THE ROLE OF EXTRACELLULAR MATRIX RIGIDITY AND ALTERED microRNA EXPRESSION IN TGF-β-MEDIATED BREAST CANCER PROGRESSION

By

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*We also certify that written approval has been obtained for any proprietary material contained therein.
Dedication

To Dan Patterson whose inquisitive nature got me interested in cancer research and whose strength and determination in fighting his own cancer teaches me the importance of cancer research every day.
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**Abbreviations**

Ago2  Argonaute  
AKT/PKB  protein kinase B  
BMDC  Bone marrow-derived cell  
BMP  Bone morphogenetic protein  
ECM  Extracellular matrix  
EGF  Epidermal growth factor  
EMT  Epithelial-mesenchymal transition  
ERK  Extracellular signal-regulated kinase  
FAK  Focal adhesion kinase  
hnRNP E1  heterogeneous nuclear ribonucleoprotein E1  
ILEI  interleukin-like EMT inducer  
JNK  c-Jun N-terminal kinas  
LOX  Lysyl Oxidase  
MAP kinase  Mitogen-activated protein kinase  
MEC  Mammary epithelial cell  
miR  microRNA  
MMP  matrix metalloproteinase  
mTOR  Mammalian target of rapamycin  
NF-κB  Nuclear factor-κB  
PDGF  Platelet-derived growth factor  
PI3K  Phosphoinositol-3-kinase  
pre-miRNA  precursor microRNA  
 pri-miRNA  primary microRNA transcript  
RISC  RNA induced silencing complex  
TβR-I  TGF-β type I receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tr>
<td>TβR-II</td>
<td>TGF-β type II receptor</td>
</tr>
<tr>
<td>TβR-III</td>
<td>TGF-β type III receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor-associated factor 6</td>
</tr>
<tr>
<td>TRBP</td>
<td>Tar RNA binding protein</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula occluden-1</td>
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The Role of Extracellular Matrix Rigidity and Altered microRNA Expression in TGF-β-Mediated Breast Cancer Progression

Abstract

By

MOLLY ANN TAYLOR

Breast cancer is the second leading cause of cancer death in women in the United States. Metastasis accounts for the death of ~90% of these patients, yet the mechanisms underlying this event remain poorly defined. Transforming growth factor-β (TGF-β) is a multifunctional cytokine that functions to suppress tumorigenesis in mammary epithelial cells (MECs). Interestingly, mammary tumorigenesis converts TGF-β from a tumor suppressor to a tumor promoter through molecular mechanisms that remain incompletely understood. Changes in tissue compliance promote the acquisition of malignant phenotypes in MECs in part through the activity of the extracellular crosslinking enzyme, lysyl oxidase (LOX), which regulates desmoplastic reactions and metastasis. We show that TGF-β induces the synthesis and secretion of LOX from normal and malignant MECs, and in breast cancers produced in mice. Additionally, antagonizing LOX activity reduces TGF-β-mediated invasion and epithelial-mesenchymal transition (EMT) in breast cancer cells. We further show that increased extracellular matrix (ECM) rigidity promotes the proliferation of malignant MECs, a cellular reaction that is abrogated by inhibiting TGF-β signaling or by antagonizing LOX activity.

As a possible mechanism for the influence of matrix rigidity on TGF-β signaling, we sought to define the microRNA expression profiles induced by TGF-β and ECM
rigidity during metastatic progression. Through global profiling analyses, we identified microRNAs whose expression was induced by TGF-β and associated with metastatic potential. In doing so, we identified miR-181a as a TGF-β-regulated “metastamir” that enhanced the metastatic potential of breast cancers by promoting their acquisition of EMT, migratory, and invasive phenotypes. Mechanistically, inactivating miR-181a elevated the expression of the pro-apoptotic molecule, Bim, which sensitized metastatic cells to anoikis. Along these lines, miR-181a activity was essential in driving pulmonary micrometastatic outgrowth and enhancing the lethality of late-stage mammary tumors in mice. Finally, miR-181a expression is dramatically and selectively upregulated in metastatic breast tumors, particularly triple-negative breast cancers, and is highly predictive for decreased overall survival in human breast cancer patients.

Collectively, our findings implicate LOX and miR-181a expression as novel diagnostic markers for metastatic progression, as well as an innovative therapeutic targets to treat metastatic breast cancers.
Chapter 1 - Introduction to TGF-β

1.1 The role of TGF-β in Breast Cancer and Metastasis

TGF-β is the prototypic member of a large family of evolutionary conserved cytokines that includes the activins, bone morphogenetic proteins, growth differentiation factors, Nodal, and inhibins [1]. Mammals express three genetically distinct TGF-β ligands (e.g., TGF-β 1–3), whose mature and biologically active forms are 97% identical and exhibit virtually indistinguishable actions in vitro [2, 3]. Individual TGF-β molecules play important roles during embryonic development and tissue morphogenesis, and in maintaining cellular and tissue homeostasis in adults [4]. TGF-β plays essential roles during the branching morphogenesis, lactation, and involution phases of postnatal mammary glands [5, 6]. TGF-β is also a powerful tumor suppressor that governs essentially every aspect of the pathophysiology of mammary epithelial cells (MECs), including their ability to proliferate, migrate, differentiate, and survive [7-9]. During mammary tumorigenesis, genetic and epigenetic events undermine the tumor suppressive functions of TGF-β, thereby enhancing the development and progression of evolving breast cancers. Mammary tumorigenesis also elicits dramatic alterations in the architecture of breast cancers and their accompanying microenvironments (e.g., desmoplastic and fibrotic reactions), which further inactivate the tumor suppressing activities of TGF-β [8, 10, 11]. These abnormal events coalesce to confer TGF-β the

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ability to stimulate the invasion and metastasis of late-stage breast cancer cells. This conversion in TGF-β function is known as the “TGF-β Paradox,” which underlies the lethality of TGF-β in metastatic breast cancer cells [8, 12, 13].

1.2 TGF-β and EMT

TGF-β is well known for its ability to promote metastatic progression through the induction of epithelial-mesenchymal transition (EMT) in MECs. This transdifferentiation process results in polarized MECs acquiring apolar and highly motile fibroblastoid-like phenotypes [7, 14]. The process of EMT is characterized by (i) changes in cytoskeletal architecture and intracellular organelle redistribution; (ii) loss of cell polarity due to downregulation of epithelial cell markers (e.g., E-cadherin, ZO-1, and β4 integrin); (iii) upregulation of fibroblastoid markers (e.g., vimentin, N-cadherin, α-smooth muscle actin); and (iv) elevated expression of invasion promoting factors [e.g., MMP-9, fibronectin; see [7, 14]]. Recently, the process of EMT has been categorized into three distinct subtypes: (i) type 1 EMT, which represents the transdifferentiation process that occurs during embryogenesis and tissue morphogenesis; (ii) type 2 EMT, which is associated with tissue regeneration during wound healing, fibrotic reactions, and inflammation; and (iii) type 3 EMT, which represents the plasticity exhibited by carcinoma cells that enables them acquire invasive, metastatic, and stem cell-like phenotypes [15]. In fact, EMT programs not only enhance the ability of carcinoma cells to invade locally as a means to exit the primary tumor, but also facilitate their survival in the circulation and ability to reinitiate proliferative programs at distant sites of metastasis
The mechanisms through which TGF-β initiates the pathophysiological activities that lead to EMT and metastasis transpire through activation of canonical Smad2/3-dependent pathways, as well as stimulation of numerous “non-canonical” signaling pathways detailed below.

1.3 TGF-β Signaling

1.3.1 Canonical TGF-β signaling

Once synthesized, TGF-β ligands are secreted into the ECM as inactive latent complexes whose conversion to active forms transpires through several mechanisms, including proteolysis, reactive oxygen species, acidic microenvironments, and binding to integrins [19, 20]. Following their activation, TGF-βs 1 and 3 bind directly to TβR-II, while TGF-β2 must first bind TβR-III to facilitate presentation to TβR-II. While TβR-III lacks intrinsic enzymatic activity, TβR-I and TβR-II both possess Ser/Thr protein kinases in their cytoplasmic domains that serve to initiate downstream signaling [4, 8]. Ligand binding to TβR-II leads to the recruitment, phosphorylation, and activation of TβR-I, which subsequently binds, phosphorylates, and stimulates Smad2/3 [8, 21]. Once activated, Smad2/3 bind to the common Smad, Smad4, exposing cryptic nuclear localization sequences and facilitating translocation to the nucleus, where Smad2/3/4 complexes function in conjunction with a host of transcription factors, enhancers, and repressors that collectively bring about dramatic alterations in gene expression in a cell- and context-specific manner [8, 21]. Collectively, this activation of Smads 2, 3, and 4 by TGF-β is referred to as “canonical TGF-β signaling”.
The amplitude and duration of Smad2/3 responses are impacted by their interaction with a variety of adapter molecules, including SARA [22], Hgs [23], PCTA [24], and Dab2 [25, 26]. Additionally, the activation of Smad2/3 by TβR-I is governed by the inhibitory Smad, Smad7, whose binding to TβR-I occludes its ability to phosphorylate Smad2/3 [27-29], as well as recruits the E3 ubiquitin ligase, Smurf1/2, to promote TGF-β receptor ubiquitination, internalization, and degradation [30, 31]. In addition, the functions of Smad7 are positively regulated by its binding to STRAP [32], but negatively regulated by its interactions with AMSH2 [33] and Arkadia [34]. Finally, canonical TGF-β signaling can be terminated through several distinct mechanisms, including (i) dephosphorylation of Smad2/3 by the nuclear phosphatase PPM1A [35]; (ii) degradation of Smad2/3 following their ubiquitination by Smurf1, Smurf2, and SCF/Roc1 [36-39]; and (iii) reversible mono-ubiquitination of Smad4 which governs its binding to activated Smad2/3 [40].

1.3.1.1 Canonical TGF-β Signaling and Metastasis

Although Smads 2 and 3 are often referred to as being a single entity in the scientific literature, recent findings have established distinct roles for Smad2 versus Smad3 in mediating the pathophysiology of TGF-β. For instance, targeted deletion of Smad2 elicits embryonic lethality [41, 42], while Smad3-deficient mice are viable and are highly susceptible to inflammation-induced colon tumorigenesis [43-45]. Along these lines, the gene expression profiles coupled specifically to Smad2 activation are readily distinguishable from those coupled to the activation of Smad3 [46-48], findings that offer unique insights into how TGF-β modulates cell growth, motility, and survival [49]. With respect to cancers of the breast, experimental inactivation of Smad2 augments breast
cancer metastasis to bone, while similar inactivation of Smad3 attenuates this metastatic response by reducing tumor angiogenesis and VEGF expression [50]. Likewise, rendering early-stage breast cancer cells deficient in Smad2/3 signaling enhances their malignancy, while similar manipulations in their late-stage counterparts prevents these breast cancer cells from colonizing the lungs [51, 52]. Interestingly, a TβR-I mutant unable to activate Smad2/3 fails to support the ability of breast cancer cells to colonize the lung [53], suggesting that canonical and noncanonical TGF-β signaling inputs coalesce in facilitating metastatic outgrowth. At present, the precise molecular mechanisms that enable these disparate signaling systems to collaborate in promoting metastatic progression by TGF-β remains unclear. Addressing this question is critical to unraveling the mysteries of the “TGF-β Paradox.” For instance, a recent study demonstrated that the administration of bisphosphonates was only effective in suppressing osteolytic bone lesions and canonical TGF-β signaling at early stages of the metastatic process, not in fully established lesions [54]. These findings indicate that canonical TGF-β signaling is differentially regulated during specific stages of the metastatic cascade. Accordingly, transient activation of Smad2/3 by TGF-β converted the migration of breast cancer cells from cohesive to single cell programs [55, 56]. Quite intriguingly, reinitiation of proliferation programs necessary for pulmonary metastatic outgrowth of these breast cancer cells required them to first inactivate Smad2/3 signaling [55, 56]. Thus, it is tempting to speculate that altered elastic moduli govern the coupling of TGF-β to Smad2/3. In support of this supposition, we recently determined that canonical TGF-β signaling is selectively silenced in (a) compliant 3D-organotypic cultures relative to rigid tissue culture plastic, and (b) pulmonary metastases relative to
their site of origin (M.A. Taylor, M.K. Wendt and W.P. Schiemann, unpublished observation). Taken together, these intriguing findings demonstrate the plasticity present in the TGF-β signaling system as carcinoma cells undergo EMT and metastatic outgrowth, presumably reflecting a shift from canonical (i.e., Smad2/3-based) to noncanonical (i.e., non-Smad2/3-based) signaling that originates from altered mechanotransduction and integrin signaling. Future studies need to define which stages of the metastatic process are dependent upon TGF-β signaling, as well as delineate which branches of the TGF-β signaling system engender these deadly events.

1.3.2 Noncanonical TGF-β signaling

Besides its ability to activate canonical Smad2/3/4 signaling, TGF-β also regulates cell behavior through its activation of a variety of Smad2/3-independent pathways, which are collectively referred to as “noncanonical TGF-β signaling.” Included in this ever-expanding list of noncanonical TGF-β effectors are the (i) MAP kinases, ERK1/2, p38MAPK, and JNK; (ii) cell survival mediators, PI3K, AKT1/2, and mTOR; (iii) inflammatory mediators, NF-κB, Cox-2, and prostaglandins; (iv) small GTP-binding proteins, Ras, RhoA, Rac1, and Cdc42; and (v) integrins and nonreceptor protein tyrosine kinases [3, 57]. Importantly, imbalances between the canonical and noncanonical TGF-β signaling systems have been associated with disease development in humans, including cancers of the breast [3, 58] (Figure 1.1). The succeeding sections highlight the roles of prominent non-canonical effectors activated by TGF-β and discuss their roles in mediating EMT and oncogenic signaling by TGF-β in cancers of the breast.
1.3.2.1 MAP Kinases

A major mechanism whereby TGF-β induces EMT and metastatic progression is through the stimulation of members of the MAP kinase family of dual-specificity protein kinases, which includes ERK1/2 (extracellular signal-related kinase 1 and 2), JNK (c-Jun N-terminal kinase, and p38 MAPK [59-62]. Indeed, TGF-β stimulation of EMT and its accompanying delocalization of E-cadherin and ZO-1 from the plasma membrane requires ERK1/2 activation, a cellular reaction abrogated by administration of MEK1/2 inhibitors [62]. Likewise, rendering MECs deficient in Dab2 expression prevents TGF-β from stimulating JNK, and from promoting MEC migration and fibronectin expression during EMT [63]. Elevated tumor expression of type I collagen has been shown to induce JNK activation [64]. As such, pharmacological inhibition of either JNK or PI3K activity abrogates the ability of type I collagen to promote the migration and metastasis of breast cancer cells [65]. The activation of p38 MAPK by TGF-β in normal and malignant MECs requires these cells to express either β1 [59] or β3 integrins [66-68]. We defined a novel signaling axis comprised of αvβ3 integrin:Src:FAK:p130Cas:TβR-II:Grb2 that is critical for TGF-β stimulation of p38 MAPK, EMT, and pulmonary metastasis of breast cancer cells [66-70]. In addition, this signaling axis also confers oncogenic activity to EGF, including its ability to facilitate p38 MAPK activation and metastatic progression in post-EMT populations of breast cancer cells [17]. Recently, an alternative mechanism operant in activating JNK and p38 MAPK by TGF-β has been elucidated. Indeed, following their activation by cytokine, TGF-β receptors interact physically with TRAF6 (TNF receptor-associated factor 6), thereby enabling this E3 ligase to ubiquitinate and activate TAK1 and its eventual stimulation of JNK and p38 MAPK [71, 72]. Importantly, depleting
MECs of TRAF6 expression fails to affect canonical TGF-β signaling; however, this same cellular condition prevents TGF-β from activating JNK and p38 MAPK, as well as from stimulating EMT [71, 72]. Collectively, these studies highlight the importance of MAP kinases in mediating the acquisition of oncogenic signaling by TGF-β, leading to the hypothesis that chemotherapeutic targeting of MAP kinase pathways may reinstate the cytostatic function of TGF-β by normalizing the inherent balance between its canonical and noncanonical signaling systems.

1.3.2.2 PI3K, AKT, and mTOR

Oncogenic TGF-β signaling is also associated with the activation of phosphoinositide-3-kinase (PI3K) and its downstream target, AKT/PKB, which collectively serve in enhancing breast cancer proliferation, survival, and motility [73]. In addition, activation of the PI3K:AKT signaling axis also enables TGF-β to induce EMT and metastatic progression in malignant MECs, doing so via either the direct coupling of TGF-β receptors to the PI3K machinery [74] or indirectly through the ability of TGF-β to transactivate the receptors for EGF [75] and PDGF [76]. In fact, dual activation of the receptors for TGF-β and EGF can produce a hyper-EMT response related to the stimulation of PI3K/AKT and ERK1/2. Interestingly, antagonizing PI3K/AKT activity pharmacologically has no effect on the morphological features of EMT, but is sufficient to prevent elevated cell motility and invasion associated with EMT phenotypes [77]. These findings suggest that the morphologic and motile phenotypes of EMT may in fact be distinct physiological entities, each coupled to unique branches of the noncanonical TGF-β signaling system. Accordingly, inactivating mTOR (mammalian target of rapamycin) fails to alter the morphological features of EMT stimulated by TGF-β, but
prevents its ability to induce the invasion of post-EMT MECs [78]. The complexities of connecting PI3K:AKT signaling to the oncogenic activities of TGF-β are further highlighted by the ability of AKT to interact physically with Smad3 and prevent its nuclear translocation in response to TGF-β, thereby diminishing the cytostatic activities of TGF-β by enhancing cell survival [79, 80]. Finally, recent studies have established AKT2 as an essential mediator of EMT stimulated by TGF-β [81, 82]. For instance, heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1) strongly binds to structural elements in the 3’-UTRs of Dab2 and interleukin-like EMT inducer (ILEI) transcripts, thereby repressing their translation and subsequent induction of EMT in polarized MECs. In response to TGF-β, AKT2 readily phosphorylates and inactivates hnRNP E1, leading to its release from Dab2 and ILEI mRNA and the initiation of EMT and metastatic progression in MECs [81, 82]. Thus, the ability of TGF-β to regulate the translation and elongation of transcripts associated with the EMT process may represent a unique clinical target to alleviate breast cancer metastasis by overriding noncanonical TGF-β signaling inputs coupled to PI3K:AKT:mTOR activation.

1.3.2.3 Nuclear Factor-κB/Cox2/Prostaglandins

NF-κB is an essential mediator of inflammation associated with the growth, survival, invasion and angiogenesis of developing neoplasms [83]. TGF-β typically represses NF-κB activity in normal cells by inducing the expression of IκBα [84, 85], or by preventing the degradation of IκBα via the formation of TβR-III:β-arrestin2 complexes [86]. In stark contrast, mammary tumorigenesis paradoxically converts TGF-β from an inhibitor to a stimulator of NF-κB activity. In doing so, TGF-β acquires the ability to form TβR-
I:xIAP:TAB1:TAK1:IKKβ complexes in both normal and malignant MECs [87-89]. Uncoupling TGF-β from NF-κB activation dramatically inhibits (i) mammary tumor development in mice in part via regulation of the innate immune system [88], and (ii) the acquisition of EMT and invasive phenotypes in normal and malignant MECs [87-90]. Along these lines, the coupling of TGF-β to NF-κB facilitates Ras-transformed breast cancer cells to undergo EMT and colonize the lung [91], as well as promotes the formation of an autocrine Cox-2:PGE2:EP2 signaling cascade essential for metastatic progression and EMT induced by TGF-β [87, 92]. Future studies need to assess the relative contribution of NF-κB in the acquisition of chemoresistant phenotypes displayed by late-stage mammary tumors, particularly those subjected to the oncogenic activities of TGF-β.

1.3.2.4 Rho-family GTPases

Recent studies have implicated members of the Rho GTPase family (i.e., RhoA/B/C, Rac1, and Cdc42) in mediating the initiation of oncogenic TGF-β signaling [14, 93, 94]. Indeed, these small plasma membrane-associated GTP-binding proteins collaborate with integrins and other receptor systems to regulate a wide array of cellular functions, including alterations in cell adhesion, morphology, and motility reflecting the generation of filopodia (e.g., Cdc42), lamellipodia (e.g., Rac1), and actin stress fibers (e.g., RhoA) [95, 96]. Thus, these findings implicate Rho-family GTPases as potential participants in all stages of the metastatic cascade. Accordingly, TGF-β stimulation of EMT requires RhoA activation, as well as that of its downstream effector, p160ROCK [97]. Additionally, the phosphorylation of Par6 by TβR-II results in the ubiquitination and
degradation of RhoA [98, 99], presumably leading to upregulated Snail expression that promotes the E-cadherin deficiencies associated with EMT phenotypes [100]. On a similar note, the ability of TGF-β to suppress RhoC expression also elicits the dissolution of junctional complexes by inducing the proteosomal degradation of E-cadherin [100]. More recently, TGF-β has been observed to induce the phosphorylation of CdGAP, a RhoGAP that only targets Rac1 and Cdc42, leading to elevated cell motility and invasion in ErbB2-positive breast cancers [101]. Independent of its ability to bind ligand, TβR-III bound to β-arrestin2 elicits constitutive activation of Cdc42, which inhibits directional migration of ovarian and breast cancer cells [102]. Finally, RhoA expression is differentially regulated by TGF-β-responsive microRNAs (miRs), such that TGF-β stimulation of miR-155 expression promotes EMT by degrading RhoA [103], while that of miR-31 suppresses breast cancer metastasis in part by reducing RhoA expression [104, 105]. Taken together, these findings highlight the functional complexities associated with the expression and activation of Rho-family GTPases during metastatic progression stimulated by TGF-β.

1.3.2.5 Integrins and nonreceptor protein tyrosine kinases

Communication within cell microenvironments is controlled in part by integrins, which govern cell adhesion, migration, and invasion, as well as cell proliferation and survival [106, 107]. Cells undergoing neoplastic transformation exhibit dramatically altered integrin expression profiles, as well as altered integrin affinities for ECM substrates, both of which enhance cancer cell invasion and metastasis [108]. Along these lines, TGF-β
induces the expression of $\alpha_3\beta_1$ and $\alpha_\nu\beta_3$ integrins, which confer migration and invasion properties to MECs [59, 66-68]. Mechanistically, upregulation of $\beta_3$ integrin by TGF-\(\beta\) results in the FAK-dependent formation of $\beta_3$ integrin:T$\beta$R-II complexes that promote the activation of Src and its phosphorylation of T$\beta$R-II at Y284 [67, 70]. Upon its phosphorylation, Y284 coordinates the recruitment and binding of the SH2-domain proteins, Grb2 and ShcA, which promote p38 MAPK activation and the initiation of EMT [66-68]. Furthermore, the FAK effector, p130Cas binds to Smad3 and prevents its phosphorylation which shifts the balance between canonical and noncanonical TGF-\(\beta\) signaling during mammary tumorigenesis [69]. Importantly, measures that disrupt this oncogenic TGF-\(\beta\) signaling axis completely abrogate the ability of TGF-\(\beta\) to induce EMT, and to promote the metastasis [68, 70, 109, 110].

Additionally, we have recently identified the non-receptor protein tyrosine kinase, c-Abl as a potent suppressor of EMT stimulated by TGF-\(\beta\) [111], which functions to reivate p53 expression and its induction of p21 (T.M.A. and W.P.S. unpublished observation).
Transmembrane signaling by TGF-β is stimulated upon its binding and activation of the Ser/Thr protein kinase receptors, TβR-I and TβR-II. The physical interaction of TGF-β with either TβR-III or TβR-II facilitates the recruitment and transphosphorylation of TβR-I, resulting in its activation and subsequent phosphorylation of the receptor-activated Smads, Smad2 and Smad3. Once activated, Smad2/3 form heterocomplexes with Smad4 and translocate to the nucleus to regulate the expression of TGF-β-responsive genes in concert with an ever expanding list of transcriptional coactivators and repressors. This branch of the bifurcated TGF-β signaling system represents the “canonical” or “Smad2/3-dependent” TGF-β pathway, which is the predominant pathway coupled to cytostasis and activated by TGF-β in normal MECs (left panel). Alternatively, TGF-β also activates a variety of “noncanonical” or “Smad2/3-independent” effectors, including Par6, NF-κB, ILK, FAK, Src, Rho-family GTPases, MAP kinases, and the PI3K:AKT:mTOR signaling axes (right panel). During EMT and mammary tumorigenesis, the balance between canonical and noncanonical TGF-β signaling systems becomes distorted and favors coupling to noncanonical effector systems, an event that manifests the “TGF-β Paradox” and the initiation of EMT, invasion, and metastasis. These events are further exacerbated by elevated deposition and eventual crosslinking of ECM molecules within tumor microenvironments (LOX, right panel), an event that promotes tumor rigidity and the activation of mechanotransduction pathways operant in amplifying noncanonical TGF-β signaling and its oncogenic activities in mammary tumors. See text for additional details. Reproduced from [3].
Figure 1.1

 Canonical
 EMT, Invasion, Metastasis
 Non-Canonical

 Nuclear
 Compliant

 Loss of TGF-βcytostasis
 Acquire EMT/Stem-like/phenotypes
 Partial TGF-βcytostasis
 EMT/stemness/disease recurrence

 Rigid
1.4. TGF-β and the Tumor Microenvironment

Tissue homeostasis in the breast is maintained by the balanced integration of signaling inputs derived from various tissue and cell architectures, and from their supporting microenvironment and ECM. Indeed, whereas normal mammary tissue specification requires reciprocal signaling inputs from distinct cell types and matrix proteins, the phenotype of developing mammary carcinomas is similarly dictated by the dynamic interplay between malignant cells and their accompanying stroma, which houses fibroblasts, endothelial cells, and a variety of infiltrating immune and progenitor cells [11, 112, 113]. Indeed, in addition to the TGF-β-mediated regulation of carcinoma cell autonomous responses detailed above, TGF-β regulates (i) angiogenesis by endothelial cells; (ii) immunosurveillance by infiltrating immune cells; and (iii) activation of cancer-associated fibroblasts (summarized in Figure 1.2 and reviewed in [114]). In addition to these activities, emerging evidence indicates that TGF-β also regulates the tumor microenvironment by influencing extracellular matrix rigidity.
Figure 1.2: TGF-β is a master regulator of MEC plasticity and microenvironmental homeostasis.

TGF-β induces malignant MECs to undergo EMT, leading to the acquisition of highly migratory, invasive, and metastatic phenotypes. TGF-β is also a potent inducer of tumor angiogenesis, which significantly enhances the growth and metastasis of late-stage mammary tumors. Through its ability to inhibit host immunosurveillance, TGF-β also plays an essential role in conferring immune privilege to developing and progressing breast cancers. Finally, TGF-β stimulates fibroblasts to synthesize and secret a variety of growth factors, cytokines, and ECM molecules that collectively create a tumor promoting microenvironment. Reproduced from [114].
1.4.1 TGF-β and ECM rigidity

Many cancers are characterized by altered tensional homeostasis. Indeed, the elastic modulus of tumor stroma is approximately 10-times more mechanically rigid than that of adjacent normal breast tissues and breast tumors are often detected by a palpable increase in stiffness [115, 116]. The formation of these rigid mammary tumor microenvironments promotes metastatic progression, as well as predicts for poor outcomes [117-120]. TGF-β potentiates these biomechanical reactions by stimulating the expression and secretion of a variety of ECM components and matrix crosslinking enzymes, which contribute to desmoplasia and fibrosis within the tumor microenvironment as detailed in the succeeding sections [3, 7, 121].

1.4.2 Fibronectin

TGF-β has long been recognized as a principle player operant in stimulating fibronectin expression and incorporation into the ECM [122]. Importantly, elevated fibronectin expression enables dormant MEC micrometastases to reinitiate cell proliferation in a β1 integrin-dependent manner [123]. Along these lines, upregulated fibronectin expression in conjunction with that of activated Ras significantly alters MEC integrin expression profiles such that epithelial α6β4 integrins are replaced by mesenchymal α5β1 integrins. Collectively, these events enhance TGF-β stimulation of EMT and its induction of MEC motility and survival signaling [124]. Interestingly, the ability of TGF-β to bestow anchorage-independent properties to responsive cells can be recapitulated by fibronectin administration, a reaction dependent upon integrin signaling [122].
production is also associated with the formation of the “premetastatic niche,” which recruits bone marrow-derived cells (BMDCs) to provide a permissive metastatic microenvironment for the arrival of disseminated breast cancer cells [120, 125]. Finally, a recent study established the importance of TGF-β secreted by mammary tumors to assemble a fibronectin matrix that is three times more mechanically rigid than matrices assembled by normal MECs [126]. Collectively, these findings implicate fibronectin as an essential mediator of metastatic progression stimulated by TGF-β, particularly its ability to respond to signaling inputs derived from integrins and mechanotransduction.

1.4.3 Collagen

In addition to its stimulation of fibronectin expression, TGF-β also induces the expression of collagen [122], whose elevated expression in mammary tumors associates with increased tumor occurrence and metastasis, as well as with poor clinical outcomes [127, 128]. Increased collagen deposition also promotes ECM rigidity, which contributes greatly to the development and progression of mammary tumors [115, 129]. Interestingly, TGF-β stimulates basal-like breast cancer cells to express Endo180, which is a cell surface receptor that promotes the (i) growth and motility of highly invasive breast cancer cells, and (ii) internalization and intracellular degradation of collagen [130]. Recently, collagen was observed to activate TGF-β receptors in a ligand-independent manner, leading to the dual stimulation of canonical and noncanonical TGF-β effectors in breast cancer cells [131]. Collectively, these findings highlight the intimate relationship between collagen and TGF-β in promoting disease progression in breast cancer patients, and suggest that measures capable of neutralizing upregulated collagen expression and
activity may offer new inroads to alleviate oncogenic TGF-β signaling.

1.4.4 Lysyl Oxidase (LOX) Family

Studies by our group (Chapter 2) and others [132-135] indicate that TGF-β upregulates the expression of the lysyl oxidase (LOX) family of extracellular matrix cross-linking enzymes and that increased LOX activity enhances tumor progression. LOX is the prototypic member of a five member gene family of copper-dependent amine oxidases (LOX, LOXL1, LOXL2, LOXL3, and LOXL4), all of which share a conserved catalytic domain (Figure 1.3 A) and primarily function in cross-linking collagen and elastin in the ECM. LOX is synthesized as a 48kDa preproprotein, which is N-glycosylated and secreted as a catalytically inactive 50kDa proenzyme, which is then cleaved into its catalytic 32kDa form by BMP-1 (procollagen C-proteinase) [136]. Whether or not other LOXL enzymes undergo similar cleavage and activation steps in vivo has not been well characterized [137]. The enzymatic activity of LOX and its family members ensues through oxidative deamination of substrate amines present in lysine residues, yielding an aldehyde product. Molecular oxygen then reduces the enzyme back to a catalytically active state, generating ammonium and hydrogen peroxide as biproducts [138] (Figure 1.3 B). This catalytic process is dependent on two cofactors: (i) a copper ion (Cu^{2+}) and (ii) a covalently integrated organic cofactor, lysyl tyrosyl quionone (LTQ) [136]. Although the most well characterized substrates for LOX are the lysine residues in collagen and elastin, LOX has been shown to readily oxidize histones H1 and H2, as well as bFGF, indicting that the enzymatic specificity is not limited to ECM proteins and that LOX my play a novel role in intracellular signaling [139].
In normal tissues, the enzymatic cross-linking activities of LOX family members serve to enhance tensile strength and structural integrity of connective tissue [136, 139]. Indeed, LOX family member activity is required for normal connective tissue function as evidenced by the fact that LOX knockout mice die parentally due to structural cardiovascular and diaphragm instability [140, 141], while LOXL1 knockout mice develop severe defects in elastic fiber deposition in the uterine tract, enlarged airspaces in the lungs, loose skin, and vascular abnormalities [142]. However, in diseased tissues, aberrant upregulation of LOX family members has been associated with tumorigenesis and metastatic progression in numerous cancers, including those of the breast [143]. Indeed, elevation of both LOX and LOXL2 is associated with more aggressive ER-negative cancers, and with the increased metastatic burden and poor survival in breast cancer patients [119, 144]. Moreover, hypoxia in breast tumors is known to drive LOX expression through a HIF1α-dependent mechanism [119]. In addition to this HIF1α-dependent mechanism of transcriptional upregulation, emerging data indicate that TGF-β drives LOX upregulation through Smad3, Akt, and MAPK-dependent mechanisms [134]. Likewise, the formation of premetastatic niches has been linked to LOX and its ability to stimulate collagen cross-linking and fibronectin production, which coalesce to recruit BMDCs to future sites of metastasis [118]. TGF-β also induces BMDC recruitment to premetastatic niches [145], suggesting a clinically important link between TGF-β and its stimulation of LOX expression in mediating the establishment of premetastatic niches.

Current evidence indicates that the enzymatic activity of LOX likely couples to non-canonical TGF-β signaling and its promotion of tumor progression through two distinct mechanisms (Chapter 2). First, the cross-linking activity of LOX leads to
increased ECM tension and rigidity in developing mammary tumors, which enhances integrin-mediated mechanotransduction coupled to the induction of breast cancer invasion and metastasis [115, 129]. Importantly, we identified an integrin-mediated oncogenic TGF-β signaling axis comprised of αvβ3 integrin, FAK, and Src that induces mammary tumor growth, invasion, and metastasis in mice [66-68, 70], suggesting that decreasing LOX ECM cross-linking activity may modulate integrin coupling to TGF-β signaling. Second, the hydrogen peroxide produced as a byproduct of LOX activity has been shown to elicit the phosphorylation and activation of FAK and Src, thereby promoting Rac1 activation through the assembly of p130Cas/Crk/Dock180 complexes and increase cell motility [146]. As activation of FAK and Src signaling pathways also lead to oncogenic signaling by TGF-β in part via the activation of p38 MAPK, hydrogen peroxide may further drive this non-canonical arm of the TGF-β signaling pathway (Figure 1.4).
Figure 1.3: Structure and Chemical Function of Lysyl Oxidase.

(A) The lysyl oxidase (LOX) family of proteins contain a conserved C-terminal domain that houses catalytic activity and contains a copper-binding motif (Cu) and a lysyl-tyrosyl-quinone (LTQ) cofactor. The N-terminal domain of both LOX and LOXL1 contain a pro sequence that allows for their secretion as inactive proenzymes, and for their interactions with the ECM [147]. LOXL2, LOXL3, and LOXL4 contain four scavenger receptor cysteine-rich (SRCR) domains, which have not been well characterized in these enzymes, but are thought to be involved in cell adhesion and protein-protein interactions [143]. (B) The stoichiometry of the LOX-catalyzed enzymatic reaction whereby LOX oxidatively deaminates a substrate amine to an aldehyde product, resulting in ammonium and hydrogen peroxide byproducts.
Figure 1.3

A

N | S
---|---
Pro-sequence | Pro-sequence
Cu | Cu
Catalytic Domain | Catalytic Domain

LOX
LOXL1
LOXL2
LOXL3
LOXL4

B

\[
\text{Peptidyl Lysine} + O_2 + H_2O \xrightarrow{\text{LOX}} \text{Peptidyl Aldehyde} + \text{NH}_4^+ + \text{H}_2\text{O}_2
\]
Figure 1.4: Coupling of oncogenic TGF-β signaling to LOX.

TGF-β upregulates LOX expression through incompletely understood mechanisms that are likely to involve both canonical and noncanonical signaling axes [134]. Upregulation of LOX reciprocally enhances noncanonical TGF-β-signaling by two distinct mechanisms (1) enhancing ECM stiffness through increased collagen and elastin cross-linking, thereby enhancing integrin signaling that couples non-canonical TGF-β signaling and its enhanced activation of p38 MAPK, and (2) generating H$_2$O$_2$ produced as a byproduct of LOX-mediated crosslinking of collagen and elastin, thereby enhancing the activation of FAK, Src, and p38 MAPK.
Figure 1.4
1.5 microRNA regulation by TGF-β

microRNAs are small (20 to 25 base pairs in length), noncoding RNAs that post-translationally regulate gene expression. microRNAs are transcribed in the nucleus by RNA polymerase II or III into primary transcripts (pri-miRNA), which are then cleaved by the Drosha-DGCR8 microprocessor complex into smaller precursor (pre-miRNA) structures and exported from the nucleus by exportin 5. Once in the cytoplasm, Dicer/TRBP binds to and cleaves the pre-miRNA to its mature length. The functional microRNA strand is then loaded together with Argonaute (Ago2) into the RNA-induced silencing complex (RISC), where it binds to the 3’UTR of target mRNAs and induces cleavage, translational repression or deadenylation, depending on sequence complementarity [148, 149]. Interestingly, the majority of microRNAs identified to date localize to fragile genomic regions associated with cancer [150], leading to the notion that microRNA-based expression signatures may be developed as diagnostic platforms for cancer patients. Accordingly, microRNA expression profiling studies have demonstrated the ability of microRNA signatures to readily distinguish normal tissues from their tumorigenic counterparts, as well as to stage and classify human mammary tumors [151-153]. Along these lines, differential microRNA expression can function in either suppressing or promoting mammary tumorigenesis, and in governing specific steps of the metastatic cascade, including the induction of MEC migration, invasion, and EMT [154-157]. Finally, loss of Dicer function has also been shown to promote metastasis [158]. The pathophysiological processes regulated by microRNAs are highly reminiscent of those controlled by TGF-β, and as such, it is not surprising to learn that microRNAs are active participants in regulating MEC response to TGF-β. For instance, TGF-β
stimulation of EMT down regulates the expression of the miR-200 family of microRNAs, which normally suppresses the expression of the EMT-responsive transcription factors, ZEB1 and ZEB2/SIP1 [159]. Consequently, elevated ZEB1 and ZEB2 expression initiate EMT in part by repressing E-cadherin expression in breast cancer cells [159]. EMT induced by TGF-β has also been linked to its stimulation of miR-21 expression [160], which enhances the migration and invasion of breast cancers by downregulating the expression of tropomyosin [156, 161]. Clinically, high miR-21 expression in early-stage breast cancers is associated with decreased disease-free survival, as well as with significantly elevated expression of TGF-β [162]. Recently, a novel mechanism involving direct association of Smad proteins with an SBE-like sequence in the stem region of a select group of microRNA precursors was shown to enhance Drosha processing upon TGF-β or BMP stimulation [163, 164]. Finally, canonical TGF-β signaling regulates the differential expression of 28 microRNAs in MECs undergoing EMT in response to TGF-β. Of these EMT-related microRNAs, the expression of miR-155 is essential in mediating MEC motility and tight junction dissolution due to a loss of RhoA expression in transitioning MECs [103]. Collectively, these studies demonstrate the role of TGF-β in directing microRNA expression in normal and malignant MECs, doing so via employment of intricate transcriptional and post-translational mechanisms.

**1.5.1 TGF-β-mediated upregulation of miR-181a**

miR-181a belongs to the miR-181 family, which contains four members (miR-181a, miR-181b, miR-181c, and miR-181d) that all contain the same seed sequence (5’ bases 2-7) thought to be responsible for mRNA translational repression or degradation (Figure
miR-181a is expressed most abundantly in the thymus, where it plays important roles in immune cell maturation. Indeed, overexpression of miR-181a in hematopoietic progenitor cells leads to an increase in mature B cell lineages [165]. Additionally, miR-181a is the most highly expressed microRNA in T-cells, where it modulates not only T-cell sensitivity and selection, but also promotes T-cell receptor-mediated activation by regulating phosphatase expression levels [166]. Given its role in haematopoiesis, it is not surprising that increased miR-181a expression has been associated with Acute Myeloid Leukemia, Non-Hodgkins lymphoma, and Multiple Myeloma [167, 168]. Paradoxically, decreased expression of miR-181a has been associated with Chronic Myelogenous Leukemia and Chronic lymphocytic leukemia [169]. Accordingly, recent evidence indicates that miR-181a expression also exhibits seemingly paradoxical roles in solid tumors. For example, miR-181a expression is decreased in several cancers, including those of the lung and brain [169]. Indeed, in glioblastomas increased miR-181a expression functions as a tumor suppressor and sensitizes cells to radiotherapy [170]. In stark contrast, aberrantly elevated expression of miR-181a is observed in cancers of the breast [171], mouth [172], liver [173], suggesting that miR-181a fulfills a tumor promoting role in these contexts. More research is needed to parse apart the context specific through which miR-181a either suppresses or promotes tumorigenesis. In Chapter 3, we provide evidence that TGF-β is a master regulator of the miR-181 family of microRNAs and that TGF-β-mediated upregulation of miR-181a promotes the acquisition of epithelial-mesenchymal transition, migratory, and invasive phenotypes leading to breast cancer metastasis.
Figure 1.5: miR-181 Family

The miR-181 family of microRNAs contains four members, miR-181a, miR-181b, miR-181c, and miR-181d all of which contain the same seed sequence (5’ bases 2-7).
Figure 1.5

5’ AACAUUCAACGCUGUCGGUGAGU 3’  miR-181a

5’ AACAUUCAUUGCUGUCGGUGGGU 3’  miR-181b

5’ AACAUUCAACCGUUGUCGGUGAGU3’  miR-181c

5’ AACAUUCAUUGUGUUGUCGGUGGGU 3’  miR-181d
1.6 Statement of Purpose

The studies herein aim to address the role that the increased biomechanical stiffening observed in the breast tumor microenvironment plays on altered TGF-β signaling in breast cancer.

First, we sought to define the role that the ECM crosslinking enzyme, LOX has on TGF-β-mediated breast cancer progression. Briefly, we found that TGF-β-mediated EMT induced the expression and secretion of LOX from normal and malignant MECs. Additionally, LOX expression in tumors correlated with the ability of TGF-β to stimulate mammary tumor growth and pulmonary metastasis. Moreover, elevating ECM rigidity enhanced TGF-β-mediated proliferation, while exposing late-stage breast cancer cells to compliant ECM signals reinstated the cytostatic activities of TGF-β (Chapter 2).

Next, we investigated the effect of increased biomechanical stiffening on the microRNA expression profiles induced by TGF-β. Utilizing 3D organotypic cultures to recapitulate the rigidity of in vivo tumor microenvironments, we performed global profiling analyses to identify microRNAs whose expression was induced by TGF-β. In doing so, we found that ECM rigidity induces differential microRNA expression by TGF-β. Moreover, we identified miR-181a as a TGF-β-regulated microRNA that is highly expressed in metastatic cells as compared to their non-metastatic counterparts. Inhibiting miR-181a activity in vitro attenuated the ability of TGF-β to induce EMT, motility and invasion. In vivo bioluminescent imaging indicated that miR-181a inhibition abrogated the pulmonary outgrowth of metastatic BC cells inoculated into the lateral tail vein of mice, and increased overall survival time. Importantly, examination of microRNA
profiles in human breast tumors demonstrated that high miR-181a expression predicts for decreased survival of human BC patients (Chapter 3).

Collectively, our findings show the importance of the ECM in regulating MEC response to TGF-β and implicate LOX and miR-181a as novel diagnostic markers for metastatic progression, as well as innovative therapeutic targets to treat metastatic breast cancers.
Chapter 2

Lysyl Oxidase Contributes to Mechanotransduction-Mediated Regulation of Transforming Growth Factor-β Signaling in Breast Cancer Cells.

2.1 Abstract

Transforming growth factor-β (TGF-β) regulates all stages of mammary gland development, including the maintenance of tissue homeostasis and the suppression of tumorigenesis in mammary epithelial cells (MECs). Interestingly, mammary tumorigenesis converts TGF-β from a tumor suppressor to a tumor promoter through molecular mechanisms that remain incompletely understood. Changes in integrin signaling and tissue compliance promote the acquisition of malignant phenotypes in MECs in part through the activity of lysyl oxidase (LOX), which regulates desmoplastic reactions and metastasis. TGF-β also regulates the activities of tumor reactive stroma and MEC metastasis. We show here that TGF-β1 stimulated the synthesis and secretion of LOX from normal and malignant MECs in vitro and in mammary tumors produced in mice. The ability of TGF-β1 to activate Smad2/3 was unaffected by LOX inactivation in normal MECs, whereas the stimulation of p38 MAPK by TGF-β1 was blunted by inhibiting LOX activity in malignant MECs or by inducing the degradation of hydrogen peroxide in both cell types. Inactivating LOX activity impaired TGF-β1-mediated epithelial-mesenchymal transition and invasion in breast cancer cells. We further show

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that increasing extracellular matrix rigidity by the addition of type I collagen to three-dimensional organotypic cultures promoted the proliferation of malignant MECs, a cellular reaction that was abrogated by inhibiting the activities of TGF-β 1 or LOX, and by degrading hydrogen peroxide. Our findings identify LOX as a potential mediator that couples mechanotransduction to oncogenic signaling by TGF-β 1 and suggest that measures capable of inactivating LOX function may prove effective in diminishing breast cancer progression stimulated by TGF-β 1.

2.2 Introduction

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates mammary gland development, as well as suppresses mammary tumorigenesis [8, 174]. In normal mammary epithelial cells (MECs), TGF-β acts as a tumor suppressor by inducing apoptosis and cell cycle arrest and by stimulating cellular differentiation. However, during breast cancer progression, TGF-β deviates from its role as a tumor suppressor to ultimately acquire tumor promoting functions, including the ability to induce breast cancer cell proliferation, invasion, and metastasis in part through the stimulation of epithelial-mesenchymal transition (EMT) [58, 70]. The molecular mechanisms that engender this switch in TGF-β function during tumorogenesis are not well defined. Signaling through Smad2/3 generally is associated with cytostasis and maintenance of normal epithelial homeostasis. However, TGF-β also signals through non-Smad-mediated pathways, such as ERK1/2 (extracellular-regulated protein kinase1/2), p38 MAPK (p38 mitogen-activated protein kinase), JNK (c-Jun N-terminal protein kinase), PI3K (phosphoinositide-3-kinase), NF-κB (nuclear factor-κB), and Akt [8, 58, 175].
Indeed, we recently showed that αvβ3 integrin interacts with the TGF-β type II receptor (TβR-II), leading to its phosphorylation by Src at Tyr284 and subsequent activation of p38 MAPK [66-68]. Collectively, these events enable TGF-β to stimulate breast cancer growth, invasion, and metastasis. In fact, studies by our group [66-70] and others [175] support the notion that inappropriate imbalances between canonical (i.e., Smad2/3–dependent) versus noncanonical (i.e., Smad2/3–independent) TGF-β pathways underlie its acquisition of oncogenic function during tumor progression.

Lysyl oxidase (LOX) is a copper-dependent amine oxidase that catalyzes the cross-linking of collagens and elastin in the extracellular matrix (ECM). LOX belongs to a five member gene family consisting of LOX, LOX-like 1 (LOXL1), LOXL2, LOXL3, and LOXL4, all of which play important roles in regulating ECM remodeling and cellular homeostasis [136, 139]. In addition, elevated LOX activity is associated with the acquisition of increased ECM tension and stiffness in developing mammary tumors, a reaction that enhances integrin-mediated mechanotransduction coupled to increased breast cancer cell invasion and dissemination from hypoxic primary tumors [115, 118, 119, 129, 176, 177]. Clinically, the aberrant expression of LOX, LOXL, and LOXL2 correlates with increased malignancy and invasiveness in human tumors, including those of the breast [119, 177, 178]. Along these lines, hydrogen peroxide produced as a byproduct of LOX-mediated collagen and elastin cross-linking stimulates Rac1 activity by promoting the assembly of p130Cas/Crk/Dock180 complexes [146]. Thus, LOX seems critical in governing the transition of tumors from indolent to aggressive disease states through the combined actions of its ECM cross-linking activities, and its production of the potential second messenger, hydrogen peroxide. Thus,
chemotherapeutic targeting of LOX may one day improve the clinical course of metastatic breast cancer patients.

Given the parallels between LOX and TGF-β in regulating ECM dynamics and promoting mammary tumorigenesis, we sought to determine the role of LOX in regulating oncogenic TGF-β1 signaling and its coupling to EMT in normal and malignant MECs.

2.3 Materials and Methods

Cell Lines and Lentiviral Vectors

Normal murine mammary gland epithelial cell (NMuMG) and metastatic 4T1 cells were cultured as previously described [111], as were human MCF10ACA1a breast cancer cells [52] and human 293T embryonic kidney cells [179]. Lentiviral particles encoding for scrambled (i.e., nonsilencing shRNA) or murine LOX shRNA (Thermo Scientific, Huntsville, AL) were prepared as described previously [111]. The extent of LOX deficiency was monitored by immunoblot analysis using anti-LOX antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). A constitutively active LOX construct, LOX-32 [180], was synthesized by polymerase chain reaction (PCR), amplifying mature and catalytically active human LOX (nucleotides 505-1251) using oligonucleotides containing Hind III (N-terminus) and Xho I (C-terminus) restriction sites. The resulting PCR product was ligated into corresponding sites in the pSecTag B vector (Invitrogen, Carlsbad, CA), which C-terminally tagged the LOX-32 complementary DNA (cDNA) with Myc- and (His)6-tags and appended the Ig κ leader sequence to its N-terminus. The
resulting LOX-32 cDNA was sequenced in its entirety on a DNA sequencing machine (3730; Applied Biosystems, Carlsbad, CA).

**Immunoblot Analyses**

LOX activity was inhibited by pretreating MECs with the irreversible LOX inhibitor, β-aminopropionitrile (βAPN; 300 μM), or with the hydrogen peroxide metabolizer, catalase (400 U/ml). Quiescent NMuMG or 4T1 cells were incubated for varying times in the absence or presence of TGF-β1 (5 ng/ml; R&D Systems, Minneapolis, MN) and, subsequently, were lysed and solubilized on ice in buffer H/Triton X-100 [181]. Clarified whole-cell extracts and conditioned medium collected from these cells before their lysis was resolved through 10% SDS-PAGE gels, transferred electrophoretically to nitrocellulose membranes, and blocked in 5% milk before incubating with the following primary antibodies (dilutions): 1) LOX (1:200; Santa Cruz Biotechnology), 2) E-cadherin (1:500; BD Biosciences, San Jose, CA), 3) phospho-Smad2 (1:1000; Cell Signaling, Danvers, MA), and 4) phospho-p38 MAPK (1:500; Cell Signaling). The resulting immunocomplexes were visualized by enhanced chemi-luminescence, and differences in protein loading were monitored by reprobing stripped membranes with anti-β-actin (1:1000; Santa Cruz Biotechnology).

**Cell Biological Assays**

The effect of antagonizing LOX activity on various TGF-β1–stimulated activities in NMuMG, 4T1, or MCF10ACa1a cells was determined as follows: 1) cell proliferation in three-dimensional organotypic cultures using either ImageJ quantitation or CellTiter 96
AQueous One Solution Cell Proliferation Assay (MTS) according to the manufacturer’s recommendations (Promega, Madison, WI), 2) cell invasion induced by 2% serum using 25,000 cells/well in a modified Boyden chamber coated with Matrigel (1:25 dilution) as described [182], 3) gene expression using 30,000 cells/well in a synthetic p3TP-luciferase reporter gene assay as described [182], and 4) p38 MAPK phosphorylation induced by expression of a constitutively active MKK6 as described [183]. In addition, the ability of TGF-β1 stimulation and LOX inhibition to alter the actin cytoskeleton was monitored using direct TRITC-phalloidin immunofluorescence as described [184]. In some cases, the cells were pretreated for 30 minutes with either β APN (300 μM) or catalase (400 U/ml) before addition of TGF-β1 (5 ng/ml) for 0 to 48 hours at 37°C. Lastly, total RNA was isolated from NMuMG and 4T1 cells using the RNeasy Plus Mini Kit according the manufacturer’s recommendations (Qiagen, Valencia, CA). Afterward, cDNA were synthesized using iScript cDNA Synthesis System (BioRad, Hercules, CA), and semiquantitative real-time PCR was conducted using iQ SYBR Green (BioRad) as described [87]. In all cases, differences in RNA concentration were controlled by normalizing individual gene signals to their corresponding GAPDH RNA signals. The oligonucleotide primer pairs used were as follows: 1) LOX forward 5′ - TGCCAGTGGATTGATATTACAGATGT and reverse 5′ - AGCGAATGTCA-CAGCGTACAA; 2) E-cadherin forward 5′ - CCCTACATACACT- CTGGTGGTTCA and reverse 5′ - GGCATCATCATCGGTCACTTTG; 3) N-cadherin forward 5′ - CCCCCCAAGTCCAACATTTTC and reverse 5′ - CGCCG TTTCATCCATACCCAC; 4) cytokeratin 19 forward 5′-TTGGGTCAGGGGGTGT TTTTC and reverse 5′ TT-
CTCATTGCCAGACAGCAGC; 5) vimentin forward 5’ -CAAGTC
CCAAGTTTGCTGACCTCTC and reverse 5’ -CTCTCCATCT- CACGCATCTGG;
6) fibronectin III forward 5’ -ACAACACCCCCAA GGAGAAG and reverse 5’ -
GCATCCTCTCTTTCTGGTTCTG; and 7) GAPDH forward 5’ -
CAACTTTGGCATTGTGGAAGGGCTC and reverse 5’ -
GCAGGGATGATGTTCTGGGCAGC.

**LOX Immunohistochemistry**

Archival 4T1 tumors that expressed GFP, WT-TGF-β type II receptor (TβR-II), or Y284F–T β R-II [68] were sectioned for histopathologic analysis in the Pathology Core at the University of Colorado Cancer Center. Afterward, LOX immunohistochemistry was performed as described [68] using two independent preparations of anti-LOX antibodies (1:50; Santa Cruz Biotechnology or Payne et al. [180]). Negative staining controls in all experiments entailed the processing of adjacent samples in the absence of added primary anti-LOX antibodies.

**Three-dimensional Organotypic Cultures**

Three-dimensional organotypic cultures were performed using the “on-top” method as described [185]. Briefly, 4T1 cells were cultured in either 48-well plates or 8-well chamber slides on Cultrex cushions (100%; Trevigen, Gaithersburg, MD) in complete medium supplemented with 5% Cultrex. ECM rigidity in these organotypic cultures was increased by adding type I collagen (3 mg/ml; BD Biosciences) to Cultrex cushions before their solidification. Where indicated, the MECs were treated with TGF-β 1 (5
ng/ml), or the TGF-β type I receptor (Tβ R-I) antagonist, Tβ R-I Inhibitor II (Calbiochem, San Diego, CA). Cell growth and acinar formation were monitored by bright-field microscopy. Measuring the secretion of TGF-β1 from 4T1 acinar structures was accomplished using a TGF-β1 ELISA assay (ElisaTech, Aurora, CO) as described [186]. To monitor alterations in E-cadherin localization, 4T1 acinar structures were stained with E-cadherin antibodies (BD Biosciences). Briefly, 4T1 organoids were propagated for 5 days, at which point they were rinsed with PBS supplemented with CaCl2 (0.5 mM) and MgCl2 (0.9 mM) before fixation in 4% paraformaldehyde/PBS. Afterward, the organoids were permeabilized with 0.1% Triton X-100/PBS for 5 minutes, thoroughly washed with PBS, and, subsequently, were blocked in 1% BSA containing 5% goat serum for 1 hour at room temperature before overnight incubation with anti–E-cadherin antibodies (1:250 dilution) at room temperature. The next morning, the organoids were washed and incubated sequentially for 1 hour with biotin-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), followed by Texas Red streptavidin D (Vector, Burlingame, CA). Afterward, the stained organoids were mounted using ProLong Gold Antifade mounting medium that contained DAPI (4′,6-diamidino-2-phenylindole (Invitrogen), and the resulting images were captured on a Leica DM5000 microscope (40×; Leica Microsystems, Bannockburn, IL).

**Statistical Analysis**

Statistical values were defined using an unpaired Student’s t test, where P < .05 was considered significant.
2.4 Results

*TGF-β1 and EMT Stimulate the Expression and Secretion of LOX in Normal and Malignant MECs*

Normal polarized NMuMG cells undergo EMT in response to TGF-β1 and, in doing so, readily acquire a fibroblastoid-like morphology (Figure 2.1, A and B). Interestingly, this same EMT protocol resulted in the significant synthesis of LOX transcripts in post-EMT NMuMG cells compared with their unstimulated counterparts (Figure 2.1A, bottom panel). In addition, TGF-β1 also elevated the expression of LOXL1, LOXL2, and LOXL3 in NMuMG cells that underwent EMT and elevated LOXL1, LOXL3 and LOXL4 in 4T1 cells (Appendix Figure 1), suggesting that TGF-β1 functions as a general regulator of LOX family member expression in normal MECs. Because LOX clearly showed the highest induction by TGF-β1 and promotes breast cancer progression [118, 119, 139, 177, 180], we restricted our analyses solely to LOX for the remainder of the studies reported herein. The ability of TGF-β1 to stimulate EMT not only was restricted to NMuMG cells but also transpired in metastatic 4T1 cells (Figure 2.1B) [67, 69, 70, 111]. Moreover, we observed TGF-β1 to also stimulate the synthesis and secretion mature LOX (32 kDa) from normal (i.e., NMuMG) and metastatic (i.e., 4T1) MECs as measured by immunoblotting the conditioned medium and whole-cell extracts prepared from these cell types (Figure 2.1C). Collectively, these findings establish TGF-β1 as an inducer of LOX expression, secretion, and proteolytic processing (i.e., an inducer of LOX activation) in normal and malignant MECs. Our results also suggest that upregulated LOX expression may play a role in regulating the malignancy of MECs,
particularly their response to TGF-β 1.

**TGF-β 1 Regulates EMT in Normal MECs through a LOX- and Hydrogen Peroxide–Dependent Pathway**

We [14, 66-68, 70] and others [59, 61, 187-189] have shown the essential function of canonical (e.g., Smad2/3) and noncanonical (e.g., p38 MAPK) TGF-β 1 effectors in mediating its stimulation of EMT in normal and malignant MECs. To determine the impact of LOX on TGF-β 1–mediated activation of Smad2/3 and p38 MAPK, we first treated normal NMuMG cells with the irreversible competitive LOX inhibitor, β APN, which has been shown to specifically inhibit the catalytic activity of LOX [136, 139, 190] and several LOXL family members [136, 178]. In complementary approaches, we also administered catalase to these cells to degrade the LOX second messenger, hydrogen peroxide [177], or stably transduced them with shRNA against LOX to deplete its expression. Figure 2.2 shows that these experimental conditions failed to alter the transcriptional activity and phosphorylation of Smad2/3 stimulated by TGF-β 1, as did depleting LOX expression in these MECs by their transduction with shRNA against LOX (Figures 2.2, A–G, and Figure 2.8). Interestingly, catalase administration inhibited TGF-β 1 stimulation of p38 MAPK in NMuMG cells (Figure 2.2 C), thereby implicating hydrogen peroxide as a mediator of p38 MAPK activation in NMuMG cells. In stark contrast, we observed both β APN administration and LOX deficiency to elevate p38 MAPK phosphorylation in resting NMuMG cells (Figure 2.2, C and E). These findings suggest that disrupting LOX expression and activity may elicit a stress response in
normal MECs. Alternatively, these results may point toward the activities of other LOXL family members in mediating the ability of TGF-β 1 to stimulate p38 MAPK in LOX-deficient MECs.

In light of the above results and those linking TGF-β 1–driven EMT to elevated LOX expression (Figure 2.1), we also examined the role of LOX in mediating EMT stimulated by TGF-β 1. As such, administration of either β APN or catalase (Figure 2.3A) or transduction with shRNA against LOX (Figure 2.3B) attenuated the formation of stress fibers stimulated by TGF-β 1, suggesting that targeting LOX activity can diminish EMT stimulated by TGF-β 1. Accordingly, TGF-β 1 induced NMuMG cells to acquire an EMT phenotype that included attenuated expression of the epithelial markers cytokeratin 19 and E-cadherin and augmented expression of the mesenchymal cell markers, N-cadherin, vimentin, and fibronectin (Figure 2.3C). Pharmacological targeting of LOX family members using β APN or administration of catalase both significantly stimulated the expression of cytokeratin-19 and E-cadherin compared with their diluent treated counterparts, suggesting that the production of hydrogen peroxide by LOX family members selectively suppressed the expression of epithelial markers, and perhaps, sensitized cells to undergo EMT in response to TGF-β 1. Similarly, β APN treatment led to a significant reduction in vimentin and fibronectin expression compared with diluent-treated controls (Figure 2.3C). Interestingly, LOX deficiency failed to fully recapitulate these responses (data not shown), suggesting that other β APN-sensitive LOXL members may play a role during EMT induced by TGF-β 1. Along these lines, β APN treatment or LOX deficiency significantly inhibited the ability of TGF-β 1 to downregulate E-
cadherin protein expression in transitioning NMuMG cells (Figure 2.3, D and E). Thus, these findings suggest that LOX and its LOXL relatives sensitizes transitioning MECs to complete the EMT program when stimulated by TGF-β 1.

**LOX Regulates Breast Cancer Cell p38 MAPK Activation and Invasion Stimulated by TGF-β 1**

Previous findings by our group demonstrated that β3-integrin interacts physically with TβR-II leading to its phosphorylation on Y284 by Src and the subsequent activation of p38 MAPK that drives the pulmonary metastasis of 4T1 tumors [66-68]. Activation of this oncogenic TGF-β signaling cascade is amplified by overexpression of wild-type (WT) Tβ R-II in 4T1 cells and, more importantly, completely inactivated by their overexpression of Y284F–Tβ R-II mutants [68]. Histopathologic analysis of sections obtained from these same tumors revealed that LOX expression was upregulated significantly and specifically in 4T1 tumors that possessed enhanced oncogenic TGF-β signaling (i.e., WT–Tβ R-II–expressing 4T1 tumors) but not in 4T1 tumors engineered to express either GFP (i.e., parental controls) or Src-resistant Y284F–Tβ R-II mutants (Figure 2.4A). Thus, LOX expression was upregulated in late-stage mammary tumors in response to oncogenic TGF-β signaling. Given the importance of p38 MAPK in driving breast cancer progression stimulated by TGF-β 1, we administered β APN or catalase to 4T1 cells before their stimulation with TGF-β 1. Similar to NMuMG cells (Figure 2.2), both experimental treatments failed to alter the coupling of TGF-β 1 to Smad2/3 in 4T1 cells (Figures 2.4, B–D, and 2.8). However, unlike NMuMG cells, administration of
either β APN or catalase significantly reduced the coupling of TGF-β 1 to p38 MAPK in 4T1 cells (Figure 2.4D), suggesting that LOX and hydrogen peroxide may play an expanded role in regulating TGF-β 1 signaling in metastatic MECs compared with their normal counterparts. In addition, transient overexpression of constitutively active LOX-32 in these cells increased basal p38 MAPK activation but failed to further augment p38 MAPK activation by TGF-β 1 (Figure 2.9). Even more surprisingly, depleting 4T1 cells of LOX expression (Figure 2.4E) not only attenuated TGF-β 1 stimulation of p38 MAPK (Figure 2.4F) but also significantly reduced Smad2/3 transcriptional activity and phosphorylation of Smad2 induced by TGF-β 1 (Figure 2.4, E–H). This reduction in Smad2/3 phosphorylation and transcriptional activity cannot be attributed to enhanced activation of p38 MAPK, which can phosphorylate the linker region of Smad2/3 [191]. For instance, Figure 2.10 shows that overexpression of constitutively active MKK6, which significantly enhanced p38 MAPK activation, had no effect on the coupling of TGF-β 1 to Smad2/3 phosphorylation and reporter gene expression in 4T1 cells.

Functionally, administration of catalase significantly inhibited 4T1 cell invasion stimulated by TGF-β 1, whereas inclusion of either β APN or catalase significantly antagonized the ability of TGF-β 1 to induce the invasion of human MCF10AC1a cells (Figure 2.4I), which were previously established as a model for metastatic progression regulated by TGF-β [52]. Similar to β APN treatment, LOX deficiency failed to affect the extent of 4T1 cell invasion stimulated by TGF-β 1 (data not shown). These findings establish TGF-β 1 as an inducer of LOX expression in vivo and suggest that up-regulated expression LOX or another LOXL family member and/or hydrogen peroxide production
may regulate the malignancy of MECs in response to TGF-β 1, including its ability to induce breast cancer cell invasion.

*Mechanotransduction Induces Autocrine TGF-β 1 Signaling Coupled to MEC Proliferation*

Tissue compliance and ECM rigidity play vital roles in mediating cellular organization during embryogenesis and in maintaining tissue homeostasis in adult tissues [176]. Moreover, increased tissue tension resulting from desmoplastic and fibrotic stromal reactions is associated with mammary tumorigenesis and its progression to metastasis, activities that have also been attributed to LOX-dependent cross-linking of collagen to elastin during neoplastic progression [115, 120, 129, 176]. At present, the role of tissue rigidity and mechanotransduction in regulating the behaviors of MECs to TGF-β remains unknown. As such, we compared the response of 4T1 cells to TGF-β 1 when propagated under compliant and rigid culture conditions. Figure 2.5A shows that in traditional two-dimensional cultures, which are extremely rigid [176], TGF-β 1 readily promoted the proliferation of 4T1 cells. In stark contrast, propagating 4T1 cells in compliant three-dimensional organotypic cultures was sufficient to restore the cytostatic activities of TGF-β 1 in these malignant MECs, which normally fail to undergo growth arrest in response to TGF-β 1 (Figure 2.5A) [67-70, 87-89, 92, 192]. Importantly, supplementing these three-dimensional organotypic cultures with type I collagen to initiate mechanotransduction uncoupled TGF-β 1 from the regulation of cell cycle progression in 4T1 cells (Figure 2.5A). Thus, ECM tension and rigidity clearly alter how
MECs respond to the cytostatic activities of TGF-β1 [111]. Along these lines, we also inhibited TGF-β1 signaling in 4T1 cells by treating them with a small-molecule TβR-I antagonist, TβR-I Inhibitor II [87-89, 92, 192], and subsequently monitored alterations in their growth and morphology. As shown in Figure 2.5B, 4T1 organoids propagated in compliant cultures formed abnormal acinar structures, which underwent dramatic expansion and branching in response to increased ECM rigidity. Interestingly, the growth and branching of 4T1 organoids elicited by mechanotransduction were abrogated by inactivating TGF-β1 signaling, which restored the formation of spherical acinar structures (Figure 2.5B). Consistent with the ability of compliant microenvironments to reinstate cytostatic signaling by TGF-β1 (Figure 2.5A), inactivation of TGF-β1 signaling in these cultures by treating them with the TβR-I antagonist was sufficient to stimulate the growth and expansion of 4T1 organoids (Figure 2.5B). Thus, autocrine TGF-β1 signaling seems to play a prominent role in regulating MEC response to TGF-β1 under compliant and rigid ECM conditions. Accordingly, 4T1 (Figure 2.5C) and MCF-7 (data not shown) organoids propagated under rigid ECM conditions produced significantly more TGF-β1 compared with their counterparts propagated under compliant ECM conditions. Taken together, these findings show for the first time that exposing late-stage breast cancer cells to compliant ECM signals reinstates the cytostatic activities TGF-β1 and, in effect, partially reestablishes the tumor suppressing functions of TGF-β1 in malignant MECs.

Mechanotransduction Induces MEC Proliferation and E-cadherin Redistribution in a
**LOX-Dependent Manner**

To examine the specific contributions of LOX activity in regulating MEC response to TGF-β, we again cultured 4T1 cells under compliant or rigid ECM conditions with or without added β APN or catalase. Figure 2.6A shows that inhibiting LOX activity or degrading hydrogen peroxide both significantly reduced the growth of 4T1 cells stimulated by rigid ECM. Consistent with the effect of compliant ECM to restore cytostasis mediated by TGF-β1, these same experimental treatments elicited little-to-no effect on the ability of 4T1 cells to undergo growth arrest in response to TGF-β1 (Figure 2.6, A and B). Similarly, LOX deficiency (Figure 2.4E) abrogated the ability of TGF-β1 to stimulate the growth of 4T1 organoids in rigid ECM (Figure 2.7A) and, instead, partially reestablished the cytostatic activities of TGF-β1 in metastatic MECs.

Finally, given the ability of LOX activity to suppress E-cadherin expression (Figure 2.3) and given the ability of E-cadherin expression to suppress the uncontrolled growth of cancer cells [193], we hypothesized that LOX deficiency may suppress the growth of 4T1 organoids by upregulating and stabilizing E-cadherin expression at the plasma membrane. Accordingly, E-cadherin expression was readily detected and localized to the cell surface in parental and LOX-deficient 4T1 organoids propagated under rigid culture conditions (Figure 2.7B). Importantly, treating these same organoids with TGF-β1 resulted in the complete loss of E-cadherin from the plasma membrane in parental and scrambled shRNA-expressing 4T1 organoids, a reaction that was not recapitulated in their LOX-deficient counterparts (Figure 2.7B). Collectively, these findings suggest that LOX expression and activity may be essential in linking the oncogenic activities of TGF-β1 and mechanotransduction in breast cancer cells, presumably by regulating the
expression and localization of E-cadherin.

2.5 Discussion

Breast cancer is the second leading cause of cancer death in women. Although the 5-year survival rate for women with localized disease is high at 98%, this number drops precipitously to only 27% once the primary tumor has metastasized [194]. For this reason, it is vitally important for science and medicine to enhance their understanding of the processes that underlie breast cancer invasion and metastasis. It has long been established that breast cancer development reflects a loss-of-tissue organization and differentiation, factors that have more recently been associated with increases in LOX expression and activity [195, 196]. We show here that TGF-β and EMT both induce the expression and secretion of LOX from normal and malignant MECs and that mammary tumors engineered to house elevated TGF-β signaling produced more LOX than did their parental counterparts, which correlated with the ability of TGF-β to stimulate mammary tumor growth and pulmonary metastasis [68]. Equally important, we provide the first evidence that the manner in which MECs respond to TGF-β can be regulated by tissue rigidity, which elicits dramatic changes in MEC acinar morphology and growth in a LOX- and hydrogen peroxide–dependent manner. Although the exact mechanisms whereby tissue tension regulates TGF-β1 function remain to be elucidated fully, it is tempting to speculate that enhanced ECM rigidity may promote the inappropriate clustering of TGF-β receptors with integrins and other growth factor receptors, thereby inducing amplified coupling of TGF-β to its noncanonical effectors [197, 198].
Accordingly, we demonstrated that antagonizing LOX activity led to the diminished ability of TGF-β to stimulate MEC invasion and EMT. Furthermore, we show that antagonizing LOX activity partially uncoupled TGF-β 1 from p38 MAPK activation in metastatic 4T1 cells, whereas only catalase administration facilitated this event in normal NMuMG cells, suggesting that the roles of LOX and hydrogen peroxide depend on the pathophysiology of MECs.

It is important to note that although our current findings support an extracellular role of LOX in mediating the oncogenic activities of TGF-β (i.e., catalase neutralizes hydrogen peroxide), we cannot exclude the possibility that LOX may promote oncogenic TGF-β signaling by acting intracellularly. Indeed, mature LOX has been detected not only in the ECM but also in the cytoplasm (Figure 2.1) and nucleus of malignant cells [136, 139]; however, the identification of specific molecules capable of interacting with and/or being targeted by LOX in either intracellular compartment remains to be elucidated fully [136]. Along these lines, LOX was shown to activate Src and promote cell adhesion through a hydrogen peroxide–dependent mechanism [180]. We show here that administering catalase to degrade hydrogen peroxide prevented TGF-β from stimulating MEC proliferation, EMT, and invasion (Figures 2.3–2.7) and from fully activating p38 MAPK (Figure 2.4). Thus, hydrogen peroxide may function as a novel “second messenger” for TGF-β in normal and malignant MECs. Collectively, our findings suggest that LOX may play an important role in initiating the conversion of TGF-β function from a suppressor to a promoter of mammary tumorigenesis. At present, the specific players targeted by LOX and hydrogen peroxide that affect TGF-β signaling
remain an active and important topic for future experimentation. In addition, it is unclear what overlapping functions other LOX family members may play. Importantly, LOXL2 is not inhibited by β APN treatment and may compensate for loss of LOX function [136, 199], and in fact, both LOX and LOXL2 mediate the ability of HIF-1α to suppress E-cadherin expression [200]. Thus, future studies need to dissect the relative contribution of individual LOX family members to the initiation of oncogenic TGF-β signaling and its coupling to canonical and non-canonical effectors.

LOX plays a critical role during the formation of premetastatic niches by stimulating collagen cross-linking and fibronectin synthesis, leading to the recruitment of bone marrow–derived cells to metastatic niches [118]. TGF-β has also been implicated in the recruitment of immature bone marrow-derived cells to drive breast cancer metastasis [145], which suggests a potential link between TGF-β and LOX in regulating the formation of premetastatic niches. Interestingly, the use of copper chelators in preclinical and phase 2 clinical trials has shown some success in diminishing metastatic burden [201], findings that are potentially important because LOX activity is absolutely dependent on copper as one of its two cofactors (the other being lysyl tyrosyl quinone [136]). Thus, it is plausible that the clinical success of copper chelators to reduce tumor metastasis lies in their ability to inhibit LOX activity and, consequently, perhaps to alleviate the oncogenic activities of TGF-β as well. Along these lines, tumor hypoxia predicts for poor prognosis and decreased survival of breast cancer patients, which is linked to hypoxia-induced expression of LOX and the generation of metastatic niches in breast cancer patients [118, 119, 202]. These findings, together with those presented herein, support the idea that LOX dictates how malignant MECs respond to the varied.
activities of TGF-\(\beta\), and as such, identify LOX as a novel participant in oncogenic TGF-\(\beta\) 1 signaling in late-stage mammary tumors. Thus, chemotherapeutic targeting of LOX may offer new inroads to alleviate breast cancer progression stimulated by TGF-\(\beta\).

Lastly, the ability of LOX to cross-link collagen to elastin results in increased tissue tension and ECM rigidity [115, 120, 176, 203]. More recently, ECM rigidity has been shown to play an important role in breast cancer development, particularly their acquisition of invasive and metastatic phenotypes [120, 176, 196, 204]. The ability of normal and malignant MECs to sense ECM stiffness transpires through integrins and other mechanotransducers, which in turn activate Src, FAK, and the GTPases, Rho, Rac, and Cdc42 [195, 196]. Importantly, we identified an oncogenic TGF-\(\beta\) signaling axis comprised in part of \(\alpha_\nu\beta_3\) integrin, FAK, and Src that induces mammary tumor growth, invasion, and metastasis in mice [68-70], as well as stimulates significant LOX expression in these same mammary tumors (Figure 2.4). We speculate that tumor-initiated MECs evolve in compliant microenvironments that favor canonical Smad2/3 signaling stimulated by TGF-\(\beta\). The continued growth of the developing neoplasm enhances ECM rigidity by upregulating TGF-\(\beta\) production (Figure 2.5) and LOX expression (Figure 2.1), which may lead to the inappropriate formation of integrin–T\(\beta\) R-II complexes [66-68]. Once formed, these complexes are also likely to interact with other growth factor receptors that presumably amplify the activation of noncanonical effectors by TGF-\(\beta\) [17]. Ultimately, these adverse events culminate in the ability of TGF-\(\beta\) to induce the acquisition of EMT, stem-like, and metastatic phenotypes in malignant MECs, leading to their metastasis at distant locales. Moreover, extending our findings to
encompass sites of micrometastases [118], which are predicted to possess compliant ECM tension, leads us to propose that the cytostatic activities of TGF-β may be partially reinstated at these newly seeded sites, perhaps contributing to tumor dormancy. Over time, this vicious microenvironmental cycle is repeated, leading to disease recurrence and poor clinical outcomes in breast cancer patients harboring metastatic disease. The basic tenets of this model are supported by the findings presented herein (Figures 2.5–2.7), and as such, this model should serve as a launching point for future studies aimed at identifying the individual effectors operant in regulating ECM tension and TGF-β1 function in distinct breast cancer subtypes.

2.6 Acknowledgments
Members of the Schiemann Laboratory are thanked for critical reading of the article.
Figure 2.1: TGF-β 1 and EMT stimulate the expression and secretion of LOX in normal and malignant MECs.

(A) Shown are representative images of TGF-β 1–treated NMuMG cells that have undergone EMT (top panel). TGF-β 1 (5 ng/ml) stimulation of EMT in NMuMG cells induced their expression of LOX as determined by semiquantitative real-time PCR. Individual transcript signals were normalized to GAPDH. Data are the mean (±SE; n = 3) fold expression of LOX transcripts relative to pre-EMT NMuMG cells. *P < .05. (B) NMuMG and 4T1 cells were incubated in the absence or presence of TGF-β 1 (5 ng/ml) for 24 hours, at which point alterations in the actin cytoskeleton were monitored by TRITC–phalloidin immunofluorescence. Insets: magnified views of boxed regions. Data are representative images from three independent experiments. (C) TGF-β 1 (5 ng/ml) stimulated LOX production and secretion from NMuMG and 4T1 cells as determined by immunoblot analysis conditioned medium (C) or detergent-solubilized whole cell extracts (L) with anti-LOX antibodies. Differences in protein loading were monitored by immunoblot analysis for β-actin. Data are representative images from three independent experiments.
Figure 2.1

A  NMuMG Cells

Pre-EMT  Post-EMT

LOX mRNA Expression (fold-stimulation)

Pre-EMT  Post-EMT

NMuMG Cells

B  Experimental Treatment

Experimental Treatment

Basal  TGF-β1

Basal  TGF-β1

NMuMG  4T1

Inset

C  Experimental Treatment

NMuMG

4T1

β-actin

-  +  -  +  TGF-β1 Treatment

MEC Cell Line

Nm  4T1

Inset
Figure 2.2: TGF-β 1 activates p38 MAPK through a hydrogen peroxide–dependent pathway in normal MECs.

NMuMG cells were transiently transfected with the TGF-β 1–responsive reporter gene, p3TP-luciferase (A) or pSBE-luciferase (B), and with pCMV–β-gal. Afterward, the transfectants were stimulated with TGF-β 1 (5 ng/ml) in the absence or presence of β APN (300 μM) or catalase (400 U/ml). Luciferase activity was measured and normalized to β-gal. Data are the mean (±SE; n = 3). (C) Quiescent NMuMG cells were pretreated with β APN (300 μM), hydrogen peroxide (H2O2, 1 mM), or catalase (400 U/ml) as indicated and, subsequently, were stimulated with TGF-β 1 (5 ng/ml) for 30 minutes. The phosphorylation and expression levels of Smad2 and p38 MAPK were monitored by immunoblot analysis with phospho-specific antibodies, and differences in protein loading were monitored by reprobing stripped membranes with antibodies against β-actin. Shown are representative images from three independent experiments. (D) Four unique shRNA sequences targeting LOX (sh#1–sh#4) were stably expressed in NMuMG cells and differences in LOX expression were analyzed by immunoblot analysis with anti-LOX antibodies to detect mature LOX. Scram indicates scrambled shRNA. β-Actin immunoreactivity is provided as a loading control. (E) Quiescent scrambled (Scram) and LOX-deficient (sh#4) NMuMG cells were stimulated with TGF-β 1 (5 ng/ml) for 30 min, at which point the phosphorylation and expression of Smad2 and p38 MAPK were monitored by immunoblot analysis as described in B. Shown are representative images from three independent experiments. Scrambled (Scram) and LOX-deficient (sh#4) NMuMG cells were transiently transfected with p3TP-luciferase (F) or pSBE-luciferase (G) pCMV–β-gal and, subsequently, were stimulated with TGF-β 1 (5 ng/ml) as described in A. Data are the mean (±SE; n = 3).
Figure 2.3: TGF-β 1 regulates EMT in normal MECs through a LOX- and hydrogen peroxide–dependent pathway.

(A) NMuMG cells were incubated in the absence or presence of either β APN (300 μ M) or catalase (400 U/ml) while undergoing EMT stimulated by TGF-β 1 (5 ng/ml). Arrowheads show strong actin fibers localized to focal adhesions in diluent-treated cells stimulated with TGF-β 1 and, conversely, stunted actin fibers in cells treated with β APN or catalase. (B) Parental (scram) or LOX-deficient (shLOX#3 and shLOX#4) NMuMG cells were stimulated by TGF-β 1 (5 ng/ml) to induce EMT. Arrowheads show strong actin fibers localized to focal adhesions in Scram cells stimulated with TGF-β 1 and, conversely, the presence of stunted actin fibers in LOX-deficient cells. Shown are representative images from three independent experiments. (C) NMuMG cells were stimulated to undergo EMT by TGF-β 1 (5 ng/ml) in the absence (i.e., diluent; Dil) or presence of either β APN (300 μ M) or catalase (400 U/ml; Cat). Altered expression of cytokeratin 19, E-cadherin, N-cadherin, vimentin, or fibronectin mRNA was determined by semiquantitative real-time PCR. Individual transcript signals were normalized to GAPDH. Data are the mean (±SE; n = 3) transcript levels normalized to corresponding unstimulated controls. *P < .05. **P < .05. (D and E) Altered E-cadherin (E-cad) expression was monitored by immunoblot analysis detergent-solubilized whole-cell extracts with anti–E-cadherin antibodies. Protein loading was controlled with anti–β -actin antibodies. Shown are representative images from two independent experiments. Accompanying graphs show the densitometric mean (±SE; n = 2) relative to corresponding basal cells. *P < .05.
Figure 2.4: LOX regulates breast cancer cell p38 MAPK activation and invasion stimulated by TGF-β 1.

(A) Elevated oncogenic TGF-β 1 signaling (i.e., WT–T β R-II expression) greatly accelerates the growth and pulmonary metastasis of 4T1 tumors in mice [8]. LOX immunohistochemistry performed on these same tumor slices showed that TGF-β 1 signaling significantly induced the expression of LOX expression in WT–T β R-II–expressing 4T1 tumors compared with their GFP- or Y284F–T β R-II–expressing counterparts. Data are representative images from two independent experiments. WT indicates wild-type. 4T1 cells were transiently transfected with p3TP-luciferase (B) or pSBE-luciferase (C) and pCMV–β-gal and, subsequently, were stimulated with TGF-β 1 (5 ng/ml) in the absence or presence of β APN (300 μ M) or catalase (400 U/ml). Luciferase activity was measured and normalized to β-gal. Data are the mean (±SE; n = 3). (D) Quiescent 4T1 cells were pretreated with β APN (300 μ M), hydrogen peroxide (H2O2, 1 mM), or catalase (400 U/ml) as indicated and, subsequently, were stimulated with TGF-β 1 (5 ng/ml) for 30 minutes. The phosphorylation and expression of Smad2 and p38 MAPK was monitored by immunoblot analysis, and differences in protein loading were monitored by reprobing stripped membranes with anti-β-actin. Shown are representative images from three independent experiments. (E) Three unique shRNA sequences targeting LOX (sh#2–sh#4) were stably expressed in 4T1 cells, and differences in LOX expression were analyzed by immunoblot analysis with anti-LOX antibodies. Scram indicates scrambled shRNA. β-Actin immunoreactivity is provided as a loading control. (F) Quiescent scrambled (Scram) and LOX-deficient (sh#4) 4T1 cells were stimulated with TGF-β 1 (5 ng/ml) for 30 min, at which point the phosphorylation status and expression of Smad2 and p38 MAPK were monitored by immunoblot analysis as described in D. Shown are representative images from three independent experiments. Scrambled (Scram) and LOX-deficient (sh#4) 4T1 cells were transiently transfected with p3TP-luciferase (G) or pSBE-luciferase (H) and pCMV–β-gal and, subsequently, were stimulated with TGF-β 1 (5 ng/ml) as described in A. Data are the mean (±SE; n = 3). (I) 4T1 or MCF10ACA1a (CA1a) cells were incubated in the absence or presence of either β APN (300 μ M) or catalase (400 U/ml) while undergoing invasion through synthetic basement membranes in response to TGF-β 1 (5 ng/ml). Data are the mean (±SE; n = 3) invasion relative to that stimulated by TGF-β 1. *P < .05.
Figure 2.4

A. Inoculated 4T1 Cell Line

- GFP
- WT-1/II
- Y284F-7II

Anti-LOX IHC

B. Bar graph showing β-TCP Luciferase Activity (fold-stimulation) for Basal and TGF-β1 conditions.

C. Bar graph showing SBE-Luciferase Activity (fold-stimulation) for Basal and TGF-β1 conditions.

D. Western blot analysis for p-Smad2, t-Smad2/3, p-p38 MAPK, t-p38 MAPK, and β-actin under different treatment conditions.

E. Western blot analysis for Nat LOX and p-actin under different treatment conditions.

F. Western blot analysis for p-Smad2, t-Smad2/3, p-p38 MAPK, t-p38 MAPK, and β-actin for 4T1 Cell Line.

G. Western blot analysis for p3TP-Luciferase Activity (fold-stimulation) under Basal and TGF-β1 conditions.

H. Western blot analysis for SBE-Luciferase Activity (fold-stimulation) under Basal and TGF-β1 conditions.

I. Western blot analysis for MEC Invasion (% of treated cells) for 4T1 and MCF10CA1a under different conditions.
Figure 2.5: Mechanotransduction induces autocrine TGF-β 1 signaling coupled to MEC proliferation.

(A) 4T1 cells were incubated in the absence or presence of TGF-β 1 (5 ng/ml) in two-dimensional tissue culture plastic or in three-dimensional organotypic cultures supplemented without (i.e., compliant) or with type I collagen (3 mg/ml; rigid). Bright-field images were captured and used to quantitate cell proliferation through ImageJ. Data are the mean (±SE; n = 3) proliferation relative to basal 4T1 cells. *P < .05. (B) Inhibition of TGF-β 1 signaling by administration of the T β R-I inhibitor (100 ng/ml) enhanced the growth of 4T1 cells in compliant three-dimensional organotypic cultures, but inhibited their growth in rigid (3 mg/ml type I collagen) three-dimensional organotypic cultures. Insets: magnified views of boxed regions. Data are representative images from three independent experiments. (C) Conditioned medium harvested from compliant or rigid three-dimensional organotypic cultures was acidified to activate total TGF-β 1. After sample neutralization, TGF-β 1 concentrations were determined by ELISA analysis. Data are the mean (±SE; n = 3) TGF-β 1 concentrations relative to those measured in compliant cultures. *P < .05.
Figure 2.5
Figure 2.6: Mechanotransduction induces MEC proliferation in a LOX-dependent manner.

(A) LOX antagonism using βAPN (300 µM) or catalase (400 U/ml) inhibited the ability of TGF-β1 to stimulate 4T1 cell growth in rigid three-dimensional organotypic cultures. Insets: magnified views of boxed regions. Shown are representative images from three independent experiments. (B) Accompanying data are the mean (±SE; n = 3) proliferation relative to the growth of basal cells in compliant cultures. *P < .05.
Figure 2.6
Figure 2.7: LOX deficiency suppresses mechanotransduction and TGF-β1 stimulation of MEC proliferation by restoring cell surface E-cadherin expression.

(A) Parental, scrambled (Scram), or LOX-deficient (sh#3 and sh#4) 4T1 cells were propagated in rigid three-dimensional organotypic cultures in the absence or presence of TGF-β1 (5 ng/ml) as indicated. Bright-field images were captured (top panel) and used to quantitate cell proliferation through ImageJ (bottom panel). Insets: magnified views of boxed regions. Data are the mean (±SE; n = 3) proliferation relative to basal 4T1 cells. *P < .05. (B) Parental, scrambled (Scram), or LOX-deficient (sh#3 and sh#4) 4T1 cells were propagated in rigid three-dimensional organotypic cultures as described in A and, subsequently, were processed to visualize the expression and localization of E-cadherin by immunofluorescence. Corresponding nuclei were detected by inclusion of DAPI as indicated. Data are representative images from three independent experiments.
Figure 2.7

A. 4T1 Cell Line

<table>
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<tr>
<th>Condition</th>
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<th>Inset</th>
<th>Rigid</th>
<th>Inset</th>
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<tr>
<td>Parental</td>
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<td></td>
<td></td>
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</tr>
<tr>
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<td></td>
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<tr>
<td>shLOX#3</td>
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<td>shLOX#4</td>
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</table>

Cell Proliferation (% basal)

- Basal
- TGF-β1

B. Rigid

<table>
<thead>
<tr>
<th>Condition</th>
<th>Parental</th>
<th>Scram</th>
<th>shLOX#3</th>
<th>shLOX#4</th>
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</thead>
<tbody>
<tr>
<td>Basal</td>
<td>E-Cad</td>
<td></td>
<td>DAPI</td>
<td>E-Cad</td>
</tr>
<tr>
<td>DAPI</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TGF-β1</td>
<td>E-Cad</td>
<td></td>
<td>DAPI</td>
<td>E-Cad</td>
</tr>
<tr>
<td>DAPI</td>
<td></td>
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</tbody>
</table>
Figure 2.8: Pharmacologic and genetic targeting of LOX fail to alter TGF-β 1 stimulation of Smad3 phosphorylation in normal and malignant MECs.

Quiescent NMuMG (A) and 4T1 (C) cells were pretreated with β APN (300 μM), hydrogen peroxide (H2O2, 1 mM), or catalase (400 U/ml) as indicated. Afterward, these MECs and their LOX-deficient (sh#4) counterparts (B, D) were stimulated with TGF-β 1 (5 ng/ml) for 30 minutes before monitoring changes in the expression (t-Smad2/3) and phosphorylation (p-Smad3) of Smad3 by immunoblot analysis. Differences in protein loading were assessed by reprobing stripped membranes with β-actin antibodies. Data are representative images from three independent experiments. Scram indicates scrambled shRNA.
Figure 2.9: Expression of constitutively active LOX stimulates p38 MAPK in normal and malignant MECs.

Human 293T (A), NMuMG (B), or 4T1 (C) cells were transiently transfected with constitutively active LOX (LOX32). Thirty-six hours after transfection, the conditioned medium (CDM) was tumbled with Ni2+-agarose beads to capture recombinant LOX32 proteins (A, C) or was precipitated with trichloroacetic/ deoxycholate (B). Recombinant LOX32 protein expression was visualized by immunoblot analysis with anti-Myc antibodies. After collecting CDM, the cells were incubated for 4 hours in serum-free medium before stimulation with TGF-β1 (5 ng/ml) for 30 minutes at 37°C as indicated. The phosphorylation status of p38 MAPK was assessed by immunoblot analysis with phospho-specific p38 MAPK (p-p38 MAPK) antibodies, whereas differences in protein loading were monitored by reprobing stripped membranes with antibodies against p38 MAPK (t-p38 MAPK) and β-actin. Data are representative images from two independent experiments.
Figure 2.9

A

HEK-293T

mock

LOX32

p-p38 MAPK

t-p38 MAPK

β-actin

Extract

myc

CDM

B

NMuMG

mock

LOX32

TGF-β1

p-p38 MAPK

p-38 MAPK

β-actin

Extract

mock

LOX32

myc

CDM

C

4T1

mock

LOX32

TGF-β1

p-p38 MAPK

p-38 MAPK

β-actin

Extract

mock

LOX32

myc

CDM
Figure 2.10: Expression of constitutively active MKK6 activates p38 MAPK but has no effect on the coupling of TGF-β to Smad2/3 in malignant MECs.

4T1 cells were transiently transfected overnight with p3TP-luciferase and pCMV-β-gal, together with either empty vector (mock) or constitutively active MKK6 as indicated. Afterward, the cells were stimulated with TGF-β1 (5 ng/ml) for 24 hours, at which point cell extracts were prepared for p38 MAPK and Smad2 immunoblot analysis (A) or luciferase and β-gal assays (B). Data are representative images or the resulting luciferase activity (mean ± SE) from three independent experiments.
Figure 2.10

**A**

<table>
<thead>
<tr>
<th>4T1 Transfection</th>
<th>Mock</th>
<th>MKK6</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>p-p38 MAPK</td>
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<td>t-Smad2/3</td>
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<tr>
<td>β-actin</td>
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</tbody>
</table>

**B**

Bar graph showing p3TP-Luciferase Activity (fold stimulation) for Basal and TGF-β1 conditions in 4T1 Transfection with Mock and MKK6 treatments.
Chapter 3

**TGF-β-mediated miR-181a expression promotes breast cancer metastasis by targeting Bim.**

3.1 Abstract

Dysregulated signaling by transforming growth factor-β (TGF-β) in late-stage breast cancers culminates in their metastatic dissemination. Precisely how TGF-β drives metastatic progression in breast cancers remains to be fully elucidated. We attempted to recapitulate tumor and metastatic microenvironments *via* the use of biomechanically compliant or rigid 3D-organotypic cultures, which were combined with global microRNA (miRNA) profiling analyses to identify miRs whose expression were upregulated in metastatic breast cancer cells by TGF-β. Here we establish miR-181a as a TGF-β-regulated “metastamir” that enhanced the metastatic potential of breast cancers by promoting their acquisition of epithelial-mesenchymal transition, migratory, and invasive phenotypes. Mechanistically, inactivating miR-181a elevated the expression of the pro-apoptotic molecule, Bim, which sensitized metastatic cells to anoikis. Along these lines, miR-181a expression was essential in driving pulmonary micrometastatic outgrowth and enhancing the lethality of late-stage mammary tumors in mice. Finally, miR-181a expression is dramatically and selectively upregulated in metastatic breast tumors, particularly triple-negative breast cancers, and is highly predictive for decreased overall survival in human breast cancer patients. Collectively, our findings strongly

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3 A version of this chapter was accepted for publication at the *Journal of Clinical Investigation* on October 11, 2012.
implicate miR-181a as a novel predictive biomarker for breast cancer metastasis and patient survival, and consequently, as a potential therapeutic target for metastatic breast cancer.

### 3.2 Introduction

Metastasis is a complex multistage process whereby primary tumor cells acquire the ability to 

1. _locally invade through the surrounding stroma_;
2. _intravasate into blood vessels_;
3. _survive transit through the vascular system_;
4. _extravasate and arrest at distant sites_; and
5. _survive in foreign microenvironments and overcome systemic dormancy to undergo metastatic outgrowth_, ultimately leading to the formation of secondary tumors in vital organ sites [205]. Metastasis of primary mammary tumors accounts for the vast majority of deaths of breast cancer patients. Indeed, the 5 year survival rate for patients with breast cancer drops precipitously from 98% for individuals with localized disease to 23% for those with metastatic disease [206]. Within normal mammary tissues, the multifunctional cytokine transforming growth factor-β (TGF-β) functions as a potent tumor suppressor through its ability to induce cell cycle arrest and apoptosis. Unlike their normal counterparts, malignant mammary tissues can transform the normal functions of TGF-β to that of a potent stimulator of breast cancer proliferation, migration, and invasion in part _via_ its ability to promote the acquisition of epithelial-mesenchymal transition (EMT) and metastatic phenotypes [3, 7, 207]. This switch in TGF-β function from that of a tumor suppressor to a tumor promoter is known as the “TGF-β Paradox,” whose mechanistic underpinnings that engender this phenomenon remain incompletely understood. Moreover, this switch in TGF-β function
is often accompanied by desmoplastic and fibrotic reactions, which elicit dramatic changes in the biomechanical properties of the tumor microenvironment. Indeed, the elastic modulus of stroma housed within breast carcinomas is approximately 10-times more mechanically rigid than that of adjacent normal breast tissues [115, 116]. TGF-β potentiates these biomechanical reactions by stimulating the expression and secretion of a variety of extracellular matrix (ECM) components, such as collagen I and fibronectin from stromal fibroblasts, and of ECM cross-linking enzymes, such as lysyl oxidase from mammary carcinoma cells [3, 7, 121]. The formation of these rigid mammary tumor microenvironments promotes metastatic progression in breast cancers, as well as predicts for poor clinical outcomes in patients harboring metastatic disease [115, 117-120]. Interestingly, normal mammary and lung tissues share similarly compliant elastic moduli, a biomechanical condition that may contribute to initiation of dormancy by disseminated breast micrometastases in the lungs [176]. We recently demonstrated that biomechanically compliant microenvironments can reinstate the cytostatic activities of TGF-β in late-stage breast cancer cells, indicating that matrix rigidity plays a vital role in mediating how cells sense and respond to the dichotomous functions of TGF-β [121]. Moreover, the ability of carcinoma cells to thrive both in rigid primary tumor microenvironments and compliant metastatic microenvironments represents an essential characteristic of fully metastatic breast cancer cells. It therefore stands to reason that enhancing our knowledge of the molecular mechanisms that mediate breast cancer metastasis may enable the development of specific metastasis-based treatments needed to improve the overall survival rates of patients harboring metastatic breast cancers.
MicroRNAs (miRNA) are small (20-30 nucleotides), non-coding RNAs that post-transcriptionally regulate gene expression through canonical base pairing between the miRNA seed sequence (nucleotides 2-8 of the 5’end) and the complementary sequence in the 3’UTR of the target mRNA. The net effect of these events elicits either translational repression or degradation of targeted mRNAs [148]. Recently, several studies have implicated aberrant miRNA expression in the development and metastatic progression of mammary tumors [171]. At present, the precise role of miRNAs in controlling metastatic progression by TGF-β remains to be fully elucidated, as does the impact of tissue compliance in altering these TGF-β- and miRNA-driven activities. Global miR expression profiling analyses enabled us to identify a variety of miRNAs whose expression are regulated by TGF-β and altered ECM rigidity, one of which was miR-181a that is upregulated by TGF-β in hepatocytes [173, 208] and breast cancer cells [171]. Importantly, we demonstrate that aberrantly high miR-181a expression enhanced the ability of TGF-β to stimulate breast cancer metastasis by inducing EMT programs by promoting resistance to anoikis by downregulating the expression of the pro-apoptotic factor, Bim. Equally important, miR-181a expression is highly associated with the development of metastatic disease in breast cancers, particularly triple-negative breast cancers (TNBCs), and is highly predictive for poor clinical outcomes in breast cancer patients. Taken together, our findings establish miR-181a as a potential predictive biomarker for breast cancer metastasis and overall survival, as well as a promising pharmacologic target for the treatment of metastatic breast cancers.

3.3 Materials and Methods
Cell culture and constructs.

NMuMG, MCF-7, and MDA-MB-231 cells were obtained from ATCC, while isogeneic 67NR, 4T07, and 4T1 cells were provided by Fred Miller (Wayne State University, Detroit, MI, USA). MDA-MB-231 cells rendered deficient in Smad4 expression were kindly provided by Yibin Kang (Princeton University, Princeton, NJ, USA). Firefly luciferase-expressing 4T1 cells were previously described [69, 209]. miRIDIAN miR-181a microRNA Mimics (20 nM final concentration) or Hairpin Inhibitors (50 nM final concentration) were obtained from Thermo Scientific and transiently transfected into cells using Lipofectamine 2000 according to the manufacturer’s recommendations (Life technologies). NMuMG and 4T1 cells were engineered to possess elevated miR-181a activity by their transduction with miExpress (Genecopia) lentiviral particles that encoded for either control (i.e., scrambled) or precursor miR-181a molecules, followed by the isolation and expansion of polyclonal cell populations by neomycin selection (200 mg/ml; Calbiochem). Alternatively, these same MECs were engineered to possess reduced miR-181a activity by their transduction with miArrest (Genecopia) lentiviral particles that encoded for either control (i.e., scrambled) or miArrest 181a, followed by the isolation and expansion of polyclonal cell populations by hygromycin selection (250 mg/ml; Invitrogen).

MicroRNA microarray.

Total RNA was prepared using the Qiagen RNeasy Plus Mini Kit and following the supplementary protocol for purification of miRNA (Qiagen). All experimental conditions were performed in duplicate prior to sending the resulting RNA preparations to Thermo Scientific for microRNA profiling analyses, whose resulting signal intensities were
subjected to statistical filtering that identified miRNA probes that had $P$-value $\leq 0.05$ in at least half of the samples. The remaining data was inter-array scaled and transformed to log$_2$ values prior to performing one-way ANOVA analyses for each cell line. Afterward, post hoc analysis was undertaken to identify miRs whose expression was significantly (*$P<0.01$) upregulated following administration of TGF-β. The miRNA expression data is MIAME compliant and have been submitted to the Gene Expression Omnibus (GEO) with accession number GSE41274.

**3D-organotypic culture and outgrowth assays.**

3D-organotypic cultures were performed using the “on-top” method [185] as previously described [121]. Briefly, cells were cultured on Cultrex cushions (100%; Trevigen) in complete medium supplemented with 5% Cultrex. ECM rigidity was increased by the addition of type I collagen (3 mg/ml; BD biosciences) to Cultrex cushions before their solidification. Where indicated, the cells were treated with TGF-β1 (5 ng/ml) for varying lengths of time and the media/Cultrex solution was replaced every 4 days. Cells were harvested for RNA analyses by digesting the Cultrex cushions with dispase (BD Biosciences) for 30 min at 37°C, followed by inactivation with EDTA (8 mM). Afterward, the dissociated organoids were collected by centrifugation and prepared for immunoblotting or real-time PCR analyses as described below. Longitudinal bioluminescent growth assays were performed as described [18]. Briefly, changes in organoid growth were detected by the addition of D-luciferin (Gold Biotechnology) to induce bioluminescence, which was quantified on a GloMax-Multi Detection System.
Cell proliferation was normalized to initial bioluminescent signals that were obtained 18 hr after plating.

**Cell invasion, migration and proliferation.**

Modified Boyden chambers coated with Matrigel (1:25 dilution) were used to monitor the invasiveness of 67NR and 4T1 cells in response to 10% serum and TGF-β1 (5 ng/ml) as described previously [182]. Alterations in 4T1 cell migration was assessed by wounding confluent cultures with a micropipette tip, which were immediately placed in serum-free media supplemented with or without TGF-β1 (5 ng/ml). Bright-field images were obtained immediately after wounding and at various times over a span of 24 h thereafter. The extent of wound closure was quantitated by measuring the wound areas obtained from 5 independent fields using ImageJ (v1.34s). Lastly, alterations in 67NR and 4T1 cell proliferation (10,000 cells/well) was determined by monitoring the incorporation of [³H]thymidine into cellular DNA as described previously [66].

**Semi-quantitative real-time PCR analyses.**

Total RNA was purified using the supplementary miRNA protocol provided by the RNeasy Plus Mini kit (Qiagen). Afterward, cDNAs were synthesized using the miScript Reverse Transcription Kit (Qiagen), which were diluted 10-fold in H₂O prior to their use in semi-quantitative real-time PCR reactions that contained 10 ml SooFast EvaGreen (Bio-Rad), 1 µl miRNA forward primer, 1 µl miScript Universal Primer (Qiagen), 3 µl H₂O, and 5 µl of diluted cDNA. miRNA expression levels were analyzed on a 7500 Fast Real-Time PCR System (Applied Biosystems). Differences in total RNA concentration
were normalized to their corresponding U6 signal. miRNA precursor levels were determined using the miScript precursor assays as recommended by the manufacturer (Qiagen). The oligonucleotide primer pairs used are provided in Supplemental Table 4.

**Table 3.1: Real-time PCR primer pairs**

<table>
<thead>
<tr>
<th>Target</th>
<th>Application</th>
<th>Sequence (5’ to 3’)</th>
</tr>
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<tbody>
<tr>
<td>miR-181a</td>
<td>PCR-Sense</td>
<td>5’-AACATTCAACGCTGTCGGTGAGT</td>
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<tr>
<td>miR-181b</td>
<td>PCR-Sense</td>
<td>5’-AACATTCAACGCTGTCGGTGAGT</td>
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<tr>
<td>miR-181c</td>
<td>PCR-Sense</td>
<td>5’-AACATTCAACGCTGTCGGTGAGT</td>
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<tr>
<td>miR-181d</td>
<td>PCR-Sense</td>
<td>5’-AACATTCAACGCTGTCGGTGAGT</td>
</tr>
<tr>
<td>U6</td>
<td>PCR-Sense</td>
<td>5’-GTGCTCGCTGCGAGCACAT</td>
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<tr>
<td>Bim</td>
<td>PCR-Sense</td>
<td>5’-TCTGAGTGTGACAGAAGGAGTGAC</td>
</tr>
<tr>
<td>Bim</td>
<td>PCR-Antisense</td>
<td>5’-CACTTCACCACCCGTCGGTACAGTAC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>PCR-Sense</td>
<td>5’-CAACCTTGGCATGTGACAGAACAGGCTC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>PCR-Antisense</td>
<td>5’-GCAGGGATGATGTTCTGGGCAGC</td>
</tr>
</tbody>
</table>

Shown are the sense and antisense primers used to amplify the indicated target gene.

*Luciferase reporter gene assays.*

Normal and malignant MECs were seeded onto 24-well plates (25,000 cells/well) and allowed to adhere overnight. The following morning, the cells were transiently transfected using TransIT-LT1 Transfection Reagent (Mirus) with 50 ng/well of pCMV-β-gal (Clontech) and 450 ng/well psi-Check2 luciferase reporter (Promega) that housed either (i) the complementary seed sequence for miR-181a (*e.g.*, miR-181a biosensor), or (ii) the 3’UTR sequence of Bim that contained either a wild-type or mutant version of the
miR-181a seed sequence (wild-type Bim reporter was kindly provided by Dr. Clark Distelhorst, Case Western Reserve University, Cleveland, OH USA). Forty-eight h post-transfection, the cells were harvested to measure the quantity of luciferase and β-gal activities present in detergent-solubilized cell extracts. Data are the mean (±SE) luciferase activities of at least 3 independent experiments.

*Immunoblotting and immunofluorescence.*

Quiescent control and miR-181a-manipulated NMuMG and 4T1 derivatives were incubated in the absence or presence of TGF-β1 (5 ng/ml) for 30 min, at which point they were solubilized in Buffer H/1% Triton X-100 and prepared for immunoblotting as described previously [121]. Antibodies and pharmacologic inhibitors used herein are described in Tables 3.2 and 3.3, respectively. Alterations in the actin cytoskeleton induced by TGF-β in parental and miR-181a-modified cells was monitored using direct TRITC-phalloidin fluorescence as described previously [184].

**Table 3.2: Immunoblotting antibodies**

<table>
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<th>Antibody</th>
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<td>Phospho-Smad 3</td>
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<td>Cell Signaling (#9520)</td>
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<td>Total Smad 2/3</td>
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<td>Cell Signaling (#3102)</td>
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<tr>
<td>Phospho-Src</td>
<td>1:500</td>
<td>Cell Signaling (#2113)</td>
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<tr>
<td>Phospho-Erk1/2</td>
<td>1:1000</td>
<td>Cell Signaling (#9101)</td>
</tr>
<tr>
<td>Phospho-Akt</td>
<td>1:500</td>
<td>Cell Signaling (#4060)</td>
</tr>
<tr>
<td>Total Src</td>
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<td>Cell Signaling (#2108)</td>
</tr>
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</table>
Shown are the antibodies and dilutions used to visualize the indicated proteins. Also provided are the vendors where these reagents were obtained.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Dilution</th>
<th>Vendor</th>
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<td>Total Erk1/2</td>
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<td>Cell Signaling (#4695)</td>
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<tr>
<td>Total Akt</td>
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<td>Cell Signaling (#9272)</td>
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<tr>
<td>E-Cadherin</td>
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<td>BD Biosciences (#610182)</td>
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<td>Caspase-3</td>
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<td>Cell Signaling (#9662)</td>
</tr>
<tr>
<td>β-Actin</td>
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<td>Santa Cruz (#sc-1616)</td>
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</table>

**Table 3.3: Pharmacological inhibitors**

<table>
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<th>Target</th>
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<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>TβR-I Inh II</td>
<td>TβR-I</td>
<td>3.5 µM</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>U0126</td>
<td>MEK1/2</td>
<td>10 µM</td>
<td>Promega</td>
</tr>
<tr>
<td>Akt Inh VIII</td>
<td>Akt</td>
<td>1 µM</td>
<td>Calbiochem</td>
</tr>
</tbody>
</table>

Shown are the pharmacological antagonists and final concentrations used inhibit the indicated protein targets. Also provided are the vendors where these reagents were obtained.

Anoikis assays.

Control and miR-181a-manipulated NMuMG and 4T1 cells were cultured over polyhydroxymethylmethacrylate (poly-HEMA)-coated plates in either 0.5% serum or serum-free media, respectively. The cells were collected at various times over a span of 48 h, at which point they were prepared for immunoblotting analyses of cleaved caspase-3. For Bim rescue experiments, a rat Bim cDNA that lacked the 3’UTR was inserted into pcDNA3.1 (Invitrogen) and transiently transfected into control or miR-181a-manipulated cells 24 h prior to initiating anoikis.

4T1 tumor growth and metastasis assays.
Firefly luciferase-expressing 4T1 cells that stably expressed miR-181a (10,000 cells/injection) or a miR-181a sponge (5000 cells/injection) were engrafted onto the mammary fat pads of 4 week old female Balb/C mice. The growth and metastasis of primary 4T1 tumors was quantified by (i) weekly bioluminescent imaging of tumor bearing animals on a Xenogen IVIS-200 (Caliper Life Sciences), and (ii) thrice weekly monitoring of primary tumor size using digital calipers (Fisher). Tumor volumes were calculated by the equation: tumor volume = (0.5)(x^2)(y), where “x” is the tumor width and “y” is the tumor length. Primary tumors were excised 4-5 weeks post-inoculation, at which point serial histological sections were prepared by the Case Comprehensive Cancer Center’s Tissue Procurement, Histology, & IHC Core, which also performed the H&E and TUNEL staining reactions. Additional immunostaining was undertaken to monitor the expression of Ki-67 (1:50; BD Pharmingen) and Bim (1:50; Cell Signaling) as described previously [68]. The resulting images were captured on a D-Metrix DX-40 slide scanner outfitted with Eyepiece Software, or on an Olympus BH2 microscope outfitted with Spot Advanced software (Diagnostic instruments Inc.).

For pulmonary outgrowth studies, the aforementioned 4T1 derivatives were injected into the lateral tail vein of 4 week old Balb/C mice. Bioluminescence imaging was performed 30 min post-inoculation (T0) and biweekly thereafter. Pulmonary tumor development was assessed by normalizing biweekly images to those obtained at T0. All animal procedures were performed in accordance to protocols approved by the Institutional Animal Care and Use Committee for Case Western Reserve University.

*Human breast cancer specimens.*
Primary tumors and matched normal tissues were collected and processed under approved IRB (Institutional Review Board) protocols from the University Hospitals Case Medical Center (Cleveland OH) and the Cleveland Clinic (Cleveland OH). All patients provided written informed consent and consented to allowing the study investigators access to their tumor specimens and clinical data.

**Analysis of human miRNA and mRNA microarray data.**

Expression and clinical outcome data was obtained from a publically available dataset of 101 human primary breast tumor samples, which contained matched genome-wide miRNA mRNA profiling (GSE19783; [210]). All samples were median-centered for miR-181a expression and denoted as having high expression if individual miR-181a signals fell above the median, and as having low expression if individual miR-181a signals fell below the median. Kaplan-Meier curves were generated using GraphPad Prism for Macintosh (v5.0). mRNA expression profiles for tumors not classified as being ErbB2 amplified were divided into high (n=41) or low (n=41) expression of miR-181a and Gene Set Enrichment Analysis (GSEA) was performed using GSEA software (v2.07) that was obtained from the Broad Institute. Genes were analyzed for enrichment using the c2 gene sets from the MSigDB, which contains 3271 curated gene sets obtained from public pathway databases and published gene signatures. $P$ values, enrichment scores (ES), and $q$ values were computed by permuting the sample labels (phenotype) 1,000 times [211, 212].

**Statistical analyses.** Statistical values were defined using a two tailed unpaired Student’s $t$-test, where a $P$ value <0.05 was considered significant.
3.4 Results

*miR-181a expression is upregulated by TGF-β and correlates with the metastatic potential of breast cancer cells.*

Several studies have recently depicted the role of TGF-β in upregulating the expression of several oncogenic miRNAs via transcriptional and miRNA processing mechanisms [103, 164, 213]. Along these lines, we recently demonstrated the ability of biomechanically rigid ECM to regulate the differential responses of normal versus malignant mammary epithelial cells (MECs) to TGF-β [121]. Likewise, increasing the rigidity of tumor microenvironments not only drives breast cancer invasion and metastasis, but also diminishes the effective delivery and penetration of chemotherapeutic agents, thereby safeguarding the survival of the developing tumor [18, 176]. However, the role of ECM rigidity in dictating miRNA expression governed by TGF-β remains undefined. As a means to fill this knowledge gap, we utilized 3D-organotypic cultures whose physical properties were altered by inclusion of type I collagen to create biomechanically rigid microenvironments that approximated those typically observed in primary mammary tumors (Figure 3.1A; [121, 176, 196]). Compliant 3D-organotypic cultures (*i.e.*, no collagen supplementation) were also generated to recapitulate the biomechanical properties of pulmonary microenvironments typically encountered by disseminated breast carcinoma cells (Figure 3.1B; [18, 121]). The murine 4T1 progression series represents an established model of TNBC development and metastasis, and consists of isogenically-derived nonmetastatic 67NR, systemically invasive 4T07, and highly metastatic 4T1 cells [214] that were propagated for 6 days in the absence or presence of TGF-β in either rigid or compliant 3D-cultures (Figure 3.1A, B). Afterward,
total RNA was extracted and subjected to miRNA profiling. It is important to note that
the ensuing discussion only relates to miRNAs whose expression was significantly
induced by TGF-β. The presentation and discussion of miRNAs whose expression was
significantly downregulated by TGF-β will be presented elsewhere. As such, Figure 3.1C
and D clearly show that the coupling of TGF-β to the induction of miRNA expression
was highly dependent upon tissue compliance. For instance, when propagated under
biomechanically rigid 3D-organotypic cultures, TGF-β significantly induced the
expression of 3 miRNAs in 67NR cells, 11 miRNAs in 4T07 cells, and 10 miRNAs in
4T1 (Tables 3.4-3.6). In stark contrast, administering TGF-β to these same cells when
propagated in biomechanically compliant 3D-organotypic cultures significantly
stimulated the expression of 27, 5, and 12 miRNAs in 67NR, 4TO7, and 4T1 cells
respectively (Figure 3.1D and Tables 3.4 - 3.6). Interestingly, only miRNAs belonging to
the miR-181 family were universally upregulated by TGF-β in all 3 isogeneic cell lines
and 3D-organotypic culture conditions, suggesting that TGF-β functions as a master
regulator of this microRNA family (Figure 3.1C, D). Accordingly, examining the
expression levels of miR-181a by semi-quantitative real-time PCR demonstrated that
TGF-β was significantly more effective in stimulating miR-181a expression in metastatic
4T1 cells as compared to their weakly metastatic 67NR counterparts. Importantly, the
coupling of TGF-β to miR-181a expression readily transpired in both rigid and compliant
3D-organotypic cultures (Figure 3.1E, F), as well as in traditional 2D-culture systems
(Figure 3.1G). Moreover, these analyses showed that the magnitude of miR-181a
expression stimulated by TGF-β correlated positively with the metastatic potential of
these isogeneic derivatives (Figure 3.1E-G). To ensure that these findings were not
limited to the 4T1 progression series, we also compared the ability of TGF-β to induce the expression of miR-181a in human MCF-7 (i.e., luminal and nonmetastatic) and MDA-MB-231 (i.e., triple-negative and metastatic) breast cancer cells. Although MCF-7 cells respond to TGF-β (Figure 3.2;[215]), miR-181a expression remained low and unresponsive to TGF-β in MCF-7 cells, an event that was in stark contrast to the significant induction of miR-181a expression in MDA-MB-231 cells both prior and after their stimulation with TGF-β (Figure 3.1H). Recently, MCF-7 cells were observed to downregulate miR-181a in response to estrogen administration [216], and as such, this finding together with those presented herein suggest that the induction of miR-181a expression by TGF-β may be selectively sustained in metastatic TNBCs. Accordingly, TGF-β treatment of nonmetastatic cells over a span of 48 hours led to a transient increase in miR-181a expression that peaked at 16 hours and thereafter declined during the ensuing time points to 48 hours (Figure 3.3A, B). In stark contrast, miR-181a expression was progressively increased and sustained in metastatic TNBCs upon completion of the TGF-β treatment (Figure 3.3A, B). Thus, metastatic human and murine breast cancers stably upregulate miR-181a in response to TGF-β. Interestingly, conflicting reports in the literature show that TGF-β-mediated upregulation of miR-181a occurs through either transcriptional [173] or post-translational mechanisms [171]. Moreover, miR-181a is transcribed from two separate genomic loci that give rise to two separate pre-miRNA, namely pre-miR-181a-1 and pre-miR-181a-2 (http://www.mirbase.org/). Our own analyses indicate that the transcription of pre-miR-181a-1 transpires through a Smad4-dependent mechanism, while that of pre-miR-181a-2 occurs independent of Smad4 (Figure 3.4A, B). However, levels of mature miR-181a are not affected by Smad4
depletion (Figure 3.4C), indicating that Smad4-independent processing steps are likely to occur after production of the pre-miR-181a transcripts.

TGF-β has previously been reported to regulate the expression of miRNAs 181a and 181b in hepatocytes [173, 208], and perhaps in cancer initiating cells of the breast as well [171]. Accordingly, TGF-β significantly upregulated miR-181b expression in the 4T1 progression series in both rigid and compliant 3D-organotypic cultures, in traditional 2D-cultures, and predominantly in metastatic MDA-MB-231 cells as compared to nonmetastatic MCF-7 cells (Figure 3.5A, B & Figure 3.6A, B). Moreover, the extent to which TGF-β coupled to the expression of the remaining miR-181 family members, namely miRNAs 181c and 181d, generally trended with those delineated for their miRNA 181a and 181b counterparts (Figure 3.5C-F & 3.6C-F). Collectively, these findings identify TGF-β as a master regulator of the expression of miR-181 family members, as well as implicate the expression of these miRNAs as potential mediators and biomarkers of breast cancer metastasis, particularly that in TNBCs.

Inactivation of miR-181a attenuates TGF-β-mediated EMT, invasion and migration. Given the parallels between miR-181a expression and metastatic potential, we next sought to investigate the probable role of miR-181a during EMT programs, which are critically involved in several aspects of metastasis stimulated by TGF-β [7]. In doing so, we utilized NMuMG (normal murine mammary gland) cells, which are used routinely as a model system for studying the molecular mechanisms whereby TGF-β promotes EMT [66, 88, 121, 217]. Figure 3.7A shows that TGF-β treatment of NMuMG cells under experimental conditions known to induce EMT programs resulted in the significant
upregulation of miR-181a expression. Interestingly, although the expression of miR-181a Mimics in NMuMG cells (Figure 3.7B) failed to alter their ability to remodel the actin cytoskeletal system during EMT reactions (Figure 3.7D), we did observe the expression of miR-181a antagonists (Figure 3.7C) to attenuate the formation of actin stress fibers in NMuMG cells stimulated with TGF-β (Figure 3.7D). Along these lines, miR-181a Mimics enhanced the extent to which TGF-β downregulated E-cadherin expression in transitioning NMuMG cells (Figure 3.7E). Likewise, inactivation of miR-181a in NMuMG cells prevented the loss of E-cadherin expression stimulated by TGF-β (Figure 3.7F). Taken together, these results indicate that miR-181a participates in mediating the induction of EMT programs stimulated by TGF-β.

An essential manifestation of EMT programs is its ability to confer transitioned cells with highly motile and invasive phenotypes, and as such, we investigated the impact of manipulating miR-181a activity on the coupling of TGF-β to these cellular processes in nonmetastatic 67NR (Figure 3.8A) and metastatic 4T1 (Figure 3.8B) cells. Figure 3.8C and D show that inactivating miR-181a decreased the ability of either cell type to invade to a serum stimulus. Indeed, nonmetastatic 67NR cells failed to invade when stimulated by TGF-β, a response that was unaffected by alterations in miR-181a activity (Figure 3.8C). However, inactivating miR-181a in metastatic 4T1 cells significantly impaired their ability acquire invasive phenotypes in response to TGF-β (Figure 3.8D). Importantly, the reduced capacity of these cell lines to invade reconstituted basement membranes was not due to differences in their rates of DNA synthesis (Figure 3.8E, F). Because expression of miR-181a Mimics failed to augment basal and TGF-β-stimulated invasion in either 67NR or 4T1 cells (Figure 3.9), these findings suggest that the
expression of miR-181 is necessary, but not sufficient in driving breast cancer invasion stimulated by TGF-β.

We also engineered 4T1 cells to stably express miR-181a, whose functionality was confirmed by detecting decreased renilla luciferase activity driven by a miR-181a biosensor (Figure 3.8G). Likewise, stable antagonism of miR-181a (i.e., miArrest 181a expression) significantly increased the activity of the miR-181a biosensor in 4T1 cells, a finding indicative of diminished miR-181a activity in these metastatic breast cancer cells (Figure 3.8H). Importantly, we observed elevated miR-181a activity to significantly increase both basal and TGF-β-stimulated wound closure (Figure 3.8I), while the converse manipulation of miR-181a activity abrogated cell migration stimulated by TGF-β (Figure 3.8J). We also found stable increases or decreases in miR-181a activity to be incapable of affecting 4T1 proliferation in traditional 2D-culture systems (data not shown). Collectively, these findings indicate that the upregulated expression of miR-181a enhances the motility and invasion of breast cancer cells, presumably by augmenting the coupling of TGF-β to the induction of EMT programs (Figure 3.8).

miR-181a expression enhances Erk1/2, Akt, and Src signaling in breast cancer cells.

We next addressed whether changes in miR-181a activity were capable of altering the coupling of TGF-β to its downstream effectors, particularly those coupled to metastatic progression. Transmembrane signaling by TGF-β transpires following its activation of canonical Smad2/3/4 signaling, as well as its stimulation of a variety of noncanonical effectors, including MAP kinases, PI3K/Akt, and NF-κB [3, 7, 207, 218]. The coupling of TGF-β to canonical Smad-based signaling is generally associated with the tumor
suppressing functions of TGF-β and predominates in normal MECs, which contrasts sharply with malignant MECs and their amplification of noncanonical TGF-β signaling that underlies its oncogenic functions in late-stage breast cancers [3]. Consistent with this model, we failed to observe any changes in the ability of TGF-β to activate Smads 2 and 3 in miR-181a-manipulated 4T1 cells (Figure 3.10A, B). Thus, alterations in miR-181a expression do not affect the initial activation of canonical signaling by TGF-β. Interestingly, elevating the activity of miR-181a enhanced the basal phosphorylation status of Src, Akt, and Erk1/2 (Figure 3.10C, E). Likewise, diminishing the activity of miR-181a decreased both the basal and TGF-β-stimulated phosphorylation of Src, Akt, and Erk1/2 in 4T1 cells (Figure 3.10D, F). Taken together, these findings suggest that elevated miR-181a functions to enhance the auto-activation of noncanonical TGF-β effectors, thereby mimicking oncogenic TGF-β signaling in metastatic breast cancer cells.

miR-181a inhibits anoikis by targeting the pro-apoptotic protein Bim for downregulation.

The ability to overcome anoikis is paramount in promoting hematogenous dissemination of carcinoma cells [219]. As such, we hypothesized upregulated miR-181a activity as a potential player in conferring breast cancer cell resistance to anoikis. We tested this supposition by culturing NMuMG and 4T1 cells over polyhydroxymethylmethacrylate (poly-HEMA) to prevent their adherence to plastic, thereby eliciting anoikis reactions. Figure 3.11A shows that parental NMuMG cells readily initiated apoptosis and caspase-3 cleavage in as little as 4 h following adhesion deprivation. Importantly, elevating miR-181a activity completely inhibited the cleavage of caspase-3 in NMuMG cells that were
suspended over a span of 24 h (Figure 3.11A), indicating that miR-181a expression was sufficient to abrogate anoikis in NMuMG cells. Accordingly, inhibiting miR-181a expression in 4T1 cells sensitized these carcinoma cells to more rapidly undergo anoikis when deprived of adherence as compared to their parental counterparts (Figure 3.11B). The administration of TGF-β during this process failed to significantly impact the induction of anoikis (Figure 3.12A, B). However, pretreating 4T1 cells with the small molecule TβR-I antagonist, TβR-I Inhibitor II, abrogated the high levels of autocrine TGF-β signaling in these cells [88, 89, 121, 220], thereby accelerating the rate of caspase-3 cleavage during anoikis in parental 4T1 cells (Figure 3.12C). Importantly, miR-181a overexpression partially protected 4T1 cells from exhibiting accelerated caspase-3 cleavage and anoikis elicited by administration of the TβR-II inhibitor II (Figure 3.12D). Thus, TGF-β and miR-181a mediate the survival of nonadherent metastatic breast cancer cells.

To identify mRNA targets of miR-181a that are relevant to anoikis, we interrogated TargetScan [221] and miRanda [222] miRNA target prediction programs in combination with Ingenuity Pathway Analysis. In doing so, we identified the pro-apoptotic BH3-only protein Bim as a possible target of miR-181a. Bim promotes apoptosis by binding to the pro-survival protein Bcl-2 and Bcl-XL, thereby engendering the release of Bax and Bak necessary to initiate programmed cell death [223]. miR-181a has previously been reported to target several Bcl-2 family members in astrocytes, glioblastomas, and hematologic malignancies [170, 224, 225]. Moreover, the 3’UTR of Bim houses a sequence that matches the 7-mer seed sequence contained in miR-181a (Figure 3.11C). Accordingly, elevating miR-181a activity in NMuMG or 4T1 cells
reduced Bim protein levels (Figure 3.11D), while diminishing miR-181a activity elevated the expression of Bim in these same cells (Figure 3.11E). Moreover, transient transfection of a Bim-3’UTR-luciferase reporter into NMuMG (Figure 3.11F) or 4T1 (Figure 3.11G) cells that stably expressed miR-181a demonstrated that miR-181a did indeed decrease the expression of luciferase driven by the Bim-3’UTR. Importantly, mutating the miR-181a seed sequence located within the Bim-3’UTR prevented miR-181a from suppressing luciferase expression driven the Bim-3’UTR in NMuMG and 4T1 cells (Figure 3.11F, G). Thus, miR-181a regulates Bim expression by binding to its complementary seed sequence in the Bim-3’UTR. It should be noted that Bim mRNA levels were not significantly impacted by increasing or decreasing miR-181a activity in these same MECs (Figure 3.13A-E), indicating that miR-181a regulates cellular levels of Bim by repressing the translation of Bim mRNA, not by inducing its cleavage and degradation. Additionally, pharmacological inhibition of Erk1/2 or Akt failed to alter the ability of miR-181a to decrease Bim protein levels (Figure 3.13 F-H), indicating the miR-181a regulates Bim expression independently of signaling inputs derived from Erk1/2 and Akt. Finally, we attempted to rescue Bim expression by transiently transfecting miR-181a-expressing NMuMG cells with a Bim expression construct that lacked its 3’UTR sequence that is targeted by miR-181a. Figure 3.11H shows that this manipulation not only restored Bim expression to NMuMG cells, but also sensitized them to anoikis and caspase-3 cleavage. Taken together, these findings suggest that miR-181a plays a critical role during the metastatic progression of mammary tumors by downregulating Bim expression, thereby engendering disseminated breast cancer cells with the ability to overcome the physiological barrier imposed by anoikis.
Inhibition of miR-181a abrogates pulmonary tumor outgrowth and increases survival in mice.

After mammary carcinoma cells escape the primary tumor, circumvent anoikis, traverse the circulation, and invade disseminated organ sites, they ultimately need to reinitiate proliferative programs operant in mediating secondary tumor lesions. As an initial means to investigate the role of miR-181a in regulating metastatic outgrowth, 4T1-luciferase cells engineered to express either miR-181a or a miR-181a sponge (miArrest 181a) were propagated at low densities onto compliant 3D-organotypic cultures to recapitulate the pulmonary microenvironment [18, 123, 127, 226]. Indeed, we recently demonstrated that TGF-β suppresses the outgrowth of 4T1 organoids in compliant 3D-culture systems [121]. Using bioluminescent growth assays, we now show that elevating miR-181a activity circumvented the cytostatic activities of TGF-β in compliant microenvironments (Figure 3.14A). Thus, upregulated miR-181a activity may play an additional and important role in overcoming metastatic dormancy. Along these lines, inactivation of miR-181a in 4T1 cells significantly impaired their ability to thrive in 3D-pulmonary cultures (Figure 3.14B), suggesting that measures capable of reducing miR-181a activity may provide a novel therapeutic mechanism to halt the outgrowth of breast cancer micrometastases.

To more rigorously test the above supposition in a preclinical setting, we inoculated the aforementioned 4T1 derivatives into the lateral tail vein of 4-wk-old female Balb/C mice. Initial carcinoma cell seeding and subsequent pulmonary outgrowth of 4T1 cells were monitored by biweekly bioluminescent imaging. Inhibiting miR-181a
activity decreased pulmonary tumor burden by 4T1 cells (Figure 3.14C), resulting in significantly increased survival time in mice (Figure 3.14D). We also investigated the affect of miR-181a expression on 4T1 tumor growth and metastasis from the mammary fat pad. Although manipulation of miR-181a levels failed to significantly alter 4T1 tumor latency, growth, and dissemination from the mammary fat pad (Figure 3.15), we were able to verify that lung metastases that arose from miArrest 181a-expressing 4T1 tumors had in fact lost expression of the miR-181a sponge as determined by elevated miR-181a biosensor activity in ex vivo cultures of 4T1 lung metastases (data not shown). This result suggests that 4T1 cells that possessed low levels of miR-181a activity were negatively selected during the development and metastasis of 4T1 tumors. Accordingly, immunostaining of 4T1 tumors revealed that those derived from miArrest 181a-expressing 4T1 cells exhibited elevated levels of apoptosis as measured by increased TUNEL staining and Bim immunoreactivity (Figure 3.14E). Similar upregulation of TUNEL staining and Bim immunoreactivity were readily detected in the lung metastases produced by miArrest 181a-expressing 4T1 cells, events that were lacking in their parental counterparts (Figure 3.14F). Collectively, these findings suggest that miR-181a antagonism sensitizes breast cancer cells to undergo enhanced apoptosis via upregulated expression of Bim, an event that may synergize with co-administration of additional DNA damaging agents to induce carcinoma cell death.

miR-181a is upregulated in TNBCs and corresponds to decreased overall survival times and increased metastasis in breast cancer patients.

To examine the prognostic value of miR-181a expression in human breast cancers, we performed semi-quantitative real-time PCR to monitor miR-181a expression levels in 41
matched cases and adjacent normal tissue samples (Table 3.7; [227]). In doing so, we determined that TNBCs harbored significantly more miR-181a as compared to non-TNBC subtypes (Figure 3.16A and Table 3.7). These findings, together with those presented in Figure 3.1H, indicate that the upregulated expression of miR-181a is significantly associated with TNBCs relative to other non-TNBC subtypes.

Finally, we also interrogated a publicly available dataset that was comprised of 101 human primary breast tumor samples that were subjected to genome-wide matched miRNA and mRNA profiling [210]. These analyses showed that the expression of miR-181a significantly predicted for shorter disease-free survival of breast cancer patients whose tumors lacked amplification of the ErbB2 locus (Figure 3.16B). Moreover, gene set enrichment analysis (GSEA) of the mRNA expression in this same dataset clearly showed that mammary tumors harboring high levels of miR-181a expression were significantly enriched for TGF-β-signaling [228, 229], thereby providing further support for the notion that TGF-β is a master regulator of miR-181a expression in breast cancers. Equally important, we also noted that mammary tumors possessing elevated expression of miR-181a were enriched for the van’t Veer breast cancer metastasis signature [230], which comprises a 70-gene signature that forms the basis for the MammaPrint test that is used clinically to identify breast cancer patients who are at greater risk of developing metastatic disease. Although additional studies are needed to define the role of miR-181a in other specific breast cancer subtypes, our findings nonetheless clearly support an essential function of miR-181a to enhance breast cancer metastasis and decrease the long-term survival of breast cancer patients harboring metastatic disease. Collectively, our
results implicate miR-181a expression as a potential predictive biomarker to identify breast cancer patients who are at high risk for developing metastatic disease.

3.5 Discussion

It is well established that miRNAs can function as tumor suppressors (e.g., miR-15a and miR-16-1) and tumor promoters (e.g., miR-155 and miR-21) [231]. More recently, miRNAs have also been implicated in regulating specific steps in the metastatic cascade. Indeed, these “metastamirs” often exhibit little-to-no effect on primary tumor development, but instead can elicit profound effects on either promoting (e.g., miR-10b, miR-143, miR-520c) or suppressing metastasis (e.g., miR-31, miR-146a/b, miR-335) [232, 233]. Because metastasis is the major cause of death for breast cancer patients, it stands to reason that defining the molecular mechanisms whereby miRNAs impact metastasis may provide novel opportunities to treat metastatic breast cancers.

We [18, 111, 121] and others [120, 234] have clearly demonstrated the ability of alterations in the biomechanics of the ECM to impact how cells sense and respond to cellular stimuli, including TGF-β. Here we significantly expand this theme by demonstrating that changes in ECM rigidity influence coupling of TGF-β to the expression of miRNAs during breast cancer progression (Figure 3.1, Tables 3.4-3.6). However, it is important to note that individual miRNAs regulate multiple mRNAs and likewise, that individual mRNAs are often targeted by multiple miRNAs. Thus, it is likely that the collective actions of numerous miRNAs acting on a host of target mRNAs ultimately contributes to the oncogenic functions of TGF-β and its stimulation of breast cancer metastasis. As such, future studies need to map the combined actions of miRNA
networks regulated by TGF-β and ECM stiffness, and to determine how these events vary between specific breast cancer subtypes and stages of their development.

Along these lines, our current study provides the initial framework to begin these analyses within the context of TNBCs and their acquisition of metastatic phenotypes in response to TGF-β. Indeed, we identified miR-181a as a “metastamir” in TNBCs and demonstrated that measures capable of inhibiting miR-181a activity abrogated TGF-β-mediated EMT, migration, invasion, and metastatic outgrowth in part by upregulating Bim expression. Besides its coupling to Bim expression, miR-181a/b has also been validated to regulate the expression of Bcl-2 [170, 224, 225, 235], ATM [171], p27 [208], K-Ras [236], and TIMP3 [173]. In the context of TNBCs and the experimental systems used herein, we were unable to associate elevated miR-181a activity to changes in the expression of KLF-6, Smad7, TIMP3, Dusp6, or Bcl-2 (data not shown), suggesting that the manifestations of aberrant miR-181a expression will be determined in a cell- and context-specific manner. Accordingly, recent evidence shows that miR-181a is capable of exhibiting seemingly paradoxical roles in cancer. For example, miR-181a expression is decreased in several cancers, including those of the lung and brain [169]. Indeed, elevating miR-181a activity in glioblastomas silences Bcl-2, thereby sensitizing these cells to radiotherapy [170]. Thus, in the context of glioblastomas, the expression of miR-181a functions as a tumor suppressor. In stark contrast, aberrantly elevated expression of miR-181a is observed in cancers of the breast [171], mouth [172], liver [173], and blood [167, 168], suggesting that miR-181a fulfills a tumor promoting role in these contexts. Collectively, these findings and those presented herein highlight the need to identify
additional miR-181a targets and define their role in promoting breast cancer development and metastatic progression, particularly that stimulated by TGF-β.

miR-181a belongs to the miR-181 family that contains three other members (i.e., miRs 181b, c, and d), all of which house identical seed sequences, suggesting that this miRNA family may exhibit redundancy in targeting mRNAs. The exact mechanism through which TGF-β controls the expression of miR-181 family members remains to be determined. Interestingly, miR-181a and miR-181b are transcribed together from two separate genomic loci, while miR-181c and miR-181d are transcribed from a third distinct locus (http://www.mirbase.org/). Our findings indicate that Smad4-dependent and independent mechanisms contribute to TGF-β-mediated transcription of miR-181a; however, miRNA transcript processing that produces mature miR-181a occurred in a Smad4-independent manner (Figure 3.4A-C). Thus, future studies need to parse out the relative contributions of transcriptional versus post-transcriptional mechanisms in mediating TGF-β stimulation of miR-181 family members, as well as to identify the TGF-β effector molecules operant in mediating these events.

Despite the aforementioned knowledge gaps, our findings do in fact demonstrate that miR-181a plays a unique role in promoting breast cancer metastasis, as the sole inactivation of miR-181a elicited dramatic effects on the ability of breast cancer cells to acquire and maintain EMT, metastatic, and anti-anoikis phenotypes. Likewise, TGF-β clearly upregulates all miR-181 family members and as such, future studies need to determine the specific function and contribution played by miR-181b/c/d during mammary tumorigenesis, as well as the extent to which these events are unique or shared amongst members of this miRNA family. This point is important because even though
related miRNAs can exhibit identical seed sequences, they nevertheless can target distinct mRNAs [237]. Thus, we suspect that miR-181b/c/d are likely to mediate distinct aspects of mammary tumorigenesis that are unique from those currently ascribed to miR-181a.

Evasion of apoptosis is a hallmark of cancer [238], while the evasion of anoikis represents a critical barrier that tumor cells must overcome to metastasize [219]. Normally, cell detachment leads to upregulation of Bim, thereby triggering anoikis [219]. Our data shows that high miR-181a expression repressed that of Bim, thus rendering normal MECs insensitive to anoikis (Figure 3.11). Conversely, inhibition of miR-181a activity sensitized malignant MECs to anoikis (Figure 3.11). Interestingly, previous reports have implicated Bim as a critical mediator of chemotherapy-induced apoptosis in numerous cancers [239]. For example, Bim expression predicts for an apoptotic response to EGFR inhibitors in EGFR-mutant lung cancers [239]. Likewise, high Bim levels are necessary in (i) sensitizing HER2-amplified breast cancer cells to undergo apoptosis in response to lapatinib [239], and (ii) mediating breast cancer apoptosis induced by paclitaxel [240]. Along these lines, chronic myeloid leukemia patients with low Bim levels have been shown to respond poorly to Imatinib [241]. Thus, diminished Bim expression not only promotes metastasis, but also supports the development of chemoresistant phenotypes. Our findings suggest that combining miR-181a antagonists with other standard-of-care chemotherapies may provide synergistic benefits by increasing Bim levels, leading to heightened tumor cell sensitivity to apoptosis. Future studies need to address this hypothesis, as well as explore the utility of miR-181a as a predictive biomarker for breast cancer metastasis and overall patient survival.
3.6 Acknowledgments

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Figure 3.1: miR-181a expression is upregulated by TGF-β and correlates with the metastatic potential of breast cancer cells.

(A&B) Nonmetastatic 67NR, systemically invasive 4T07, and broadly metastatic 4T1 cells were propagated in the absence or presence of TGF-β1 (5 ng/ml) for 6 days in either rigid (3 mg/ml collagen) or compliant 3D-organotypic cultures (20X magnification). Total RNA was harvested and hybridized to miRIDIAN miR arrays by Dharmacon. (C&D) Venn diagrams depicting miRNAs induced by TGF-β in 67NR, 4T07, and 4T1 cells revealed that the miR-181 family was upregulated by TGF-β1 treatment in all breast cancer cell lines and treatment conditions. (E&F) TGF-β1 (5 ng/ml) treatment of 67NR, 4T07, and 4T1 cells propagated in either rigid (3 mg/ml collagen, E) or compliant (F) 3D-organotypic cultures increased miR-181a expression in a manner correlated with the metastatic potential of individual breast cancer cell lines. (G&H) TGF-β1 (5 ng/ml) treatment of mouse 67NR, 4T07, and 4T1 (G), and of human MCF-7 and MDA-MB-231 (H) cells propagated in traditional 2D-cultures stimulated miR-181a expression in a manner correlated with the metastatic potential of the individual cell lines. Individual miR signals were normalized to U6 and the data are presented as the mean (±SE; n=3) fold expression of miR-181a relative to basal 67NR or MCF-7 cells (*P<0.05; Student’s t-test).
Figure 3.1

**A** Rigid (Primary Tumor Microenvironment)

**B** Compliant (Pulmonary Microenvironment)

**C** Rigid

**D** Compliant

**E** 3D Rigid

**F** 3D Compliant

**G** 2D Cell Culture

**H** 2D Cell Culture

- mmu-miR-181a
- mmu-miR-181b
- mmu-miR-181d
Table 3.4: miRs upregulated by TGF-β in 67NR cells

<table>
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<th>Compliant</th>
<th>Fold Upregulation</th>
<th>P-value</th>
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Table 3.5: miRs upregulated by TGF-β in 4T07 cells

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Table 3.6: miRs upregulated by TGF-β in 4T1 cells

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Figure 3.2: MCF 7 Cells respond to TGF-β

MCF-7 cells were stimulated with TGF-β1 (5 ng/ml) for 30 min. Afterward, the phosphorylation of Smad2 (arrowhead) was measured by immunoblotting, and differences in protein loading were monitored by reprobing stripped membranes with anti-β-actin antibodies. Images are representative of 2 similar experiments.
Figure 3.2

\[
\begin{array}{c}
\text{TGF-}\beta \\
\hline
- & + \\
\text{pSmad2} & \text{} \\
\text{}\beta\text{-Actin} & \text{} \\
\end{array}
\]

MCF-7
Figure 3.3: High levels of miR-181a are maintained in malignant cells over time.

Time-course of miR-181a expression induced by TGF-β in nonmetastatic and metastatic human and murine breast cancer cells. Murine 67NR, 4T07, and 4T1 cells (A) or human MCF-7 or MDA-MB-231 cells (B) were stimulated with TGF-β1 (5 ng/ml) for varying times over a span of 48 h as indicated, at which point the expression of miR181a was determined by semi-quantitative real-time PCR.
Figure 3.3
**Figure 3.4: Smad4 depletion does not effect mature miR-181a levels.**

Regulation of pre-miR-181a expression and processing by canonical Smad4 signaling. Control (shScram) and Smad4-deficient (shSmad4) MDA-MB-231 cells were stimulated with TGF-β1 (5 ng/ml) for 48 h, at which point the expression of pre-miR-181a-1, pre-miR-181a-2, and miR-181a was determined by semi-quantitative real-time PCR. Individual signals were normalized to those of U6. Data are the mean (±SE; n=3) fold expression of pre-miR-181a-1 (A), pre-miR-181a-2 (B), or miR-181a (C) relative to basal expression levels. (*P<0.05; Student’s t-Test).
Figure 3.4
Figure 3.5: TGF-β is a master regulator of the miR-181a family in 3D culture.
TGF-β stimulates the expression of miR-181 family members in 3D-organotypic breast cancer cultures. TGF-β1 (5 ng/ml) treatment of 67NR, 4T07, and 4T1 cells for 6 days in rigid (A, C, E) or compliant (B, D, F) 3D-organotypic cultures universally induced the expression of miR-181b (A&B), miR-181c (C&D), and miR-181d (E&F) as determined by semi-quantitative real-time PCR. Individual miR signals were normalized to those of U6. Data are the mean (±SE; n=3) fold expression of miR-181 family members relative to those detected in basal 67NR cells (*P<0.05; Student’s t-Test).
Figure 3.5
Figure 3.6: TGF-β is a master regulator of the miR-181a family in 2D culture.

TGF-β stimulates the expression of miR-181 family members in murine and human breast cancer cells. Murine 67NR, 4T07, and 4T1 cells (A, C, E) or human MCF-7 or MDA-MB-231 (B, D, F) were stimulated with TGF-β1 (5 ng/ml) 48 h, at which point the expression of miR-181b (A&B), miR-181c (C&D), and miR-181d (E&F) was determined by semi-quantitative real-time PCR. Individual miR-181 signals were normalized to those of U6. Data are the mean (±SE; n=3) fold expression of miR-181 family members relative to those detected in basal 67NR (A, C, E) or MCF-7 (B, D, F) cells (*P<0.05; Student’s t-Test).
Figure 3.6
Figure 3.7: Inhibition of miR-181a attenuates TGF-β-mediated EMT in normal MECs.

(A) NMuMG cells were stimulated to undergo EMT with TGF-β1 (5 ng/ml), which upregulated miR-181a expression as determined by semi-quantitative real-time PCR where individual miR signals were normalized to U6. Data are the mean (±SE; n=3) fold-expression relative to basal cells (*P<0.05; Student’s t-test). (B&C) Transient transfection of miR-181a Mimic (B) or hairpin inhibitors (C) resulted in elevated or diminished miR-181a expression in NMuMG cells. Data are the mean (±SE; n=3) fold-expression relative to corresponding controls (*P< 0.05; Student’s t-test). (D-F) miR-181a expression levels were manipulated in NMuMG cells as above and subsequently stimulated with TGF-β1 (5 ng/ml) for 48 h to induce an EMT program, which was monitored by phalloidin staining to visual alterations in the actin cytoskeleton (D), or by immunoblotting to monitor E-cadherin expression (E&F). Inactivation of miR-181a (Anti miR-181a) blunted TGF-β stimulation of EMT programs in NMuMG cells. Original images were obtained at 20X magnification and insets represent a 10X magnification of the original, all are representative findings observed in 3 independent experiments.
Figure 3.7

A

B

C

D

E

F
Figure 3.8: Inhibition of miR-181a attenuates TGF-β-mediated EMT, invasion and migration.

(A&B) An antagomiR against miR-181a (Anti-miR-181a) was transiently transfected into 67NR or 4T1 cells, resulting in decreased expression of miR-181a as determined by semi-quantitative real-time PCR. (C&D) The invasiveness of miR-181a-manipulated 67NR and 4T1 cells in response to TGF-β1 (5 ng/ml) treatment was significantly reduced by miR-181a inactivation. (E&F) The proliferation of miR-181a-manipulated 67NR and 4T1 cells in response to TGF-β1 (5 ng/ml) treatment was unaffected by miR-181a inactivation. (G&H) 4T1 cells engineered to stably express miR-181a (G) or a miR-181a sponge (H) were transiently transfected with a renilla luciferase miR-181a biosensor and CMV-b-gal, which was used to control for differences in transfection efficiency. miR-181a was shown to significantly elevate miR-181a activity, while miR-181a antagonists were shown to significantly reduce miR-181a activity. (I&J) The ability of TGF-β1 (5 ng/ml) to induce the migration of 4T1 cells with elevated (I) or reduced (J) miR-181a activity was significantly stimulated by miR-181a activation (I), or was significantly inhibited by miR-181a inactivation (J). (20X magnification). All data are the mean (±SE; n=3; *P<0.05; Student’s t-test).
Figure 3.9: Overexpression of miR-181a mimics fail to enhance breast cancer cell invasion and proliferation.

(A&B) Transient transfection of miR-181a mimics elevated miR-181a expression in 67NR (A) and 4T1 (B) cells as measured by semi-quantitative real-time PCR. Individual miR-181a signals were normalized against those measured for U6.  (C-F) The aforementioned 67NR and 4T1 variants were incubated in the absence (i.e., basal) or presence of TGF-β1 (5 ng/ml) to monitor changes in cell invasion (C&D) or DNA synthesis (E&F). All data are the mean (±SE; n=3) relative to corresponding basal activity (*P<0.05; Student’s t-Test).
Figure 3.9
Figure 3.10: miR-181a expression enhances Erk1/2, Akt, and Src signaling in breast cancer cells.

(A-F) 4T1 cells that harbored manipulated miR-181a activity as indicated were stimulated with TGF-β1 (5 ng/ml) for 30 min. Afterward, the phosphorylation and expression of Smad2/3, Erk1/2, Akt, and Src were measured by immunoblotting as indicated. Shown are representative images from 3 independent experiments. Scr, scrambled control vector. miArr, miArrest vector. Lanes in Panels B, E, & F were run on the same gel, but were noncontiguous (white lines).
Figure 3.10

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Figure 3.11: miR-181a inhibits anoikis by targeting the pro-apoptotic protein Bim for downregulation.

(A&B) NMuMG and 4T1 cells with manipulated miR-181a activity were suspended over poly-HEMA-coated culture dishes for 0-48 h to induce anoikis. Caspase-3 cleavage was monitored by immunoblotting detergent-solubilized whole-cell extracts with anti-caspase-3 antibodies, while differences in protein loading were controlled with anti-β-actin antibodies. (C) miRanda alignment of mmu-miR-181a, Bcl2l11, and mutated Bim sequences demonstrates perfect complementation between the miR-181a seed sequence and the Bim 3’UTR. *, mutated miR-181a seed sequence binding bases. (D&E) Immunoblotting NMuMG and 4T1 cell extracts demonstrated that elevated miR-181a activity decreased Bim protein levels (D), while diminished miR-181a activity elicited increased Bim protein levels. Differences in protein loading were assessed by β-actin immunoblotting. (F&G) NMuMG (F) or 4T1 (G) cells were transiently transfected with a renilla luciferase reporter gene whose expression was driven by either wild-type or mutant (mut) Bim 3’UTR seed sequences Panel C. Stable miR-181a expression suppressed that of luciferase driven by the wild-type Bim-3’UTR, an event that was lacking in cells transfected with a mut-Bim-3’UTR vectors. (H) NMuMG cells were transiently transfected with miR-181a Mimics with or without a 3’UTR-deficient Bim cDNA. Afterward, the transfectants were suspended over poly-HEMA coated culture dishes for 24 h to induce anoikis. Bim expression (left panel) and caspase-3 cleavage (right panel) were monitored as described in above. All data are representative of 3 independent experiments, or are the mean (±SE; n=3; *P<0.05; Student’s t-test). Lanes in Panels D & E were run on the same gel, but were noncontiguous (while lines).
Figure 3.11

A

B

C

D

E

F

G

H

3' uagaggggucugcagcACUJACAta 5' mmu-miR-181a
204: 5' auauaaauuuugagUGAUUCu 3' Bim
204: 5' auauaaauuuugAGAUUCu 3' Bim mut
Figure 3.12: Abrogation of TGF-β signaling sensitizes normal and malignant MECs to undergo anoikis.

(A&B) NMuMG (A) or 4T1 (B) cell derivatives were suspended over poly-HEMA-coated culture dishes and treated with TGF-β1 (5 ng/ml) for 0-24 h as indicated. The extent of anoikis was monitored by immunoblotting for cleavage of caspase-3. (C&D) 4T1 derivatives indicated were pre-treated for 48h with the TβR-I inhibitor (100 ng/ml) as indicated prior to their being suspended for 24 h over poly-HEMA-coated culture dishes to induce anoikis. Afterward, caspase-3 cleavage was monitored by immunoblotting detergent-solubilized whole-cell extracts with anti-caspase-3 antibodies. Differences in protein loading were assessed with anti-β-actin antibodies. Shown are representative images from 3 (A&B) or 2 (C&D) independent experiments.
Figure 3.12

A

\[ \text{cont. mimic} + \text{TGF-}\beta \]

\[ \text{181a mimic} + \text{TGF-}\beta \]

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NMuMG

B

\[ \text{miArrest Scram} + \text{TGF-}\beta \]

\[ \text{miArrest 181a} + \text{TGF-}\beta \]

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4T1

C

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\[ \text{T}_{\beta} \text{RI - I} \]

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4T1 Scram

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\[ \text{T}_{\beta} \text{RI - I} \]

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4T1 181a
Figure 3.13: miR-181a suppresses Bim expression by repressing the translation of its mRNA.

(A) Stable overexpression of miR-181a in NMuMG cells decreased miR-181a biosensor activity indicative of elevated miR-181a activity. (B&C) Neither stimulation of miR-181a expression (B), nor inhibition of its activity (C) in NMuMG cells affected Bim mRNA expression levels as measured by semi-quantitative real-time PCR. Bim transcript levels were normalized to those for GAPDH. (D&E) Neither stimulation of miR-181a expression (D), nor inhibition of its activity (E) in 4T1 cells affected Bim mRNA expression levels as measured by semi-quantitative real-time PCR as above. Data in Panels A-E are the mean (±SE; n=3; *P<0.05; Student’s t-Test). (F-H) Immunoblotting 4T1 cell extracts demonstrated that TGF-β decreased Bim protein levels (F), while neither MEK inhibition (U0126, 10 mM; G) or Akt inhibition (Akt Inh VIII, 1 mM; H) abrogated the ability of TGF-β or miR-181a to decrease Bim protein levels. Differences in protein loading were assessed by β-actin immunoblotting. Shown are representative images from 2 independent experiments.
Figure 3.13

A. miR-181a reporter activity in NMuMG cells. Scrambled (Scram) and miR-181a (181a) expression.

B. Bim mRNA expression in NMuMG cells. Control (cont. miR) and miR-181a (miR-181a).

C. Bim mRNA expression in NMuMG cells. Anti-control (Anti cont.) and anti-miR-181a (Anti-181a).

D. Bim mRNA expression in 4T1 cells. Scrambled (Scram) and miR-181a (miR-181a).

E. Bim mRNA expression in 4T1 cells. miR-181a arrest (mi Arrest) and scrambled (Scram).

F. Western blot analysis of TGFβ, Bim, and β-Actin in 4T1 cells.

G. Western blot analysis of TGFβ, Bim, and β-Actin in 4T1 cells treated with Scramble, 181a, and U0126 Rx.

H. Western blot analysis of TGFβ, Bim, and β-Actin in 4T1 cells treated with Scramble, 181a, and Akt Inh VIII Rx.
Figure 3.14: Inhibition of miR-181a abrogates pulmonary tumor outgrowth and increases survival in mice.

(A&B) The ability of TGF-β1 (5 ng/ml) to suppress 4T1 organoid growth was abrogated by elevated miR-181a activity (A), while basal 4T1 organoid growth was significantly suppressed by miR-181a inactivation (B). Data are the mean (±SE) bioluminescent signals obtained in 3 independent experiments completed in triplicate. (*P<0.05; Student’s t-test). Images shown are 20X magnification. (C) Luciferase-expressing 4T1 cells engineered to stably express a miR-181a antagonist (i.e., miArrest 181a) were injected into the lateral tail vein of Balb/C mice (n=5) and pulmonary tumor outgrowth was monitored by intravital bioluminescent imaging. Data are the mean (±SE; *P<0.05) pulmonary photon flux readings 24 days post-injection, while the inset shows representative bioluminescent signals of pulmonary outgrowth measured on day 24. (D) Kaplan–Meier survival curves of Balb/c mice (n=5) from Panel C. (E&F) Histopathological analyses of H&E, Ki67, TUNEL, and Bim in 4T1 primary tumors (20X magnification; E) and their pulmonary metastases (40X magnification; F) indicate that inactivation of miR-181a resulted in increased Bim expression and elevated apoptosis.
Figure 3.14

A

Fold 2D Growth

Basal

miArrest

TGF-β

miArrest

Scram

181a

B

Fold 3D Growth

miArrest

Scram

181a

C

Proliferative Outgrowth

(Average Area Flux)

Day 0

Day 24

D

Percent survival

E

H&E

Ki67

TUNEL

Bim

Scram

181a

miArrest

Scram

181a

F

H&E

Ki67

TUNEL

Bim

Scram

181a

miArrest

Scram

181a

miArrest

181a
Figure 3.15: miR-181a expression fails to affect primary tumor growth and metastatic dissemination.

(A&B) 4T1 cells engineered to overexpress miR-181a (A) or possess diminished miR-181a activity (B) were engrafted onto the mammary fat pads of 6-week-old Balb/c mice. Data are the mean (± SEM; n=5) tumor volumes quantified at the indicated times post engraftment. (C&D) Data are the mean (± SEM; n=5) bioluminescent tumor area flux units detected in the aforementioned tumor-bearing mice. (E&F) Data are the mean (±SE) bioluminescent pulmonary metastasis area flux at the indicated time points.
Figure 3.15

A. 

B. 

C. 

D. 

E. 

F.
Figure 3.16: miR-181a is upregulated in TNBCs and corresponds to decreased overall survival times and increased metastasis in breast cancer patients.

(A) Semi-quantitative real-time PCR of miR-181a expression in human TNBC (n=16) as compared to other non-TNBC (n=25) tumors. Expression levels of miR-181a were normalized to U6 levels and plotted as average fold change detected between the tumor and corresponding normal tissue. Data from 16 triple negative and 25 non-triple negative tumors, displayed as a dot plot alongside the mean and SEM (P=0.05; Student’s t-Test).

(B) In a miRNA expression data set of 82 breast cancer patients that were negative for ErbB2 amplification [210], patients that possessed high tumor expression of miR-181a exhibited significantly reduced disease-free survival times. The median value for miR-181a was used to divide the samples into high (above the median) and low (below the median) miRNA expression and the corresponding P-value was calculated by log-rank analysis.

(C) Gene Set Enrichment Analysis (GSE19783) plots for patients with breast tumors that were negative for ErbB2 amplification and expressed high levels of miR-181a demonstrated enrichment for the TGF-β signaling system [228, 229], and for the van’t Veer breast cancer metastasis signature [230].
Table 3.7: Detection of miR-181a expression levels in human breast cancers

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Tumor Grade</th>
<th>ER</th>
<th>PR</th>
<th>HER2</th>
<th>Fold miR-181a expression*</th>
</tr>
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<tbody>
<tr>
<td><strong>Triple-Negative Tumors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>III</td>
<td>-</td>
<td>-</td>
<td>Absent</td>
<td>1.39 ± 0.44</td>
</tr>
<tr>
<td>T6</td>
<td>III</td>
<td>-</td>
<td>-</td>
<td>Absent</td>
<td>2.46 ± 0.43</td>
</tr>
<tr>
<td>T13</td>
<td>III</td>
<td>-</td>
<td>-</td>
<td>Absent</td>
<td>1.54 ± 0.83</td>
</tr>
<tr>
<td>T14</td>
<td>III</td>
<td>-</td>
<td>-</td>
<td>Absent</td>
<td>14.63 ± 0.23</td>
</tr>
<tr>
<td>T15</td>
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<td>-</td>
<td>-</td>
<td>Absent</td>
<td>18.05 ± 11.72</td>
</tr>
<tr>
<td>1160</td>
<td>II</td>
<td>-</td>
<td>-</td>
<td>Absent</td>
<td>1.82 ± 0.30</td>
</tr>
<tr>
<td>1202</td>
<td>III</td>
<td>-</td>
<td>-</td>
<td>Absent</td>
<td>1.90 ± 0.55</td>
</tr>
<tr>
<td>3503</td>
<td>III</td>
<td>-</td>
<td>-</td>
<td>Absent</td>
<td>0.90 ± 0.61</td>
</tr>
<tr>
<td>1195</td>
<td>III</td>
<td>-</td>
<td>-</td>
<td>Absent</td>
<td>1.67 ± 1.46</td>
</tr>
<tr>
<td>1108</td>
<td>III</td>
<td>-</td>
<td>-</td>
<td>Absent</td>
<td>18.67 ± 18.14</td>
</tr>
<tr>
<td>3478</td>
<td>III</td>
<td>-</td>
<td>-</td>
<td>Absent</td>
<td>0.30 ± 0.11</td>
</tr>
<tr>
<td>1361</td>
<td>I</td>
<td>-</td>
<td>-</td>
<td>Absent</td>
<td>2.56 ± 0.61</td>
</tr>
<tr>
<td>3347</td>
<td>II</td>
<td>-</td>
<td>-</td>
<td>Absent</td>
<td>60.95 ± 58.19</td>
</tr>
<tr>
<td>3439</td>
<td>III</td>
<td>-</td>
<td>-</td>
<td>Absent</td>
<td>1.66 ± 1.50</td>
</tr>
<tr>
<td>3472</td>
<td>II</td>
<td>-</td>
<td>-</td>
<td>Absent</td>
<td>69.31 ± 22.34</td>
</tr>
<tr>
<td><strong>Non-Triple Negative Tumors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T12</td>
<td>III</td>
<td>-</td>
<td>-</td>
<td>Equivocal</td>
<td>10.62 ± 4.79</td>
</tr>
<tr>
<td>T2</td>
<td>III</td>
<td>-</td>
<td>-</td>
<td>Present</td>
<td>0.69 ± 0.18</td>
</tr>
<tr>
<td>3304</td>
<td>III</td>
<td>-</td>
<td>+</td>
<td>Absent</td>
<td>4.48 ± 0.21</td>
</tr>
<tr>
<td>T19</td>
<td>I</td>
<td>+</td>
<td>-</td>
<td>Absent</td>
<td>8.25 ± 3.82</td>
</tr>
<tr>
<td>T20</td>
<td>III</td>
<td>+</td>
<td>-</td>
<td>Absent</td>
<td>0.60 ± 0.19</td>
</tr>
<tr>
<td>3167</td>
<td>II</td>
<td>+</td>
<td>-</td>
<td>Absent</td>
<td>1.13 ± 0.33</td>
</tr>
<tr>
<td>3371</td>
<td>III</td>
<td>+</td>
<td>-</td>
<td>Absent</td>
<td>6.79 ± 5.44</td>
</tr>
<tr>
<td>1142</td>
<td>I</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>T1</td>
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<td>+</td>
<td>+</td>
<td>Absent</td>
<td>0.35 ± 0.22</td>
</tr>
<tr>
<td>T3</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td>Absent</td>
<td>0.78 ± 0.34</td>
</tr>
<tr>
<td>T7</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td>Absent</td>
<td>0.25 ± 0.12</td>
</tr>
<tr>
<td>T8</td>
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<td>+</td>
<td>+</td>
<td>Absent</td>
<td>3.03 ± 2.74</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>Absent</td>
<td>0.84 ± 0.38</td>
</tr>
<tr>
<td>T17</td>
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<td>+</td>
<td>+</td>
<td>Absent</td>
<td>1.04 ± 0.43</td>
</tr>
<tr>
<td>T18</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td>Absent</td>
<td>0.26 ± 0.12</td>
</tr>
<tr>
<td>T5</td>
<td>III</td>
<td>+</td>
<td>+</td>
<td>Absent</td>
<td>0.86 ± 0.50</td>
</tr>
<tr>
<td>T10</td>
<td>III</td>
<td>+</td>
<td>+</td>
<td>Absent</td>
<td>2.70 ± 0.37</td>
</tr>
<tr>
<td>3418</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td>Absent</td>
<td>2.07 ± 1.58</td>
</tr>
<tr>
<td>3458</td>
<td>III</td>
<td>+</td>
<td>+</td>
<td>Absent</td>
<td>1.51 ± 1.16</td>
</tr>
<tr>
<td>1162</td>
<td>II</td>
<td>+</td>
<td>+</td>
<td>Absent</td>
<td>3.96 ± 3.39</td>
</tr>
<tr>
<td>3380</td>
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<td>+</td>
<td>+</td>
<td>Absent</td>
<td>0.30 ± 0.03</td>
</tr>
<tr>
<td>3438</td>
<td>II</td>
<td>+</td>
<td>+</td>
<td>Absent</td>
<td>2.05 ± 0.25</td>
</tr>
<tr>
<td>3292</td>
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<td>+</td>
<td>+</td>
<td>Absent</td>
<td>19.77 ± 0.75</td>
</tr>
<tr>
<td>T11</td>
<td>III</td>
<td>+</td>
<td>+</td>
<td>Heterogeneous</td>
<td>1.66 ± 0.57</td>
</tr>
<tr>
<td>3374</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td>Unknown</td>
<td>2.31 ± 1.21</td>
</tr>
</tbody>
</table>

*Fold-change in miR-181a expression in human breast tumors was normalized against those detected in matched normal tissue.
Chapter 4 - Discussion

4.1 The role of the tumor microenvironment in mediating TGF-β signaling

Traditionally, cancer has been understood as a disease of the cell, whereby mutations are accumulated in proto-oncogenes and/or tumor suppressor genes that lead to sustained tumor growth and eventually invasion and metastasis. However, tumor biology is complex and extends beyond this cell-centric view. The role of the microenvironment is emerging as an essential player in influencing the pathology of tumor development. Indeed, microarray profiling studies of the ECM component of breast tumors has shown that an ECM signature alone can predict for breast cancer outcomes [242], and conversely, normalized environments can suppress tumorigenesis [243, 244]. TGF-β is a master regulator of the tumor microenvironment, influencing angiogenesis, enhancing fibrosis, modulating fibroblast differentiation, and mediating immune suppression. In addition, TGF-β plays an important role in modulating the biomechanical properties of the ECM through the secretion of scaffolding proteins like fibronectin and collagen, as well as through the induction of MMPs and LOX. Our findings clearly indicate that the microenvironment reciprocally regulates TGF-β signaling. Indeed, we show that exposing late-stage mammary tumors to normal (compliant) microenvironment can reinstate the cytostatic effects of TGF-β (Figure 2.5) and that this reaction is partially dependent on LOX (Figures 2.6 and 2.7). Moreover, changes in microenvironmental rigidity alter the milieu of microRNAs regulated by TGF-β (Figure 3.1 and Tables 3.4, 3.5, 3.6). As such, we have identified LOX and miR-181a as novel regulators of oncogenic signaling by TGF-β.
4.2 LOX

We have shown here that TGF-β induces the synthesis and secretion of LOX from malignant MECs and in 4T1 mammary tumors produced in mice. Moreover, this increase in LOX expression and activity enhances the tumor promoting properties of TGF-β (Chapter2). Additionally, antagonizing the expression and activity of LOX impairs the ability of TGF-β to induce EMT and invasion, as well as partially uncouples TGF-β from the activation of p38 MAPK in metastatic cells (Figure 2.4). Even more remarkably, we demonstrated that exposing late-stage breast cancer cells to compliant microenvironments is sufficient in reinstating the cytostatic function of TGF-β, a reaction that is readily reversed by rendering these same 3D-organotypic cultures mechanically rigid by the inclusion of type I collagen (Figure 2.5). Importantly, inhibiting the activities of TGF-β or LOX, or degrading hydrogen peroxide in rigid cultures abrogates oncogenic TGF-β signaling, thereby implicating LOX as an important mediator of breast cancer progression stimulated by TGF-β (Figures 2.6 and 2.7).

Given these findings, we hypothesize that tumor-initiated MECs evolve in compliant microenvironments that favor canonical Smad2/3 signaling stimulated by TGF-β. The continued growth of the developing tumor leads to molecular changes that enhance ECM rigidity by upregulating TGF-β production (Figure 2.5), which enhances LOX expression (Figure 2.1) and further promotes matrix stiffening. This increase in matrix rigidity may then lead to the inappropriate clustering of TβR-II with integrins [66-68, 115]. Once formed, these complexes recruit and interact with other growth factor
receptors that then amplify the activation of noncanonical TGF-β signaling pathways [17]. Ultimately, these events culminate in TGF-β-mediated EMT and metastasis. Distant sites of metastasis, such as the lung, possess compliant ECM tension [176], which likely reinstates the cytostatic function of TGF-β and contributes to tumor dormancy [18]. Increased LOX expression coupled with other molecular events, may reinitiate ECM stiffening, thus repeating this vicious microenvironmental cycle (Figure 4.1). The basic principles of this model are supported by the data presented in Chapter 2. However, more research is needed to identify the individual effectors that regulate ECM tension and alter cell response to TGF-β in distinct breast cancer subtypes.
Figure 4.1: Microenvironment alters TGF-β signaling and promotes tumor progression.

Tumor-initiated MECs evolve in compliant microenvironments that favor canonical Smad2/3 signaling stimulated by TGF-β. Increased rigidity, enhanced by LOX activity, leads to the inappropriate formation of integrin–TβR-II complexes and interactions with other growth factor receptors. This amplifies the activation of noncanonical effectors by TGF-β. Ultimately, these adverse events culminate in the ability of TGF-β to induce the acquisition of EMT, stem-like, and metastatic phenotypes in malignant MECs, leading to their metastasis at distant locales. Sites of metastasis possess compliant ECM tension, which may reinstate the cytostatic activities of TGF-β, perhaps contributing to tumor dormancy. Over time, this vicious microenvironmental cycle is repeated, leading to disease recurrence and poor clinical outcomes in breast cancer patients harboring metastatic disease.
Figure 4.1
4.2.1 LOX family members

Importantly, LOX is only one member of a five member gene family of copper-dependent amine oxidases (LOX, LOXL, LOXL2, LOXL3, and LOXL4) that have all been shown to function in cross-linking collagen and elastin in the ECM. At present very little is known about the specific functions of LOXL, LOXL3, and LOXL4. However, LOXL2, like LOX has been shown to play important roles in cancer progression and metastasis. Indeed, LOXL2 has been shown to be upregulated by hypoxic conditions and to promote EMT through the stabilization of Snail, leading to E-cadherin downregulation [245]. Moreover, LOXL2 has been shown to be upregulated in triple-negative breast cancers and to be clinically correlated with metastasis and decreased survival [246]. In addition, inhibition of LOX and LOXL2 has been shown to decrease primary growth and reduce metastatic burden in preclinical models of breast and ovarian cancer [246, 247].

Given our findings that TGF-β acts as a master regulator of the LOX family (Figure 2.1, Appendix Figure 1), it is likely that other family members play a role in mediating oncogenic signaling events stimulated by TGF-β. In order to study the roles of individual LOX family members in TGF-β-mediated tumor progression, future studies will need to examine which family members are upregulated by TGF-β treatment in normal (i.e. NMuMG and MCF10A), nonmetastatic (i.e. 67NR, MCF10AT1k and MCF10ACA1h), lymph node metastasizing (i.e. 168 FARN), and broadly metastatic (i.e. 4T1, MDA-MB-231, and MCF10ACa1a) cells. After determining which family members are regulated at each stage of metastasis, the creation of cell lines expressing either constitutively-active and dominant-negative forms of each LOX family member for use in functional assays
will be useful in determining the consequences of LOX family member manipulation on TGF-β-mediated proliferation, invasion, EMT, and metastasis.

4.2.2 Extracellular Roles of LOX

Collagen cross-linking and ECM stiffness have been shown to enhance invasive behavior by elevating focal adhesions and increasing PI3K signaling [115]. Indeed, women with mammographically dense breasts have an increased relative risk of developing cancer, suggesting that ECM stiffness itself acts as a molecular rheostat to potentiate oncogenic signals from the microenvironment [248]. Antibody inhibition of LOX, which specifically targets extracellular LOX, has been shown to decrease fibrosis and tumor progression, which suggests that the extracellular crosslinking activities of LOX represent a major mechanism through which LOX expression drives tumor progression and metastasis [115]. Finally, the formation of the premetastatic niche has been linked to LOX and its ability to stimulate collagen cross-linking and fibronectin production, which coalesce to recruit BMDCs to future sites of metastasis [118]. TGF-β also induces BMDC recruitment to premetastatic niches [145], suggesting a clinically important link between TGF-β and its stimulation of LOX expression in mediating the establishment of these premetastatic niches. Future studies need to demonstrate the validity of this supposition, as well as determine the chemotherapeutic effectiveness of LOX inhibition in the context of TGF-β as a novel means to alleviate oncogenic TGF-β signaling in cancers of the breast.

4.2.3 Intracellular Roles of LOX
In addition to the role of LOX in mediating ECM cross-linking reactions, recent evidence suggests that LOX also mediates intracellular signaling events. Indeed, hydrogen peroxide produced as a byproduct of LOX-mediated collagen and elastin cross-linking has been shown to act as a novel “second messenger” leading to the activation of Src and FAK, which stimulate the assembly of p130Cas/Crk/Dock180 complexes coupled to cell migration [146, 180]. Additionally, secreted LOX has been shown to be translocated from the extracellular space back into the cell [249] and has been detected in the cytoplasm and nucleus of malignant cells [136, 139]. More recently, LOXL2 was shown to catalyze the removal of H3K4me3 through deamination of the methyl lysine [250]. This suggests that LOX family members may play novel roles in mediating intracellular signaling and gene regulation. Our findings support an extracellular role for LOX in mediating the oncogenic activities of TGF-β. However, future research using expression of constitutively-active LOX in combination with catalase treatment or expression of dominant-negative forms of LOX in combination with hydrogen peroxide treatment will be useful in defining the role that LOX produced H₂O₂ plays in TGF-β-mediated EMT and metastasis. Additionally, the use of secretion-deficient LOX and nuclear localization-specific forms of LOX, will be useful to identify the specific nuclear and cytoplasmic molecules capable of interacting with LOX and to determine if these interactions are capable of altering TGF-β signaling.

4.2.4 Therapeutic targeting of LOX

Given that dysregulated signaling in the tumor microenvironment promotes tumor growth and metastasis, targeting the tumor microenvironment represents a novel method for
treating breast cancer. LOX represents an attractive candidate for therapeutic targeting of the tumor microenvironment. Indeed, numerous studies have shown in vivo efficacy of using either βAPN or a LOX-targeting antibody in preclinical disease models reducing metastatic disease in breast cancer [115, 118, 119]. Moreover, a humanized version of a LOXL2 inhibitory antibody is currently undergoing Phase I clinical trials [143] in patients with advanced solid tumors. Additionally, copper chelators have shown success in diminishing metastatic burden in preclinical and phase 2 clinical trials, which may be in part due to their ability to inhibit LOX activity [201, 251]. Our findings support the idea that LOX activity enhances oncogenic signaling by TGF-β in late-stage mammary tumors. Thus, chemotherapeutic targeting of LOX may offer a new opportunity to target TGF-β-mediated breast cancer progression.

4.3 The role of ECM rigidity on TGF-β-mediated microRNA expression

At the onset of this research there was virtually nothing known about the role of the ECM in inducing changes in microRNA expression. We have shown herein that alterations in ECM rigidity influences coupling of TGF-β to the expression of microRNAs during breast cancer progression (Figure 3.1, Tables 3.4-3.6). Interestingly, examination of the number of microRNAs differentially regulated between compliant and rigid microenvironments by TGF-β decreased with increasing metastatic potential, indicating the impact of the microenvironment on TGF-β-mediated microRNA expression decreases as malignancy increases. Indeed, 40 microRNAs were differentially regulated by the microenvironment in response to TGF-β in 67NR cells, while only 3 were commonly regulated. In contrast, in 4T1 cells only 8 microRNAs were differentially regulated by the
microenvironment, while 10 were commonly regulated by TGF-β in both rigid and compliant microenvironments (Appendix Figure 2). Along these same lines, hierarchical clustering indicated that the non metastatic 67NR and 4TO7 cells clustered based on the microenvironments in which they were cultured, while metastatic 4T1 cells only clustered on the basis of TGF-β treatment (Appendix Figure 2). These results also indicate that TGF-β induces fewer microRNAs in the more metastatic 4T1 cell line compared to the non-metastatic 67NR cell line, which fits with previous findings that there is often a global decrease in microRNA expression with tumor progression [252].

Importantly, individual microRNAs regulate multiple mRNAs and individual mRNAs are often targeted by multiple microRNAs. Thus, it is likely that TGF-β induces a complex network of microRNAs that coordinately contribute to its oncogenic functions during breast cancer progression. Ingenuity pathway analysis of the predicted mRNA targets of this TGF-β-mediated network of microRNAs indicated a collective and striking enrichment in targets involved in cell death (Appendix Figure 3 & 4). Thus, numerous TGF-β-induced microRNAs are likely to play an important role in modulating the apoptotic balance within the cell, which may serve to either promote or suppress TGF-β-mediated breast cancer progression in a context-dependent manner. Future studies need to identify the function of individual microRNAs operant in this process, as well as to map the combined actions of these TGF-β- and ECM-regulated microRNAs during breast cancer progression.

4.3.1 The Role of microRNAs in mediating ECM-induced TGF-β-mediated cytostasis
Given our initial finding that compliant ECM reinstates the cytostatic activities of TGF-β (Figure 2.5), a perplexing question that remains to be answered relates to the exact role that microRNAs play in this process. We hypothesized that microRNAs that were induced by TGF-β only in a rigid microenvironment would likely play a role in promoting tumorigenesis and metastasis, while those that were only upregulated by TGF-β under compliant conditions would likely contribute to TGF-β-mediated cytostasis. We observed that miR-99b* and miR-132 were microRNAs uniquely upregulated by TGF-β only under rigid conditions in 4T1 cells. However, individual overexpression of these microRNAs had no effect on TGF-β-mediated EMT, invasion or proliferation (Appendix Figure 5). Thus, the combined action of numerous TGF-β-mediated microRNAs may be required for robust effects on tumorigenesis. Future work will need to characterize microRNAs upregulated only under compliant conditions to investigate their possible role in TGF-β-mediated cytostasis under these microenvironmental conditions. Additionally, knockdown of Dicer in the 4T1 progression series, followed by analysis of growth in compliant and rigid 3D-organotypic cultures is necessary to address the question as to whether microRNAs are necessary for the differential growth effects of TGF-β in 3D culture.

4.3.2 TGF-β-mediated upregulation of miR-181a promotes breast cancer metastasis

We have shown that TGF-β is a master regulator of the miR-181 family, independent of ECM rigidity (Figures 3.1, 3.5, 3.6). Interestingly, the miR-181 family of microRNAs were the only microRNAs universally regulated by TGF-β independent of ECM rigidity in all cell lines examined. The findings presented in Chapter 3 identify miR-181a as a
breast cancer metastamiR. Our data show that miR-181a functions in the early stages of metastasis, enhancing TGF-β-mediated EMT, invasion and metastasis, and in the later stages of metastasis by protecting metastatic cells from anoikis by targeting Bim. Moreover, inhibition of miR-181a sensitized metastatic cells to undergo anoikis by increasing Bim expression. In addition miR-181a inhibition abrogated in vivo pulmonary outgrowth of 4T1 cells. As metastasis is responsible for the death of the majority of breast cancer patients, this suggests that miR-181a may provide an innovative therapeutic target to treat metastatic breast cancers.

4.3.3 TGF-β regulation of miR-181 family

miR-181a belongs to the miR-181 family, which contains three other members (i.e. miRs 181b, c, and d), all of which house identical seed sequences, suggesting that there may be functional redundancy in the mRNA targets of this microRNA family. miR-181a and miR-181b are transcribed together from two separate genomic loci, while miR-181c and miR-181d are transcribed from a third distinct locus. Interestingly, although expression levels of all of the other miR-181 family members were tightly coupled to TGF-β treatment (Figures 3.1, 3.5, 3.6), only expression of miR-181a highly corresponded with metastatic potential (Figure 3.1). Accordingly, examination of miR-181 family expression levels in a publicly available dataset indicated that only miR-181a significantly predicted for shorter disease-free survival of breast cancer patients whose tumors lacked ErbB2 amplification (Figure 3.16 and Appendix Figure 8). This indicates that, while all miR-181 family members are induced by TGF-β, miR-181a plays a unique role in the promotion of metastasis in breast cancer. Furthermore, although all miR-181
family members contain the same seed sequence, it is likely that they may target unique mRNAs. Further support for this idea comes from the recent creation of miR-181 family knockout mice, where miR-181a/b-1 knockout attenuated the development of NOTCH-induced acute lymphoblastic leukemia, while knockout of miR-181a/b-2 or miR-181c/d did not recapitulate this response [253]. Future work, utilizing breast cancer cells where each family member is overexpressed or inhibited, is needed to identify the functional effects of miR-181b, c, and d expression on TGF-β-mediated tumor promotion in breast cancer as well as to identify their individual and overlapping mRNA targets.

4.3.4 mRNA targets of miR-181a

4.3.4.1 Bim as a target of miR-181a

We have shown that miR-181a exerts its biological effects in part by promoting the downregulation of the pro-apoptotic protein Bim (Figures 3.12, 3.13). Normally, cell detachment leads to upregulation of Bim, triggering anoikis. Our data shows that high levels of miR-181a repress Bim, thus rendering malignant cells insensitive to anoikis and allowing them to overcome a critical barrier to metastasis. Conversely, inhibition of miR-181a activity was capable of sensitizing malignant MECs to anoikis (Figure 3.11). This finding is interesting in light of the idea that microRNAs play a role in influencing how cells respond to environmental stress [254, 255]. As the tumor microenvironment is an inherently stressed milieu, it stands to reason that even small changes in microRNA expression levels may shift the balance of protein expression within the cell, leading to dramatic phenotypic changes. Along these lines, while miR-181a overexpression alone is not sufficient to confer metastatic capability to nonmetastatic 4TO7 cells (Appendix
Figure 6), miR-181a inhibition can dramatically shift the homeostatic balance of a cell from pro-survival to pro-apoptotic by increasing Bim protein levels (Figures 3.11). Furthermore, examination of metastatic explants from miArrest 181a-expressing 4T1 tumors indicated that lung metastases had lost expression of the miR-181a sponge, as determined by decreased miR-181a biosensor activity in *ex vivo* cultures of 4T1 lung metastases (Appendix Figure 7). This suggests that low levels of miR-181a are negatively selected during metastatic dissemination.

4.3.4.2 Additional mRNA targets of miR-181a

Our work (Chapter 3), clearly indicates that Bim modulation by miR-181a is important for engendering cells with the ability to overcome anoikis. However, microRNAs generally regulate multiple targets [256]. Indeed, using four of the most common microRNA target prediction programs, microT, microRNA.org, miRDB and TargetScan, gives anywhere from 731-6,890 predicted targets of miR-181a depending on which algorithm is employed [257-261]. Of this long list of predicted miR-181a targets, we investigated KLF-6, Smad7, TIMP3, Dusp6, and Bcl-2 as possible miR-181a targets known to be involved in cancer or TGF-β signaling. Although, KLF-6, TIMP3, Dusp6 and Bcl-2 have all been experimentally validated as targets of miR-181a in gastric cancer, endometrial cancer, T-cell selection and lung cancer, respectively [166, 235, 262, 263], we did not observe downregulation of any of these targets in the 4T1 cells harboring enhanced miR-181a expression (data not shown). These findings suggest that the manifestations of aberrant miR-181a expression are determined in a highly cell- and context-specific manner. Interestingly, although KLF-6 expression was not altered by
miR-181a expression in 4T1 cells, it was downregulated by miR-181a overexpression in NMuMG cells (Appendix Figure 9), again illustrating the context-specific regulation of mRNA targets by miR-181a.

In an attempt to identify additional targets of miR-181a, we performed a microarray on NMuMG cells transiently transfected either with a miR-control or miR-181a. Likewise, we also performed a microarray on 4T1 cells engineered to stably express control microRNA or miR-181a sponges. Unfortunately, none of these experimental conditions resulted in statistically significant changes in mRNA expression levels (data not shown), highlighting the fact that miR-181a likely regulates additional mRNAs at the level of translational repression not mRNA degradation, which is analogous to its regulation of Bim. Future experiments utilizing miR-181a affinity capture assays, followed by microarray analysis [264] may prove useful in identifying additional miR-181a targets that are relevant to metastatic breast cancers.

4.3.5 Smad-dependent vs. Smad-independent roles in TGF-β-mediated regulation of microRNAs

Smad-mediated transcription has been shown to be operant in regulating a number of microRNAs. Indeed, Smad4 knockdown in NMuMG cells resulted in deregulation of 28 microRNAs [103]. Not surprisingly, many of these microRNAs have been shown to play roles in tumor progression. More recently, a novel role for Smads2/3 in post-transcriptionally regulating microRNA biogenesis has been identified. Smad2/3 bind to a conserved RNA sequence, termed R-SBE, in the stem region of pri-miRNAs and promote microRNA maturation by Drosha [163, 164]. Taken together, this suggests that
TGF-β regulates microRNAs through both transcriptional and post-transcriptional mechanisms.

The exact mechanism through which TGF-β controls the expression of miR-181 family members remains to be determined. Our work indicates that the transcription of pre-miR-181a-1 transpires through a Smad4-dependent mechanism, while that of pre-miR-181a-2 occurs independently of Smad4 activity (Figure 3.4). Interestingly, despite the decrease in pre-miR-181a-1 induced by Smad4 depletion, expression levels of mature miR-181a remain unaffected, indicating the presence of Smad4-independent post-transcriptional processing of miR-181a. However, it is important to note that the pri-miR-181a stem region does not contain a canonical R-SBE site. Thus, future studies utilizing cell lines deficient in singular and multiple combinations of Smad2, 3, and 4 are needed to parse out the relative contributions of transcriptional versus post-transcriptional mechanisms in mediating TGF-β stimulation of miR-181a expression, as well as in promoting the expression of other TGF-β-induced microRNAs.

4.3.6 microRNAs as biomarkers

Numerous reports indicate that microRNAs play functional roles as both tumor promoters and tumor suppressors during carcinoma development and metastatic progression. Additionally, many microRNAs are located at fragile sites or regions in the genome that are frequently amplified or deleted in cancer [150]. As such, microRNAs hold great promise as prognostic biomarkers. Indeed, microRNAs have been shown to be expressed in a tissue- and developmental stage-specific manner [252]. Moreover, single microRNAs have been shown to associate specifically with breast cancer subtypes [265], indicating
that microRNA signatures may have greater predictive power than mRNA signatures that require hundreds of mRNAs to classify molecular subtype. Along these lines, microRNA profiles have been shown to be more effective in identifying tumors of unknown origin than mRNA profiles [252]. Our work (Chapter 3) indicates that miR-181a predicts for metastatic disease and decreased survival times in non-ERBB2 amplified tumors (Figure 3.16), and as such suggests that miR-181a upregulation may be enhanced in triple-negative mammary tumors (Table 3.7). Given the role of miR-181a in targeting Bim, as well as the correlation of Bim expression levels to therapeutic response in numerous cancers [239-241], it is tempting to speculate that miR-181a could also function as a predictive biomarker for response to chemotherapies. Future research utilizing larger clinical data sets will be needed to parse apart the predictive value of miR-181a expression in each of the individual breast cancer subtypes, as well as to determine its role in predicting therapeutic response.

In addition to the identification of miR-181a as a potential biomarker for disease progression to metastasis, we have identified a unique set of microRNAs that are aberrantly upregulated by TGF-β in late-stage, triple-negative breast cancers. Indeed, this subset of differentially expressed microRNAs readily distinguishes triple-negative breast cancer cells from their luminal counterparts (Appendix Figure 10). Future studies utilizing patient tumor samples need to be undertaken to examine the expression of these microRNAs in combination with clinicopathological parameters associated with patient outcome and survival to determine the utility of this novel microRNA based diagnostic platform.
4.3.7 microRNAs as therapeutics

The small size, high stability and potent biological effect of microRNA oligonucleotides offer great promise of microRNAs as therapeutic agents. Two basic approaches exist for manipulating microRNAs in vivo. First, oncogenic microRNAs can be deactivated through the use of anti-miRNA oligonucleotides (AMOs), microRNA sponges and microRNA masking [266]. Second, overexpression of tumor suppressive microRNAs can be achieved through the use of viral vectors that encode microRNAs or transfection of chemically altered double stranded microRNA mimics [266]. Systemic treatment using anti-miR oligonucleotides against miR-10b have shown promise in vivo in several pre-clinical models of breast cancer [267]. Moreover, an inhibitor of miR-122 is currently in Phase II trials for the treatment of hepatitis, becoming the first microRNA-based therapeutic in humans [255]. A current challenge in the design of microRNA therapeutics for cancer lies in the fact that the majority of systemically administered microRNAs accumulate in the liver and kidney. As such, future studies need to develop efficient and effective delivery mechanisms, such as the use of nanoparticles, capable of bypassing kidney and liver clearance mechanisms.

Our work indicates that inhibition of miR-181a may sensitize cells to stress signals that induce anoikis by increasing expression of Bim. Given that previous reports have implicated Bim as a critical mediator of chemotherapy induced apoptosis in numerous cancers [241], including those of the breast [239, 240], future experiments designed to test the effects of antisense oligonucleotides against miR-181a [267] in combination with other standard-of-care chemotherapies will be necessary to determine if miR-181a inhibition can re-sensitize chemotherapy resistant cells to apoptosis.
4.4 Future Directions

Through the work described herein, we discovered a novel role for mechanotransduction in regulating the behaviors of MECs to TGF-β and importantly, in recapitulating the “TGF-β Paradox” in 3D-organotypic cultures (Chapter 2). This novel in vitro “Mechanotransduction Model of the TGF-β Paradox” system recapitulates the TGF-β paradox by modulating the tension sensed by MECs. Utilizing this system, we defined the microRNAome regulated by TGF-β and mechanotransduction. Characterization of miR-181a, identified in this screen, indicated that inhibition of this microRNA exhibited significant antitumor activity against triple-negative tumors propagated in mice, providing “proof of principle” that microRNA targeting can abrogate metastasis (Chapter 3). Despite these insights, several unanswered questions remain to be addressed, including (i) can microRNA signatures be used as a diagnostic platform to distinguish TNBCs from other subtypes, (ii) what are the effector molecules and pathways that couple TGF-β to the expression of metastamiRs, and (iii) can metastamiRs be chemotherapeutically targeted to decrease metastatic progression? We hypothesize that (i) metastamiR signatures can readily detect the development, progression, and recurrence of triple-negative breast cancers in patients, and (ii) chemotherapeutic targeting of metastamiRs identified in this screen will abrogate breast cancer metastasis by phenotypically and morphologically reverting the malignant behaviors of mammary tumors.

Future studies will be aimed at first, developing a sensitive diagnostic platform to delineate TNBCs based on metastamiR profiles. Our preliminary data (Appendix Figure 10) indicates that our metastamiR signature can indeed distinguish TNBCs from their
luminal counterparts. To determine if this metastamiR signature is indeed unique to TNBCs we will perform real-time PCR to monitor changes in expression of these microRNAs across several genetically distinct human breast cancer cell lines of different subtypes, such as normal (e.g. HMLE, MCF10A), luminal (e.g. BT47, MCF7, T47D), basal A (e.g BT20, MDA-MB-468, SUM149), and basal B (e.g. BT549, MDA-MB-157, MDA-MB-231). Additionally, we will determine if these metastamiR signatures are unique to breast cancers by examining their expression in other human cancer cell lines such as colon (SW480), liver (Hep G2), prostate (LNCaP), ovarian (SKOV-3), glioma (U87MG) and fibrosarcoma (HT1080). Finally, these studies will be extended to include circulating tumor cells obtained from breast cancer patients. Thus, metastamiRs identified to be specific to TNBC and detectable in CTCs could be used in a “liquid bioassay” to distinguish patients with TNBC, as well as to monitor chemotherapeutic effectiveness and disease recurrence.

Second, it will be necessary to determine the function of identified metastamiRs in mediating tumor progression and the mechanisms whereby TGF-β couples to the expression and activity of these microRNAs. Using metastamiRs identified in Chapter 3, we will overexpress these microRNAs using lentiviral pre-miR mimetics in nonmetastatic cells (e.g. 67NR or MCF7) or inhibit these microRNAs using lentiviral anti-miR sponge inhibitors in metastatic cells (e.g. 4T1 or MDA-MB-231). Next, the effect of selected microRNAs on malignant MEC behavior will be assessed using (i) cell proliferation (ii) cell invasion (iii) EMT, and (iv) mouse xenograft assays described in Chapters 2 and 3. Finally, real-time PCR for pre-microRNA transcripts, as well as mature microRNA levels in cells deficient in Smads 2, 3, and 4 will be used to determine the
transcriptional and post-transcriptional mechanisms through which TGF-β regulates metastamiRs.

Lastly, future studies need to design and assess the preclinical efficacy of multifunctional metastamiR nanoparticles to alleviate breast cancer tumor growth and metastasis, thereby creating efficacious miR-based therapies that can be utilized in the clinic. Our lab has previously shown that initiation of oncogenic signaling by TGF-β requires MEC upregulation of β3-integrin. More recently, nanoparticles that incorporate integrin-binding RGD motifs have been used to target siRNAs to inhibit glioblastoma growth in vivo [268, 269]. As such, we will use a similar strategy to incorporate an integrin-binding RGD motif into targeted nanoparticles containing an anti-miR. These nanoparticles will be tested in vitro utilizing real-time PCR for their ability to decrease microRNA expression levels. Additionally, the ability of these nanoparticles to impact MEC behavior will be assessed using traditional proliferation, invasion, and EMT assays. These studies will then be extended to in vivo analyses to assess the ability of microRNA containing nanoparticles to decrease microRNA expression in vivo, as well as their ability to halt tumor growth and metastasis.

4.5 Concluding Remarks

The challenge in designing pharmaceuticals effective in targeting TGF-β signaling lies in the ability of these agents to circumvent the principles of the “TGF-β Paradox.” For instance, an ideal anti-TGF-β agent would be expected to specifically inactivate the oncogenic activities of TGF-β, while simultaneously preserving and/or enhancing its
tumor suppressing functions. Currently, all chemotherapeutics developed against the TGF-β pathway universally function as pan-TGF-β antagonists, and as such, the preclinical and clinical use of these drugs has been shown to enhance the tumorigenicity of early-stage cancers as predicted by the “TGF-β Paradox” [12]. We previously hypothesized that the specific targeting of noncanonical TGF-β effectors might provide a novel means to restore its cytostatic function in developing mammary tumors [12]. The research presented here offers two novel approaches to selectively inhibit noncanonical TGF-β signaling. First, the activation of mechanotransduction by ECM rigidity clearly plays an important role in promoting breast cancer development and progression [118, 129, 176, 196], including that stimulated by TGF-β [121]. These findings suggest that pharmacological targeting of the tumor microenvironment and its accompanying desmoplastic reactions may provide novel avenues to treat metastatic progression stimulated by TGF-β. Indeed, co-administration of LOX and integrin inhibitors may simultaneously abrogate the oncogenic activities of TGF-β and mechanotransduction in developing mammary tumors. Second, the development of microRNA-based therapies may permit the fine-tuning of TGF-β behavior by targeting the microRNAome in mammary tumors. Our findings suggest that combining miR-181a antagonists with other standard-of-care chemotherapies may provide synergistic benefits by increasing Bim levels, leading to heightened tumor cell sensitivity to apoptosis.
Appendix
Appendix Figure 1: TGF-β induces LOX family member expression.

(A) TGF-β1 (5 ng/ml) stimulation of EMT in NMuMG cells induced their expression of LOX and associated LOXL family members as determined by semi-quantitative real-time PCR. Individual transcript signals were normalized to GAPDH. Data are the mean (± SE; n=3) fold expression of LOX relative to pre-EMT NMuMG cells (*, P < 0.05; Student’s T-Test). (B) LOX family member expression is increased in malignant MECs. Transcript levels in NMuMG cells were also compared to transcript levels in 4T1s after 36 hours in culture by semi-quantitative RT-PCR and normalized to GAPDH expression levels. LOX family member expression was increased in the malignant 4T1 MECs. Data are mean (± SE; n=3)
Appendix Figure 2: The effect of Mechanotransduction on microRNA expression decreases with malignancy.

The number of microRNAs differentially regulated by TGF-β treatment under either Rigid or Compliant growth conditions in each cell line (left). Hierarchical clustering indicates that the non-metastatic 67NR and 4TO7 cells cluster based on rigidity, while the metastatic 4T1 cells cluster based on cell treatment (right).
Appendix Figure 3: TGF-β treatment in compliant culture induces microRNAs that target cell death pathways.

Ingenuity pathway analysis of predicted mRNA targets of TGF-β upregulated microRNAs indicates that targets involved in cell death are highly enriched in 67NR (A), 4TO7 (B) and 4T1 (C) cells.
Appendix Figure 4: TGF-β treatment in Rigid culture induces microRNAs that target cell death pathways.

Ingenuity pathway analysis of predicted mRNA targets of TGF-β upregulated microRNAs indicates that targets involved in cell death are highly enriched in 67NR (A), 4TO7 (B) and 4T1 (C) cells.
Appendix Figure 5: miR-99b* and miR-132 expression does not influence TGF-β mediated EMT, invasion, or proliferation.

(A & D) NMuMG cells transfected with a control microRNA, miR-132 (A), or miR-99b* (D) were incubated in the absence or presence of TGF-β1 (5 ng/ml) for 24h, at which point alterations in the actin cytoskeleton were monitored by TRITC-phalloidin immunofluorescence. (B & E) 4T1 cells transfected with a control microRNA, miR-132 (B), or miR-99b* (E) then induced to undergo invasion through synthetic basement membranes in response to TGF-β1 (5 ng/ml). (C & F) 4T1 cells transfected with a control microRNA, miR-132 (C), or miR-99b* (F) were stimulated with TGF-β1 (5 ng/ml) for 48h at which point cellular DNA was radiolabeled with [3H] thymidine and quantified by scintillation counting.
Appendix Figure 6: miR-181a expression is not sufficient to induce metastasis.

(A) 4TO7 cells engineered to overexpress miR-181a were engrafted onto the mammary fat pads of 6-week-old Balb/c mice. Tumor volume was quantified as the mean bioluminescent tumor area flux as detected by bioluminescent imaging at indicated times post engraftment and after the primary tumor was resected at 6 weeks post engraftment (n=3). (B) Pulmonary metastasis of the tumors described in (A) was quantitated as area flux at the indicated time points (n=3).
Appendix Figure 7: Metastasis selects for diminished 181a activity.

(A) 4T1 cells engineered to possess diminished miR-181a activity (miArrest 181a) were engrafted onto the mammary fat pads of 6-week-old Balb/c mice. Data are the mean (± SEM; n=5) tumor volumes quantified at the indicated times post engraftment and post tumor resection at 2 weeks post engraftment. (B) Renilla luciferase activity of the miR-181a biosensor, indicates reduced miR-181a activity in 4T1 miArrest 181a cells prior to injection into Balb/c mice, while cells harvested from pulmonary metastases have increased miR-181a activity. Renilla luciferase signal was normalized to CMV-β-gal.
Appendix Figure 8: miR-181b, c, and d family members do not predict for decreased survival in human breast cancer patients.

(A-D) Using a microRNA expression data set of 82 breast cancer patients that were negative for ErbB2 amplification [210], the median value for miR-181 family (A), miR-181b (B), miR-181c (C), and miR-181d (D) was used to divide the samples into high (above the median) and low (below the median) microRNA expression and the corresponding P-value was calculated by log-rank analysis.
Appendix Figure 9: KLF6 is downregulated by miR-181a in NMuMG but not in 4T1 cells.  
(A & B) NMuMG (A) or 4T1 (B) cells were transiently transfected with a control miR mimic (Dye mimic), miR-181a, or and anti miR-181a. Afterward, protein levels of KLF6 were measured by immunoblotting. Shown are representative images from 3 independent experiments.
Appendix Figure 10: A microRNA signature for triple-negative breast cancers.
Real-time PCR of microRNAs distinguishing triple negative breast cancer (MDA-MB-231) cells from luminal breast cancer (MCF7) cells. Blue numbers represent fold-difference in expression between luminal A and TNBCs.
References


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