Critical role for lysyl oxidase in mesenchymal stem cell-driven breast cancer malignancy

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Mesenchymal stem cells (MSCs) are multipotent progenitor cells with the ability to differentiate into multiple mesoderm lineages in the course of normal tissue homeostasis or during injury. We have previously shown that MSCs migrate to sites of tumorigenesis, where they become activated by cancer cells to promote metastasis. However, the molecular and phenotypic attributes of the MSC-induced metastatic state of the cancer cells remained undetermined. Here, we show that bone marrow-derived human MSCs promote de novo production of lysyl oxidase (LOX) from human breast carcinoma cells, which is sufficient to enhance the metastasis of otherwise weakly metastatic cancer cells to the lungs and bones. We also show that LOX is an essential component of the CD44-Twist signaling axis, in which extracellular hyaluronan causes nuclear translocation of CD44 in the cancer cells, thus triggering LOX transcription by associating with its promoter. Processed and enzymatically active LOX, in turn, stimulates Twist transcription, which mediates the MSC-triggered epithelial-to-mesenchymal transition (EMT) of carcinoma cells. Surprisingly, although induction of EMT in breast cancer cells has been tightly associated with the generation of cancer stem cells, we find that LOX, despite being critical for EMT, does not contribute to the ability of MSCs to promote the formation of cancer stem cells in the carcinoma cell populations. Collectively, our studies highlight a critical role for LOX in cancer metastasis and indicate that the signaling pathways controlling stroma-induced EMT are distinct from pathways regulating the development of cancer stem cells.

Neoplastic epithelial cells within breast carcinomas are often greatly outnumbered by a variety of connective tissue cell types, which collectively form the tumor-associated stroma (1). This mesenchymal microenvironment is integral for tumor initiation and growth, because it regulates the survival and proliferation of neoplastic cells and the overall dynamics of tumor development (2). Therefore, defining the nature of the signals exchanged between the stromal niche and the cancer cells should provide insights into how breast cancers develop and progress and reveal therapeutic modalities based on intercepting the tumor–stroma cross-talk.

Mesenchymal stem cells (MSCs) are connective tissue progenitor cells that contribute to fibrotic reactions during tissue remodeling and repair in places of wounding and inflammation (3). They reside primarily in the bone marrow, and are mobilized to the circulation and recruited to their destination in response to systemic signals emanating from injured tissues (4). Emulating wounds (5), breast tumors also emit systemic signals that attract MSCs into the tumor stroma (6). Indeed, we and others have shown previously that bone marrow-derived MSCs home to and become incorporated into the stroma of developing breast carcinoma xenografts (7–9). MSCs were also found at elevated levels in the circulation of patients with advanced breast cancer (10) and in the stroma of human primary breast carcinomas (11), observations that are consistent with the systemic mobilization of MSCs by and their recruitment into breast tumors in the clinical setting.

The functions of MSCs in breast cancer pathogenesis, however, have not been fully elucidated, but accumulating evidence indicates that they play prominent roles in supporting tumor development (6). Indeed, bone marrow-derived MSCs within the stroma of breast cancer xenografts were shown to enhance the growth kinetics of the ensuing tumors and their metastasis to lungs and bones (7, 9). The abilities of MSCs to serve these malignant functions have now been described in multiple models of breast cancer, and are mediated by a number of MSC-derived factors, such as chemokine (C-C motif) ligand 5 (CCL5), IL-17B, IL-6, or IL-8 (7, 9, 11), with paracrine actions on the neighboring breast cancer cells that cause their growth, motility, invasion, and/or distant metastasis. However, in contrast to the increasingly detailed understanding of the cross-talk operating between MSCs and cancer cells and the characteristics of cancer-associated MSCs, little is known regarding the molecular features of how breast cancer cells respond to the influences of MSCs. We, therefore, set out to determine the molecular and phenotypic attributes of MSC-activated cancer cells and explore whether such traits contribute to breast cancer progression.

Results

Bone Marrow-Derived MSCs Stimulate de Novo Production of Lysyl Oxidase in Breast Cancer Cells. GFP-labeled MDA-MB-231 or MCF7/Ras breast cancer cells (BCCs) were cultured alone or with human bone marrow-derived MSCs (hereafter called MSCs) for 72 h. GFP-BCCs were then recovered using FACS, and their RNA was processed for gene expression analysis using Affymetrix-based arrays (Fig. S1A). Compared with cancer cells cultured alone, MSC-stimulated MDA-MB-231 (MDA-MB-231MSC) and MCF7/Ras (MCF7/RasMSC) cells exhibited significant (more than twofold; P < 0.05) expression changes in 87 and 55 genes, respectively, the great majority of which was induced. However, seven of these genes were increased in both the MDA-MB-231MSC and MCF7/RasMSC cells (Fig. S1B). Pathway analysis of the up-regulated genes using gene set enrichment analyses (GSEAs) revealed a significant enrichment for multiple pathways involved in cancer progression, predominantly in the ECM receptor interaction gene set (Fig. S1C). In light of its critical regulation of ECM maturation and remodeling (12) and its...
ranking among the highest induced genes in the transcription arrays of BCC\textsuperscript{MSC}, our attention focused on l-lysyl oxidase (LOX).

LOX is a copper-dependent amine oxidase that catalyzes the cross-linking of collagens and elastins in the ECM (13). It is secreted to the extracellular space as a 50 kDa proenzyme, then cleaved by bone morphogenetic protein-1 into an 18 kDa propeptide (called PP) and a 32 kDa active enzyme (called LOX) (14). LOX is the prototype for four additional proteins that share sequence and functional similarities called LOX-like or LOXL proteins, all implicated in tumorigenesis (15). In breast cancer, LOX was described to promote cancer cell invasion (16) and LOX is the prototype for four additional proteins that share highly related LOX-like genes (Fig. S2). Importantly, LOX expression is only induced in the BCC\textsuperscript{MSC} (Fig. S2) and in vivo (Fig. 1) and not in the cocultured MSCs (Fig. S2). Indeed, LOX overexpression caused EMT, we assessed the levels of fibronectin, α-smooth muscle actin, vimentin, and N-cadherin in BCC\textsuperscript{LOX}. LOX caused a multifold up-regulation in these mesenchymal markers (Fig. 3A and B) and a significant reduction in E-cadherin protein in the E-cadherin-rich MCF7/Ras cells (Fig. 3B). The stimulation of EMT by LOX was consistent with its ability to notably trigger the EMT master transcriptional regulator Twist by 20-fold in both MDA-MB-231 and MCF7/Ras.

LOX Overexpression Promotes Breast Cancer Invasion, Metastasis, and Epithelial-to-Mesenchymal transition. Next, we examined whether LOX overexpression in BCCs (BCC\textsuperscript{LOX}) accentuated their invasive and metastatic traits. Indeed, forced expression of the cDNA coding for full-length LOX in BCCs (Fig. S3A) resulted in the accumulation of the active enzyme (Fig. S3B-D), which caused twofold enhancement in BCC motility in Boyden chamber assays (Fig. 2A) as well as promoted two- to fourfold increases in their average migration velocity on collagen-coated microchannel lattices (Fig. 2B and C and Fig. S3E). In addition, LOX overexpression enhanced wound-healing assays (Fig. S3F) and delayed anokis (Fig. 2D). Most importantly, BCC\textsuperscript{LOX} formed s.c. tumors (Fig. 2E and Fig. S4A and B) that were approximately five to eight times more metastatic than controls (Fig. 2E and F and Fig. S4C), with a marked predilection of disseminated cells to form metastases in bone (Fig. 2G). Collectively, these results indicate that elevated levels of LOX were sufficient in enhancing the abilities of weakly metastatic cancer cells to complete the metastasis cascade. The loss of epithelial phenotypes and the acquisition of mesenchymal traits through the process of epithelial-to-mesenchymal transition (EMT) have been shown to enhance the invasive and metastatic abilities of carcinoma cells in a variety of cancer models, including breast cancer (21). To determine whether LOX overexpression caused EMT, we assessed the levels of fibronectin, α-smooth muscle actin, vimentin, and N-cadherin in BCC\textsuperscript{LOX}. Indeed, LOX caused a multifold up-regulation in these mesenchymal markers (Fig. 3A and B) and a significant reduction in E-cadherin protein in the E-cadherin-rich MCF7/Ras cells (Fig. 3B). The stimulation of EMT by LOX was consistent with its ability to notably trigger the EMT master transcriptional regulator Twist by 20-fold in both MDA-MB-231 and MCF7/Ras.
cells (Fig. 3C), indicating that LOX regulates a critical driver of the EMT machinery.

In line with this finding, MSCs triggered strong EMT phenotypes in cocultured BCCs (Fig. 3A). This transition invoked more potent up-regulation of Twist mRNA than other EMT-linked transcription factors (25- to 50-fold) (Fig. 3C and Fig. S5A) and was concomitant with the localization of the Twist protein to the nuclei of BCCMSC (Fig. S5 B and C). Accordingly, we proceeded to assess the extent to which LOX contributed to the MSC-elicited Twist induction in BCCs. First, inhibition of LOX expression in the cancer cells by ~80% using shRNAs (Fig. S5D) abrogated the ability of MSCs to trigger Twist in BCCs (Fig. 3 D and E), and effectively inhibited MSC-induced EMT altogether (Fig. S5 E and F). Second, inhibition of the enzymatic activity of LOX by βAPN (100 μM) crippled its ability to induce Twist in BCCLOX (Fig. 3 F and G), neutralized the ability of MSCs to induce Twist in admixed BCCs (Fig. 3G and Fig. S5G), and compromised the induction of EMT in BCCLOX (Fig. S5H). These observations provided strong indications that LOX is a critical mediator of MSC-induced EMT in BCCMSC and that the role of LOX in inducing EMT depended largely on its enzymatic functions. Of note is that βAPN (100 μM) significantly reduced the ability of MSCs to trigger LOX in the cancer cells (Fig. 3G) and that MSCs were not able to induce LOX overexpression in cancer cells devoid of Twist (Fig. S6). These results suggest that Twist is required for LOX induction by the MSCs. How this regulation is exerted is at present undetermined.

LOX Does Not Mediate the MSC-Induced Expansion of Cancer Stem Cells. The EMT program has been described to endow cancer cells with certain stem cell properties thought to be conducive to metastasis (22). Indeed, BCCs prompted to undergo EMT acquire certain stem cell characteristics, such as the ability to form mammospheres and an increased capacity to form tumors in limited dilution analyses (23, 24). Conversely, stem cell-like cells derived from mammary epithelium or BCC lines exhibit EMT phenotypes (23). Most importantly, cancer stem cells (CSCs) derived from human breast cancer specimens are enriched for EMT markers (23). These and other similar observations (25) suggested that the EMT and CSC programs are intertwined and that acquisition of EMT traits by cancer cells is sufficient to bestow on them CSC characteristics. Because LOX was a critical regulator of EMT, we asked whether it played similar roles in regulating the CSC program in BCCs. Aldehyde dehydrogenase 1 (ALDH1)-positive breast cancer cells are enriched for CSCs (26); however, we found that BCCLOX exhibited no augmentation in their ALDH1-positive populations as determined by ALDEFLUOR-based assays (Fig. S7A). Along the same lines, LOX overexpression did not provide cancer cells with any significant advantage in mammosphere-forming assays in vitro (Fig. S7B and C), suggesting that, although LOX could promote robust EMT, it was not sufficient to promote entrance into the CSC state.

Recent work showed that MSCs were able to promote a fourfold increase in the ALDH1-positive population of SUM159 BCCs cocultured with MSCs (11). In our own experiments with the MDA-MB-231 and MCF7/Ras cells, we found that MSCs caused a manifold rise in the ALDH1 positivity of BCCMSC (Fig. S7D), a phenotype mediated by MSCs through contact-dependent mechanisms (Fig. S7 E and F). Furthermore, BCCMSC exhibited an ~2- to 12-fold enhancement in the primary (Fig. S7 G and H) and secondary (Fig. S7I) mammosphere-forming capacities, consistent with the acquisition of CSC traits. These observations prompted us to examine whether LOX, although not sufficient on its own in driving the CSC program, was necessary for MSC-induced CSC phenotypes. Surprisingly, βAPN did not affect MSC-induced increases in the ALDH1 positivity of BCCMSC (Fig. S7D) and did not affect their mammosphere-forming activities (Fig. S7 G–I). The MSC-induced CSC phenotype did not require LOX expression either, because MSCs were still able to promote the mammosphere-forming abilities (Fig. S7) and enhance the ALDH1 positivity (Fig. S7) of cancer cells lacking significant LOX expression. Interestingly, however, inhibition of LOX expression significantly compromised the ability of MSCs to enhance tumor growth and metastasis (Fig. S8), suggesting that LOX-dependent pathways played important roles in MSC-induced metastasis. However, these results also suggest that LOX-independent pathways (such as those pathways inducing CSC phenotypes) contributed substantially to MSC-induced malignancy, perhaps in an equivalent fashion to those pathways governed by LOX. Collectively, these findings ascribe significant importance to both LOX-dependent (e.g., EMT) and -independent (e.g., CSC) signaling in MSC-induced metastasis and suggest that separate stromal triggers feed into such processes.
Hyaluronan–CD44 Interactions Mediate MSC-Induced LOX and Twist Expression. We focused on determining the nature of the MSC-triggered upstream signaling pathways regulating LOX expression in the cancer cells. Previous reports described the regulation of LOX by hypoxia (18) or mechanical transduction (27), but we found that neither of these mechanisms mediated MSC-triggered LOX overexpression in our BCCs (SI Results and Fig. S9 A–E). Furthermore, we found that the ability of MSCs to induce LOX in BCCs was not mediated by soluble factors (SI Results and Fig. S9 E–I) but resided instead in the ECM of the MSCs. Indeed, culture of BCCs on ECM deposited by MSC monolayers (Fig. S9f) resulted in a significant up-regulation of LOX in the cancer cells (Fig. S9k). These data indicate that the observed heterotypic signaling responsible for the induction of LOX in the cancer cells originates from the ECM of MSCs. This influenced our attention focused particularly on the glycosaminoglycan hyaluronan (HA) as being a potential trigger for LOX in BCCMSC for a number of reasons. First, HA/hyaluronidase is one of the most abundant proteoglycans present in the ECM of mammalian cells, particularly the ECM of MSCs (28, 29). Second, GSEA strongly highlighted the ECM–receptor interaction pathway in BCCMSC (Fig. S1i), in which HA is significantly represented. Third and most importantly, HA was shown to be a critical modulator of EMT phenotypes in normal and cancerous epithelial cells, and it is abundant in the stromal environment of cancer cells that cause the invasion and metastasis (30). Indeed, treatment of cocultures of MSCs and cancer cells with hyaluronidase inhibited MSC-induced LOX (and Twist) expression in BCCMSC (Fig. 4A), suggesting a necessary role for HA in regulating LOX induction. To investigate whether HA was sufficient in inducing LOX in the cancer cells, we expanded MDA-MB-231 cells on culture surfaces coated with HA molecules in different sizes from 4–8 to >950 kDa. Interestingly, only the high-molecular weight HA substrate was able to cause the up-regulation of LOX transcription (~120-fold), and it did so to levels comparable with the levels triggered by admixed MSCs (Fig. 4B), a phenotype that paralleled the activation of the LOX promoter by HA (Fig. 4C). These observations indicated that HA was sufficient and necessary for the ability of MSCs to trigger LOX expression in the admixed cancer cells.

HA acts through multiple receptors, with CD44 being its major partner in cancer (30). We, therefore, proceeded to explore whether it mediated the actions of MSCs on the LOX–Twist axis. Indeed, inhibition of CD44 expression in the cancer cells (Fig. 4D, Inset) abolished the ability of MSCs to trigger LOX expression in the MDA-MB-231MSC (Fig. 4D). Furthermore, expression of CD44 shRNAs in the cancer cells impaired MSC-induced Twist expression (Fig. 4D), further corroborating the link between LOX and Twist and placing CD44 as a critical upstream molecule that mediates the actions of MSCs on the LOX–Twist pathway in the cancer cells.

How HA–CD44 interactions caused de novo transcription of LOX, however, was undetermined. Previous work by Tammi et al. (31) indicated that the association of HA with CD44 causes internalization of CD44 fragments, and some translocate to the nucleus (32) and modulate gene transcription by direct association with and activation of gene promoters (33). To investigate whether such a mechanism operated in our cocultures, we probed for localization of CD44 in MDA-MB-231 and MDA-MB-231MSC. Strikingly, Western blot and immunofluorescence analyses indicated that the interaction of MSCs with cancer cells caused a marked increase in CD44 localization to the nucleus of the cancer cells (Fig. 4 E and F). Particularly, we observed a preferential enrichment (more than fivefold) of an ~50-kDa fragment of CD44 in the nucleus of the MSC-stimulated cancer cells, consistent with a potential role for this fragment in modulating gene transcription. These results suggested that CD44 might play a more proximal role in modulating LOX transcription by affecting its promoter activity. To test this possibility, we conducted ChIP on the nuclear preparations derived from MSC-stimulated cancer cells or their control counterparts using a polyclonal anti-CD44 or isotype control antibodies followed by PCR using primers specific to the LOX promoter or control human satellite 2 (SAT2). We found that CD44 associated specifically with the LOX promoter, a coupling that seems to be markedly accentuated by admixed MSCs (Fig. 4G). Collectively, our results are consistent with a model in which the interaction of MSCs with cancer cells causes translocation of CD44 to the nucleus, which associates with and activates the LOX promoter, leading to Twist-dependent induction of EMT phenotypes conducive to cancer metastasis (Fig. 4H).
Discussion

Our findings uncover a pivotal role for LOX in regulating the MSC-induced EMT phenotypes of cancer cells and reveal a previously undescribed functional link between LOX and Twist. This link extends to the clinical setting, because the expression of these two genes statistically correlated across a large group (n = 2,158) of human cancers (Fig. 5A), including the subgroup (n = 333) of breast cancers within this cohort (Fig. 5B). Twist and LOX also significantly correlated across three additional and commonly used sets of breast cancer array databases (Fig. 5C). Furthermore, subsets of breast cancer patients with tumors that displayed elevated expression levels of both genes also exhibited lower chances of survival (Fig. 5D). Finally, subsets of particularly aggressive triple negative breast tumors—called metastatic breast cancers—previously reported to be enriched for EMT markers (34, 35) exhibited preponderant expressions of both Twist and CD44 (Fig. 5E) and displayed ~7- to 60-fold upregulation of LOX mRNA compared with controls (Fig. 5F). These results are consistent with the premises of our experimental findings, and they suggest that concerted assessment of LOX/Twist levels bears a prognostic value in human breast cancers.

On the mechanistic level, this report describes a direct role for CD44 in the transcriptional regulation of LOX and suggests an alternative mechanism of LOX induction that is not mediated by hypoxia-inducible factor 1α (HIF1α) and does not seem to involve tенsgery. Considering that the latter two stimuli only trigger small increases in LOX transcription in the cancer cells (27, 36) in contrast to the multifold increases that we observed with MSCs, we posit that the HA-CD44 trigger might be a more efficient means to regulate LOX expression in vivo. That said, how CD44 becomes internalized as a result of BCC/MSC contact and the specific CD44 protein sequences that couple to the LOX promoter have not been determined and are currently under investigation.

On the cellular level, LOX expression endowed cells with multiple phenotypes of malignancy, including enhanced abilities to resist anoikis, increased motility, and enhanced ability to metastasize to mouse lungs and bones (Fig. 2 and Figs. S3 E and F and S4). These results, together with the observation that LOX was potently induced by MSCs in different BCC lines (Fig. 1F), suggest that LOX represents a conduit for the generalized response of cancer cells to the prometastatic signals of stromal MSCs. In this respect and in light of the fact that most (but not all) of the tested prometastatic actions of MSCs were sensitive to the LOX inhibitor BAPN or shLOX, we posit that anti-LOX–based therapeutic approaches may be effective in combating metastatic disease, not only by targeting premetastatic niche formation (19) but also by targeting cancer cells.

Previous work indicated that induction of EMT caused induction of CSC phenotypes, and conversely, CSCs were found to be enriched in EMT markers (22–24, 37). These observations indicated that EMT and CSC phenotypes are tightly regulated by the same framework and suggested that the signal transduction pathways that govern these two programs are intimately intertwined. However, we found that, although LOX induction was sufficient to cause Twist up-regulation (and EMT) to levels comparable with those levels observed in BCC MSC, it was not sufficient in driving the CSC phenotype (Fig. 5F). Taken together, these data argue that the upstream stroma-initiated signaling pathways governing the propagation of CSCs may not be identical to those pathways fostering EMT.

Emerging concepts in cancer metastasis emphasize the role of the tumor stroma in regulating the metastatic cascade and suggest that cancer cells are highly responsive to the contextual signals of nearby stromal cells (2). In this context, the induction of EMT and CSC phenotypes in the cancer cells by stromal MSCs is consistent with the transient nature of such contextual signals, because both EMT and CSC programs may need to be reversed to allow for the completion of the metastatic cascade (22). Indeed, BCC MSC exhibited a gradual decrease in their LOX content over time (Fig. S10), which corroborates this hypothesis and presents LOX-elicited signaling as a prominent molecular feature of this transient state. Because the protumorigenic and prometastatic functions of stromal MSCs have now been recognized in multiple tumor settings (7, 38), it would be important to exploit such a platform to further characterize the molecular nature of the heterotrophic cross-talk that takes place at the tumor-stroma interface.

Materials and Methods

Cell lines and culture conditions, Affymetrix arrays and GSEA analyses, matrix preparations, migration assays, constructs, reagents, primer sequences, LOX enzyme assays, ALDEFLUOR assays, mammosphere assays, immunohistochemical methods, animal work, ChIP, and clinical analyses are discussed in SI Materials and Methods.
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Supporting Information

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SI Materials and Methods

Cell Lines and Cell Cultures. Breast cancer cell (BCC) lines MDA-MB-231, MCF7, MCF7/Ras, T47D, and MDA-MB-435 and normal human embryonic lung fibroblasts (WI-38) or HEK-293T cells were cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin. Bone marrow-derived human mesenchymal stem cells (BM-MSCs) were obtained from the Institute for Regenerative Medicine at Scott and White, Texas A&M Health Science Center (Temple, TX). These cells originate from bone marrow aspirates of healthy donors, and they are purified first by density centrifugation and then by expansive culture conditions conducive to the growth of MSCs. Before release to users, these cells are analyzed over three passages for CFUs, cell growth, differentiation potential into fat, bone, and chondrocytes, and various cell marker expression by flow cytometry (negative for CD36, CD34, CD19, CD11b, CD45, CD49b, CD3, and CD184; positive for CD166, CD90, CD105, CD147, CD49c, and CD29). Adipose-derived MSCs (Ad-MSCs) were purchased from ScienceCell Research Laboratories (7510). The cells were isolated from human adipose tissue and characterized as being uniform for CD73, CD90, and CD105 and lipid staining after differentiation tests. BM-MSCs and Ad-MSCs were cultured in α-MEM supplemented with 15.6% FBS, 1% glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. All cells were maintained at 37 °C under 5% CO2.

Affymetrix Arrays and Gene Set Enrichment Analyses. One microgram total RNA was used to prepare cDNA using the Genechip HT One-Cycle cDNA Synthesis Kit (900687; Affymetrix) and the GeneChip HT IVT Labeling Kit (900688; Affymetrix). cRNA targets were cleaned up, fragmented, and hybridized to Affymetrix HT-HG U133 2.0 Plus Arrays (900751; Affymetrix). The hybridization and subsequent washing and staining were performed on the Affymetrix GeneChip Array Station automation platform. Samples that present with the range of 40-60% of genes and GAPDH and β-actin ratios less than three passed and were incorporated in the analyses. Gene set enrichment analyses (Broad Institute of Harvard and Massachusetts Institute of Technology: www.broadinstitute.org/gsea) were performed on the genes found up-regulated in the array sets of BCCMSC vs. BCC alone.

Direct Coculture, Sorting, and Long-Term Reculture. BM-MSCs, Ad-MSCs, or WI-38 cells were seeded at a density of 3 × 10⁶ cells in a 15-cm tissue culture plate and allowed to adhere overnight. GFP-expressing BCCs were then overlaid on top of the monolayers at a density of 1 × 10⁶ cells. All cell types were cultured individually in parallel as controls. Cells were maintained in MSC-specific media and incubated for 3 d. Based on the experiment, cells were treated with β-aminopropionitrile (βAPN; 100 µM, A3134; Sigma Aldrich), hypoxia-inducible factor 1α (HIF1α) inhibitor LW6 (10 µM; EMD Chemicals), Rho-associated kinase (ROCK) inhibitor (10 µM, Y27632; Calbiochem), or hyaluronidase (150 U/mL; EMD Chemicals) for the duration of the incubations. After 3 d incubation, cocultured cells were trypsinized, filtered to obtain single-cell suspensions, and separated by flow cytometry. GFP-expressing BCCs were recovered, and the samples were divided into portions processed for RNA and/or protein extraction using PARIS kit (Ambion); where applicable, they were recultured for additional analysis.

Indirect Coculture. BM-MSCs were cultured in α-MEM with 2% FBS, 1% glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin for 3 d. Media were then collected and transferred to BCCs to determine the effects of MSC-secreted soluble factors on BCC gene expression. After culture in MSC-conditioned media for 3 d, BCCs were lysed, and RNA was extracted and used in quantitative RT-qPCR (RT-qPCR) gene expression analyses. BCCs grown in α-MEM media served as controls. Alternatively, MSCs and BCCs were grown in a Boyden chamber, where the two populations were separated by a 0.4-µm porous membrane that allows for soluble factor exchange. MSCs were cultured alone or together with BCCs in the top insert in α-MEM with 2% FBS, 1% glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin, whereas BCCs were cultured in the bottom well for 3 d. After incubation, BCCs seeded in the bottom chamber were lysed, and RNA was extracted and used in RT-qPCR gene expression analyses.

Conditioned ECM Assay. BM-MSCs were cultured in α-MEM with 2% FBS, 1% glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin for 3 d with or without hyaluronidase (150 U/mL) treatment. Cells were then washed three times with PBS and incubated with 25 mM EDTA in PBS (pH 7.4) at 4 °C overnight to allow for gentle cell detachment with minimal effect on the MSC-deposited matrix. After cell detachment, the solution was aspirated, and the plate was washed repeatedly with PBS. BCCs were then seeded onto that plate and incubated for 3 d in MSC media. After incubation, BCCs were lysed, and RNA was extracted to determine the effect of MSC ECM proteins on BCC gene expression. For preparation of the hyaluronic acid-coated culture surfaces, hyaluronan produced by microbial fermentation of Streptococcus spp. was purchased from R&D Systems at different molecular masses [GLR003, ultra low (4–9 kDa); GLR001, low (15–40 kDa); GLR004, medium (75–350 kDa); GLR002, high (>950 kDa)] and reconstituted in water as per the manufacturer’s recommendations. Products had >99% purity and were used at a concentration of 100 µg/mL as described in the work by Martchenko et al. (1). Tissue culture plates were preincubated with 100 µg/mL hyaluronidase overnight at 4 °C. The next day, 1 × 10⁶ MDA-MB-231 cells were overlaid on top of the hyaluronic acid layer and incubated for 3 d at 37 °C. After incubation, cells were lysed, and their RNA was extracted and used in RT-qPCR analysis.

Micromechanical Reconfigurable Culture (Combs). This system consists of micromachined silicon substrates suitable for tissue culture. It has two parts that can be repositioned to allow for dynamic manipulation of cell–cell interactions. Using tweezers, the device parts can be locked in two different configurations: gap, where cell populations are separated by a narrow 80-µm distance, or contact, where direct intimate contact is allowed between two cell populations. MSs and BCCs were seeded onto combs at a density of 500,000 cells/mL in a 12-well plate and the cell-coated combs were incubated in gap or contact mode for 3 d. After incubation, cells were scraped, lysed, and RNA-extracted for gene expression analysis by RT-qPCR.

Hypoxia Cultures. To generate a positive control for HIF1α Western blot experiments and validate the effectiveness of the HIF1α chemical inhibitor (LW6; 10 µM; Calbiochem), cells were placed in a reduced oxygen chamber for 2 d and immediately lysed for RNA and protein isolation followed by RT-qPCR and
Western blot analysis of HIF1α levels (610958; BD Transduction). Hypoxic conditions were achieved using INVIVO 400 from Ruskin Technology.

**Rho Kinase (ROCK) Inhibition and Focal Adhesion Kinase (FAK) Immunoblot.** The effectiveness of the ROCK chemical inhibitor (Y27632, 10 μM; EMD Chemicals) was validated by Western blot analysis of p-FAK in treated and untreated cells (p-FAK-Tyr397, 3283; Cell Signaling).

**pBabe-puro-LOX Construct.** pCR4-TOPO-LOX was purchased from Open Biosystems (MHS1010-98052123). Primers for PCR amplification were purchased from Eurofins MWG Operon and included BamHI/EcoRI restriction sites. Primer sequences for full-length human LOX are hLOX-5PRIME-BamHI-CGGGAT-CCATTCGCTTGCGCTGTA and hLOX-3PRIME-EcoRI-CGGGATCTTCCATATACGTTGAAATTTGTC. PCR products were subcloned into pBabe-puro vector and verified by sequencing. HEK-293T cells were transfected with pBabe-LOX using retroviral packaging and Fugene HD (Roche). The viral supernatants were collected at 48 h, passed through a 0.45-μm filter, and used to infect BCCs (MDA-MB-231 and MCF7/Ras) in the presence of polybrene (10 μg/mL). Stable cell lines were generated after puromycin selection (2 μg/mL). LOX mRNA expression and protein levels were verified by RT-qPCR and Western blot analysis, respectively. LOX promoter-driven luciferase reporter was purchased from SwitchGear Genomics (S703087).

**shRNA Constructs.** LOX and Twist shRNA constructs were obtained from the Dana-Farber Cancer Institute (Boston, MA) through the RNAi Consortium. The CD44 shRNA constructs were obtained from S. Godar (University of Cincinnati Cancer Institute, Cincinnati, OH) (2). HEK-293T cells were transfected with shRNA constructs and (F) 5′-GCCATCACTGTTGAGAAAAC-3′ and (F) 5′-GGTTACAGATGCAGCTG-3′ primers were purchased from Qiagen. Primer sequences for full-length human β-actin were purchased from Sigma. Primer sequences for full-length human Vimentin were (R) 5′-AGAGGAAACATCGCTGTTG-3′ and (F) 5′-GGTTACAGATGCAGCTG-3′. PCR products were subcloned into pBabe-puro vector and verified by sequencing. HEK-293T cells were transfected with pBabe-LOX using retroviral packaging and Fugene HD (Roche). The viral supernatants were collected at 48 h, passed through a 0.45-μm filter, and used to infect BCCs (MDA-MB-231 and MCF7/Ras) in the presence of polybrene (10 μg/mL). Stable cell lines were generated after puromycin selection (2 μg/mL), and gene silencing was verified by RT-qPCR and Western blot analysis.

**Amplite LOX Enzymatic Activity Assay.** BCC lysates and conditioned media from direct cocultures with BM-MSCs or BCCs were collected at 48 h, β-actin served as loading control. BCC lysates and conditioned media from direct cocultures with BM-MSCs or BCCs were incubated for 5 d and imaged every day, and the mammospheres subsequently fixed with 4% paraformaldehyde (PFA). The inner part of the membrane was cleaned with a cotton swab, and the bottom surface was stained with DAPI and mounted on a glass slide. The number of cells that migrated to the lower surface of the membrane was determined by nuclei counts under the microscope.

**Transwell Migration Assays.** Transwell migration assays were performed as standard. Briefly, 50,000 BCCs were seeded in the top chamber of a Boyden chamber apparatus and allowed to migrate overnight across 8-μm porous membranes toward media with 2% FBS placed in the bottom chamber. The membranes were subsequently fixed with 4% paraformaldehyde (PFA). The inner part of the membrane was cleaned with a cotton swab, and the bottom surface was stained with DAPI and mounted on a glass slide. The number of cells that migrated to the lower surface of the membrane was determined by nuclei counts under the microscope. Western blots. Cells were lysed using 150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM

**Microfluidic Migration on Collagen I and IV.** Microfluidic devices composed of microchannels coated with collagen I and collagen IV were used to test the ability of BCCs to invade and migrate in the presence or absence of βAPN (100 μM). Devices were placed in a multiwell plate, and 2 μL suspension was loaded at a density of 10^4 cells/mL. The plate was mounted on the automated stage of a Zeiss Axiovert microscope equipped with an environmental chamber set at 37 °C and 5% CO2. Cells were imaged using a 10× objective, and phase contrast with individual frames was acquired from each device every 10 min for 8 h. The resulting movies were analyzed by manual tracking of 50 cells per frame using ImageJ (National Institutes of Health).

**Anoikis Assays.** Cancer cells (30,000) were starved in 2% FBS/DMEM for 24 h and then trypsinized and suspended in serum-free media. Tubes were continuously rotated at 37 °C for the duration of the experiments. Viable cells were counted using the trypan blue exclusion assay.

**Real-Time PCR Analysis.** RT-qPCR on cultured cells was performed as standard. Most primers were purchased from Eurofins MWG Operon. LOX primers were purchased from Qiagen. Primer sequences used are as follows: Human Lysyl Oxidase: Qiagen Quantitect primer assay hs LOX_1 SG QT00017311, Lot Number 9132351. LOX-like 1: (R) 5′-AGCCACCCGGGAGTACTCT-3′ and (F) 5′-GCTGAATCCATGAGGAACAA-3′. LOX-like 2: (R) 5′-TCCCTGCTTCCGGCTGTAT-3′ and (F) 5′-GGATCCCTGAAACAAAGG-3′. LOX 3: (R) 5′-CCACAAAGGCTGATGTA-3′ and (F) 5′-GAGTCAGATGGTCTCAGGGAT-3′. ROCK 1: (R) 5′-GATCTCTGTGGGCAAGATC-3′ and (F) 5′-TTGCGGCTATGGAATATCTGCG-3′. HIF1α: (R) 5′-CACTGGTAACTCAGATGC-3′ and (F) 5′-GGGAGGAGAAGCCCTTTTCCT-3′. Zeb1: (R) 5′-CCCTGTTAACAATCCTGCTG-3′ and (F) 5′-AAGAAGATTGTACAGATGCAGTCTG-3′. Vimentin: (R) 5′-GTCCTGCTTGATTGGGCAACATC-3′ and (F) 5′-GAGAATCTTGCCGGTGAAACG-3′. Fibronectin: (R) 5′-TCCCTCGGAAATCAGTCAACAGG-3′ and (F) 5′-CGTGGGAGACCTCTGGAAGA-3′. E-cadherin: (R) 5′-CATCTGATCGTTACCTGATC-3′ and (F) 5′-AGAACGCTATTGCCACATACCTC-3′. N-cadherin: (R) 5′-CCGAGATGTTGTAATAGT-3′ and (F) 5′-ACATGGGACACCTTTACAAAGG-3′. Smooth Muscle Actin: (R) 5′-GCGTTCAAGCAGCATAGAGG-3′ and (F) 5′-ACTGGGAGACATGGAGAAGG-3′. CD44: (R) 5′-ACTGTTGGGTTGAATGTTGTCCT-3′ and (F) 5′-AGGAGACGACTTCAGGAAGTTAC-3′. HIF-1α: (R) 5′-ACAGTCCACTTGGTTGCTGA-3′ and (F) 5′-AATGTGCTAGAGAGCAGAAGCAG-3′. GAPDH: (R) 5′-GCCAAATGGACCAAAACTCC-3′ and (F) 5′-GACCCATCGTCTAGACAC-3′. Actin: (R) 5′-TCCAATGACCTTGGTGGTGG-3′ and (F) 5′-AGATGATTCCGACCTTGG-3′. Mammosphere Assays. BCCs were suspended at a density of 10,000 cells/well in ultra low attachment six-well plates. Cells were incubated for 5 d and imaged every day, and the mammospheres were quantified. For secondary mammospheres, the primary spheres were dissociated with Trypsin, counted, and reseeded at a density of 1,000 cells/well for another 5 d.

**ALDEFLUOR Assay and Analysis.** The ALDEFLUOR kit (StemCell) was used to measure aldehyde dehydrogenase 1 (ALDH1) enzymatic activity in BCCs from direct coculture with GFP-labeled MSCs. Sorted MDA-MB-231 and MCF7/Ras cells previously exposed to MSCs were suspended in ALDEFLUOR assay buffer containing ALDH1 substrate and incubated for 1 h at 37 °C. Treatment with diethylaminobenzaldehyde (DEAB), an ALDH inhibitor, served as negative control. Stained cells were analyzed using the FITC channel on the BD-LSR II flow cytometer.

**Mammosphere Assays.** BCCs were suspended at a density of 10,000 cells/well in ultra low attachment six-well plates. Cells were incubated for 5 d and imaged every day, and the mammospheres were quantified. For secondary mammospheres, the primary spheres were dissociated with Trypsin, counted, and reseeded at a density of 1,000 cells/well for another 5 d.
Tris-HCl, pH 7.5, and 2 mM EDTA supplemented with Protease Inhibitor Mixture kit (Roche) and phosphatase inhibitor mixture (Roche). Wells were loaded with 50 μg protein. After separation by denaturing gel electrophoresis, proteins were transferred to Immobilon-P transfer membranes (IPVH0010; Millipore) and probed overnight at 4 °C using primary antibodies against LOX (ab31238; Abcam), Twist1 (sc-15393; Santa Cruz), fibronectin (610078; BD Transduction), E-cadherin (610182; BD Transduction), N-cadherin (610921; BD Transduction), smooth muscle actin (ab5694; Abcam), vimentin (NCL-VIM-V9; NOVOCAS-TRA), CD44 (3578; Cell Signaling), GFP (A6455; Invitrogen), histone H1 (MA1-35403; Pierce), and β-actin (Cell Signaling). Primary antibody incubations were followed by incubation with HRP-labeled secondary antibodies to detect the immune complexes. Signals were developed using Immobilon-Western chemiluminescent-HRP substrate from Millipore.

**Immunofluorescent Staining.** Cells cultured alone or admixed with MSCs were seeded into four-well chamber glass slides at a 1:3 ratio and incubated for 3 d. Cultures were then washed two times with PBS, fixed with 2% PFA for 15 min, permeabilized (0.1% Triton X-100, PBS, 0.5% albumin, 0.02% NaN₃) for 10 min, and blocked in normal goat serum for 30 min at room temperature. After blocking, cells were incubated in primary antibody solution against Twist1 (ab50887; Abcam) and CD44 (3570; Cell Signaling) overnight at 4 °C. The next day, the chambers were washed with PBS, and cells were incubated in FITC- or tetramethyl rhodamine isothiocyanate (TRITC)-labeled secondary antibody solution for 1 h at room temperature. Slides were mounted in Vectashield mounting solution containing DAPI and covered with a coverslip. Pictures were taken under fluorescent microscopy (Nikon).

**Animal Experiments.** Nude mice were injected s.c. with BCCs (10⁶ cells) overexpressing LOX or containing vector controls. Another set of mice was injected with BCCs harboring LOX-shRNA cells alone or admixed with MSCs. Cells with Luciferase-shRNA were used as controls. Tumor growth was monitored over weeks and assessed by caliper measurements. At the end of the study, tumors, lungs, and ribs were harvested. Metastasis to lung was assayed by counting the number of GFP-positive nodules under fluorescence microscopy. Metastasis to bone was assessed using immunohistochemistry and counting the number of colonies that stained positive for GFP. The number of GFP-positive colonies was averaged for each group and reported as fold change relative to control. Where indicated, tumors were dissociated with collagenase, sorted based on GFP, and analyzed by RT-qPCR to probe for LOX mRNA expression. Lungs and ribs were formalin-fixed and processed for immunohistochemical analysis as described below.

**Immunohistochemistry.** Tumors, lungs, and ribs were fixed overnight in 10% formalin. Ribs were additionally softened in decalifying solution for 2 d. Fixed tissues were embedded in paraffin and sectioned. Antigen retrieval was done in a pressure cooker by boiling sections immersed in sodium citrate for 1 h. Sections were blocked in normal goat serum for 1 h and incubated in 1:500 dilution of anti-LOX (ab31238; Abcam) or anti-GFP primary antibody (A6455; Invitrogen) overnight at 4 °C followed by secondary anti-rabbit biotinylated antibody (Vector Laboratories). Avidin–biotin solution was added on the slides followed by detection with DAB and then counterstaining with hematoxylin.

For staining of snap-frozen tissues obtained from patients with metastatic breast cancer, sections were fixed in freshly prepared 4% PFA solution for 10 min and then dipped in hot citrate buffer solution (10 mM) for 12 min. Sections were chilled on ice, washed two times with PBS, and blocked in 7% horse serum for 30 min at room temperature. Primary antibody incubation with Twist1 (1:100, ab49254; Abcam) and CD44 (1:100, ab6124; Abcam) was done overnight at 4 °C. This incubation was followed by AlexaFluor-labeled donkey anti-rabbit 594 or goat anti-mouse 488 antibodies (1:300; Molecular Probes) for 1 h at room temperature. Hoechst solution was used for nuclear staining (1:10,000) for 3 min. Slides were then rinsed with distilled water and covered with polynvinyl alcohol mounting medium (Fluka).

**Chip Analyses.** GFP-labeled MDA-MB-231 cells were cocultured with MSCs for 72 h and then sorted, lysed, and processed for ChIP using the MAGnify ChIP System (Invitrogen). Briefly, 10⁶ cells were treated with formaldehyde to cross-link chromatin, lysed using buffer provided in the kit, and incubated with polyclonal anti-CD4 antibody (3578; Cell Signaling) overnight at 4 °C. The chromatin was then washed, the cross-linking was reversed, and the DNA was purified before PCR analysis with LOX promoter primers: full-length forward 5′-CGCGAATTCTGTAAGTGTCCTTCTGAGTG-3′ and reverse 5′-CGCGGATCCGGGAAAGGAGAAATCTTCCAACC-3′; SAT2 primers forward 5′-CTGCAATCATCCAAATGCGTGCTG-3′ and reverse 5′-GATTCCATTGGGTCCATTCC-3′ served as controls.

**Statistical Analysis.** All in vitro and in vivo data were analyzed using Student’s t test. Significance interval was set at P < 0.05.

**Clinical Correlation Studies.** Raw datasets of GSE2109 with 2,158 various human cancer samples were downloaded from the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE2109). There are 33 breast cancer samples in this dataset. Raw array intensity CEL files were normalized with Affymetrix package from bioconductor (http://www.bioconductor.org) software. Normalized intensity value for both TWIST1 (probe set ID 213943_at) and LOX (probe set ID 204298_s_at and 215446_s_at) were pulled out for correlation analysis with all 2,158 cancer samples as well as only breast cancer samples. The expression level of LOX was calculated as an average of the two probe sets. The correlation plot with r and P values between log₂ TWIST1 and log₂ LOX expression intensity was calculated by EXCEL.

Raw datasets of GSE1456, GSE2034, and GSE4922 were also downloaded from the National Center for Biotechnology Information Gene Expression Omnibus website, log-transformed if not already done so, and median-centered. Pearson correlation was calculated between LOX (probe set 215446_s_at) and TWIST1 (probe set 213943_at), and a linear model was fit. The r values for the three studies were GSE1456 = 0.48, GSE2034 = 0.41, and GSE4922 = 0.41.

**Patient Survival Studies.** Using median-centered log ratios from GSE2034, samples were partitioned into groups of high or low levels of LOX and TWIST1 by comparison with the median of all samples. Samples with high or low levels of both LOX and TWIST1 were selected for additional analysis. Together with clinical parameters from the National Center for Biotechnology Information Gene Expression Omnibus, survival analysis was performed using Kaplan–Meier estimates using the log-rank test and the proportional hazard model to compare survival curves.

**SI Results**

The LOX promoter harbors HIF1α-response elements (3), and hypoxic conditions were shown to stimulate LOX transcriptional activation (4). Interestingly, de novo stimulation of LOX mRNA by MSCs was not accompanied by HIF1α up-regulation (Fig. S9).4. In addition, treatment of the MDA-MB-231:MSC cocultures with 10 μM of a cell-permeable amidoephonic compound that prevents HIF1α accumulation (5, 6) (Fig. S9 B and C) was not sufficient in blocking the ability of MSCs to trigger
LOX expression in the cancer cells (Fig. S9A). These results indicated that MSC-triggered LOX up-regulation in the cancer cells may be independent of HIF1α.

Most recently, the work by Levental et al. (7) described the induction of LOX protein expression in mammary epithelial cells that invaded firm matrices and suggested that stiff stroma may be regulating LOX in the cancer cells by some form of mechanical signaling (7). Furthermore, previous reports indicated that mechnano-transduction signaling critically relies on the activity of the Rho effector ROCK and that the ROCK inhibitor Y27632 was sufficient to disrupt the ability of cells to sense mechanical tension in the tumor microenvironment (8). However, treatment of MSC + BCC cultures with Y27632 (10 μM) (Fig. S9D and E) did not affect LOX mRNA levels in MSC-stimulated cancer cells, suggesting that LOX induction in our BCCMSC model does not result from tension potentially exerted on the cancer cells by juxtaposed MSCs.

We next explored whether MSC-derived soluble factors were responsible for LOX up-regulation in the cancer cells. In this context, we found that neither the conditioned media derived from MSC cultures (Fig. S9F) nor culture of cancer cells and MSCs on opposite sides of membranes in 0.4-μm transwell chambers (Fig. S9G) elicited any LOX overexpression in the cancer cells. To investigate the possibility that the contact of MSCs with cancer cells enables the release of soluble mediators that then prompt cancer cells to overexpress LOX, we allowed BCCs to exchange factors with BCC:MSC cocultures plated across 0.4-μm membranes (Fig. S9G). However, RT-qPCR on the BCCs stimulated by BCC:MSC media (Fig. S9G, Bottom Left) revealed no stimulation of LOX expression. Similarly, the culture of BCCs with MSCs across combs separated by 80-μm gap (9) (Fig. S9H) did not result in the activation of LOX transcription in the BCCs (Fig. S9I), strongly showing that the MSC-derived signal responsible for robust LOX stimulation in cancer cells is not soluble in nature.

Fig. S1. Identification of MSC-modulated genes in BCCs. (A) GFP-labeled MDA-MB-231 and MCF7/Ras cells were cultured alone or on a monolayer of MSCs for 3 d (BCC:MSC ratio was 1:3). Cells were then sorted based on GFP, and their RNA was purified and processed for gene expression analysis using Affymetrix-based arrays. (B) When all BCCMSC samples were analyzed together, seven genes were found to be increased in both MDA-MB-231MSC and MCF7/RasMSC. (C) Gene set enrichment analysis categorizing the overall modulated genes into the indicated canonical pathways.
Fig. S2. Induction of LOX is specific and occurs in MSC-activated cancer cells and not the cancer-activated MSCs. (A) GFP-labeled BCCs were cultured alone or on a monolayer of MSCs for 3 d (BCC:MSC ratio was 1:3). Cancer cells were then sorted, lysed, and analyzed for LOX, LOX-L1, LOX-L2, LOX-L3, and LOX-L4 mRNA expression by RT-qPCR. Results were normalized to GAPDH. Data are expressed as fold induction ± SEM (n = 3). (B) MSCs were sorted out of cocultures with the indicated GFP-labeled BCCs, and their RNA was isolated and analyzed for LOX mRNA expression by RT-qPCR. Results were normalized to GAPDH. Data are expressed as fold induction ± SEM (n = 3). (C) RT-qPCR data showing the difference (∆Ct) between LOX amplification cycles in cDNA derived from the indicated groups.
**Fig. 53.** βAPN reduces the ability of exogenous LOX to promote cellular migration. (A) LOX mRNA expression levels detected by RT-qPCR on RNA derived from MDA-MB-231 and MCF7/Ras cells constitutively expressing LOX. Results were normalized to GAPDH. Data are expressed as fold induction ± SEM (n = 3). (B) Western blot analyses of the total lysates of MDA-MB-231 and MCF7/Ras cells overexpressing LOX. GAPDH was used as a loading control. The (C) culture media and (D) lysates of control (Ctrl) MDA-MB-231 and MCF7/Ras cells or cells overexpressing LOX (LOX) were harvested, and the enzymatic activity of LOX was measured using Amplitite-based fluorometric analysis. Data are expressed as fold change in relative fluorescence units (RFU) ± SEM (n = 3). (E) Cells overexpressing LOX with or without βAPN (100 μM) were allowed to migrate on collagen types I and IV across microchannels and recorded for 24 h. Movies were analyzed using manual tracking (ImageJ; National Institutes of Health), and average cell velocities (in micrometers per min) were calculated. Data are representative of 50 cells/frame. (F) Wound healing assays initiated by a straight streak in a confluent monolayer of controls or MDA-MB-231LOX and MCF7/RasLOX cells treated with or without βAPN (100 μM). Cells were imaged at 0 and 12 h.

**Fig. 54.** Exogenous LOX is sufficient in promoting metastasis. (A) A total of 10⁶ MDA-MB-231-control and MDA-MB-231LOX cells were injected s.c. into nude mice. Tumor kinetics were followed for 9 wk. Data represent mean ± SEM (n > 7 for each group). (B) Average tumor weight of xenografts in A ± SEM. (C) Representative pictures of GFP-positive nodules in the lungs of mice described in A and B. There was a 5.132-fold increase in the number of GFP-positive nodules observed in the lungs of the MDA-MB-231LOX-bearing mice compared with their controls.

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Fig. S5. LOX activity is required for MSC-triggered and Twist-dependent epithelial-to-mesenchymal transition (EMT). (A) The expression levels of EMT transcription factors were analyzed by RT-qPCR on RNA derived from MDA-MB-231MSC and MCF7/RasMSC and compared with controls. Results were normalized to 18S and are expressed as fold ± SEM. (B) Anti-Twist-TRITC immunofluorescence analyses of MDA-MB-231 control and MDA-MB-231MSC cells treated with or without βAPN (100 μM). DAPI is used to stain the nuclei. (C) Western blot analysis of nuclear extracts of MDA-MB-231 or MDA-MB-231MSC probed with anti-Twist and anti-histone H1 antibodies. (D) MDA-MB-231 and MCF7/Ras cells stably expressing the indicated LOX-shRNA plasmids (shLOX) were stimulated with MSCs for 3 d, after which LOX silencing was analyzed by RT-qPCR on sorted BCCs. shRNA against luciferase (shLuc) served as the control. Data were normalized to β-actin and are represented as fold induction in LOX mRNA expression ± SEM. (E and F) GFP-labeled BCCs harboring shLuc or shLOX-2 were cultured alone or with MSCs for 3 d. Sorted BCCs were subjected to RT-qPCR for the indicated genes. Results are expressed as fold induction ± SEM (n = 3). (G) GFP-MCF7/Ras cells cultured alone or with MSCs were treated with vehicle or βAPN (100 μM). BCCs were sorted as described earlier, lysed, and subjected to Western blot analysis for Twist and Actin. (H) Western blots on control (Ctrl) or LOX-overexpressing cells (LOX) using antibodies for the indicated proteins. Actin was used as loading control.
Fig. 56. Twist is required for MSC-triggered LOX induction in the cancer cells. (A) RT-qPCR on sorted cancer cells stably expressing the indicated shRNA constructs after culture with or without MSCs for 3 d. Data were normalized to GAPDH and are presented as fold of Twist mRNA expression ± SEM. (B) Western blot results on select groups in A. Actin was used as a loading control. (C) GFP-MDA-MB-231 control and GFP-MDA-MB-231-shTwist cells sorted out from MSC cocultures were analyzed by RT-qPCR for LOX mRNA expression levels. Data were normalized to GAPDH and are presented as fold induction ± SEM.
Fig. S7. Induction of the cancer stem cell phenotype by MSCs is not dependent on LOX. (A) ALDH1 activity determined on control cells and BCCLOX. Panels are representative of three independent repeats and compared with DEAB-treated samples. (B) Mammosphere formation of control cells (Ctrl) and BCCLOX (LOX) seeded in low-attachment conditions. (C) Total numbers of spheroids in B were quantified on day 5 after culture and averaged from three independent repeats, each performed in triplicates (mean ± SEM). (D) ALDH1 activity assays on sorted cancer cells cultured alone, from MSCs, or stimulated by MSCs in the presence of βAPN (100 μM) for 3 d. Samples were normalized to DEAB-treated controls. (E) MSCs were cultured with cancer cells in Boyden chambers across 0.4-μm filters, allowing for exchange of soluble factors. (F) Cancer cells in E were recovered and subjected to ALDEFLUOR assays to determine ALDH1 activity. Panels are representative of three independent repeats and compared with DEAB-treated controls. (G) Primary mammosphere formation of BCC controls or BCC sorted from 3-d cultures with MSCs or 3-d cultures with MSCs in the presence of βAPN (100 μM). (H) Spheroids of low-attachment cultures in the indicated groups were quantified on day 5 after culture and averaged from three independent repeats, each performed in triplicates (mean ± SEM). (I) Secondary mammospheres were generated from primary spheres of the same groups that have been dissociated and reseeded at a density of 1,000 cells/well in low attachment plates. Bar graphs represent the number of spheres per group categorized by size (<500 or >500 μm) and counted at day 5 after culture. (J) ALDH1 activity determined by ALDEFLUOR assays on MDA-MB-231 controls or MDA-MB-231-shLOX-2 sorted out of cocultures with MSCs. Results were normalized to DEAB-treated controls.
**Fig. S8.** MSC-induced metastasis depends, in part, on LOX activity. Nude mice were injected with $0.7 \times 10^6$ MDA-MB-231 cells expressing shLuc or MDA-MB-231 cells expressing shLOX-2 alone or together with $2.1 \times 10^6$ MSCs. Tumor kinetics were measured for 3 wk, after which the mouse lungs were excised and analyzed for GFP-positive nodules. Indicated values represent the average number of GFP-positive clusters per lung section. $n = 10$ mice per group.
Fig. 59. Induction of LOX by MSCs is mediated by ECM factors. (A) RT-qPCR of LOX and HIF1α on sorted MDA-MB-231 cells cultured alone or on a monolayer of MSCs for 3 d (BCC:MSC ratio was 1:3) in the presence or absence of HIF1α inhibitor (LW6; 10 μM). Results were normalized to β-actin. Data are expressed as fold induction ± SEM (n = 3). (B) RT-qPCR of HIF1α on MDA-MB-231 cells cultured under hypoxia in the presence or absence of HIF1α inhibitor (C) Western blot analysis of cell lysates of MDA-MB-231 cells cultured alone or sorted out of cocultures with MSCs with or without HIF1α inhibitor under normoxia or hypoxia. (D) RT-qPCR for LOX on sorted BCCs cultured alone or along with MSCs in the presence or absence of the ROCK inhibitor Y27632 (10 μM). Data are expressed as fold induction ± SEM (n = 3). (E) Western blot analysis of cell lysates of MDA-MB-231 or MCF7/Ras cells cultured alone or sorted out of cocultures with MSCs with or without the ROCK inhibitor. Antiphospho-FAK antibody was used as a measure of ROCK activation. (F) RT-qPCR analyses on MDA-MB-231 and MCF7/Ras cells treated for 3 d with the conditioned media (CM) derived from the indicated cultures. Data represent mean ± SEM. Data were normalized to GAPDH. (G) RT-qPCR analyses on MDA-MB-231 and MCF7/Ras cells cultured in the Boyden chamber set up (Left, bottom wells) for 3 d with the indicated culture groups on the membrane top (Left, top wells). Data represent mean ± SEM and were normalized to GAPDH. (H) GFP-labeled cancer cells were cultured on combs and clipped together with partner combs carrying MSCs in two positions: gap and contact. (I) RT-qPCR analysis for LOX on cancer cells recovered from H. Data were normalized to GAPDH and are represented as fold induction ± SEM (n = 3). (J) MSC monolayers were cultured for 3 d and then gently lifted with EDTA solution. The remaining ECM tethered to the culture dish was washed with PBS. Cancer cells were then plated on top of the conditioned ECM for 3 d. (K) MDA-MB-231 controls or cells harboring shLOX-2 were recovered after 3 d of culture on MSC-ECM in J and subjected to RT-qPCR analyses on LOX. Values were normalized to GAPDH and are represented as fold induction ± SEM of duplicate experiments (n = 2).

Fig. S10. The induction of LOX in the cancer cells by MSCs is reversible. RT-qPCR analysis of LOX mRNA expression in MDA-MB-231 or MCF7/Ras cells sorted out after 3-d coculture with MSCs and put back in culture alone for 21 d. Values were normalized to GAPDH and are represented as fold induction ± SEM (n = 2).