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Lysyl Oxidase Contributes to Mechanotransduction-Mediated Regulation of Transforming Growth Factor-β Signaling in Breast Cancer Cells[1,2]

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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 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-β1 or LOX, and by degrading hydrogen peroxide. Our findings identify LOX as a potential mediator that couples mechanotransduction to oncogenic signaling by TGF-β1 and suggest that measures capable of inactivating LOX function may prove effective in diminishing breast cancer progression stimulated by TGF-β1.

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Introduction

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates mammary gland development, as well as suppresses mammary tumorigenesis [1,2]. 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) [3,4]. The molecular mechanisms that engender this switch in TGF-β function during tumorigenesis are not well defined. Signaling through Smad2/3 generally is associated

Abbreviations: βAPN, β-aminopropionitrile; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; LOX, lysyl oxidase; MEC, mammary epithelial cell; NMuMG, normal murine mammary gland epithelial cell; TGF-β1, transforming growth factor-β1

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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 [2,3,5]. Indeed, we recently showed that $\alpha_v\beta_3$ 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 [6–8]. Collectively, these events enable TGF-β to stimulate breast cancer growth, invasion, and metastasis. In fact, studies by our group [4,6–9] and others [5] 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 [10,11]. 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 [12–17]. Clinically, the aberrant expression of LOX, LOXL, and LOXL2 correlates with increased malignancy and invasiveness in human tumors, including those of the breast [16–18]. 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 [19]. 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.

Materials and Methods

Cell Lines and Lentiviral Vectors

Normal murine mammary gland epithelial cell (NMuMG) and metastatic 4T1 cells were cultured as previously described [20], as were human MCF10ACA1a breast cancer cells [21] and human 293T embryonic kidney cells [22]. Lentiviral particles encoding for scrambled (i.e., nonsilencing shRNA) or murine LOX shRNA (Thermo Scientific, Huntsville, AL) were prepared as described previously [20]. 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 [23], 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 Igκ 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 [24]. 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 chemiluminescence, 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 [25], 3) gene expression using 30,000 cells/well in a synthetic p3TP-luciferase reporter gene assay as described [25], and 4) p38 MAPK phosphorylation induced by expression of a constitutively active MKK6 as described [26]. 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 [27]. In some cases, the MECs cells were pretreated for 30 minutes with either βAPN (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 [28]. 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′-AGCGAATGTCACAGCGTA CAA; 2) E-cadherin forward 5′-CCCTACATACACTCTGGTGGTTCA and reverse 5′-GGCATCATC ATCGGTCACTTTG; 3) N-cadherin forward 5′-CCCCCCAAGTCCAACATTTC and reverse 5′-CGCCG TTTCATCCATACCAC; 4) cytokeratin 19

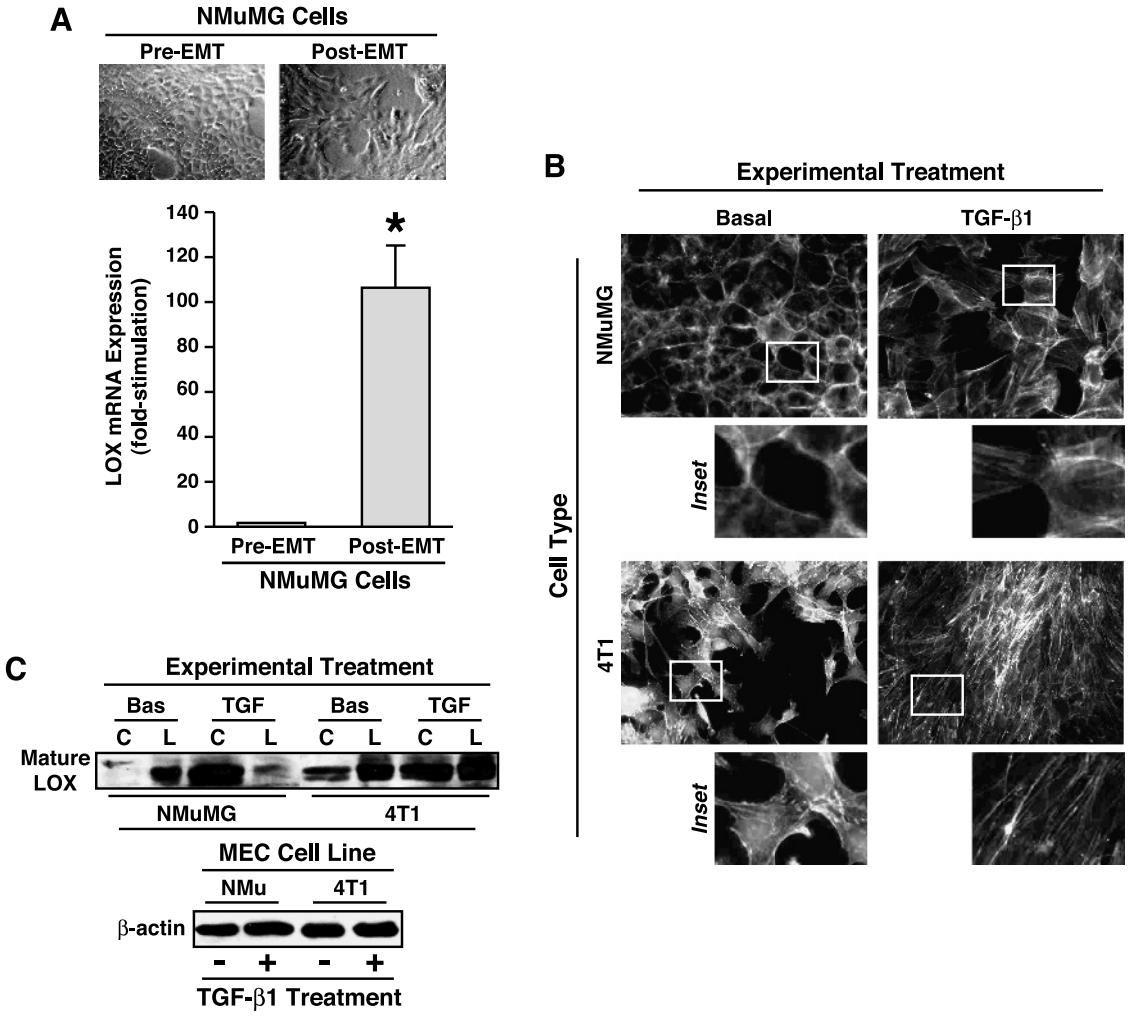


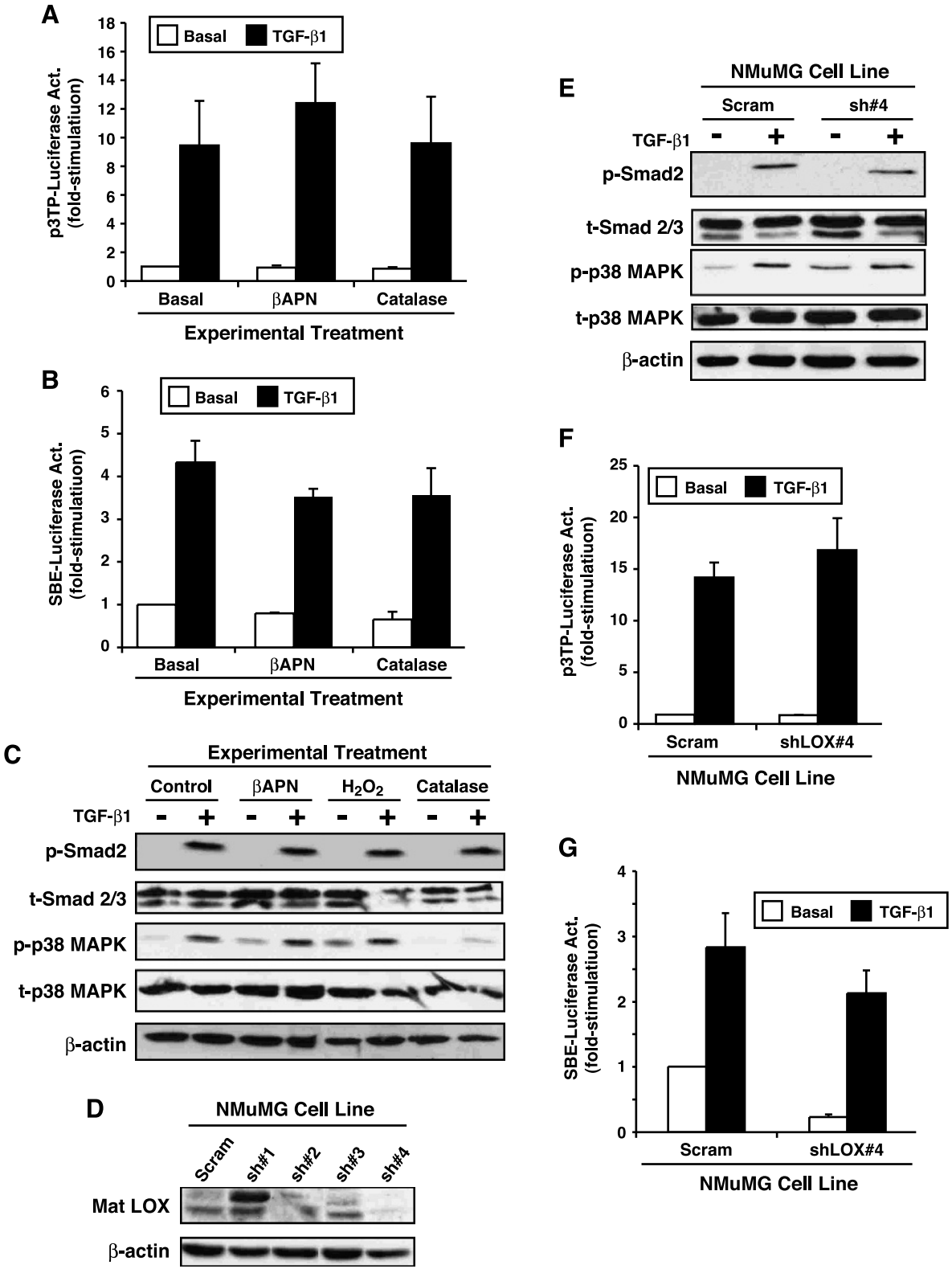
Figure 1. TGF-β1 and EMT stimulate the expression and secretion of LOX in normal and malignant MECs. (A) Shown are representative images of TGF-β1–treated NMuMG cells that have undergone EMT (top panel). TGF-β1 (5 ng/ml) stimulation of EMT in NMuMG cells induced their expression of LOX as determined 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-β1 (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-β1 (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. TGF-β1 activates p38 MAPK through a hydrogen peroxide–dependent pathway in normal MECs. NMuMG cells were transiently transfected with the TGF-β1–responsive reporter gene, p3TP-luciferase (A) or pSBE-luciferase (B), and with pCMV–β-gal. Afterward, the transfectants were stimulated with TGF-β1 (5 ng/ml) in the absence or presence of βAPN (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 βAPN (300 μM), hydrogen peroxide (H_2O_2, 1 mM), or catalase (400 U/ml) as indicated and, subsequently, were stimulated with TGF-β1 (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-β1 (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-β1 (5 ng/ml) as described in A. Data are the mean (±SE; $n = 3$).

forward 5′-TTGGGTCAGGGGGTGTTTTC and reverse 5′-TTCTCATTGCCAGACAGCAGC; 5) vimentin forward 5′-CAAGTCCAAGTTTGCTGACCTCTC and reverse 5′-CTCTTCCATCTCACGCATCTGG; 6) fibronectin III forward 5′-ACAACAACCCCAA GGAGAAG and reverse 5′-GCATCCTCTCTTCTGGTTCTG; and 7) GAPDH forward 5′-CAACTTT GGCATTGTGGAAGGGCTC and reverse 5′-GCAGGGATGATGTTCTGGGCAGC.

LOX Immunohistochemistry

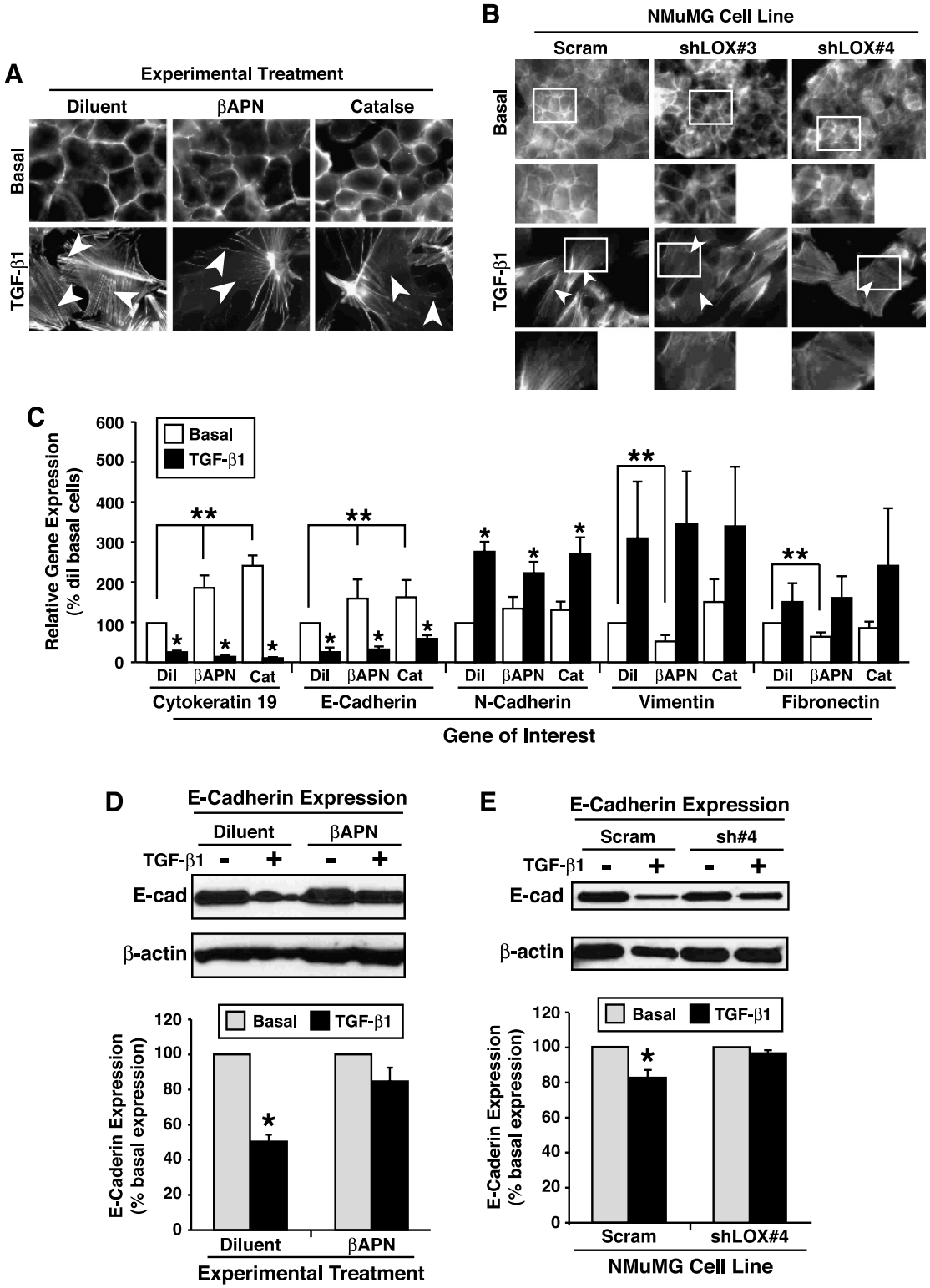
Archival 4T1 tumors that expressed GFP, WT-TGF-β type II receptor (TβR-II), or Y284F–TβR-II [8] were sectioned for histopathologic analysis in the Pathology Core at the University of Colorado Cancer Center. Afterward, LOX immunohistochemistry was performed as described [8] using two independent preparations of anti-LOX antibodies (1:50; Santa Cruz Biotechnology or Payne et al.



[23]). 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 [29]. 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-β1 (5 ng/ml), or the TGF-β type I receptor (TβR-I) antagonist, TβR-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 [30].

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 $CaCl_2$ (0.5 mM) and $MgCl_2$ (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.

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 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 1*A*, *bottom panel*). In addition, TGF-β1 also elevated the expression of LOXL1, LOXL2, and LOXL3 in NMuMG cells that underwent EMT (data not shown), 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 [10,13,16,17,23], 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 1*B*) [4,7,9,20]. 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 1*C*). 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-β1.

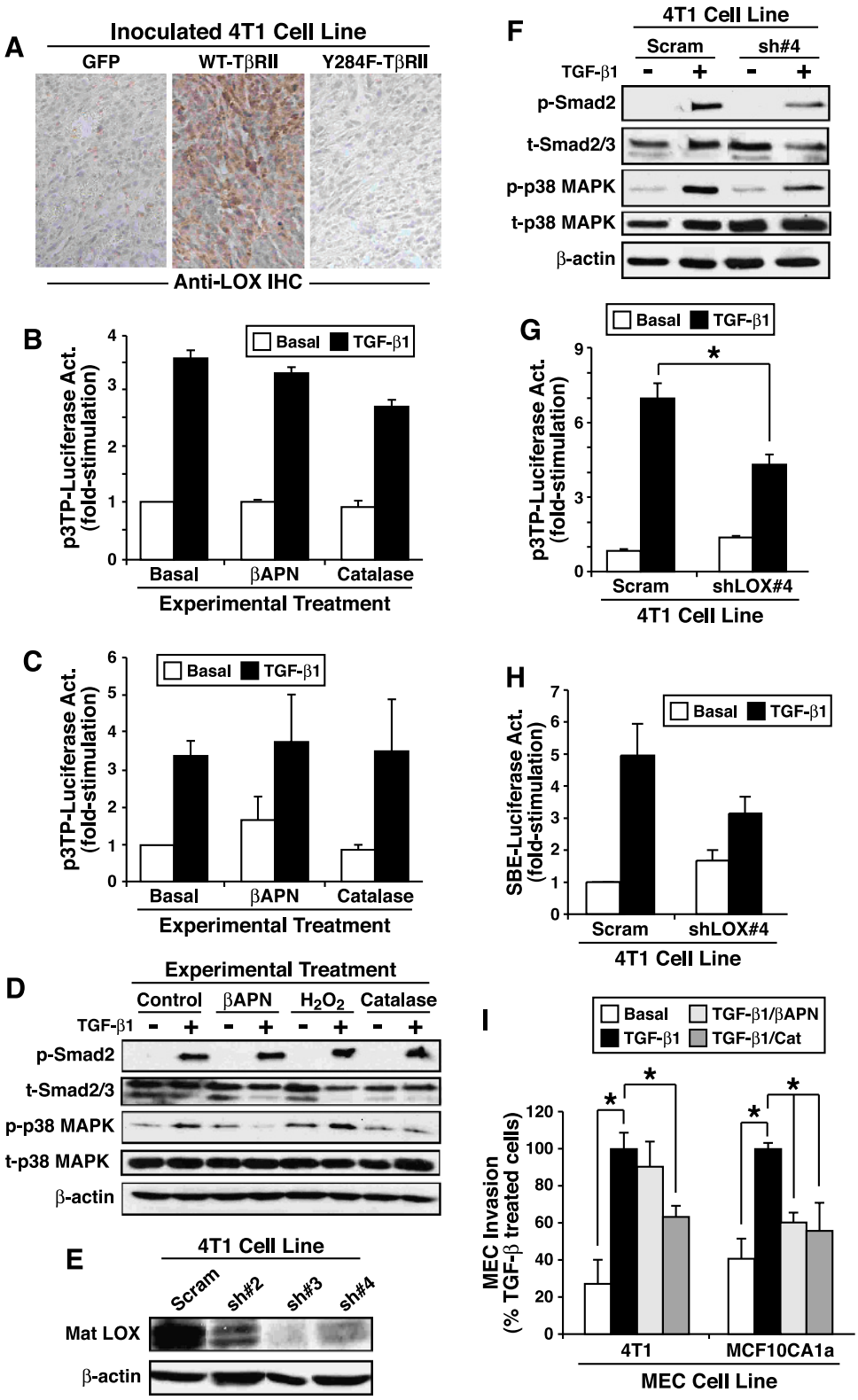
TGF-β1 Regulates EMT in Normal MECs through a LOX- and Hydrogen Peroxide–Dependent Pathway

We [4,6–8,31] and others [32–36] have shown the essential function of canonical (e.g., Smad2/3) and noncanonical (e.g., p38 MAPK) TGF-β1 effectors in mediating its stimulation of EMT in normal and malignant MECs. To determine the impact of LOX on TGF-β1–mediated activation of Smad2/3 and p38 MAPK, we first treated normal NMuMG cells with the irreversible competitive LOX inhibitor, βAPN, which has been shown to specifically inhibit the catalytic activity of LOX [10,11,37] and several LOXL family members [11,18]. In complementary approaches, we also administered catalase to these cells to degrade the LOX second messenger, hydrogen peroxide [17], or stably transduced them with shRNA against LOX to deplete its expression. Figure 2 shows that these experimental conditions failed to alter the transcriptional activity and phosphorylation of Smad2/3 stimulated by TGF-β1, as did depleting LOX expression in these MECs by their transduction with shRNA against LOX (Figures 2, *A*–*G*, and W1). Interestingly, catalase administration inhibited TGF-β1 stimulation of p38 MAPK in NMuMG cells (Figure 2*C*), thereby implicating hydrogen peroxide as a mediator of p38 MAPK activation in NMuMG cells. In stark contrast, we observed both βAPN administration and LOX deficiency to elevate p38 MAPK phosphorylation in resting NMuMG cells (Figure 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-β1 to stimulate p38 MAPK in LOX-deficient MECs.

In light of the above results and those linking TGF-β1–driven EMT to elevated LOX expression (Figure 1), we also examined the role of LOX in mediating EMT stimulated by TGF-β1. As such, administration of either βAPN or catalase (Figure 3*A*) or transduction with shRNA against LOX (Figure 3*B*) attenuated the formation of stress

Figure 3. TGF-β1 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 βAPN (300 μM) or catalase (400 U/ml) while undergoing EMT stimulated by TGF-β1 (5 ng/ml). Arrowheads show strong actin fibers localized to focal adhesions in diluent-treated cells stimulated with TGF-β1 and, conversely, stunted actin fibers in cells treated with βAPN or catalase. (B) Parental (scram) or LOX-deficient (shLOX#3 and shLOX#4) NMuMG cells were stimulated by TGF-β1 (5 ng/ml) to induce EMT. Arrowheads show strong actin fibers localized to focal adhesions in Scram cells stimulated with TGF-β1 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-β1 (5 ng/ml) in the absence (i.e., diluent; Dil) or presence of either βAPN (300 μM) 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. *$P < .05$.

fibers stimulated by TGF-β1, suggesting that targeting LOX activity can diminish EMT stimulated by TGF-β1. Accordingly, TGF-β1 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 3*C*). Pharmacological targeting of LOX family members using βAPN 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-β1. Similarly, βAPN treatment led to a significant reduction in vimentin and fibronectin expression compared with diluent-treated controls (Figure 3*C*).



Interestingly, LOX deficiency failed to fully recapitulate these responses (data not shown), suggesting that other βAPN-sensitive LOXL members may play a role during EMT induced by TGF-β1. Along these lines, βAPN treatment or LOX deficiency significantly inhibited the ability of TGF-β1 to downregulate E-cadherin protein expression in transitioning NMuMG cells (Figure 3, *D* and *E*). Thus, these findings suggest that LOX and its LOXL relatives sensitize transitioning MECs to complete the EMT program when stimulated by TGF-β1.

LOX Regulates Breast Cancer Cell p38 MAPK Activation and Invasion Stimulated by TGF-β1

Previous findings by our group demonstrated that β_3-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 [6–8]. Activation of this oncogenic TGF-β signaling cascade is amplified by overexpression of wild-type (WT) TβR-II in 4T1 cells and, more importantly, completely inactivated by their overexpression of Y284F–TβR-II mutants [8]. 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–TβR-II–expressing 4T1 tumors) but not in 4T1 tumors engineered to express either GFP (i.e., parental controls) or Src-resistant Y284F–TβR-II mutants (Figure 4*A*). 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, we administered βAPN or catalase to 4T1 cells before their stimulation with TGF-β1. Similar to NMuMG cells (Figure 2), both experimental treatments failed to alter the coupling of TGF-β1 to Smad2/3 in 4T1 cells (Figures 4, *B–D*, and W1). However, unlike NMuMG cells, administration of either βAPN or catalase significantly reduced the coupling of TGF-β1 to p38 MAPK in 4T1 cells (Figure 4*D*), suggesting that LOX and hydrogen peroxide may play an expanded role in regulating TGF-β1 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-β1 (Figure W2). Even more surprisingly, depleting 4T1 cells of LOX expression (Figure 4*E*) not only attenuated TGF-β1 stimulation of p38 MAPK (Figure 4*F*) but also significantly reduced Smad2/3 transcriptional activity and phosphorylation of Smad2 induced by TGF-β1 (Figure 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 [38]. For instance, Figure W3 shows that overexpression of constitutively active MKK6, which significantly enhanced p38 MAPK activation, had no effect on the coupling of TGF-β1 to Smad2/3 phosphorylation and reporter gene expression in 4T1 cells. Functionally, administration of catalase significantly inhibited 4T1 cell invasion stimulated by TGF-β1, whereas inclusion of either βAPN or catalase significantly antagonized the ability of TGF-β1 to induce the invasion of human MCF10ACA1a cells (Figure 4*I*), which were previously established as a model for metastatic progression regulated by TGF-β [21]. Similar to βAPN treatment, LOX deficiency failed to affect the extent of 4T1 cell invasion stimulated by TGF-β1 (data not shown). These findings establish TGF-β1 as an inducer of LOX expression *in vivo* and suggest that upregulated expression LOX or another LOXL family member and/or hydrogen peroxide production may regulate the malignancy of MECs in response to TGF-β1, including its ability to induce breast cancer cell invasion.

Mechanotransduction Induces Autocrine TGF-β1 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 [12]. 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 [12,14,15,39]. 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-β1 when propagated under compliant and rigid culture conditions. Figure 5*A* shows that in traditional two-dimensional cultures, which are extremely rigid

Figure 4. LOX regulates breast cancer cell p38 MAPK activation and invasion stimulated by TGF-β1. (A) Elevated oncogenic TGF-β1 signaling (i.e., WT–TβR-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-β1 signaling significantly induced the expression of LOX expression in WT–TβR-II–expressing 4T1 tumors compared with their GFP- or Y284F–TβR-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-β1 (5 ng/ml) in the absence or presence of βAPN (300 μM) 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 βAPN (300 μM), hydrogen peroxide (H_2O_2, 1 mM), or catalase (400 U/ml) as indicated and, subsequently, were stimulated with TGF-β1 (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 antibodies against 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-β1 (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-β1 (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 βAPN (300 μM) or catalase (400 U/ml) while undergoing invasion through synthetic basement membranes in response to TGF-β1 (5 ng/ml). Data are the mean (±SE; $n = 3$) invasion relative to that stimulated by TGF-β1. *$P < .05$.

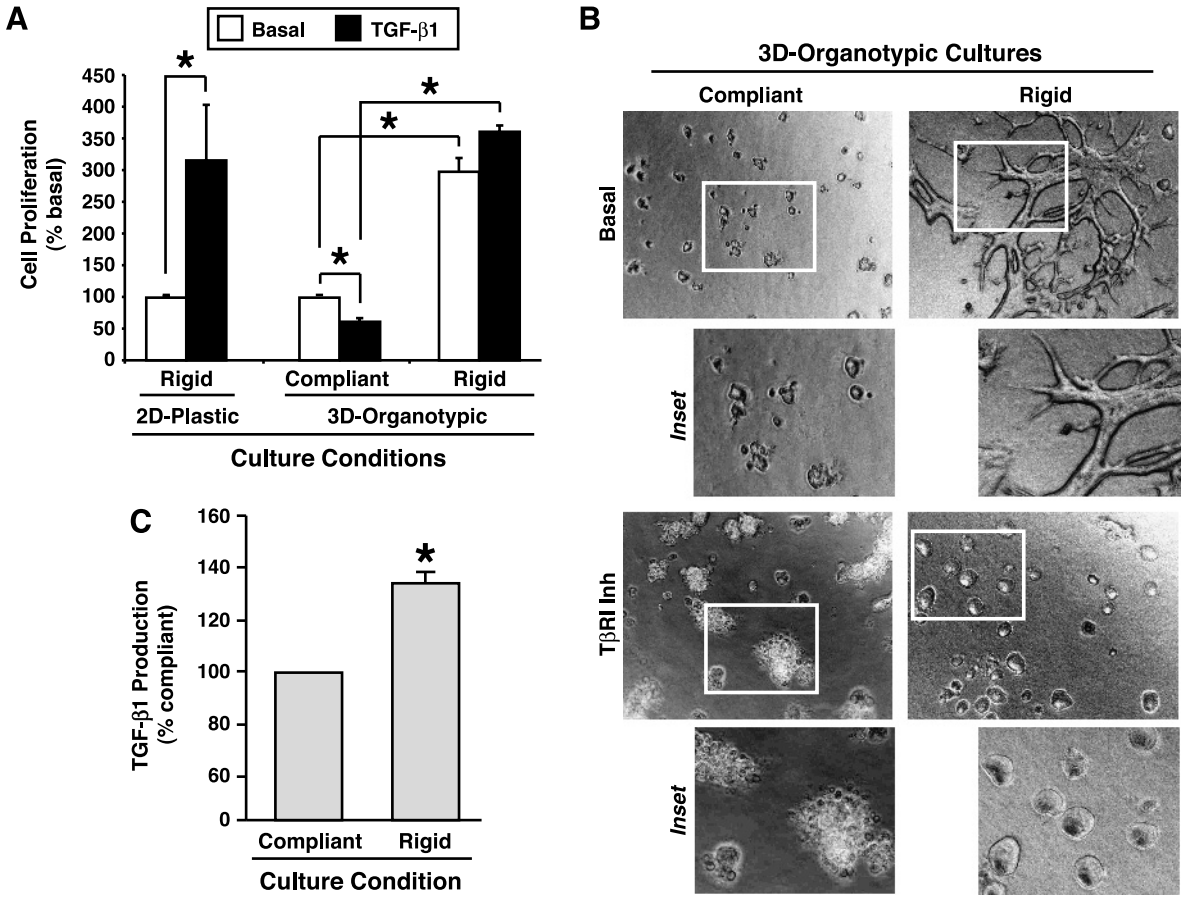


Figure 5. Mechanotransduction induces autocrine TGF-β1 signaling coupled to MEC proliferation. (A) 4T1 cells were in incubated the absence or presence of TGF-β1 (5 ng/ml) in two-dimensional tissue culture plastic or in three-dimensional organotypic cultures supplemented without (i.e., compliant) or with 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-β1 signaling by administration of the TβR-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-β1. After sample neutralization, TGF-β1 concentrations were determined by ELISA analysis. Data are the mean (±SE; $n = 3$) TGF-β1 concentrations relative to those measured in compliant cultures. *$P < .05$.

[12], TGF-β1 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-β1 in these malignant MECs, which normally fail to undergo growth arrest in response to TGF-β1 (Figure 5*A*) [4,7–9,28,40–43]. Importantly, supplementing these three-dimensional organotypic cultures with type I collagen to initiate mechanotransduction uncoupled TGF-β1 from the regulation of cell cycle progression in 4T1 cells (Figure 5*A*). Thus, ECM tension and rigidity clearly alter how MECs respond to the cytostatic activities of TGF-β1 [20]. 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 [28,40–43], and subsequently monitored alterations in their growth and morphology. As shown in Figure 5*B*, 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 5*B*). Consistent with the ability of compliant microenvironments to reinstate cytostatic signaling by TGF-β1 (Figure 5*A*), 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 5*B*). 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 5*C*) 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 βAPN or catalase. Figure 6*A* shows that inhibiting LOX activity or degrading hydrogen

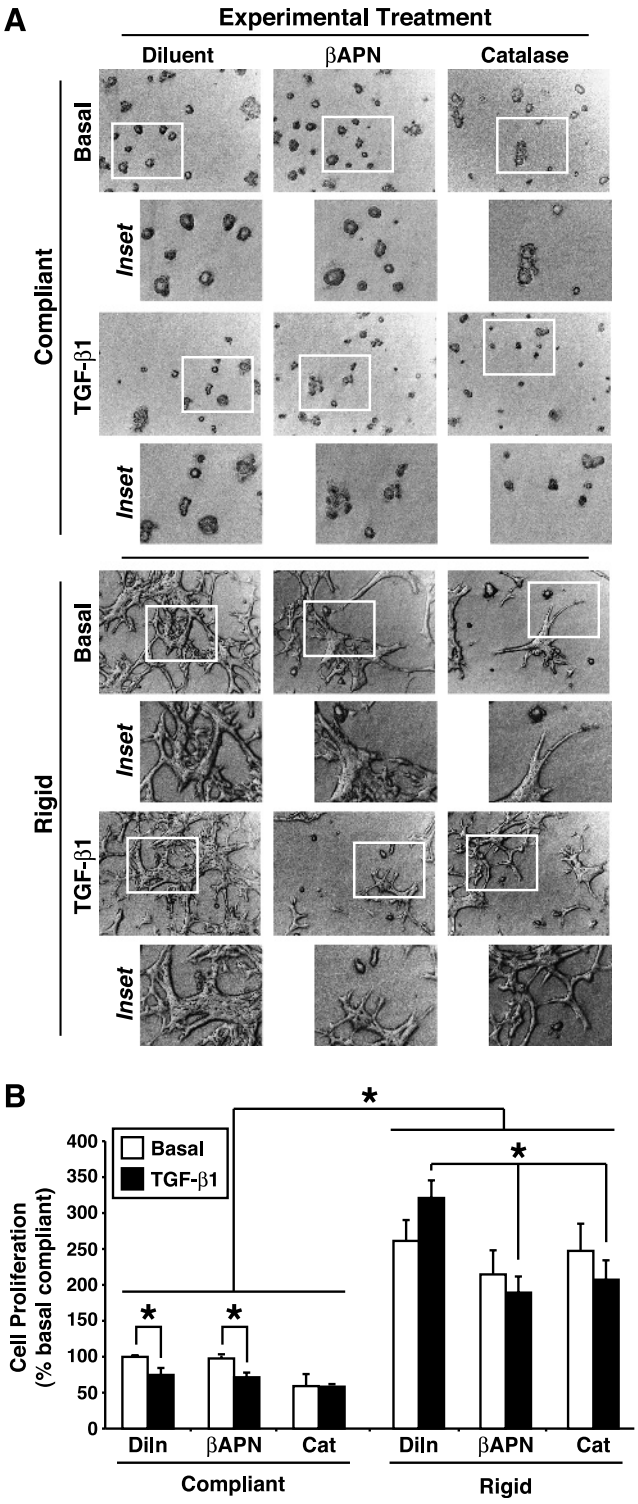


Figure 6. Mechanotransduction induces MEC proliferation in a LOX-dependent manner. (A) LOX antagonism using βAPN (300 μM) or catalase (400 U/ml) inhibited the ability of TGF-β1 to stimulate 4T1 cell growth in rigid three-dimensional organotypic cultures. Insets: magnified views of boxed regions. Shown 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. *$P < .05$.

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 6, *A* and *B*). Similarly, LOX deficiency (Figure 4*E*) abrogated the ability of TGF-β1 to stimulate the growth of 4T1 organoids in rigid ECM (Figure 7*A*) 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 3) and given the ability of E-cadherin expression to suppress the uncontrolled growth of cancer cells [44], 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 7*B*). 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 7*B*). 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.

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 [45]. 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 [46,47]. 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 [8]. 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 [48,49]. 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-β1 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.

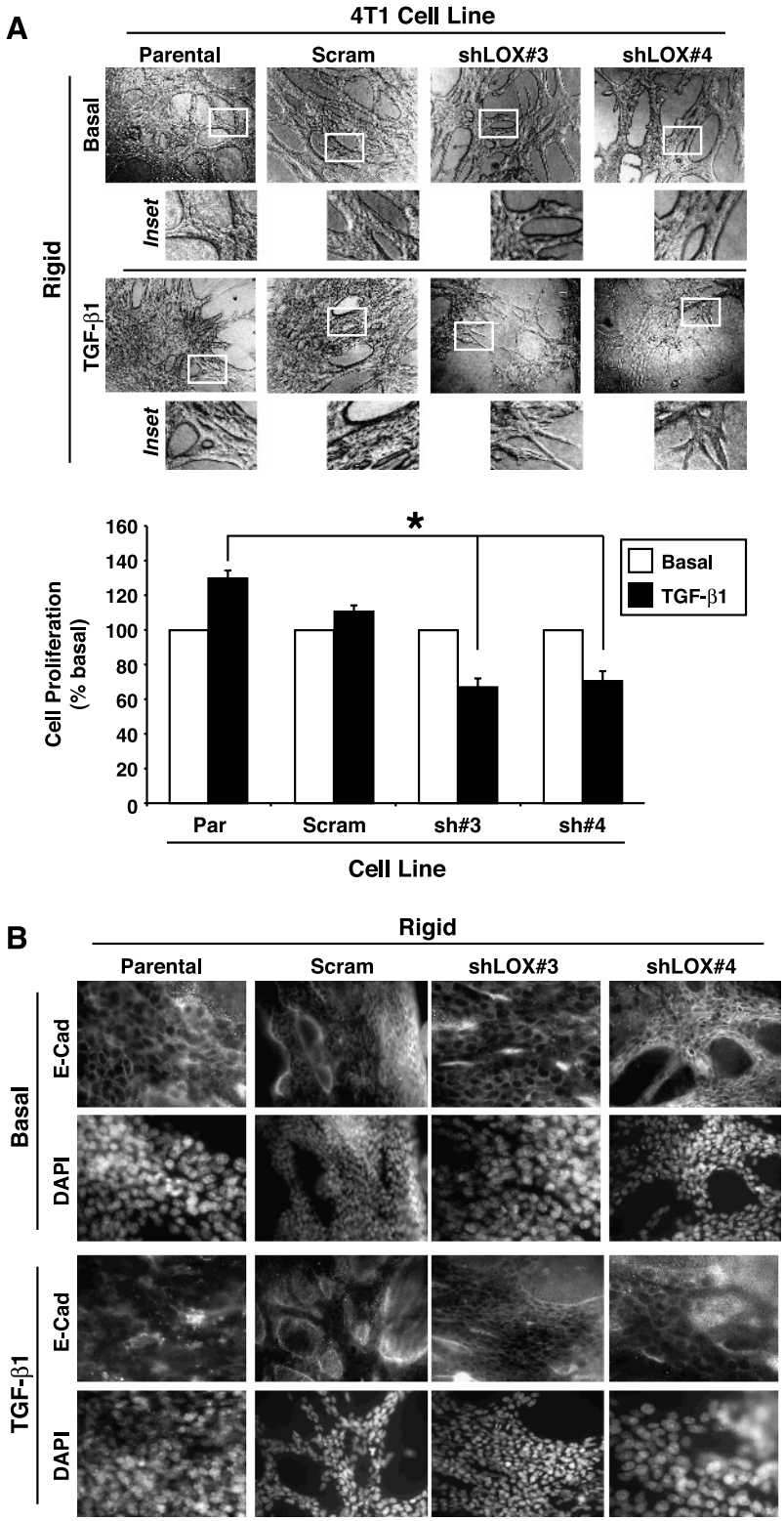


Figure 7. LOX deficiency suppresses mechanotransduction and TGF-β1 stimulation of MEC proliferation by restoring cell surface E-cadherin expression. (A) Parental, scrambled (Scram), or LOX-deficient (sh#3 and sh#4) 4T1 cells were propagated in rigid three-dimensional organotypic cultures in the absence 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.

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 1) and nucleus of malignant cells [10,11]; 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 [11]. Along these lines, LOX was shown to activate Src and promote cell adhesion through a hydrogen peroxide–dependent mechanism [23]. We show here that administering catalase to degrade hydrogen peroxide prevented TGF-β from stimulating MEC proliferation, EMT, and invasion (Figures 3–7) and from fully activating p38 MAPK (Figure 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 βAPN treatment and may compensate for loss of LOX function [11,50], and in fact, both LOX and LOXL2 mediate the ability of HIF-1α to suppress E-cadherin expression [51]. 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 noncanonical 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 [13]. TGF-β has also been implicated in the recruitment of immature bone marrow–derived cells to drive breast cancer metastasis [52], 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 [53], 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 [11]). 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 [13,16,54]. These findings, together with those presented herein, support the idea that LOX dictates how malignant MECs respond to the varied activities of TGF-β, and as such, identify LOX as a novel participant in oncogenic TGF-β1 signaling in late-stage mammary tumors. Thus, chemotherapeutic targeting of LOX may offer new inroads to alleviate breast cancer progression stimulated by TGF-β.

Lastly, the ability of LOX to cross-link collagen to elastin results in increased tissue tension and ECM rigidity [12,15,39,55]. More recently, ECM rigidity has been shown to play an important role in breast cancer development, particularly their acquisition of invasive and metastatic phenotypes [12,39,47,56]. 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 [46,47]. Importantly, we identified an oncogenic TGF-β signaling axis comprised in part of $\alpha_v\beta_3$ integrin, FAK, and Src that induces mammary tumor growth, invasion, and metastasis in mice [4,8,9], as well as stimulates significant LOX expression in these same mammary tumors (Figure 4). We speculate that tumor-initiated MECs evolve in compliant microenvironments that favor canonical Smad2/3 signaling stimulated by TGF-β. The continued growth of the developing neoplasm enhances ECM rigidity by upregulating TGF-β production (Figure 5) and LOX expression (Figure 1), which may lead to the inappropriate formation of integrin–TβR-II complexes [6–8]. Once formed, these complexes are also likely to interact with other growth factor receptors that presumably amplify the activation of noncanonical effectors by TGF-β [57]. 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. Moreover, extending our findings to encompass sites of micrometastases [13], 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 5–7), 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.

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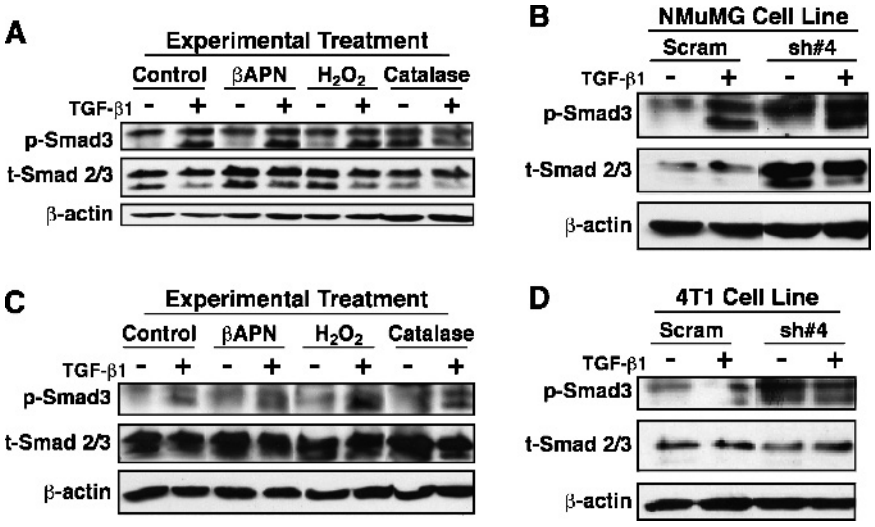

Figure W1. Pharmacologic and genetic targeting of LOX fail to alter TGF-β1 stimulation of Smad3 phosphorylation in normal and malignant MECs. Quiescent NMuMG (A) and 4T1 (C) cells were pretreated with βAPN (300 μM), hydrogen peroxide (H_2O_2, 1 mM), or catalase (400 U/ml) as indicated. Afterward, these MECs and their LOX-deficient (sh#4) counterparts (B, D) were stimulated with TGF-β1 (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.

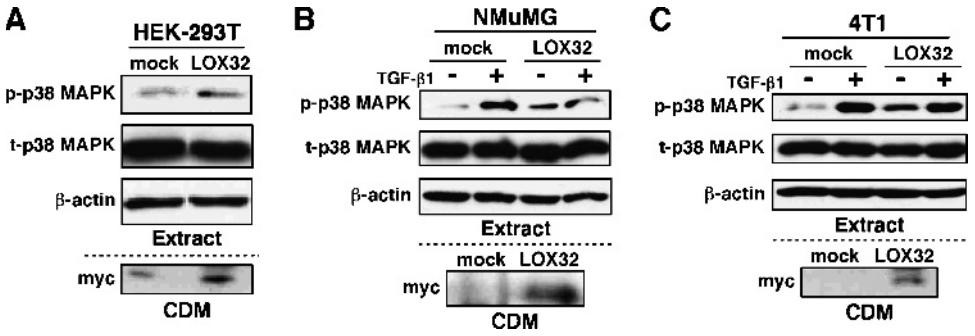

Figure W2. Expression of constitutively active LOX stimulates p38 MAPK in normal and malignant MECs. Human 293T (A), NMuMG (B), or 4T1 (C) cells were transiently transfected with constitutively active LOX (LOX32). Thirty-six hours after transfection, the conditioned medium (CDM) was tumbled with Ni^{2+}-agarose 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.

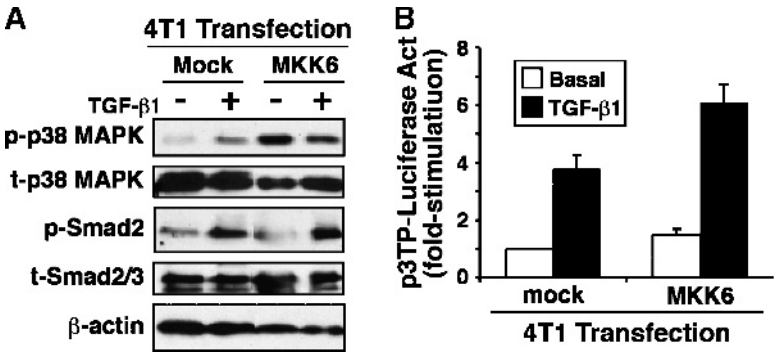

Figure W3. Expression of constitutively active MKK6 activates p38 MAPK but has no effect on the coupling of TGF-β to Smad2/3 in malignant MECs. 4T1 cells were transiently transfected overnight with p3TP-luciferase and pCMV–β-gal, together with either empty vector (mock) or constitutively active MKK6 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.