial TGF-β impairment will be discussed in the next section of this chapter as TGF-β has significant effects on numerous cell types found in the tumor microenvironment.

Recent work addresses the contribution of stromal changes, primarily in chemokine secretion, due to attenuation of TGF-β signaling in malignant mammary epithelium, on the outcome of human disease (Bierie et al. 2009). In this paper epithelial loss of TGFβR2 results in increased expression of CXCL1, CXCL5, and bone marrow stromal cell antigen 2 (BST2) while downregulating expression of genes such as CXCL12, platelet derived growth factor β (PDGFβ) and CTGF (Fig.2). These gene
expression changes significantly correlated with worse patient outcome in patients with lymph node positive, estrogen receptor positive (ER+) Luminal A type breast cancer.

These findings introduce an interesting concept explaining many of the counterintuitive findings regarding impairment of epithelial TGF-β signaling ultimately promoting enhanced metastasis. If, as many have shown, the stromal microenvironment is in and of itself a prognostic factor in driving tumor metastasis, it could override the previously established cell autonomous signaling events associated with tumor cell migration, invasion and metastasis (Finak et al. 2008). Thus, even though tumor cells lacking TGF-β responsiveness no longer gain any epithelial centric metastatic advantages through the induction of EMT and migratory changes, the stromal alterations resulting from gene expression changes upon loss of TGF-β responsiveness compensate and overcome this loss to promote metastasis. As tumors are heterogeneous, likely consisting of cells with and without the ability to respond to TGF-β, the contribution of stromal changes brought on by TGF-β signaling null epithelial cells to the metastasis of TGF-β responsive epithelium and vice versa is a concept yet to be delineated and is an important step in completing our understanding of TGF-β signaling dynamics and their effects on tumor progression.

**Effects of TGF-β Signaling in Stromal Fibroblasts on Tumor Progression**

Classically, increased expression of TGF-β1 in the bulk tumor, as well as increased serum levels of TGF-β ligands, is associated with poor patient prognosis (Gonzalez-Santiago et al. 2011; Smirne et al. 1999). While the effects of this increased
ligand expression on epithelial tumor progression has been well established, consideration of the tumor stroma has only recently garnered a significant amount of momentum. Although giving no indication of being causal or merely an effect of progression, specific gene expression changes in the stroma are noted at defined steps of breast cancer progression (Knudsen et al. 2012). Thus, much like the “Vogelgram” laid out specific genetic changes associated with the stages of colon cancer progression (Vogelstein et al. 1988), we are beginning to appreciate that gene expression changes are also occurring in the stroma of tumors that correspond with tumor progression and staging. However, mechanisms behind these changes and the pathways driving specific gene expression changes have yet to be fully elucidated. Given, the pleotropic nature of TGF-β and its established role in manipulating the development and function of numerous stromal cells known to affect tumor progression, the homeostatic functions of these cells and how they are altered by TGF-β signaling to effect tumor progression is an important consideration in determining the pro- or anti-tumorigenic role of stromal TGF-β signaling. In particular, the role of TGF-B in manipulating the function of fibroblasts is well known. Given that TGF-B signaling is essential to determining their phenotypic responses as well as fibroblasts having a significant role in tumor progression, these cells became a focal point for our studies.

Being one of the most well studied non-immune cells in the tumor microenvironment, carcinoma associated fibroblasts have been shown to play an important role in nearly every hallmark of tumor progression (Hanahan and Coussens 2012). These functions are performed through the secretion of a myriad of growth
factors and cytokines but primarily through the secretion and remodeling of extracellular matrix proteins. TGF-β has previously been shown to induce myofibroblast differentiation in fibroblasts leading to increased collagen deposition and ECM remodeling (Desmouliere et al. 1993; Sime et al. 1997). In fact, one of the most common features of carcinomas overexpressing TGF-β is a desmoplastic stromal environment (Border and Noble 1994; Verona et al. 2007; Walker et al. 1994). Increased ECM deposition corresponds well with known functions of TGF-β in fibroblast activation and desmoplasia independently corresponds with development of breast cancer (Bierie and Moses 2006a; Boyd et al. 2007; Leask and Abraham 2004; Massague 2012). Similarly, TGF-β has been shown to drive the expression of matrix remodeling genes such as lysyl oxidase and SPARC to promote the maturation of secreted collagens from fibroblasts (Peyrol et al. 1997; Reed et al. 1994). Recently, it has been appreciated that the act of maturing these collagen fibers has its own effect on tumor progression through the induction of mesenchymal epithelial characteristics via increased matrix stiffness (Levental et al. 2009; Paszek et al. 2005). Thus, through corollary evidence we can appreciate that TGF-βs action on the extracellular matrix through fibroblasts acts to promote tumor progression. Direct testing to address this connection indeed results in the expected outcome. Expression of a constitutively active Tgfbr1 in dermal fibroblasts results in increased fibrosis and increased expression of known fibroblast TGF-β target genes in chemically induced tumors in mice (Sonnylal et al. 2007). Thus, an abundance of TGF-β in a tumor would likely elicit a similar response. This has been shown to be true given that tumors that have been characterized as having increased levels of TGF-β
Figure 3. TGF-β Modulates Fibroblast Microenvironmental Function. Fibroblasts have numerous functions that can affect tumor progression including chemokine and growth factor release, secretion of matrix components, and induction of matrix remodeling. TGF-β activity in these fibroblasts mediates these effects through promotion or suppression of various mediators of the resulting phenotype. Studies analyzing TGF-β effects on gene expression through microarray analysis and proteomic analysis of fibroblasts with intact or defective TGF-β signaling pathways are summarized. Green boxes indicate gene expression changes that are induced by TGF-β signaling and red boxes are those genes that are suppressed by TGF-β.
Figure 3. TGF-β Modulates Fibroblast Microenvironmental Functions

[Diagram showing the modulation of fibroblast functions by TGF-β, with categories for Chemokines, Matrix Deposition, Growth Factors, and ECM Remodeling, listing various molecules and their interactions.]
are associated with increased fibroblast activation and collagen deposition. Adding to this, fibroblasts derived from small cell lung carcinomas show enrichment for TGF-β signaling compared with normal lung fibroblasts and the TGF-β enriched gene signature derived from these fibroblasts predicted poor patient outcome (Navab et al. 2011). In fact, it has been shown that this increased TGF-β activation is caused through interactions between stromal fibroblasts and colon carcinoma cells inducing expression of numerous MMPs and known TGF-β target genes (Hawinkels et al. 2012). As discussed above, numerous studies illustrate TGF-β mediated signaling mechanisms involved in the reciprocal interactions of tumor cells and the stroma. However, it wasn’t until recently that the effects of these interactions in the context of tumor cell metastasis has been appreciated. Calon et al. show overexpression of TGF-β in a TGF-β nonresponsive carcinoma cell line drives enhanced metastasis of these tumor cells. This is through the stimulated expression of known TGF-β target genes such as interleukin 11 (IL-11), angiopoietin-like 4 (ANGPTL4), and CTGF from stromal fibroblasts (Figure 3). Importantly, the TGF-β driven signature from these fibroblasts was able to predict recurrence in human colon cancer patients (Calon et al. 2012). As the authors comment, such TGF-β induced gene expression changes have also been ascribed to epithelial carcinoma cells, thus, regardless of the tumors cells ability to respond to TGF-β, the pro-tumorigenic effects of this gene expression profile are seen in tumors expressing high levels of TGF-β.

Similar to epithelial TGF-β signaling, work examining the role of fibroblast TGF-β on tumor progression has revealed a contradictory role for the pathway with respect to
tumor progression. While there is a preponderance of evidence supporting a tumor promoting role of TGF-β in stromal fibroblasts, there is also a significant amount of work showing a tumor suppressive role. The foundation of this work lies in the paper from Bhowmick et al. showing that specific deletion of Tgfbr2 in fibroblasts systemically results in spontaneous carcinoma initiation (Bhowmick et al. 2004). Further work has shown that fibroblasts lacking TGF-β signaling increase the progression of breast, prostate, and squamous cell cancer, as well as melanoma (Cheng et al. 2005; Franco et al. 2011; Meng et al. 2011). The major gene expression changes associated with the loss of this signaling axis in fibroblasts are in cytokine and chemokine expression. In particular, increased expression of CXCL1, CXCL5, CXCL12, and TGF-β1 is observed in fibroblasts with abrogated TGF-β signaling (Xu et al. 2010) (Figure 3). This would indicate that modulation of the tumor microenvironment through increased infiltration of immune cells is a primary driver behind enhanced tumor progression seen when fibroblasts lose TGF-β responsiveness (Bacman et al. 2007; Hazelbag et al. 2002). Supporting this is recent work from Achyut et al. in which a significant increase in inflammation is observed in spontaneous forestomach carcinomas resulting from abrogation of TGF-β signaling in fibroblasts (Achyut et al. 2013). Interestingly, this inflammatory response appears to drive tumor formation through the silencing of CDKN1A via epigenetic promoter methylation. Administration of anti-inflammatory drugs significantly delayed tumor onset and increased overall survival, supporting immune cell infiltration as a facilitator of tumor development. This work has correlated TGFBR2 expression with progression of breast cancer from normal to ductal carcinoma.
in situ (DCIS) to invasive ductal carcinoma (IDC) and shows that stromal TGFBR2 expression goes down as tumor progress towards invasiveness (Knudsen et al. 2012). Interestingly, immunohistochemical studies in colon cancer have shown a similar trend but have gone one step further to show that low expression of TGFBR2 in the stroma is an independent predictor of poor patient prognosis (Bacman et al. 2007). This work indicates that the systemic use of TGF-B inhibitors on cancer patients may elicit beneficial effects from the context of the tumor cell itself, but could induce protumorigenic effects when modulating carcinoma associated fibroblasts.

**Identified Microenvironmental Effects of TGF-β Targeted Therapeutics in Preclinical Models**

Given the significant effects seen in tumor progression on manipulation of TGF-β responsiveness, efficacious intervention in the TGF-β pathway remains a highly sought after goal. With evidence supporting loss of TGF-β receptors in epithelial cells having a profound effect on the stroma, as well as increased ligand expression by the tumor being associated with poor patient prognosis, the tumor microenvironmental effects of this pathway are increasingly becoming relevant to the implementation of therapeutics targeting the TGF-β pathway. In particular, the use of treatments which inhibit the TGF-β pathway act to reverse many pro-tumorigenic effects active TGF-β signaling has on the tumor microenvironment. Preclinical studies using drugs which inhibit TGF-β signaling through either ligand sequestration or inhibitory receptor binding are well tolerated and efficacious in inhibiting both epithelial and stromal TGF-β signaling activation.
Interestingly, numerous studies note these treatments are efficacious in slowing tumor progression and correlate these findings with significant changes in the tumor microenvironment (Table 1). Based on similar findings found across numerous studies, it appears that inhibition TGF-β signaling significantly impedes tumor progression through its effects on the immune system. Systemic treatment of tumor bearing mice with inhibitors blocking TGF-β signaling results in increased maturation and activity of dendritic cells thus leading to increased tumor antigen presentation as well as increased T cell mediated tumor cell cytotoxicity (Diaz-Valdes et al. 2011; Tanaka et al. 2010).

Other studies have shown similar results in that treatments inhibiting TGF-β signaling present with increased T cell infiltration or loss of chemotaxis of myeloid cells which could impede anti-tumor immunity (Terabe et al. 2009; Zhong et al. 2010). Regardless of the mechanism identified, the end result of these experiments in many cases is an increase in T cell mediated cytotoxicity and decreased tumor growth and progression (Hardee et al. 2012; Schlingensiepen et al. 2011; Uhl et al. 2004).

Others have noted anti-tumorigenic effects of anti-TGF-β treatments which occur through decreased endothelial cell proliferation, blood vessel formation, and ultimately inhibition of angiogenesis (Hardee et al. 2012; Mazzocca et al. 2009; Noma et al. 2008; M. Zhang et al. 2011). This phenotype aids in slowing tumor progression not only by limiting nutrients to tumor cells, but also by increasing the delivery of chemotherapeutic agents. Popularized by Rakesh Jain, the hypothesis of stromal normalization postulates that inhibition and reversal of pro-tumorigenic
### Table 1. Microenvironmental Effects of TGFβ Inhibitors in Preclinical Tumor Models

<table>
<thead>
<tr>
<th>TGFβ Target</th>
<th>Drug Name</th>
<th>Cancers Tested</th>
<th>Microenvironmental Effects</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β Ligand Trap</td>
<td>P144</td>
<td>Melanoma</td>
<td>↑Dendritic Cell, Natural Killer, and T cell activity</td>
<td>(Diaz-Valdes et al. 2011)</td>
</tr>
<tr>
<td>1D11</td>
<td>Lung, Breast, and Glioblastoma</td>
<td>↑Natural Killer cell infiltration and CD8+ mediated cell killing ↓Blood Vessel Area</td>
<td>(Hardee et al. 2012; J. Liu et al. 2012a; Terabe et al. 2009)</td>
<td></td>
</tr>
<tr>
<td>TGF-β Antisense</td>
<td>AP12009</td>
<td>Pancreatic Cancer</td>
<td>↑Immune Cell Mediated Cytotoxicity</td>
<td>(Schlingensiepen et al. 2011)</td>
</tr>
<tr>
<td>Receptor Inhibitors</td>
<td>GW788388</td>
<td>Esophageal Squamous Cell Carcinoma</td>
<td>↓Fibroblast activation ↓vascular development</td>
<td>(Noma et al. 2008)</td>
</tr>
<tr>
<td>LY2109761</td>
<td>Hepatocellular carcinoma, Glioblastoma</td>
<td>↓Angiogenesis ↑periocyte coverage</td>
<td>(Mazzocca et al. 2009; M. Zhang et al. 2011)</td>
<td></td>
</tr>
<tr>
<td>LY364947</td>
<td>Glioblastoma</td>
<td>↑vascular permeability</td>
<td>(Hardee et al. 2012)</td>
<td></td>
</tr>
<tr>
<td>LY3022859</td>
<td>Breast, Pancreas, Colon Cancer</td>
<td>↑Natural killer cell and Cytotoxic T cell mediated tumor cell killing ↓Treg cell presence ↓MDSC infiltration</td>
<td>(Zhong et al. 2010)</td>
<td></td>
</tr>
<tr>
<td>SB431542</td>
<td>Breast Cancer</td>
<td>↑Dendritic Cell maturation</td>
<td>(Tanaka et al. 2010)</td>
<td></td>
</tr>
<tr>
<td>SD-208</td>
<td>Breast Cancer Bone Metastasis, Glioma</td>
<td>↓Osteoclast differentiation ↑Immune Cell Mediated Cytotoxicity</td>
<td>(Uhl et al. 2004)</td>
<td></td>
</tr>
<tr>
<td>SX-007</td>
<td>Glioma</td>
<td>↑CD3+ Cell Presence</td>
<td>(Tran et al. 2007)</td>
<td></td>
</tr>
<tr>
<td>SM-16</td>
<td>Lung Cancer</td>
<td>↑Activated CD8+ T cells, ↑CD4+ Presence</td>
<td>(Garrison et al. 2012)</td>
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</table>
microenvironmental changes would be beneficial to patients due to enhanced delivery of cytotoxic chemotherapies. In an elegant series of experiments, Dr. Jain showed that TGF-β inhibition fit the role of a stromal normalizer in that 1D11 treatment of mammary tumor implants reduced collagen deposition and increased blood vessel perfusion which ultimately results in an increased efficacy of doxorubicin uptake (J. Liu et al. 2012a). While not explicitly shown, there is also the potential of anti-TGF-β treatments to free cells from cell cycle arrest induced by active TGF-β signaling and thus enhance the efficacy of chemotherapeutics even further.

**Overview of Current TGF-β Targeting Clinical Trials for Cancer Treatment**

Several phase I/II clinical trials of TGF-β neutralizing antibodies and small molecule inhibitors to both ligand and receptor have shown to be safe and efficacious with many trials still accruing patients (Bogdahn et al. 2011; Mead et al. 2003; Roldan Urgoiti et al. 2012; Schlingensiepen et al. 2011). TGF-β inhibitory treatment regimens across numerous cancer types in mouse models as well as early stage clinical trials have shown benefits from the treatment with a mechanism ascribed being increased activity of antitumoral adaptive immune cells (Kim et al. 2008; Uhl et al. 2004). Treatments targeting the TGF-β pathways fall into three categories of inhibitors: extracellular trapping of TGF-β ligands, antisense knockdown of TGF-β ligand expression, and inhibitors of activation of TGF-β receptor kinase activity. GC-1008, a ligand trap developed by Genzyme, has shown to be well tolerated in human Phase I trials with 5 of the 23 patients treated showing partial response. The antisense oligo AP12009, which
targets the TGF-β2 ligand, has also been used in phase I clinical trials and provides a significant survival benefit over conventional chemotherapy in high grade glioma patients (Bogdahn et al. 2011). Interestingly, the mechanism behind this response is thought to derive from enhanced anti-tumoral immunity in these patients. Yet, as more is learned about the effects of TGF-β signaling pathway in specific components of the microenvironment, we begin to see potential reasons behind a lack of response in some patients. For example, loss of TGF-β signaling in fibroblasts diminishes the induction of a desmoplastic response from these cells yet still promotes tumor progression through augmentation of inflammatory cell infiltration (Achyut et al. 2013; Calon et al. 2012). Heterogeneous cell populations within the primary tumor must also be given consideration as inhibition of TGF-β may prevent tumors from undergoing EMT and invading into the surrounding tissue, but this may occur at the cost of freeing cells from the growth inhibitory effects of TGF-β. In spite of this, clinical trials involving TGF-β inhibition through either genetic abrogation of TGF-β sensitivity, such as through the adoptive transfer of T lymphocytes expressing dominant negative forms of TGF-β receptor, or the use of inhibitory antibodies have pushed forward. Though modest and mixed results are consistently obtained from these trials, this is probably due to varying stromal compositions and the pleotropic effects of TGF-β.

Without a thorough understanding of the contextual dependence of TGF-β signaling actions in the stroma it is likely that the implementation of systemic therapeutics targeting the TGF-β pathway will yield inconclusive results due to tumor heterogeneity. Thus, novel methods of delivery that allow for specific targeting to a
cellular type or evasion of pathway activation are being explored, including the adoptive transfer of T lymphocytes with abrogated TGF-β response thus circumventing its antitumorigenic response. This highly specific manipulation should provide beneficial effects on tumor progression without introducing controversial effects by effecting TGF-β signaling in other stromal and epithelial cells. There must also be a greater emphasis placed upon the understanding of how stromal components interact with each other and with tumor cells to drive tumor progression. Such studies which would include how TGF-β modulates this interaction would provide a clear picture of the context in which tumor progression is occurring. A greater understanding of the context driving TGF-β signaling effects in tumor progression will provide invaluable information on how and when to target the TGF-β pathway in patients.

**Summary**

Primary work supporting the dichotomous nature of TGF-β signaling in cancer progression includes early studies involving epithelial overexpression of TGF-β ligand and abrogation of epithelial TGF-β signaling through receptor knockout. While supporting dual roles for TGF-β signaling in late stage tumor progression both these models present with two similarities, increased metastasis and increased secretion of TGF-β ligand. While TGFBR2 knockout epithelium is unable to respond to this increased ligand secretion, the microenvironmental changes would still be present. Observations from our laboratory and others support microenvironmental changes representing a significant portion of the effects of epithelial TGF-β ligand overexpression and TGF-β
signaling abrogation. This led us to hypothesize that stromal TGF-β signaling was driving enhanced metastasis in our TGFBR2 knockout tumors. As described above, TGF-β signaling in stromal components supports tumor progression and metastasis through the induction of pro-tumorigenic microenvironmental functions, through MSC, fibroblast, and myeloid cells mediators, and inhibition of anti-tumorigenic immune cell function, particularly those involving the adaptive immune system. Thus, we investigated the role of TGF-β signaling in stromal fibroblasts as a mediator of the resultant microenvironmental changes which could be responsible for this phenotype. Interesting, our findings once again support a dichotomous role for TGF-β signaling, but this time in the context of a fibroblast rather than an epithelial cell. Presented herein we demonstrate that TGF-β can drive expression of matrix remodeling genes and that this matrix remodeling promote tumor cells metastasis. Conversely, abrogation of TGF-β signaling in fibroblasts also promotes tumor progression through an altered gene expression profile that enhances tumor progression. Given that once again TGF-β signaling is shown to have dichotomous effects on tumor progression, our results support the use of highly selective TGF-β inhibition, such as through the adoptive transfer of cells lacking the ability to respond to TGF-β, or targeting the downstream targets of the TGF-β pathway identified as driving the pro-tumorigenic changes, such as inhibition of the mediators of matrix remodeling or immune cell chemotaxis.
Chapter II

TGF-β INDUCED FIBROBLAST MATRIX REMODELING PROMOTES TUMOR CELL METASTASIS

Introduction

The stromal microenvironment of a tumor is an essential component of tumor progression (Finak et al. 2008). Comprised of various resident and recruited cell types as well as extracellular proteins, the stromal components can determine phenotypic characteristics and ultimately patient outcome. By providing growth factors and other migratory signals as well as depositing scaffolding proteins, the tumor stroma can effectively drive or impede a tumor cell toward intravasation and metastatic colonization (Khamis et al. 2012). Specifically, matrix deposition and remodeling, largely facilitated through fibroblast mediators, promotes tumor growth and migration (Levental et al. 2009). While stromal influence is acknowledged, a full understanding of the signals driving the formation of a tumor promoting stroma as well as the reciprocal response of the epithelium to these changes has yet to be obtained. Insights into these interactions will provide the backbone for future therapeutic interventions specifically targeting tumor-stromal crosstalk.

Extracellular matrix (ECM) proteins, and in particular collagen, are a major component of the tumor microenvironment and exert significant effects on the tumor
epithelium (Garamszegi et al. 2009). Through its integrin mediators, extracellular matrix proteins encourage tumor growth and invasiveness. Increased mammographic density, which is significantly associated with collagen levels, independently predicts increased probability of occurrence of breast cancer in patients (Boyd et al. 2007). These results are mimicked in murine models of breast cancer progression in which deposition of collagen that is unable to be proteolytically cleaved results in increased tumor formation as well as increased lung metastasis (Provenzano et al. 2008). Recently, it has been appreciated that ECM-epithelial crosstalk is not only mediated by the ECM proteins themselves, but by the orientation and crosslinking status of the collagen fibers. Lysyl Oxidase (LOX) is a matrix modifying enzyme that cross-links and stiffens collagen fibers to promote their stability (Levental et al. 2009). LOX has garnered interest in breast cancer as an important enzyme regulating stromal modification to drive malignant progression (Levental et al. 2009). Although epithelial LOX has been implicated in tumor metastasis through the promotion of a TGF-β driven Epithelial to Mesenchymal Transition (EMT) and integrin mediated epithelial invasion (Taylor et al. 2011), the role of stromal LOX in tumor metastasis has yet to be examined.

Previous work from our laboratory showed that abrogation of TGF-β signaling in epithelia results in a significant increase in PyMT driven mammary carcinoma metastasis (Forrester et al. 2005). Looking at the primary tumor for a potential cause of this phenotype, one of the most striking observations was an expansion of the stromal microenvironment (Bierie et al. 2008). As these tumors presented with increased levels of TGF-β derived from infiltrated myeloid derived suppressor cells and an increase in
αSMA positive fibroblasts, we hypothesize that these activated fibroblasts are driving stromal expansion through increased matrix remodeling. Work we have published in Pickup et al. 2013 aimed to identify differentially regulated matrix associated genes resulting in increased stromal expansion in the PyMT$^{mgko}$ model of breast cancer and to examine their role in driving epithelial cell phenotypes which ultimately results in metastasis. To address this question, we used our established PyMT$^{mgko}$ model of mammary tumor progression in which the PyMT ongogene is selectively expressed in the mammary epithelium through the mouse mammary tumor virus (MMTV) promoter. Our findings show that TGF-β secreted by myeloid cells induces expression of LOX by carcinoma-associated fibroblasts, which in turn increases matrix crosslinking and stiffness to drive Keratin 14 cell FAK signaling, carcinoma cell intravasation and metastasis.

**Materials and Methods**

**Mouse Model**

TβRII(fl/fl) mice were crossed with MMTV-PyVmT/MMTV-Cre/ TβRII(fl/fl) transgenic mice to produce the TβRII(fl/fl)/PyMT (PyMT$^{fl/fl}$) and TβRII(fl/fl)/PyMT/MMTV-Cre (PyMT$^{mgko}$) mice. Cell lines were isolated from these spontaneous tumors and used in further *in vitro* experimentation. LOX inhibition studies used Beta-Aminopropriontrile (3mg/ml, Sigma) dissolved in the drinking water. Mice were housed and handled according to approved Institutional Animal Care and Use Committee protocols.
**Lung Whole Mount and Circulating Tumor Cell analysis**

Lungs were fixed in 10% neutral buffered formalin overnight at 4°C. The next day, lungs were dehydrated, placed in xylene for 1 h, and then changed to fresh xylene overnight. Lungs were rehydrated before dipping in Mayer's hematoxylin for 2 min and then washed in running tap water for 5 min. Tissues were destained in HCl (fresh 1% v/v from a 12 N solution) for 20 min, rinsed in running tap water overnight, dehydrated, and placed in xylene overnight before counting of stained metastatic tumor foci under a dissecting light microscope.

Circulating blood was isolated from the left ventricle of tumor bearing mice upon sacrifice. 200uL of the blood was plated into a well of a gelatin coated 6 well dish and allowed to grow for 3 to 4 weeks. After the growth phase, colonies larger than 150um were counted and quantified.

**Picrosirius Red Staining and Quantification**

Five micron sections of paraffin-embedded mammary tumors were stained with 0.1% Picrosirius Red (Direct Red 80; Sigma Aldrich). Stained sections were imaged on a Zeiss Axiophot equipped with a cross-polarizer. Images were quantified for pixel density of thresholded light intensity (Levental et al. 2009).

**In situ hybridization**

The following protocol was performed on sections of fresh frozen tumor tissue. In brief, sections were digested with 0.125mg/ml of pronase, fixed in 10% formalin and blocked
with 0.2% glycine. Sections were probed with Digoxigenin labeled sense and anti-sense probes, each ~300bp in length. Probes were obtained from digestion of full length mouse LOX cDNA with HindIII and XbaI (New England Biolabs). Following overnight probe incubation, staining was visualized through staining sections with 1:500 AP-labeled anti-DIG (Roche). Sections were counterstained with DAPI (Invitrogen) for nuclei visualization (Gorden et al. 2007).

Collagen Contraction Assay

Assay was performed via Cell Biolabs Protocol for Cell Contraction Assay CBA-201. Briefly, 100,000 fibroblasts are suspended in a 0.5mL collagen matrix and allowed to solidify in a well of a 24 well plate. After solidification, the collagen mixture is freed from the plate into a floating state at which point contraction of the collagen gel is monitored and recorded. Pictures at various timepoints are quantified for area relative to the 0 hour time point.

Tissue preparation for AFM measurements of ECM stiffness:

Mammary glands were analyzed following cryopreservation. Fresh glands were embedded in OCT (Tissue-Tek) aqueous embedding compound within a disposable plastic base mold (Fisher) and were snap frozen by direct immersion into liquid nitrogen. Frozen tissue blocks were then cut into 20 µm sections using disposable low profile microtome blades (Leica, 819) on a cryostat (Leica, CM1900-3-1). Prior to the AFM measurement, each section was thawed by immersion in PBS at room temperature. The samples were maintained in proteinase inhibitor in PBS (PROTEASE INHIBITOR COCKTAIL...
Roche Diagnostics, 11836170001), with Propidium Iodide (SIGMA P4170, 20 µg/ml) during the AFM session.

**AFM measurements of ECM stiffness on tissue sections:**

All AFM indentations were performed using an MFP3D-BIO inverted optical AFM (Asylum Research) mounted on a Nikon TE2000-U inverted fluorescent microscope, as previously described (Lopez et al. 2011). Briefly, we used silicon nitride cantilevers with spring constant of 0.06 N/m with borosilicate glass spherical tip with 5 µm in diameter (Novascan Tech). The cantilever was calibrated using the thermal oscillation method prior to each experiment. Samples were indented at 20 µm/s loading rate, with a maximum force of 2 nN. Five AFM force maps were typically obtained on each sample, each map as a 20x20 µm raster series of indentations utilizing the FMAP function of the IGOR PRO build supplied by Asylum Research. The Hertz model was used to determine the elastic properties of the tissue. Tissue samples were assumed to be incompressible and a Poisson’s ratio of 0.5 was used in the calculation of the Young’s elastic modulus.

**Two-photon microscopy image acquisition and analysis:**

For two-photon imaging, we used custom resonant-scanning instruments based on published designs containing a five-PMT array (Hamamatsu, C7950) operating at video rate (Bullen et al. 2009). The setup was used with two channel simultaneous video rate acquisition via two PMT detectors and an excitation laser (2W MaiTai Ti-Sapphire laser, 710-920nm excitation range). Second harmonics imaging was performed on a Prairie Technology Ultima System attached to an Olympus BX-51 fixed stage microscope.
equipped with a 25× (NA 1.05) water immersion objective. Unfixed, hydrated samples were exposed to polarized laser light at a wavelength of 830nm and emitted light was separated with a filter set (short pass filter, 720nm; dichroic mirror, 495nm; band pass filter, 475/40nm). Images of x-y planes of 284 by 284μm at a resolution of 0.656μm/pixel were captured using Micro-Manager Open Source Microscopy Software (Micro-Manager) in at least 3 locations on each mammary gland. Quantification of collagen fibers was achieved by setting a minimal threshold in the second harmonic signal. The threshold was maintained for all images across all conditions. The area of regions that was covered by the minimal threshold was calculated and 3 images per sample were averaged together (Image J, Image Processing and Analysis in Java). Collagen fiber diameters data were visualized and analyzed using Imaris (Bitplane AG) and MATLAB (MathWorks).

**RT-PCR**

RNA was purified with RNeasy Mini kit including DNasel treatment (Qiagen). cDNA synthesis was performed using VILO cDNA kit (Life Technologies). SYBR green master mix is LuminoCt (Sigma). Primers designed using NCBI-Primer Blast (Supplementary Table S1), melting curves inspected after every run performed on BioRad CFX96 real time cyclers. All primers were optimized for 60 degree annealing and two-step cycling was performed from 95 degrees (10s) to 60 degrees (30s) for 40 cycles. GAPDH was used to calculate normalized fold change.
**Western Blotting**

Total protein was isolated using Complete LysisM Buffer (Roche). Protein was diluted to equal concentrations and equally loaded on 10% polyacrylamide gels prior to transfer to a nitrocellulose membrane. Blots were incubated overnight with LOX (1:1000, Pierce), pFAK397 (1:1000, Invitrogen), Total FAK (1:1000 Cell Signaling), Collagen Type I (), and LOXL2 () antibodies. HRP-conjugated secondary antibodies were used to visualize band intensity via x-ray film exposure.

**Immunofluorescence**

BSA (12%) was used to block all sections as well as dilute primary and secondary antibodies. Keratin 14 (1:500, Covance), pFAK397 (1:100, Millipore), Gr1 (BD), and αSMA (Sigma) were incubated on 5mm tumor sections overnight at 4°C. Secondary antibodies were all goat derived, highly cross-adsorbed, and used at 1:500. Slides were mounted in SlowFade +DAPI (Molecular Probes/Invitrogen). Sections were imaged on a Zeiss LSM510 inverted confocal microscope with a 40X/1.3 Plan-NEOFLAUR objective.

**LOX activity assay**

Normal mouse mammary fibroblasts were treated with conditioned media from Gr1+ and Gr1- myeloid cells for 24 hours with or without TGF-ß inhibitor SB431542 (Sigma). Gr1+ and Gr1- cell conditioned media was prepared from magnetically isolated (Miltenyi Biotec) cells from a tumor bearing mouse. Fibroblast conditioned media was obtained by conditioning fresh phenol red free DMEM with 10% adult bovine serum. Conditioned
media was prepared in a final volume of 1 mL containing 1.2 M urea (Amresco), 0.05 M sodium borate (Sigma, pH 8.2), 0.1 units/mL of horseradish peroxidase (Fluka), 50 μM Amplex Red (Invitrogen) and 10 mM 1,5-diaminopentane (Sigma-Aldrich) and were incubated at 37°C for 1 hour. Fluorescence intensity was read on a Spectramax M5 at an excitation/emission of 560/590nm. Intensity values were normalized to BAPN treated conditioned media to evaluate LOX specific fluorescence intensity (Levental et al. 2009).

Microarray Data Analysis

Publically available human datasets, GSE33692 and GSE9014, were downloaded from NCBI Gene Expression Omnibus and analyzed via Agilents’ GeneSpring GX microarray analysis software. GSE33692 dataset represents microarray data files from RNA extracted from either matched epithelial or stromal tissue from 3 normal, 9 DCIS, and 10 IDC breast cancer patients[11]. GSE9014 is microarray data derived from the stromal captured tissue of 53 breast cancer patients[1].

Statistics

Statistical analysis was performed using Graphpad Prism after consultation with the Vanderbilt Biostatistics Department.
Results

TGF-β Drives Fibroblast Activation and Matrix Remodeling in Mouse Mammary Fibroblasts

The role of TGF-β signaling in fibroblast activation has been previously established (Reed et al. 1994). However, work concerning the functional role of activated fibroblasts in the context of cancer progression has delineated several functional hallmarks (Calon et al. 2012). Of particular interest was the role TGF-β played in driving the maturation and remodeling of the extracellular matrix. In normal physiological as well as in diseased states, TGF-β has been shown to drive the expression of numerous matrix remodeling genes from cells of mesenchymal origin, including fibroblasts. Validating these findings in mouse mammary fibroblasts, we see similar results to those previously reported in that treatment of fibroblasts with TGF-β significantly induced the expression of genes associated with an activated myofibroblast, including alpha-smooth muscle actin (αSMA), fibroblast activated protein (FAP), connective tissue growth factor (CTGF), and the alpha 2 subunit of type I Collagen (Col1a2) (Figure 4A). Having established that mouse mammary fibroblast phenocopy the established role in fibroblast activation, we sought to address whether this affected the cells ability to interact with and remodel a collagenous matrix. To accomplish this, we cultured mouse mammary fibroblasts embedded in a type I collagen matrix free floating in media and monitored the size of the collagen plug over a course of 72 hours. Over this time we observed that treatment with TGF-β induced a more dramatic
Figure 4. TGF-β Induction of ECM Remodeling Gene Expression in Fibroblasts.
A) Q-PCR analysis of αSMA (1.0±0.07, 1.66±0.085), FAP (1.00±0.11, 2.99±0.2), CTGF (1.00±0.04, 17.57±1.15), and Col1a2 (1.00±0.05, 6.17±0.37) expression in mouse mammary fibroblasts untreated compared to fibroblasts treated 10ng/ml of TGFβ1. Values represent relative fold change normalized to untreated fibroblasts. B) Representative images of a matrix contraction assay in which mouse mammary fibroblasts were allowed to contract a type 1 collagen matrix over a 72 hour period. Quantification included average diameter of the collagen plug at each time point for the cell lines at each condition. C) Time course Q-PCR analysis of TGF-β induction of matrix remodeling gene LOX (1.00±0.22, 2.94±0.39, 2.25±0.3). Values represent relative fold change normalized to untreated fibroblasts. D) Time course fluorescent activity assay for LOX normalized to BAPN treated sample of the same treatment at each timepoint.
contraction of the collagen matrix compared with the untreated control cells (Figure 4B). And similar to this induced phenotypic change, this effect was abrogated upon treatment of these cells with an ALK5 inhibitor, SB431542 (Figure 4B). Interestingly, inhibition of TGF-β signaling abrogated even basal levels of collagen contraction by the mouse mammary fibroblasts without stimulation of TGF-β. With these results in mind, we sought to address the gene expression changes induced by TGF-β specifically involved in collagen maturation and remodeling. Lysyl oxidase (LOX) expression was significantly altered in fibroblasts upon treatment with TGF-β (Figure 4C). LOX was of particular interest due to the large degree of recent work supporting LOX promoting the progression of numerous cancers through the induction of epithelial changes (Leight et al. 2012; Shih et al. 2012). Validating these finding of increased LOX gene expression, we see that LOX activity in conditioned media of fibroblasts treated with TGF-β was indeed higher than untreated control cells (Figure 4D). The data indicates that the activation of fibroblasts by TGF-β in mammary tissue not only drives matrix production but also the active remodeling of the synthesized matrix and particularly the production of LOX.

**LOX Expression is Enriched in the Stroma of Aggressive Breast Cancer**

Having established a TGF-β as a driver for LOX expression from fibroblasts, we sought to address whether stromal LOX expression was relevant to the progression of human breast cancer. Recent work has emphasized the production of LOX by
Figure 5. LOX Expression is Enriched in the Stroma of PyMT Mouse Mammary Tumors as well as Human Invasive Ductal Carcinoma. A) qPCR analysis of LOX expression in fibroblasts (1±0.084, n=3), PyMT<sup>mgko</sup> tumor cells (0.14±0.1, n=3), and PyMT<sup>fl/fl</sup> tumor cells (0.3±0.12, n=3). Values represent relative fold change normalized to untreated fibroblasts. B) qPCR analysis for LOX from RNA extracted from epithelium (1±0.17, n=6) or stroma (9.15±0.9, n=6) from PyMT<sup>mgko</sup> tumors. Values represent relative fold change normalized to LCM Epithelium. C) Representative image of in situ hybridization of PyMT<sup>mgko</sup> tumor counterstained with DAPI. D) Analysis of microarray data from patient matched epithelium and stromal isolated from human invasive ductal carcinoma via LCM for ACTA2, CTGF, and LOX expression (GSE33692). Green = downregulated, Red = upregulated. E) Quantification of normalized expression values for ACTA2, CTGF, and LOX microarray data presented in (D).
Tumor cells particular when under hypoxic stress (Erler et al. 2006). Tumor cells cultured under hypoxic conditions or expressing high levels of HIF1α express higher levels of LOX than their non-hypoxic counterparts (Erler et al. 2006). Alternately, in human breast carcinoma, LOX expression is also a stromally produced gene that is expressed along with type I collagen (Levental et al. 2009; Peyrol et al. 1997). To determine the relative contribution of either epithelial or fibroblast cells to LOX production we performed gene expression analysis on cell lines for each cell type. Our results indicate that fibroblasts consistently expressed significantly more LOX when compared with epithelial tumor cells (Figure 5A). To validate the relevance of these in vitro findings to disease progression in vivo, laser capture microdissection (LCM) was performed on tumor sections to isolate RNA from either epithelial or stromal regions of the tumor. Upon qPCR analysis, the stroma of PyMT^{mgko} tumors expressed approximately 10 fold more LOX than the neighboring epithelium (Figure 5B). In situ hybridization for LOX mRNA again showed the stromal regions of PyMT^{mgko} tumors were highly enriched for LOX mRNA compared to the epithelium (Figure 5C). To verify the validity and relevance of such findings to human disease, publically available datasets of LCM epithelium and stroma from invasive ductal carcinoma (IDC) were analyzed to localize LOX expression. Indeed, matching the finding in the PyMT^{mgko} tumors, IDC tumors quantitatively showed a marked increase in LOX expression in the stroma compared to the epithelium. LOX expression also aligned with genes known to be expressed in activated myofibroblasts, connective tissue growth factor (CTGF) and α-smooth muscle actin (ACTA2) all of whose expression was increased in the stroma.
(Figure 5D-E) (Arora and McCulloch 1994). These results indicated that in both murine PyMT tumors as well as human IDC, the stroma is an abundant source of LOX.

**Infiltrating Myeloid Cells Drive Stromal Lysyl Oxidase Expression in a TGF-β Dependent Manner**

In numerous disease states including wound healing, fibrosis, and cancer, a large source of TGF-β production and secretion comes from infiltrating myeloid cells. Previous studies in our laboratory have shown that knockout of the type II transforming growth factor beta receptor in mammary tumor cells induced numerous stromal changes including increased CD11b+Gr1+ myeloid derived suppressor cell (MDSC) infiltration (L. Yang et al. 2008). These tumors also present with an increased level of available TGF-β ligand. As there is no difference in tumor cell secretion of TGF-β1 and a significant increase in TGFB1 expression from MDSCs infiltrating tumors compared to those found in the spleen, it is probable that the increased TGF-β1 ligand production seen in PyMT^MGKO_ tumors is due to increased infiltration of MDSCs. Having previously established TGF-β as a driver of LOX expression in fibroblasts, we sought to determine if this infiltrating myeloid cell population could also activate fibroblasts to drive LOX expression. We treated mouse mammary fibroblasts with conditioned media from either Gr1 negative cells, representing lymphocytic and myeloid cell lineages excluding the Gr1+ MDSC population, or Gr1 positive cells which includes the MDSC population. While both Gr1- and Gr1+ conditioned media induced expression of αSMA from the
Figure 6. Infiltrating Myeloid Cells Drive Stromal LOX expression in a TGF-β dependent manner. A) qPCR analysis of αSMA (also known as ACTA2) and LOX expression from fibroblasts treated with conditioned media from Gr1- or Gr1+ cells isolated from PyMT\textsuperscript{mgko} mice with or without SB431542 treatment. Values represent relative fold change normalized to untreated fibroblasts. B) Dual Immunofluorescence localizing Gr1+ myeloid cells (Green) and αSMA expressing fibroblasts (Red) in PyMT\textsuperscript{mgko} tumors. C) Scatter plot correlating stromal TGF-β1 expression with stromal LOX expression in human breast cancer patients (GSE9014).
treated fibroblasts, only Gr1+ conditioned media significantly increased the expression of LOX (Figure 6A). As any number of factors could be responsible for the induced gene expression change, we combined the conditioned media treatments with the TGF-β inhibitor SB431542 to determine if the induction of αSMA and LOX expression was dependent upon TGF-β. Indeed, the gene expression changes elicited by both Gr1- and Gr1+ conditioned media were dependent upon TGF-β. Validating these gene expression changes, we see that conditioned media from fibroblasts treated with conditioned media from Gr1+ myeloid cells had increased levels of lysyl oxidase activity as measured by the detection of hydrogen peroxide, a byproduct of LOX activity (Figure 6B). To provide in vivo significance to these findings, we colocalized Gr1+ myeloid cells infiltrating PyMTMGKO tumors with activated αSMA expressing fibroblasts. As shown in the representative images presented in Figure 6C, areas of Gr1+ cell infiltration colocalized with αSMA actin positive cells (Figure 6C). Additional support for the premise of TGF-β derived from stromal cells driving fibroblast activation and LOX expression was gained from analysis of human patient microarray datasets. Gene expression analysis on RNA extracted from the stroma of breast cancer patients shows that there is a positive correlation between TGFβ1 expression and the expression of LOX (Figure 6D). The data would indicate that in both mouse and human breast cancer, TGF-β derived from infiltrating myeloid cells can promote the activation of stromal fibroblasts and the expression of LOX.
PyMT<sup>MGKO</sup> Tumors Have Increased Maturation of the Collagenous Extracellular Matrix

The generation of mammary tumors with and without the type II transforming growth factor beta receptor has been previously described (Bierie et al. 2008; Forrester et al. 2005). As previously noted, mouse mammary tumors lacking Tgfbr2 gene expression are characterized by increased infiltration of MDSCs as well as increased levels of TGF-β ligand. As presented previously, our work suggests that these infiltrating myeloid cells can promote the activation of stromal fibroblasts and drive matrix remodeling. Prior work showed this mouse model presents with an enhanced reactive stroma and increased myofibroblast presence compared to tumors with intact TGF-β signaling (Bierie et al. 2008). However, the differences in the extracellular matrix of these tumors remained unexplored thus we sought to investigate whether the previously noted in vitro induction of matrix remodeling translated into LOX expression and collagenous deposition in in vivo tumors. Picrosirius Red staining analyzed under polarized light was used to further define the deposition of collagen fibers. This stain also provides insight into collagen stability as positively stained collagen fibers have been described as thicker, more aligned, and more cross-linked (Gorden et al. 2007). Picrosirius Red staining showed enhanced deposition of mature collagen in PyMT<sup>mgko</sup> tumors (Figure 7A). These data were quantified to show this difference as statistically significant between the PyMT<sup>fl/fl</sup> and PyMT<sup>mgko</sup> tumors (Figure 7A). Second harmonics generation, the phenomenon of collagen scattering incoming light to a specific emission wavelength of exactly half that of the incoming light, revealed a trend of more abundant linearized, thick collagen fibers dispersed throughout the stroma, consistent with a
Figure 7. PyMT<sup>MGKO</sup> Tumors Have Increased Maturation of the Collagenous Extracellular Matrix. B) Representative images (20X) of Picrosirius Red staining of PyMT<sup>fl/fl</sup> and PyMT<sup>mgko</sup> tumor sections. Quantification of thresholded pixel density representing positive picrosirius staining for PyMT<sup>fl/fl</sup> (8.8*10<sup>6</sup> A.U.± 1.4*10<sup>6</sup>, n=16) and PyMT<sup>mgko</sup> (2.1*10<sup>7</sup> A.U.± 2.3*10<sup>6</sup>, n=16) tumors. B) Second harmonic generation (SHG) for label free imaging of fibrillar collagen. Scatter plot of SHG signal indicate a 30% increase of SHG signal in PyMT<sup>mgko</sup> (7140 A.U.±5696, n=5) compared to PyMT<sup>fl/fl</sup> (4314 A.U.±2753, n=5) (P=0.1753 ns). C) Bulk tumor qPCR analysis of Col1α2 expression in Control (1±0.49, n=6) and PyMT<sup>mgko</sup> tumors (0.9±0.3, n=6). Bulk tumor qPCR analysis of Col4α1 expression in Control (1±0.69 n=6) and PyMT<sup>mgko</sup> (0.62±0.12, n=6) tumors. Values represent fold change relative to PyMT<sup>fl/fl</sup> expression. D) qPCR and western blot analysis of LOX expression in PyMT<sup>fl/fl</sup> (1±0.19, n=8) and PyMT<sup>mgko</sup> (2.32±0.14, n=8) using whole tumor RNA and protein extracts. Values represent fold change relative to PyMT<sup>fl/fl</sup> expression. E) Scatter plot of Young’s Elastic Modulus of PyMT<sup>fl/fl</sup> and PyMT<sup>mgko</sup> showing an increase of the elastic modulus of PyMT<sup>mgfl</sup> stromal mammary glands (840 ± 21.61 Pa) compared to PyMT<sup>fl/fl</sup> (550 ± 10.54 Pa).
greater amount of cross-linked collagen present in the PyMT\textsuperscript{mgko} tumors, presented and quantified in Figure 7B. There are several factors that can drive the expansion of a collagen matrix including increased collagen synthesis or increased maturation of collagen that is synthesized. Interestingly, Q-PCR analysis indicated no increased expression of Collagen Type I or Type 4 implicating collagen stabilization as the driver of increased collagen deposition (Figure 7C). Consistently, tumor gene expression and protein levels showed that expression of the collagen crosslinking enzyme LOX was increased in the PyMT\textsuperscript{mgko} tumors compared to PyMT\textsuperscript{fl/fl} tumors (Figure 7D). This protein cross-links collagen fibers to regulate their stability (Payne et al. 2007). LOX was of particular interest due to its significant role in matrix remodeling and tumor progression (Erler et al. 2006). Concordant with increased expression of LOX, atomic force microscopy indentation quantified a significant increase in the stiffness of the extracellular matrix associated with the PyMT\textsuperscript{mgko} tumors (Figure 7E). These results indicate that rather than enhanced collagen synthesis, PyMT\textsuperscript{mgko} tumors promote increased matrix remodeling through collagen stabilization culminating in an overall increase in collagen deposition and stiffness in the stroma associated with PyMT\textsuperscript{mgko} tumors.

The LOX Inhibitor, BAPN, Attenuates Collagen Maturation/Crosslinking in PyMT\textsuperscript{MGKO} Tumors.

Several studies have linked LOX activity with collagen crosslinking and increased matrix stiffness, however others have also noted that increased matrix stiffness can be
Figure 8. The LOX Inhibitor, BAPN, Attenuates Collagen Maturation/Crosslinking in PyMT^MGKO Tumors. 
A) Representative AFM force maps of PyMT^fl/fl, PyMT^mgko and PyMT^mgko + BAPN stromal areas, indicating stiffer collagen in PyMT^mgko. BAPN treatment significantly decreased the elastic modulus by 69.52% compared to PyMT^mgko (P<0.0001).  
B) Top row: Representative images of ECM fibrillar collagen diameter (color-coded) and cell nuclei (purple) of PyMT^fl/fl, PyMT^mgko and PyMT^mgko + BAPN visualized with second harmonic generation (SHG) microscope. Bottom row: collagen fibers diameter quantification shows a distribution in the PyMT^fl/fl samples with 80% of fibers with diameter falling in the range 0.4 - 2.2 µm. PyMT^mgko shows a wider distribution (80% of fibers with diameter falling in the range 0.6 - 3.8 µm) with respect to the PyMT^fl/fl. BAPN treatment reduced collagen accumulation and fiber thickness in PyMT^mgko (80% of fibers with diameter falling in the range 0.5 – 1.8 µm).  
C) ECM stiffness distribution of PyMT^fl/fl (0.1 to 2 kPa), PyMT^mgko (0.1 to 10 kPa) and PyMT^mgko + BAPN (0.1 to 0.5 kPa).
driven by factors such as increased collagen secretion in an enclosed space. To test whether LOX activity was responsible for the increased matrix stiffness observed in PyMT\textsuperscript{mgko} mice, we treated tumor bearing mice with an irreversible chemical inhibitor of LOX activity, Beta-Aminoproprionitrile (BAPN). BAPN is a synthetic chemical inhibitor of LOX activity that mimics the efficacy of LOX inhibitory antibodies when used in in vivo treatments (Bondareva et al. 2009; Erler et al. 2006; Wilmarth and Froines 1992). To examine the effects of BAPN treatment on the matrix stiffness of our tumors, several analyses were performed. Atomic force microscopy indentation mapping performed on those tumors showed a significant increase in stromal matrix stiffness in PyMT\textsuperscript{mgko} tumors compared to PyMT\textsuperscript{fl/fl}, validating the previously shown results, as well as a reduction in the tensile strength of the stromal regions of these tissues upon treatment with BAPN when compared to untreated controls (Figure 8A). These results indicate that a significant amount of the increased matrix stiffness observed in PyMT\textsuperscript{mgko} tumors is due to the actions of LOX. Consistent with the regional distribution and co-localization of Gr1+ cells and αSMA fibroblasts was the non-uniform spread of linearized collagen fibers we observed at the tumor periphery by second harmonic generation imaging (Figure 8B). Supporting the idea of regional infiltration of MDSCs inducing local collagen remodeling, we quantified a spectrum of focally stiffened extracellular matrix by atomic force microscopy indentation (Figure 8C). Reinforcing LOX’s role in this phenotype, we observed that BAPN treatment significantly reduced this variability, normalizing collagen fiber thickness and reducing stromal stiffness. These results show
that LOX is the primary driver of the enhanced matrix stiffness observed in PyMT$^{mgko}$ tumors as well as validate the efficacy of BAPN to inhibit this matrix stiffness.

BAPN Reduced Tumor Cell Metastasis Through Decreased Tumor Cell Extravasation in PyMT$^{MGKO}$ Tumors.

TGF-β has long been known to promote metastasis in late stage tumors through the induction of an EMT, however epithelial loss of TGF-β signaling in our mammary tumors presents with increased metastatic burden (Forrester et al. 2005). There is yet no concrete evidence to support a mechanism behind the increased metastasis seen upon abrogation of TGF-β signaling in tumor epithelium, but the myriad of stromal changes observed in this model are independently linked to tumor progression. Lysyl oxidase expression in human breast cancer has been shown to be predictive of worse outcome in human patients as well as increased metastasis in mouse models of the disease (Erler et al. 2006). As matrix stiffness and LOX expression are significantly increased in PyMT$^{mgko}$ tumors, the effect of this increased expression on the enhanced lung metastasis of the PyMT$^{mgko}$ tumors was examined. Treatment of PyMT$^{mgko}$ mice with BAPN resulted in no significant change in time to tumor palpation (Figure 9A). Histological analysis of the primary tumor showed no changes to the composition or characteristics of the tumor epithelium or stromal infiltrates (Figure 9B). BAPN treatment also did not cause any significant changes in tumor volume in PyMT$^{mgko}$ mice (Figure 9C). However, upon examination of lung whole mounts from PyMT$^{mgko}$ mice with and without BAPN treatment, there was a significant decrease in the incidence of
Figure 9. BAPN Reduced Tumor Cell Metastasis Through Decreased Tumor Cell Extravasation in PyMTMGKO Tumors. A) Survival curves from birth to first palpable tumor in PyMTmgko (n=8 per group) mice with and without BAPN treatment. B) Representative images (20X) of Hematoxylin and Eosin staining PyMTmgko tumors treated with and without BAPN. C) Tumor volume at the time of sacrifice in PyMTmgko mice control (3.26±0.07 mm², n=8) and BAPN treatment (3.07±0.36 mm², n=8). D) Incidence of tumor metastasis PyMTmgko mice treated with and without BAPN presented as a percentage of the total group. E) Quantification of the number of lung metastases per control (5.5±1.45, n=8) and BAPN treated group (1.17±0.6, n=8) F) Quantification of the number of circulating tumor cells cultured from the circulating blood of PyMTfl/fl and PyMTmgko mice treated with (1±0.37, n=8) and without BAPN (4±0.68, n=8). Values represent the average number of 100mm or greater colonies formed for each experimental group. G) Quantification of lung metastasis in mice 3 weeks after tail vein injection of PyMTmgko tumor cells into mice treated with (5.625±2.51) or without (3.875±0.55) the LOX inhibitor BAPN.
lung metastasis following BAPN treatment (Figure 10D). Additionally, in those PyMT\textsuperscript{mgko} mice that did harbor lung metastasis upon BAPN treatment, the number of lung metastasis was significantly lower than in the untreated PyMT\textsuperscript{mgko} mice (Figure 9E). In the cascade of metastatic progression, intravasation from the primary tumor into the vasculature is one of the first steps (Pantel and Brakenhoff 2004). Upon examination, we noted a reduced numbers of viable tumor cells in the circulation suggesting that LOX inhibition significantly reduced metastasis by inhibiting tumor cell intravasation (Figure 9f). As premetastatic niche effects of LOX have been established (Erler et al. 2009), tail vein injection experiments into mice with and without BAPN treatment were performed and showed no differences in metastatic colonization potential (Figure 9G). Thus, these data suggest that matrix remodeling promotes tumor cell metastasis in PyMT\textsuperscript{mgko} tumors by enhancing intravasatation into the vasculature.

**Matrix Stiffness Enriches for a Basal Keratin 14 Cell Population.**

Since we showed that increased matrix stiffness promoted tumor cell escape from the primary tumor into the vasculature, we next looked for correlations between matrix remodeling and phenotypic molecular changes associated with highly aggressive, metastatic tumors. Previous work has shown that tumor cells cultured on stiff collagenous matrix have increased mesenchymal characteristics compared to tumor cells cultured on softer matrix. While there are numerous potential reasons behind this phenotypic observation, one of the more notable ones includes the augmentation of a cells ability to respond to TGF-β dependent upon the stiffness of the matrix. On a “soft”
Figure 10. Matrix Stiffness Enriches for a Basal Keratin 14 Cell Population. A) qPCR analysis of Keratin 14 expression from whole tumor RNA of PyMT<sup>mgko</sup> mice with (0.52±0.054) and without (1.33±0.076) BAPN treatment. Values Represent Normalized ΔΔ<sub>Ct</sub>. B) Representative images (40X) of immunofluorescent staining for Keratin 5 (Green:488nm) and Keratin 14 (Red:594nm) counterstained with DAPI. Yellow seen in Overlay represents colocalization of staining. C) Immunofluorescent images of PyMT<sup>mgko</sup> (1.2*10<sup>7</sup>±1.1*10<sup>6</sup>, n=16) and PyMT<sup>mgko</sup>+BAPN (6.9*10<sup>6</sup>±9.07*10<sup>5</sup>, n=16) tumors stained for keratin 14 were quantified via thresholded pixel density measurement.
matrix, treatment of cells with TGF-β induces cell cycle arrest eventually leading to apoptosis. When these same cells are cultured on stiff matrix, they instead undergo an EMT in response to TGF-β compared to the cytostatic effects observed on soft matrix (Leight et al. 2012). As our model utilizes tumor cells which are unresponsive to TGF-β, we observed no differences in the induction of mesenchymal characteristics upon inhibition of matrix stiffness with BAPN (data not shown). Analysis of whole tumor mRNA revealed that the stiffer tumors had increased levels of the myoepithelial/basal keratin 14 expression compared to those tumors where LOX activity was inhibited (Figure 10A). Immunofluorescent imaging of tumor sections validated these findings. Particularly in regions of stromal/epithelial interface there is an enrichment of keratin 14 positive cells in untreated PyMTMGKO tumors. However, reduction of tumor stiffness through BAPN treatment resulted in an abrogation of keratin 14 positive cells at the tumor/stromal interface (Figure 10B). These results were quantified to show significant loss of keratin 14 staining in PyMTMGKO tumors treated with BAPN (Figure 10C). Thus, while we do not see an increase cells with a mesenchymal phenotype we do see an expansion of cells that have been associated with more aggressive basal tumors and poor patient prognosis.

**Focal Adhesion Kinase Activation is Enriched in Keratin 14 Cells on Stiff Matrix**

One of the hallmark features noted of epithelial cells interactions with mature, crosslinked, collagenous matrix is the importance of integrins and focal adhesions in governing the epithelial cells response to these matrix changes. Integrins, as mediators
Figure 11. Focal Adhesion Kinase Activation is Enriched in Keratin 14 Cells on Stiff Matrix. A) Immunoblot analysis of lysates from PyMT^mgko and PyMT^mgko +BAPN tumors for phosphor-FAK(397) and Total FAK. Integrated pixel intensity shows decreased phosphorylated FAK in BAPN treated PyMT^MGKO (0.47±0.09 a.u., n=16) tumors compared with untreated PyMT^MGKO (0.76±0.017 a.u., n=16) relative to total FAK levels. B) Representative images (40X) of immunofluorescent staining of phospho-FAK397 (Green) and Keratin 14 (Red) counter staining with TOPRO3 (Blue). C) Immunofluorescent images of PyMT^mgko (237956±29571 a.u., n=16) and PyMT^mgko +BAPN (181259±15658 a.u., n=16) tumors stained for phospho397-FAK were quantified via thresholded pixel density measurement.
of tumor cells interactions with the extracellular matrix, promote the afore-mentioned phenotypes observed in epithelial cells in response to alterations in the extracellular matrix. Valerie Weaver’s group has shown that alpha 5 beta 1 integrin is essential to a cell’s ability to detect and respond to matrix changes. One of these changes most important to the induction of changes to epithelial characteristics is the formation of focal adhesions at the site of integrin binding to extracellular matrix proteins. Weaver and others have noted that as matrix stiffness increases the number and size of focal adhesions formed is significantly increased in tumor cells in stiff matrix environments compared with softer environments (Baker et al. 2012). Immunoblot analysis confirms this finding in our model as well, showing that inhibition of LOX activity in PyMT<sup>mgko</sup> tumors reduces the phosphorylation of focal adhesion kinase, which is a necessary event for the formation of proper focal adhesion complexes (Figure 11A). While there is less total FAK in the tumors as well, quantification of the relative amount of phosphorylated focal adhesion kinase to total focal adhesion kinase shows that there is less FAK phosphorylation regardless of the decreased total FAK levels (Figure 11A). We sought to address the localization of focal adhesion formation in our tumors and how this localization changed in response to inhibition of matrix stiffness. Imaging of PyMT<sup>mgko</sup> tumors treated with and without BAPN revealed an interesting observation in that the majority of phosphorylated FAK found in the untreated tumors was found at the tumor/stromal interface and seems to localize in keratin 14 positive cells. Upon inhibition of LOX activity with BAPN, we see not only a loss of these keratin 14 positive cells, as noted above, but we also see that there is a loss of focal adhesion kinase
phosphorylation, validating the immunoblot data (Figure 1B). Quantification of phosphorylation FAK in PyMT<sup>mgko</sup> tumors indeed shows that treatment with BAPN results in a significant reduction in focal adhesion formation upon inhibition of matrix stiffness (Figure 11C). These results validate previously reported finding as to the effects of matrix stiffness on epithelial-ECM interactions as well as provide an intriguing possibility of the responsiveness to matrix stiffness be localized in a particular population of tumor cells.

**Discussion**

The microenvironment is an essential component in promoting tumors towards metastasis (Hanahan and Coussens 2012). While significant effort has been placed on the cellular components of the microenvironment, such as immune cells, fibroblasts and endothelial cells, one of the most interesting components is the extracellular matrix (ECM). The ECM acts a foundation upon which tumors build themselves, a scaffold for blood and lymphatic vessels, and a trigger for integrin mediate cellular changes to promote growth and migration (P. Lu et al. 2012). As breast cancer with abrogated TGF-β signaling have substantially worse disease free survival, we sought to address the role an altered collagen matrix played in the aggressiveness of tumors (Bierie et al. 2009; Paiva et al. 2012). In PyMT<sup>mgko</sup> tumors, a significant increase in collagen stiffness and LOX expression was observed (Figure 7). Matrix remodeling through collagen crosslinking has been linked with increased tumor progression and invasiveness (Levental et al. 2009). LOX, as a primary mediator of collagen crosslinking, is increased
Figure 12. Proposed model of PyMT^mgko promotion of matrix stiffness and enhancement of tumor cell metastasis. PyMT^sgko tumor cells have been shown to secrete increased levels of CXCL1 and CXCL5 ligands. This enhanced chemokine secretion promotes the infiltration of myeloid cells, in particular Gr1+CD11b+ myeloid derived suppressor cells. These cells act as a major source of TGF-β1 ligand which coupled with the increased infiltration result in a tumor with increased total TGF-β1 availability compared to PyMT control tumors. TGFβ1 acts on stromal fibroblasts to promote their activation to myofibroblasts as well as their secretion of matrix remodeling protein lysyl oxidase. Lysyl oxidase promote the maturation of collagen fibers through the covalent crosslinking of individual strands of collagen into a cohesive trimer. Collagen crosslinking is correlated with numerous epithelial changes but in particular the promotion of tumor cell metastasis.
| PyMT$^{Mgko}$ Cells |  
|---------------------|---|
| Enhanced CXCL1/5 Expression |  
| Increased MDSC Infiltration |  
| Increased TGFβ1 Expression |  
| Presence of Activated Fibroblasts |  
| Enhanced Lysyl Oxidase Expression |  
| Increased Collagen Crosslinking |  
| Promotion of Tumor Cell Metastasis |  

Metastasis
in breast cancer and associated with poor patient prognosis/metastasis [NextBio]. We observed that BAPN treatment to inhibit LOX mediated matrix remodeling significantly reduced tumor metastasis through decreased tumor cell intravasation. Our data indicates that LOX is stromally derived in PyMT\textsuperscript{mgko} tumors, thus LOX acts as a promoter of tumor metastasis independent of the cellular source and potentially through similar mechanisms. Interestingly, it appears that stromal TGF-β signaling drives this increase in LOX expression. The evidence for TGF-β promoting LOX expression and matrix remodeling thus adds another layer of complexity to the premise of therapeutically targeting TGF-β in the context of cancer.

Various tumor characteristics have been attributed to alterations of the collagenous microenvironment in tumors. Notably, hypoxic conditions drive the expression of LOX from tumor epithelium (Erler et al. 2006). As a driver for many of the phenotypes observed in our PyMT\textsuperscript{mgko} tumors, hypoxia was a prospective candidate for our enhanced tumorigenesis. However, no difference in hypoxia was seen (data not shown) leading us to look elsewhere. Previous work from our laboratory has shown that epithelial loss of functional TGFβR2 expression results in an increased recruitment of MDSCs and that this promotes tumor progression to metastasis (L. Yang et al. 2008). These cells are a major source of TGF-β in tumors. We show that these immature myeloid cells localize to areas of αSMA expression in PyMT\textsuperscript{mgko} tumors and can promote tumor matrix remodeling through TGF-β mediated stimulation of stromal fibroblasts, specifically via induced expression of LOX. It has been previously established that lung fibroblasts, as well as cardiac myofibroblasts, can express LOX upon TGF-β stimulation.
(Boak et al. 1994; Choudhary et al. 2009). We show that this LOX promoting source of TGF-β from fibroblasts can be tumor infiltrating immune cells. Our study suggests that the tumor epithelium can indirectly promote an aggressive microenvironment through the facilitation of interactions of various components of the tumor microenvironment.

LOX is known to promote focal adhesion formation in mammary carcinoma cells, and in vivo inhibition of LOX suppresses both hypoxic and non-hypoxic tumor metastasis (Levental et al. 2009). Using a different mouse model of breast cancer, we have recapitulated these findings of decreased focal adhesion formation and metastasis upon inhibition of LOX with BAPN. While it should be noted that BAPN has been reported to have effects on members of the LOX family, we did not see any appreciable changes in LOXL expression (data not shown) and while certainly not quantitative, the relative C_t’s for the LOXL qPCR were significantly lower than those for LOX indicating lower gross expression in our tumors. As LOX acts to crosslink extracellular collagen and elastin, the induction of these phenotypes is likely due to its ability to promote stiffness in the tumor microenvironment (Baker et al. 2012; Levental et al. 2009). Increased matrix stiffness leads to an EMT in tumor epithelium, which led us to ask if inhibition of LOX in our spontaneous tumors resulted in decreased circulating tumor cells and metastasis through the prevention of this transition (Leight et al. 2012). While no difference in the induction of EMT was observed, most likely due to a lack of TGF-β responsiveness in the carcinoma cells of the model used, we did see an increase in keratin 14 expression. While previously basal breast cancer cells have been shown to secrete LOX, this was the first instance in which we see LOX activity regulating this tumor cell phenotype (Chu et
al. 2012; Erler et al. 2006). Live cell imaging of Keratin 14+ mammary epithelium has shown these cells to be highly protrusive and migratory specifically in response to a collagen matrix (Nguyen-Ngoc et al. 2012). As collagen was shown to be the main driver of this migratory phenotype, modulation of the collagen matrix could abrogate this effect. This indeed turned out to be the case in that inhibition of LOX resulted in fewer Keratin 14+ cells. Linking this aggressive basal phenotype with previous findings regarding the effects of LOX on the tumor epithelium, we find that K14 cells are enriched for the formation of focal adhesions in our PyMT^{mgko} tumors. The ability to adhere and respond to the extracellular matrix is an essential step in obtaining a migratory phenotype and promoting metastasis of tumor cells. As expected, inhibition of LOX activity diminished this focal adhesion enrichment. This, potentially, links not only the epithelial phenotype of these cells with their ability to respond to matrix cues, in particular matrix crosslinking and stiffness, but also to the decrease in metastasis through diminished tumor cell intravasation.

The data presented in this study show that microenvironmental changes have significant effects on tumor progression. We show that the aggressiveness of tumor epithelium is not only dictated by the genetic programming of the tumor cell but also by the state of the extracellular matrix. By demonstrating that inhibition of matrix remodeling can not only inhibit tumor cell metastasis, but also modulate the phenotypic characteristics of the tumor cells, we have further refined the conceptual framework used to think about stromally targeted therapeutics. Showing that myeloid cell infiltrates can arouse stromal activation should widen the breadth of patients...
considered for immune modulating treatments and also provide new readouts for the efficacy of treatment options. However, while addressing many pressing issues in the field of cancer biology, our data also raise some interesting questions. We show that extracellular matrix modifications can drive phenotypic changes in basal cells, thus it is now necessary to identify the molecular basis for this interaction. Both growth factor responsiveness as well as adhesion have been shown to regulate cellular phenotypes, therefore the ability of ECM modification to promote these signaling pathways could begin to address this phenotypic switch. It is also unclear what the specific role these basal cells play in the invasive and metastatic phenotype observed. Addressing the specific migratory and invasive capacity of these different populations of cells will aid in pushing forward our knowledge of tumor metastasis. With metastasis representing the major cause of morbidity and mortality in breast cancer patients, a thorough understanding of stromal cues present in the tumor microenvironment and reciprocal epithelial responses to these cues in the context of tumor metastasis is essential.
STROMAL LOSS OF *TGFBR2* EXPRESSION PREDICTS POOR PROGNOSIS IN BREAST CANCER PATIENTS

**Introduction**

While aspects of the tumor microenvironment have been shown to play a role in influencing tumor progression, Morag Parks group solidified the role of the stroma in manipulating patient outcome by showing that stromal gene expression signatures can predict breast cancer patient survival (Finak et al. 2008). However, this study does not address the factors in the microenvironment regulating the interaction between stromal cells and the tumor epithelium or specific gene expression changes driving poor patient prognosis. Numerous functions of fibroblasts have been shown to influence tumor progression, particularly in the context of TGF-β induced changes to gene expression and cellular phenotype (Lewis et al. 2004; Verona et al. 2007). As mentioned previously, TGF-β induces numerous stromal changes predominantly through the manipulation of the collagenous extracellular matrix (Reed et al. 1994). In lung cancer, carcinoma associated fibroblasts present with a gene expression signature enriched in TGF-β responsive genes and showed that treatment of normal fibroblasts with TGF-β induced a similar gene expression pattern (Navab et al. 2011). Despite the preponderance of evidence supporting a pro-tumorigenic role of active TGF-β signaling in the tumor microenvironment, experimental and clinical evidence supports a loss of TGF-β signaling being worse for cancer progression. In colon cancer, stromal *TGFBR2* expression is an
independent predictor of patient survival with lower stromal *TGFBR2* resulting in worse patient prognosis (Bacman et al. 2007). Immunohistochemical analysis of oral and squamous cell carcinoma stroma shows that TβRII is lost specifically in fibroblasts found in the tumor stroma as tumor progress from dysplasia to carcinoma (Meng et al. 2011). Thus, much like epithelial TGF-β signaling, there appears to be opposing effects of TGF-β signaling on fibroblasts in the context of influencing tumor progression.

Previous work from our laboratory has established loss of *Tgfbr2* expression in FSP expressing fibroblasts as a driver of tumorigenesis in adjacent epithelium (Bhowmick et al. 2004). This seminal work supported two emerging topics in the field of cancer biology: (1) the importance of stromal gene expression in the influence of tumor formation and (2) that loss of TGF-β signaling in a stromal cell population can promote tumor progression. Further *in vivo* analysis of tumors lacking *Tgfbr2* expression in fibroblasts shows that this loss of TGF-β signaling promotes cell growth and migration through increased HGF secretion (Cheng et al. 2005). Interestingly, the abrogation of TGF-β signaling in fibroblasts has a similar effect to abrogation in epithelial cells in that numerous cytokines regulating immune cell recruitment are increased (Hembruff et al. 2010; Li et al. 2012). This is supported by *in vivo* evidence showing tumors where fibroblast lack expression of *Tgfbr2* has increased infiltration of myeloid cells. The infiltration of these myeloid cells promotes tumor growth through a variety of direct and indirect mechanisms. Given the significance of fibroblast *Tgfbr2* expression on the progression of mouse model of cancer as well as the evidence supporting a pro-tumorigenic role of TGF-β target genes in fibroblasts, we sought to address the
functional role TGF-β plays in these cells in human disease progression. To address this question, we used in vitro and in vivo experimental models to correlate gene expression and phenotypic changes in fibroblasts upon loss of TGFBR2 to gene expression changes identified in human patients.

**Experimental Procedures**

**Cell lines**

Fibroblast cell lines were derived from C57BL/6 mice containing LoxP sites flanking exon 2 of the TGFBR2 gene. Knockout fibroblasts were made by crossing the above mouse with a FSP-Cre mouse to direct recombination to specifically fibroblasts. Mouse mammary glands from these mice were harvested and grown in 10% FBS containing DMEM. Selective trypinization was used to eliminate epithelial cells from the cultures to generate a cell population of fibroblasts. Experiments involving tumor cells were performed with tumor cells derived from Polyoma Middle T tumor bearing mice.

**qPCR**

RNA was purified with RNeasy Mini kit including DNaseI treatment (Qiagen). cDNA synthesis was performed using VILO cDNA kit (Life Technologies). SYBR green master mix is LuminoCt (Sigma). Primers designed using NCBI-Primer Blast, melting curves inspected after every run performed on BioRad CFX96 real time cyclers. All primers were optimized for 60 degree annealing and two-step cycling was performed from 95 degrees (10s) to 60 degrees (30s) for 40 cycles. GAPDH was used to calculate normalized fold change.
Western Blot

Total protein was isolated using Complete LysisM Buffer (Roche). Protein was diluted to equal concentrations and equally loaded on 10% polyacrylamide gels prior to transfer to a nitrocellulose membrane. Blots were incubated overnight with Phospho-SMAD2, αSMA, ACTIN antibodies. HRP-conjugated secondary antibodies were used to visualize band intensity via x-ray film exposure.

RayBio Chemokine Array/ECM qPCR Array

Membrane bound antibody arrays Mouse Cytokines 3,4,5 Cat# AAM-CYT-2000-4 were obtained from RayBio and incubated with conditioned medium. Exposure was performed with ECL plus, images were scanned at high resolution and analyzed for intensity using NIH image J software. RT-PCR focused arrays were purchased from SABiosciences/Qiagen and performed as instructed by manufacturers protocol, including RNA purification, cDNA synthesis, real time instrumentation protocol and analyzed via web based tools provided (http://www.sabiosciences.com/pcrarraydataanalysis.php). Specifically, the ECM array was used.

Immunohistochemistry

Deparafinized slides underwent pH6.0 Citrate Buffer antigen retrieval. These slides were blocked with serum derived from the host animal of the primary antibody for 1 hour before overnight incubation with the primary antibody: pSmad2, Ki67, Caspase3, and F4/80. After washing with PBS to remove the primary, strepavadin
conjugated secondary antibody against the primary host species was added to the sections for 30 minute. Another round of washing preceded the addition...

**Immunofluorescence**

BSA (12%) was used to block all sections as well as dilute primary and secondary antibodies. Fibronectin and Collagen Type IV antibodies were incubated on fibroblasts plated in a 4 well culture slide overnight at 4°C. Secondary antibodies were all goat derived, highly cross-adsorbed, and used at 1:500. Slides were mounted in SlowFade +DAPI (Molecular Probes/Invitrogen). Sections were imaged on a Zeiss LSM510 inverted confocal microscope with a 40X/1.3 Plan-NEOFLAUR objective.

**Migration Assay**

24 hours prior to seeding of tumor cells into the transwells, 50,000 fibroblasts with or without Tgfbr2 expression were seeded into a 24 well plate. After allowing these cells to establish for 24 hours, transwells with 25,000 PyMT cells seeded into the upper chamber with placed in the wells containing either of the fibroblasts. PyMT tumor cells were allowed to migrate for a 24 hour period before being fixed and stained with DAPI for immunofluorescence imaging. Total number of DAPI+ nuclei were quantified for each 20X image using Metamorph.

**Tumor Implantation/Metastasis Counts**

PyMT tumor cells and fibroblasts with and without the expression of TGFB2 were mixed at a ratio of 2.5:1 and suspended in collagen mixture. These collagen plugs were allowed to solidify and then implanted into the cleared #4 mammary gland of
C57/B6 mice. Tumors were allowed to grow for approximately 28 days before sacrifice. At time of sacrifice, tumors were weighed and total size was measured with calipers. Lungs were also removed for analysis of lung metastasis.

Lungs were fixed in 10% neutral buffered formalin overnight at 4°C. The next day, lungs were dehydrated, placed in xylene for 1 h, and then changed to fresh xylene overnight. Lungs were rehydrated before dipping in Mayer's hematoxylin for 2 min and then washed in running tap water for 5 min. Tissues were destained in HCl (fresh 1% v/v from a 12 N solution) for 20 min, rinsed in running tap water overnight, dehydrated, and placed in xylene overnight before counting of stained metastatic tumor foci under a dissecting light microscope.

**Microarray Data Analysis**

Publically available human datasets, GSE33692 and GSE9014, were downloaded from NCBI Gene Expression Omnibus and analyzed via Agilents’ GeneSpring GX microarray analysis software. GSE33692 dataset represents microarray data files from RNA extracted from either matched epithelial or stromal tissue from 3 normal, 9 DCIS, and 10 IDC breast cancer patients (Sharma et al. 2010). GSE9014 is microarray data derived from the stromal captured tissue of 53 breast cancer patients (Finak et al. 2008). Kaplan Myer plots for relapse free patient survival were generated using kmplot.com (Gyorffy et al. 2010).
Statistics

Statistical analysis was performed using Graphpad Prism after consultation with Dr. Fei Yi of the Vanderbilt Biostatistics Department.

Results

Decreased stromal TGFR2 expression correlates with poor prognosis in breast cancer patients.

As indicated above, the effects of TGF-β signaling in stromal fibroblasts have been shown to promote tumor progression and metastasis. However, as breast cancer progresses from an in situ disease to an invasive disease, gene expression analysis shows that stromal expression of TGFR2 decreases. In colorectal cancer, decreased stromal expression of TGFR2 correlated with poor patient survival thus we sought to address whether stroma with intact TGF-β signaling components predicted better survival for breast cancer patients. Using publically available data mining tools, decreased expression of TGFR2 from bulk tumor predicts for poor survival in breast cancer patients. Break down of these patients into those with aggressive LN+ disease or Grade 3 cancer shows a similar trend with decreased TGFR2 expression again predicting poor patient survival (Figure 13A). To address the functional loss of TGF-β signaling in stromal cells, we next performed immunohistochemistry for phosphorylated Smad2 on a tissue microarray containing patient tissue ranging from normal to invasive ductal carcinoma (Figure 13B). Gross histological analysis of stromal cells in these tissue samples reveals that Smad2 phosphorylation in fibroblastic cells in the tumor
Figure 13. Decreased Stromal TGFBR2 Expression Correlates with Poor Patient Prognosis. A) In silico analysis of breast cancer patient survival upon segregation of whole tumor expression of TGFBR2 into high or low expression in all patients, LN+ patients, and patients with Grade 3 breast cancer. B) IHC staining for phosphorylated Smad2 in a) adenosis of the breast b) hyperplasia of ductal epithelium c) Grade 2 Invasive Ductal Carcinoma and d) Grade 3 Invasive Ductal Carcinoma. Arrows indicate stromal cells with fibroblastic morphology. C) Analysis of breast cancer patient data (GSE ) based upon segregating stromal expression of TGFBR2 into high vs. low groups.
A. TGFβR2 in All Patients

- HR = 0.67 (0.58 - 0.78)
- logrank P = 1.5e-07

B. TGFβR2 in LN+ Patients

- HR = 0.69 (0.5 - 0.93)
- logrank P = 0.015

C. TGFβR2 in Grade 3 Patients

- HR = 0.68 (0.51 - 0.9)
- logrank P = 0.0064

B. Images:
(a) TGFβR2 in All Patients
(b) TGFβR2 in LN+ Patients
(c) TGFβR2 in Grade 3 Patients
(d) TGFβR2 in Grade 3 Patients

C. Graphs:
- TGFβR2 Tumor Size: N.S.
- TGFβR2 Patient Outcome: P=0.024
- TGFβR2 Patient Recurrence: P=0.005
stroma decreases as the disease progresses towards IDC. Given the indication that TGF-β signaling in the stroma is indeed decreased as tumors progress, we sought to address the impact this would have on patient survival. Publically available datasets of laser capture microdissected tumor stroma from breast cancer patients was segregated into TGFBR2 high and low expressing groups. From here, the changes in the available patient data of these two groups were compared. No difference was observed in tumor size from patients with low or high TGFBR2 stroma. However, patients with TGFBR2 low stroma did show a statistically significant increase in poor outcome and tumor recurrence (Figure 13C). These data indicate that stromal loss of TGFβR2 expression is likely to occur in fibroblasts and that this decreased expression is correlated with poor patient outcome.

Fibroblasts lacking Tgfbr2 expression present with an altered gene expression profile

Given that our data indicate fibroblastic cells in the stroma show decreased TGF-β signaling as tumor progress and we have previously published that loss of TβRII in fibroblasts promotes epithelial tumorigenesis, we sought to address the functional significance of loss of Tgfbr2 expression in fibroblasts. To do this, we isolated and characterized fibroblasts from Floxed Tgfbr2 mice with (Fibro^fl/fl) and without (Fibro^R2KO) FSP-Cre expression to drive fibroblast specific recombination of the type II transforming growth factor beta receptor. Fibroblasts were selected based on degree of recombination. Northern blot analysis was utilized to select the fibroblast culture to utilize for our studies based on degree of recombination of Tgfbr2 (data not shown).
Figure 14. Fibro^{R2KO} Lose TGF-β Responsiveness and Present an Altered Gene Expression Profile. A) Immunoblot analysis of for TGF-β responsiveness based on phosphorylation of Smad2 as well as changes in fibroblast marker alpha smooth muscle actin. B) qPCR quantification of changes in expression of TGF-β responsive genes CTGF (1.00±0.11, 19.27±2.13, 0.03±0.003, 0.04±0.005), Smad7 (1.00±0.07, 2.19±0.12, 0.39±0.014, 0.34±0.015), and Col1α2 (1.00±0.04, 2.23±0.22, 1.04±0.03, 1.16±0.06) upon TGF-β stimulation of both Fibro^{fl/fl} and Fibro^{R2KO} fibroblasts. Values represent fold change relative to Fibro^{fl/fl} fibroblast expression. * = p<0.05. C) Representative immunofluorescent images of ECM deposition in in vitro cultures of Fibro^{fl/fl} and Fibro^{R2KO} fibroblasts with and without TGF-β stimulation.
A.  

<table>
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<tr>
<th>Protein</th>
<th>Fibro^0/0</th>
<th>Fibro^M</th>
<th>TGF-β1</th>
<th>Fibro^R2KO</th>
<th>TGF-β1</th>
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</tr>
</tbody>
</table>

Intensity values normalized to actin: 1089360, 993480, 848895, 716040

C.  

Fibronectin  
Fibro^0/0  
Fibro^0/0 + TGF-β  
Fibro^R2KO  
Fibro^R2KO + TGF-β

Collagen 4  
Fibro^0/0  
Fibro^0/0 + TGF-β  
Fibro^R2KO  
Fibro^R2KO + TGF-β
Immunoblot analysis for canonical TGF-β signaling components as well as alpha-smooth muscle actin indicate that Fibro^{R2KO} fibroblasts lose the ability to respond to TGF-β as indicated by loss of induction of Smad2 phosphorylation (Figure 14A). Interestingly, there is no indication to any change in typical fibroblast genes such as α-SMA or collagen I. However, quantitative PCR analysis of Fibro^{fl/fl} fibroblasts indeed shows these cells can respond to TGF-β through the increased expression of known TGF-β responsive fibroblast genes such as CTGF, Smad7, and Col1α2 (Figure 14B). Validating these observations, immunofluorescence for ECM proteins was performed on in vitro cultures. As expected, fibronectin deposition was increased upon treatment of Fibro^{fl/fl} fibroblasts with TGF-β1 and completely absent in Fibro^{R2KO} fibroblast cultures. As well, there was minimal collagen 4 expression in normal fibroblast cultures and this is significantly increased in Fibro^{R2KO} fibroblasts (Figure 14C). These data would indicate that loss of Tgfbr2 expression in fibroblasts does indeed attenuate the responsiveness of fibroblasts to TGF-β as well as prevent the induction of known TGF-β responsive genes.

Loss of Fibroblast Tgfbr2 expression promotes tumor cell metastasis

As we have previously shown that stromal loss of TGFBR2 is associated with poor patient prognosis, we sought to address the functional impact of TβRII loss in fibroblasts on tumor progression. To do this, we implanted tumor cells with either Fibro^{fl/fl} or Fibro^{R2KO} fibroblasts into the cleared #4 mammary fat pad in C57BL/6 mice. These tumors were allowed to grow for 28 days prior to sacrifice and isolation of tumors and
Figure 15. Fibroblasts lacking Tgfbr2 expression promote tumor cell metastasis. A) Quantification of tumor weight at the tumor of sacrifice 28 days after tumor implantation of Fibro^{fl/fl} or Fibro^{R2KO} fibroblasts with PyMT tumor cells. B) Quantification of lung metastasis in mice with either Fibro^{fl/fl} or Fibro^{R2KO} fibroblasts with PyMT tumor cells 28 days after implantation. C) Representative images of Ki67 staining in tumors implants with either Fibro^{fl/fl} or Fibro^{R2KO} fibroblasts. Graph shows quantification of Ki67 staining Fibro^{fl/fl} + PMTB6-2 implants (0.685±0.014 A.U., n=16) and Fibro^{R2KO} + PMTB6-2 implants (0.72±0.016 A.U., n=16) normalized to the total number of cell based on hematoxylin staining. D) Representative images of cleaved caspase 3 staining in tumor implants with either Fibro^{fl/fl} or Fibro^{R2KO} fibroblasts. Graph shows quantification of cleaved caspase 3 staining of Fibro^{fl/fl} + PMTB6-2 implants (0.026±0.0016 A.U., n=16) and Fibro^{R2KO} + PMTB6-2 implants (0.023±0.0036 A.U., n=16) normalized to total cell number based on hematoxylin staining. D) Representative images of DAPI staining of transwells after 24 hours of cell migration stimulated by either Fibro^{fl/fl} or Fibro^{R2KO} fibroblasts. Graph represents quantification of total number of migrated PyMT cells towards either Fibro^{fl/fl} (138.18±4.71, n=12) or Fibro^{R2KO} (242.5±13.53, n=12) fibroblasts per 20X image.
lungs. Gross analysis of tumor weight indicates no difference in the growth of tumor cells when implanted with TβRII knockout fibroblasts (Figure 15A). Previously in our laboratory we showed fibroblasts lacking Tgfbr2 expression promoted tumor growth via increased Ki67+, proliferative cells (Cheng et al. 2005). Tumor cells implanted with Fibro^{R2KO} fibroblasts do not show a similar trend as we found no differences in Ki67 staining (Figure 15C). We also do not observe any differences in cell death, as quantified through staining for cleaved caspase3 (Figure 15D). The major phenotypic difference observed between these tumors was the enhancement of tumor cell metastasis by Fibro^{R2KO} fibroblasts. Whole mount analysis of lung metastasis in these mice showed that Fibro^{R2KO} fibroblasts promote the lung metastasis of tumor cells (Figure 15B). With Fibro^{R2KO} fibroblasts promoting tumor cell metastasis upon implant, the effect of fibroblasts directly on the epithelial cells ability to migrate was addressed. Fibro^{fl/fl} and Fibro^{R2KO} fibroblasts were seeded onto the bottom of a 24 well plate prior to the use of non-coated transwells with PyMT cells in the chamber. The number of PyMT which migrated towards the fibroblasts was assessed and quantified by counting DAPI positive cells on the underside of the transwell. From this experiment we see that there was a significant increase in the number of tumor cells which migrate towards the Fibro^{R2KO} fibroblasts (Figure 15E). Such data lends itself towards TGF-β signaling, in the context of fibroblast-epithelial interactions, being suppressive of the induction of tumor cell migration which could play a role in tumor metastasis.
Gene signature derived from fibroblasts lacking Tgfb2 expression correlates with poor patient prognosis.

With TGF-β signaling playing such a pivotal role in so many aspects of fibroblast function, we performed microarray analysis on Fibro^{fl/fl} and Fibro^{R2KO} fibroblasts. This analysis allowed us to do an unbiased survey of significant gene expression changes dictating the phenotypic differences between these cells. Q-PCR analysis validated many of the top genes up and down regulated. Analysis of patient gene expression data using the top 10 genes that were up-regulated in the Fibro^{R2KO} fibroblasts (Table 2), we observe that increased expression of these genes is correlated with poor patient survival in breast cancer patients, particularly in LN+ patients. The same is also true for genes down-regulated in Fibro^{R2KO} fibroblasts (Table 1, Figure 1C). Using the same analysis for down-regulated genes, we observe that patients with low expression of the identified genes correlate with poor patient prognosis. Interestingly, analysis of expression data from LN+ patients does not maintain this trend showing that increased expression of genes that are downregulated in Fibro^{R2KO} fibroblasts correlates with poor patient prognosis (Figure 16C). To begin to address the functional changes that effect patient outcome, we performed pathway analysis on genes that were significantly altered by 5 fold between Fibro^{fl/fl} and Fibro^{R2KO}. Validating the model, we observe that the TGF-β pathway was identified as a significantly altered pathway using the gene expression that was decreased in the Fibro^{R2KO} fibroblasts (Table 3). Also, supportive of our results showing the Fibro^{R2KO} gene expression signature correlates with poor patient prognosis, we observe that genes that
Table 2. Top 10 genes that were either increased or decreased in the 1362 fibroblasts compared to the 1340 fibroblasts.

<table>
<thead>
<tr>
<th>UP Gene List</th>
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Figure 16. Gene Expression Signature of Fibroblasts which lack TβRII Correlates with Poor Patient Prognosis. A) Clustering analysis of gene significantly changed between fibroblasts with and without Tgfbr2 expression. B) qPCR validation of top gene identified as being increased: Il-5 (1.00±0.17, 5.32±0.1, n=3), Serpinb5(1.00±0.17, 820.2±187.2), and Ctsc(1.00±0.17, 1588.1±139.2), or decreased: Ogn(1.00±0.19, 0.012±0.002), Dlk1(1.00±0.17, 0.00007±0.00001), and Thbd(1.00±0.18, 0.0052±0.006), in fibroblasts lacking TGF-β responsiveness. Values represent fold change relative to Fibrofl/fl. C) Relapse Free Survival (RFS) of patients upon segregation into high or low expression of top 10 genes whose expression is increased or decreased in fibroblast lacking Tgfbr2 expression. Red = High expression; Black = Low Expression.
### Table 3. Pathway Analysis of Fibroblasts Lacking Tgfbr2 Expression

#### Pathways Increased Upon Tgfbr2 Loss

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<tr>
<th>Gene Set Name [# Genes (K)]</th>
<th>Description</th>
<th># Genes in Overlap (k)</th>
<th>k/K</th>
<th>p-value</th>
<th>FDR q-value</th>
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<td>KEGG_ARRHYTHMOMEGNIC_RIGHT_VENTRICULAR_AR_CARDIOMYOPATHY_ARVC [76]</td>
<td>Arrhythmogenic right ventricular cardiomyopathy (ARVC)</td>
<td>5</td>
<td></td>
<td>2.13e-5</td>
<td>1.2e-3</td>
</tr>
<tr>
<td>KEGG_FC_EPSILON_RI_SIGNALING_PATHWAY [79]</td>
<td>Fc epsilon RI signaling pathway</td>
<td>5</td>
<td></td>
<td>2.58e-5</td>
<td>1.2e-3</td>
</tr>
<tr>
<td>KEGG_ECM_RECEPTOR_INTERACTION [84]</td>
<td>ECM-receptor interaction</td>
<td>5</td>
<td></td>
<td>3.47e-5</td>
<td>1.29e-3</td>
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<tr>
<td>KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION [267]</td>
<td>Cytokine-cytokine receptor interaction</td>
<td>7</td>
<td></td>
<td>1.62e-4</td>
<td>5.64e-3</td>
</tr>
<tr>
<td>KEGG_RENAL_CELL_CARCINOMA [76]</td>
<td>Renal cell carcinoma</td>
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<td></td>
<td>2.55e-4</td>
<td>6.77e-3</td>
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<tr>
<td>KEGG_CELL_ADHESION_MOLECULES_CAMS [134]</td>
<td>Cell adhesion molecules (CAMs)</td>
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<td></td>
<td>3.15e-4</td>
<td>7.32e-3</td>
</tr>
<tr>
<td>KEGG_HYPERTROPHIC_CARDIOMYOPATHY_HCM [85]</td>
<td>Hypertrophic cardiomyopathy (HCM)</td>
<td>4</td>
<td></td>
<td>5.35e-4</td>
<td>1.09e-2</td>
</tr>
<tr>
<td>KEGG_ERBB_SIGNALING_PATHWAY [87]</td>
<td>ErbB signaling pathway</td>
<td>4</td>
<td></td>
<td>5.84e-4</td>
<td>1.09e-2</td>
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</table>

#### Pathways Decreased Upon Tgfbr2 Loss

<table>
<thead>
<tr>
<th>Gene Set Name [# Genes (K)]</th>
<th>Description</th>
<th># Genes in Overlap (k)</th>
<th>k/K</th>
<th>p-value</th>
<th>FDR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KEGG_FOCAL_ADHESION [201]</td>
<td>Focal adhesion</td>
<td>6</td>
<td></td>
<td>3.46e-5</td>
<td>6.43e-3</td>
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<tr>
<td>KEGG_TGF_BETA_SIGNALING_PATHWAY [86]</td>
<td>TGF-beta signaling pathway</td>
<td>4</td>
<td></td>
<td>1.37e-4</td>
<td>1.27e-2</td>
</tr>
</tbody>
</table>
that are identified in pathway involved in cancer are significantly found in those genes increased in the Fibro$^{R2KO}$ fibroblasts. From this we conclude that similar to the results presented previously in which TGFBR2 low stroma predicts worse patient prognosis, the gene expression changes associated with Tgfbr2 loss in fibroblasts are also predictive of poor patient prognosis.

**Fibroblasts with Abrogated TGF-β Signaling have Altered ECM Related Gene Expression**

As the functional role of TGF-β signaling in the induction of fibroblast ECM secretion and deposition, we anticipated that Fibro$^{R2KO}$ fibroblasts would have a significantly altered ECM expression profile. Thus, the BROAD pathway analysis of gene expression changes in these fibroblasts showing significant differences in ECM Receptor interactions gave credence to looking further into these effects (Table 3). In order to get a broad overview of changes specifically centered on ECM changes, we performed a qPCR array specifically for genes involved in the deposition and modification of the extracellular matrix (Figure 17A). The results of this array supports that abrogation of TGF-β signaling in fibroblasts does indeed induce significant changes to the cells ability to deposit and manipulate the ECM. As expected, we observe that several genes previously identified were validated such as increased expression of Col4a1 and a loss of CTGF expression in
Figure 17. Fibroblasts lacking Tgfr2 Expression have altered ECM related gene expression profile. A) qPCR results for gene expression analysis of ECM related genes in either the Fibro^fl/fl^ mouse mammary fibroblasts or the Fibro^R2KO^ mouse mammary fibroblasts. All genes are shown relative to the Fibro^fl/fl^ mouse mammary fibroblasts. B) Analysis of patient relapse free survival after segregation of patients based on those genes shown to be increased in the Fibro^R2KO^ fibroblasts relative to the Fibro^fl/fl^ fibroblasts.
A. Genes with Increased Expression in Fibro$^{RKO}$

B. All Patients

- HR = 0.79 (0.69 - 0.9)
- logrank P = 0.00052

LN+ Patients

- HR = 2.09 (1.57 - 2.79)
- logrank P = 2.5e-07
Fibro \(^{R2KO}\) fibroblasts. As the gene expression changes in the Fibro \(^{R2KO}\) fibroblasts were previously established to be predictive of poor patient survival, we want to know the gene expression changes specifically influencing the ECM had the same effect on patient survival. Specifically using those genes whose expression is increased in the Fibro \(^{R2KO}\) fibroblasts, we do not see high expression of these genes predicting poor patient response (Figure 17B). Rather we observe these genes being predictive of overall better survival in non-segregated breast cancer patients. However, when we segregate these patients into those that presented with LN+ disease, we again see that the increased expression of these ECM genes does predict poor survival in these patients (Figure 17B).

**Loss of Tgfb2 in Fibroblasts Promotes Myeloid Infiltration through Increased Cytokine Expression**

Another of the functional changes in Fibro \(^{R2KO}\) fibroblasts identified by the BROAD pathway analysis that garnered our attention was the cytokine receptor interactions (Table 2). As abrogation of TGF-β signaling in epithelial cells promotes the expression of numerous chemokines and others have identified cytokine changes in fibroblasts with low Tgfb2 expression, we sought to identify whether we saw similar changes in our cells. For this, we performed a cytokine array on conditioned media comparing Fibro \(^{fl/fl}\) and Fibro \(^{R2KO}\) fibroblasts (Figure 18A). The conditioned media from Fibro \(^{R2KO}\) fibroblasts had increased levels of the chemokines CXCL5, CXCL12, and CCL9. Also present was an increased level of decorin and MMP3 validating findings from both the microarray expression results as well as the ECM qPCR array. Given that there was
Figure 18. Fibroblasts lacking Tgfbr2 expression have altered interactions with myeloid cells correlating with a different cytokine profile. A) Image of representative cytokine array profile examining differences in cytokine presence in the conditioned media of Fibro$^{fl/fl}$ and Fibro$^{R2KO}$ fibroblasts. Graphs show quantification of changes in CXCL5, CCL9, CXCL12, DCN, PTX3, and MMP3 based on altered pixel density normalized to positive control. B) Representative images of F4/80 staining of tumors resulting from implants of PMTB6-2 tumors cells with either Fibro$^{fl/fl}$ or Fibro$^{R2KO}$ fibroblasts. C) Altered relapse free survival of all (left) and LN+ (right) patients based on expression those cytokines differentially expressed in the Fibro$^{R2KO}$ fibroblasts.
significant up-regulation of chemokines that have been associated with influencing the infiltration of myeloid cells into the tumor microenvironment, we turned to our in vivo implants presented in Figure 16 to analyze macrophage infiltration. Staining for F4/80, a marker of activated macrophages shows a trend of more activated macrophages present in the stromal areas of the tumors with Fibro\textsuperscript{R2KO} fibroblasts (Figure 18B). Increased infiltration of macrophages, particularly those with the M2 polarization phenotype, is associated with increasingly aggressive tumors and poor prognosis in breast cancer patients. To establish that these gene expression changes were significant in the progression of breast cancer in patients, we looked at the survival of patients with high expression of the identified cytokines. Once again increased expression of these genes was predictive of better survival compared to patients with low expression (Figure 18C). Although similar to the altered ECM genes, increased expression of these cytokines did predict for poor prognosis in patients with LN+ disease (Figure 18C). While we are unable to correlate differential expression of these genes with infiltration of myeloid cells in breast cancer patients, this data does support that the cytokine gene expression changes in Fibro\textsuperscript{R2KO} fibroblasts promotes disease progression in both mouse model of cancer as well as breast cancer patients.

Discussion

Previous studies have established that \textit{Tgfbir2} expression is decreased as many tumors progress (Achyut et al. 2013; Paiva et al. 2012). Here we present a similar
finding showing that phosphorylated SMAD levels in fibroblastic cells in the stroma of breast cancers decreases as the disease progresses. However, there is no data correlating stromal expression of Tgfbr2 with patient prognosis. This has been shown by immunohistochemistry in oral and squamous cell carcinoma and now we have shown that decreased expression of Tgfbr2 in the stroma is predictive of poor prognosis in breast cancer patients (Bacman et al. 2007). Interestingly, our data shows that low Tgfbr2 expression not only predicts poor patient outcome but also predicts disease recurrence. This data supports the functional significance of fibroblast TGF-β signaling in tumor progression, potentially acting as a tumor suppressor in more advanced disease (Figure 19). Also, with Tgfbr2 low stroma being associated with recurrence there is the potential for fibroblast TGF-β signaling to play a role in the regulation of tumor cell dormancy or cancer stem cells, both implicated in breast cancer recurrence.

Using implantation of tumor cells and Fibro\textsuperscript{fl/fl} and Fibro\textsuperscript{R2KO} fibroblasts as a model for the effects of fibroblast TGF-β signaling, we were able to study the effects of modifying this signaling on tumor progression. Contrary to previous reports, we do not see any significant differences in tumor cell proliferation or cell death both by analysis of gross tumor weight as well as quantification of Ki67 and cleaved caspase3 immunohistochemistry (Cheng et al. 2005). However, as there was a difference in tumor cell metastasis, we looked into the effects of Fibro\textsuperscript{fl/fl} and Fibro\textsuperscript{R2KO} fibroblasts on epithelial cell migration. Not surprisingly, given our \textit{in vivo} data, fibroblasts lacking TGF-β responsiveness induced more tumor cell migration compared to fibroblasts with intact TGF-β signaling. We performed microarray analysis to address gene expression...
Figure 19. Proposed model for Fibro<sup>R2KO</sup> fibroblast promotion of tumor cell metastasis. Fibroblasts lacking the ability to respond to TGF-B have lost the functional characteristics demonstrated by fibroblasts with TGFBR2 expression, notably the ability to secrete collagen and fibronectin as well as contract a collagenous matrix. Additionally, these cells have a significantly altered secretome such that expression of growth factors and other cytokines are enhanced. This altered secretome is associated with the phenotypic response of enhanced infiltration of myeloid cells into the tumor microenvironment as well as the induction of tumor cell migration. Ultimately, these phenotypic changes are associated with increased metastasis seen in mice upon implantation of tumor cells with Fibro<sup>R2KO</sup> cells compared with Fibro<sup>fl/fl</sup> cells.
<table>
<thead>
<tr>
<th>In the tumor microenvironment</th>
<th>![Image of tumor microenvironment]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast unable to respond to TGF-β</td>
<td>![Image of fibroblast]</td>
</tr>
<tr>
<td>Loss of normal matrix secretion and remodeling</td>
<td>![Image of matrix remodeling]</td>
</tr>
<tr>
<td>Significantly altered secretome</td>
<td>![Image of secretome]</td>
</tr>
</tbody>
</table>
| 1) Promote Myeloid Cell Infiltration  
2) Promote Tumor Migration | 1) ![Image of myeloid cell infiltration]  
2) ![Image of tumor migration] |
| Promotion of Tumor Cell Metastasis |  

**Metastasis**
differences in gene expression in these fibroblasts which may lead to these phenotypic changes in epithelial cells. Pathway analysis of gene expression changes in Fibro$^{R2KO}$ fibroblasts revealed enrichment in several functional categories, but of particular interest to us, based on previously published reports on the function of TGF-β in fibroblasts, is alterations of extracellular matrix interactions as well as cytokine interactions. With these two areas of focus in mind, we were able to show that not only does gene expression in these categories significantly change, but that these changes are associated with poor prognosis of LN+ patients. While this significance does not remain true for all patients, this could be indicative of this gene expression signature’s importance in invasive disease.
Tumor Microenvironment in the Progression of Breast Cancer

Similar to the evolution of the study of epithelial tumor cells, we are beginning to appreciate the numerous effects stromal cells have on tumor progression and breakdown essential pathways in regulating their pro- or anti-tumorigenic function (Hanahan and Coussens 2012). The majority of studies into the stromal tumor microenvironment focus on a particular cell type and how a specific gene expression change in that cell type alters epithelial tumor cell biology. However, the field is moving towards an understanding that interactions of stromal components are an essential aspect in understanding what drives differential gene expression programs identified in particular stromal cell populations. Interactions and influences of immune cells on each other have been established for a long time. The results of these interactions have significant roles in determining cellular functions as well as influence the progression of numerous disease states. While not centered on immune cell interactions, bleomycin induced lung fibrosis is a prime example of how stromal interactions can drive disease progression. In this model, bleomycin treated lungs in mice undergo a characteristic infiltration of immune cells leading to the activation of stromal fibroblasts and excessive deposition of a collagenous matrix (Wynn 2011). It is not a stretch to imagine that such
interactions could also be found in the tumor microenvironment driving many characteristics of tumor progression. In our studies we present data that indicate that infiltrating myeloid cells can drive the activation of stromal fibroblasts and the expression of matrix remodeling proteins which are associated with increased tumor progression (Pickup et al. 2013). Similar to our studies, work in murine models of scleroderma shows that fibroblasts in this system have increased TGF-β signaling activity as described by nuclear localization of Smad3 (Takagawa et al. 2003). While our work currently stands as a correlative interaction, future studies in which myeloid cell infiltration or functions are modulated would provide invaluable mechanistic insight into these cellular interactions. Such interactions are important in order to fully understand cancers from a systems level and implement therapeutics which account for and exploit the interactions promoting the observed phenotypes.

One particular aspect of the tumor microenvironment which could be promoted through the interaction of stromal cells is the desmoplastic response seen in many tumors. The drivers of collagenous expansion in mammary tissue and tumors are an important consideration due to the significance this phenotype has on tumor progression. Dr. Norman Boyd has pioneered work which identifies dense mammographic tissue as a predictive factor in the development of breast cancer. High mammographic density, as determined by routine mammography, correlates with a 12.2% increased risk in developing ductal hyperplasia and a 9.67% increased risk in ductal carcinoma in situ (Boyd et al. 2007). Patricia Keely’s group has extended this work showing that mouse models of breast cancer which present with a high degree of
stromal collagen deposition have increased disease incidence and progression of cancer towards metastasis (Provenzano et al. 2008). While this is in a genetically engineered mouse model, it would be interesting if high mammographic density breast tissue and human breast cancer, which presents with increased collagen deposition, are correlated with increased presence of myeloid cells. It should be noted that this type of stromal interaction influencing tissue homeostasis and disease progression is not a de novo idea, but rather an idea co-opted from the proposed interaction that drive the stages of wound healing. Modulating the presence or function of various microenvironmental components can have dramatic effects on re-epithelialization and wound closure in experimental models such as depletion of macrophage populations which results in a significant loss of fibroblast activation and collagen deposition in wound healing (Rodero and Khosrotehrani 2010). Interesting with regards to the work presented above, recombination of Tgfbr2 in murine dermal fibroblasts inhibits fibroblast function of collagen deposition and remodeling and also inhibits the infiltration of macrophages into experimental wounds (Martinez-Ferrer et al. 2010). The crosstalk between stromal fibroblasts and infiltrating myeloid cells is only one example of a multitude of stromal interactions which could potentially influence the progression of numerous cancers. Given the pleotropic nature of many of the potential mediators of these interactions, it is important to elucidate the function of these interactions in terms of tumor progression to determine whether therapeutic intervention will have an overall positive effect for the patient. The potential for these interactions to have a significant impact
on tumor progression underlies the importance of approaching not only the tumor microenvironment but all aspects of tumor biology from a systems approach.

Pro and Anti Tumorigenic Functions of TGF-β Signaling in Fibroblasts

A key concept emphasized by the data presented above is that TGF-β signaling has dichotomous roles in tumor progression. Given that this is the established dogma for epithelial TGF-β signaling, it is interesting to note similar findings in stromal fibroblasts (L. Yang and Moses 2008). Numerous studies have expounded the role of TGF-β in fibroblast function. TGF-β signaling in fibroblasts promotes the induction of myofibroblast differentiation, stimulation of collagen synthesis, and expression of cytokines and chemokines to drive immune cell infiltration (Berie and Moses 2006a). Independently, each of these functions has been linked to the progression of numerous diseases, but in particular TGF-β driven overactivation of fibroblasts is essential in fibrosis (Border and Noble 1994; Leask and Abraham 2004). Sustained production or release of bioactive TGF-β ligands in sites of wound healing is a major contributor to the excessive deposition of collagen fibers characteristic of fibrosis in many organ sites. Supporting this notion, TGF-β1 deficient mice show a lack of collagen deposition in late stage cutaneous wound healing (Kulkarni et al. 1993). With so many similarities between aberrant wound healing and cancer, it is not surprising that activation of TGF-β signaling in fibroblasts has been shown to influence the progression of each.

As shown above, we have identified Lysyl Oxidase as a key target for active TGF-β signaling in fibroblasts that influences metastatic progression in our PyMT^{mgko} model.
Individually, LOX has been linked to both stromal expression in invasive breast cancer as well as a key component in metastasis (Erler et al. 2006; Peyrol et al. 1997). However, given that more recently LOX has been mainly studied in the context of hypoxic tumor microenvironments, these studies should now expand into tumors that lack a high degree of tumor hypoxia but have a large degree of stromal infiltration and expansion of collagenous matrix deposition. Use of LOX inhibitors in patients that have a large degree of stromal expansion and high LOX expression could be of benefit due to the effects identified in our studies as well as others. An interesting, yet to be observed, effect from the use of LOX inhibitors could be the improvement of therapeutic efficacy. Given that TGF-β inhibitors have been identified by Rakesh Jain as a stromal normalizer, it is possible that LOX inhibitors could fall into the same category, particularly since LOX is a TGF-β target (J. Liu et al. 2012a). One of the primary components identified by Dr. Jain’s lab is that therapeutic targets that affect the vasculature of tumors significantly enhance therapeutic delivery. This hypothesis works well with the known actions of LOX which have been shown to promote VEGF secretion as well as promote the migration of endothelial cells and angiogenic sprouting in subcutaneous tumors (Baker et al. 2013). Combining the previously identified angiogenic phenotype with the identification of LOX mediated matrix crosslinking as a factor in the expansion of a basal cell population in breast cancers, LOX inhibitors could not only enhance delivery of chemotherapeutic agents but also slow the expansion of a cell population which correlates with worse prognosis in breast cancer patients.
Despite the fact that the effects of active TGF-β signaling in fibroblasts have been shown to promote the progression and metastasis of breast cancers, experimental and informatics data show that loss of TGFBR2 expression in stromal fibroblasts correlates with more advanced cancer progression (Achyut et al. 2013; Bhowmick et al. 2004). Importantly, analysis of expression data from human breast cancer stroma shows that those patients with lower stromal expression of TGFBR2 have a significantly worse prognosis as well as an increased risk of cancer recurrence. The pro-tumorigenic effects of fibroblasts lacking TGF-β responsiveness seem to stem from manipulation of immune cell infiltration as well as induction of changes to neighboring epithelial cell populations. Previous studies from our laboratory have shown that loss of TGF-β responsiveness enhances the expression of HGF to drive epithelial proliferation and migration (Cheng et al. 2008). Additionally, abrogation of TGF-β responsiveness induces changes to the infiltration of inflammatory cells through enhanced chemokine expression, similar to the effects of abrogation of TGF-β signaling in epithelial tumor cells (Achyut et al. 2013). The data presented above supports our previous work showing that fibroblasts lacking Tgfbr2 expression promote the migration and metastasis of tumor cells. And while not yet established, our data supports TGF-β suppressing a secreted factor in fibroblasts which promotes migration and invasion through the induction of changes to epithelial cell morphology and signaling. Based on previously published data as well as data shown above, it is clear the abrogation of TGF-β signaling in stromal fibroblasts promotes the progression of breast cancer. This is an intriguing and important concept.
as such data could confound results from pre-clinical and clinical trials using systemic inhibitors of the TGF-β signaling pathway.

A potential resolution to these seemingly dichotomous results lies in the distinct mechanisms by which fibroblasts with and without TGF-β signaling promote tumor cell metastasis. Specifically, I have shown that active TGF-β signaling can promote tumor cell metastasis through modifications of the extracellular matrix while suppressing TGF-β signaling in fibroblasts promotes an altered secretome to act on myeloid cells promoting their infiltration as well as directly on epithelial cells promoting a migratory phenotype. These two different paths by which fibroblasts promote tumor cell metastasis also underlie a clinically relevant situation given that suppression of fibroblast TGF-β signaling is not a homogenous event. As we see in our analysis of stromal TGF-β signaling, while there is an enrichment of fibroblastic cells lacking active TGF-β signaling in advanced tumors, there are also fibroblastic cells with active TGF-β signaling. Given this, these two distinct mechanisms of action could not only promote tumor cell metastasis on their own, but work synergistically to ultimately lead to an even greater metastatic event (Figure 20). Supporting this is data from the Bhowmick lab in which a 50/50 mixture of fibroblasts with and without TGF-β signaling lead to a significant enhancement of tumor growth when compared with each population on its own (Kiskowski et al. 2011). This suggests that distinct mechanisms of tumor progression work either independently or synergistically to drive enhanced tumor growth. Applying this concept to the work presented here, the potential for cooperation by these fibroblasts in promoting tumor metastasis is apparent.
Figure 20. Proposed model for synergistic effects of fibroblasts with and without TGF-β signaling on the promotion of tumor cell metastasis. TGF-β responsive fibroblasts can secrete ECM components as well as enzymes which remodel collagen fibers, such as lysyl oxidase, which promote have been shown to promote tumor progression. TGF-β non-responsive fibroblasts promote tumor metastasis through an altered secretome which promote myeloid cell infiltration as well as tumor cell migration. It appears that this mechanism acts to promote metastasis to a greater degree than TGF-β responsive fibroblasts given that fibroblasts lacking Tgfbr2 expression increased metastasis over TGF-β responsive fibroblasts upon implantation. As crosslinked collagen has been shown to promote tumor cell EMT and metastasis, there is a potential for these two mechanisms to act synergistically. Thus when combined, the crosslinked collagen from TGF-β responsive fibroblasts acts as a track promoting the invasion of the high migratory tumor cells which are stimulated by TGF-β non-responsive fibroblasts.
Specifically, fibroblasts with intact TGF-β signaling create a microenvironment which allows for enhancement of a basal cell population and fibroblasts lacking TGF-β signaling secrete growth factors which promote the migratory ability of those cells (Figure 21). With this in mind, it will be interesting to observe if in a tumor in which there are fibroblasts that can and cannot respond to TGF-β, whether inhibition of phenotypic changes associated with either population, either matrix crosslinking or secretome changes, would modulate the promotion of metastasis by the other fibroblast population.

Microenvironmental consideration for therapeutic targeting of TGF-β

The data presented above supports targeting genes suppressed by TGF-β in fibroblasts. However, while there is a significant enrichment for poor prognosis in patients with lower levels of stromal TGFBR2 expression, there are also a large number of patients with low TGFBR2 expression in the stroma which do not succumb to the disease. Such data implies that further segregation of these patients could refine those that would benefit from treatment with a TGF-β targeting agent. This is an important consideration, as mentioned above, for the dynamic range of effects TGF-β has on numerous cells in the tumor microenvironment can confound the efficacy of systemic treatment TGF-β treatment. Thus, successful implementation of TGF-β targeted therapies will rely on finding those patients who present with phenotypic characteristics of active TGF-β signaling whose abrogation will outweigh the potentially pro-tumorigenic effects of abrogating TGF-β.
As such delineation of patients has yet to be achieved, work has progressed on targeting TGF-β signaling specifically in cell populations which would benefit from TGF-β abrogation. A prime example of this is the adoptive transfer of T cells expressing a dominant negative TGFBR2 to eliminate the suppressive function of TGF-β on T cell activity (Q. Zhang et al. 2005). As the fibroblast population in tumors are not as clearly defined as immune cells and abrogation of TGF-β signaling has yet to show any anti-tumorigenic function in fibroblasts, it is unlikely that such a therapeutic strategy would be beneficial. Even if such targeting was possible, evidence shows that maximal promotion of tumorigenic features does not occur upon complete knockdown of TGFBR2 in all stromal fibroblasts, but rather when fibroblasts have a mixed percentage of cells that can and cannot respond to TGF-β (Kiskowski et al. 2011). This would suggest that while both active TGF-β signaling and loss of TGF-β signaling have tumor promoting effects, these effects act in distinct mechanisms to allow for a potentially additive effect upon combination of these two cell populations. It is also feasible that these tumor promoting activities are not only additive but rather supportive of the activities of the other. For instance, above we have shown that myeloid derived TGF-β1 can drive fibroblast activation and secretion of LOX (Pickup et al. 2013). If fibroblasts lacking Tgfbr2 expression promote immune cell infiltration, these immune cells could secrete high levels of TGF-β1 which could active those fibroblasts in the tumor microenvironment which can still respond to TGF-β. However, such data do not preclude the implementation of TGF-β targeted therapeutics but rather emphasizes the need for a thorough understanding of the crosstalk between different populations of
stromal cells as well as delineation of patients and tumor characteristics prior to treatment selection.
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