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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 mesodermal lineages in the course of normal tissue homeostasis or during injury. We have previously shown that MSCs migrate to sites of tumorogenesis, 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. 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. 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 interfering 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. 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. 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 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 (GSEA) 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...
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 (16–18) and contribute to premetastatic niche formation (19). However, how LOX exerts its functions and whether it serves a role in direct epithelial to stromal interactions has not been determined. We, therefore, proceeded to investigate the role of LOX in how cancer cells respond to activated MSCs.

Quantitative assessment of LOX mRNA levels in BCCMSC confirmed the ability of MSCs to trigger multifold induction of LOX in the cancer cells in vitro (Fig. 1A). Similar in vivo analyses conducted on xenografts containing human MSCs revealed a strong enrichment (>50-fold) for LOX mRNA in cancer cells sorted out from the dissociated tumors (Fig. 1B) as well as marked induction of LOX protein detected by immunohistochemistry on the tumor sections (Fig. 1C). LOX up-regulation in the cancer cells was not triggered by other cocultured mesenchymal cells, such as human lung embryonic fibroblasts (WI-38), and it was weakly induced by adipose-derived MSCs (M4 MSCs) (approximately fourfold induction above background) (Fig. 1D). Of note, MSCs did not significantly induce the expression of any of the highly related LOX-like genes (Fig. S2A), suggesting that the effects of MSCs on LOX induction are specific.

Importantly, LOX expression was only induced in the BCCMSC (and not in the cocultured MSCs) (Fig. S2 B and C), and it resulted in the enrichment of the active 32 kDa mature form of the protein (14) (Fig. 1E). Indeed, the MSC-induced LOX was shown to be enzymatically active in the media of the cancer cells using AmphiCell-based fluorimetric assays, an effect that was significantly down-regulated by the LOX inhibitor β-aminopropionitrile (APN) (100 μM βAPN) (Fig. 1F). Furthermore, cancer cells expressing a LOX promoter-driven luciferase reporter construct displayed more than sevenfold increase in luciferase activity when stimulated by MSCs both in vitro (Fig. 1G) and in vivo (Fig. 1H), suggesting that MSCs induced de novo transcriptional up-regulation of LOX in carcinoma cells. Finally, in addition to their actions on the MDA-MB-231 and MCF7/Ras, MSCs were also able to drive LOX expression in T47D, MCF7, and MDA-MB-435 human BCCs (Fig. 1J), suggesting that the induction of LOX by MSCs in BCCs is a general phenomenon. Taken together, these observations indicate that bone marrow-derived MSCs induce specific de novo expression of LOX in BCCs, resulting in potent accumulation of the 32 kDa mature form of the protein.

**LOX Overexpression Promotes Breast Cancer Invasion, Metastasis, and Epithelial-to-Mesenchymal transition.** Next, we examined whether LOX overexpression in BCCs (BCCLOX) 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. S3 B–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. 2 B and C and Fig. S3E). In addition, LOX overexpression enhanced wound-healing assays (Fig. S3F) and delayed anoikis (Fig. 2D). Most importantly, BCCLOX formed s.c. tumors (Fig. 2E and Fig. S4 A and B) that were approximately five to eight times more metastatic than controls (Fig. 2 E 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 BCCLOX. Indeed, LOX caused a multifold up-regulation in these mesenchymal markers (Fig. 3 A 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.
Twist is required for LOX induction by the MSCs. How this regulation is exerted is at present undetermined. 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 multifold rise in the ALDH1 positivity of BCCs (Fig. 3 D and E), and effectively inhibited MSC-induced EMT altogether (Fig. 3 F and G). Neutralized the ability of MSCs to induce Twist in admixed BCCs (Fig. 3 G and Fig. S5G), and compromised the induction of EMT in BCC-LOX (Fig. S5H). These observations provided strong indications that LOX is a critical mediator of MSC-induced EMT in BCCs 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 Twist 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 promoted 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 LOX inhibited any 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. S7 B and C), suggesting that, although LOX could promote robust metastasis, it was not sufficient to promote entrance into the CSC state.

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We focused on determining the nature of the MSC-induced transcription by affecting its promoter activity. Twist expression in the LOX is essential for MSC-induced EMT of BCCs. (Fig. 4A) RT-qPCR for the indicated genes conducted on sorted GFP-BCC cultured alone, with MSCs or WI-38 cells, or on BCCLOX or their controls. (b) Western blots on BCC controls or BCCLOX. (c) Twist RT-qPCR on samples in A. (D) RT-qPCR for LOX and Twist on MDA-MB-231 controls or MDA-MB-231-shLOX-2 cultured ± MSCs. (E) Western blots on MDA-MB-231 controls or MDA-MB-231-shLOX-2 cells cultured ± MSCs. (F) Western blots on BCC controls or BCCLOX ± β-APN (100 µM). (G) RT-qPCR for LOX and Twist on BCCs cultured ± MSCs ± β-APN (100 µM) for 3 d. Data in A, C, D, and G were normalized to β-actin and are expressed as fold induction ± SEM (n ≥ 3).

**Fig. 3.** LOX is essential for MSC-induced EMT of BCCs. (A) RT-qPCR for the indicated genes conducted on sorted GFP-BCC cultured alone, with MSCs or WI-38 cells, or on BCCLOX or their controls. (B) Western blots on BCC controls or BCCLOX. (C) Twist RT-qPCR on samples in A. (D) RT-qPCR for LOX and Twist on MDA-MB-231 controls or MDA-MB-231-shLOX-2 cultured ± MSCs. (E) Western blots on MDA-MB-231 controls or MDA-MB-231-shLOX-2 cells cultured ± MSCs. (F) Western blots on BCC controls or BCCLOX ± β-APN (100 µM). (G) RT-qPCR for LOX and Twist on BCCs cultured ± MSCs ± β-APN (100 µM) for 3 d. Data in A, C, D, and G were normalized to β-actin and are expressed as fold induction ± SEM (n ≥ 3).

**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 heterogeneous signaling responsible for the induction of LOX in the cancer cells originates from the ECM of MSCs. Influnence by these results, 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/hyaluronic acid is one of the most abundant proteoglycan 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. S1), 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 its abundance in the stromal environment of cancer cells causes their 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 grouped in different sizes from 4–8 to >950 kDa. Interestingly, only the high-molecular weight HA substratum 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 and activation of gene promoters (33). To investigate whether such a mechanism operated in our cocultures, we probed for the 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. 5H).
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 metaplastic 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 premise 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 tenseness. 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. 1I), 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 BCCMSC, it was not sufficient in driving the CSC phenotype (Fig. S7). 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, BCCMSC 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 heterotopic cross-talk that takes place at the tumor to 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.
Fig. 5. The LOX–Twist axis is a powerful prognostic indicator in breast cancer. Correlation analyses of the expression intensity of Twist and LOX in GSE2109 (adjusted $R^2 = 0.12, P = 2.26 \times 10^{-10}$), GSE2227 (adjusted $R^2 = 0.15, P = 8.27 \times 10^{-13}$), and in three cohorts of breast cancers GSE1456 ($R^2 = 0.38, P = 2.87 \times 10^{-13}$), GSE4922 ($R^2 = 0.17, P = 4.6 \times 10^{-13}$), and GSE2034 ($P = 0.037$). (C) Kaplan–Meier patient survival curves for the indicated breast cancer pools in GSE2034. (D) CD44 and Twist staining of metastatic breast cancer tissues. (E) LOX mRNA expression in clinical metastatic breast cancer tissues. 1–6, Normal matched controls; 7, epithelial; 8, squamous; 9, glandular; 10, chondroid; 11–20, fusiform.

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