LOX-Mediated Collagen Crosslinking Is Responsible for Fibrosis-Enhanced Metastasis

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Abstract

Tumor metastasis is a highly complex, dynamic, and inefficient process involving multiple steps, yet it accounts for more than 90% of cancer-related deaths. Although it has long been known that fibrotic signals enhance tumor progression and metastasis, the underlying molecular mechanisms are still unclear. Identifying events involved in creating environments that promote metastatic colonization and growth are critical for the development of effective cancer therapies. Here, we show a critical role for lysyl oxidase (LOX) in creating a milieu within fibrosing tissues that is favorable to growth of metastatic tumor cells. We show that LOX-dependent collagen crosslinking is involved in creating a growth-permissive fibrotic microenvironment capable of supporting metastatic growth by enhancing tumor cell persistence and survival. We show that therapeutic targeting of LOX abrogates not only the extent to which fibrosis manifests, but also prevents fibrosis-enhanced metastatic colonization. Finally, we show that the LOX-mediated collagen crosslinking directly increases tumor cell proliferation, enhancing metastatic colonization and growth manifesting in vivo as increased metastasis. This is the first time that crosslinking of collagen I has been shown to enhance metastatic growth. These findings provide an important link between ECM homeostasis, fibrosis, and cancer with important clinical implications for both the treatment of fibrotic disease and cancer. Cancer Res; 73(6); 1721–32. ©2012 AACR.

Introduction

Metastases are responsible for more than 90% of cancer-related deaths (1), and there is a critical need to develop more effective therapies to combat the metastatic process (2–4). This will be achieved through a better understanding of the underlying molecular processes enabling metastatic colonization to take place and the identification of key events responsible for creating a milieu that promotes metastatic growth. There is extensive evidence across multiple models that there is a fundamentally critical role for the microenvironment and also remodeling of the ECM in promoting tumor growth and metastasis (5–13). At the same time, the diverse and more importantly, dynamic nature of the complex reciprocal signaling networks between both malignant and nonmalignant cells in these environments plays a crucial role in mediating disease progression (14, 15). Thus, tumor metastasis is no longer being seen as a cell autonomous event, but a continuing process, which relies upon these complex networks of microenvironmental cues. It is also widely becoming accepted that the microenvironment is of major importance in determining the survival and growth of disseminated tumor cells at preferential metastatic sites (16, 17). Many of these microenvironmental cues come from the structural components of the ECM in terms of both biochemical and biophysical properties (18) as well as the dynamic remodeling processes that continually reshape the ECM.

The mechanisms contributing to primary tumor development, metastatic progression, and tissue fibrosis share many commonalities including increased matrix deposition and remodeling (19–22). ECM remodeling is particularly prevalent during fibrosis leading to functional changes in biochemical and biomechanical properties of the ECM and resulting microenvironment, which in turn supports the activation of pathogenic signaling pathways and further tissue remodeling (6, 7, 23). Lysyl oxidase (LOX) is an extracellular amine oxidase whose primary function is to posttranslationally modify collagens and elastin in the extracellular matrix, thereby catalyzing the covalent crosslinking of fibers (24, 25). This crosslinking is essential for the stabilization of collagen fibrils and fibers, and for the integrity and elasticity of mature elastin. One of the major substrates for LOX is collagen I, a chief component of both desmoplastic tumor stroma and organ fibrosis. The hallmark of organ fibrosis is the increased ECM protein synthesis by activated fibroblasts, the most abundant of which
is fibrous collagen type I. When secreted at high levels in vital organs, collagen I leads to severe morbidity and sometimes mortality. Fibroblasts in healthy adult lung are quiescent, synthesizing little collagen, yet during fibrosis they activate becoming key producers of ECM components, such as collagen and fibronectin (26–28). Collagen I has also been shown to be a key component of both the primary tumor and metastatic microenvironment (29), and changes have been identified in gene expression signatures associated with poor prognosis and metastases in breast cancers (30–33). Elevated levels of the collagen I precursor, procollagen I, can also be readily identified in the serum of patients with recurrent breast cancer (34). Recently, Barkan and colleagues have elegantly shown that increasing collagen I secretion can drive the activation of dormant D2.0R cells seeded to the lung (35).

LOX-mediated crosslinking of collagen I at the primary tumor site has already been implicated in cell invasion and malignant progression (6, 8). LOX expression has also been shown to be closely correlated with elevated collagen I expression during development (36). However, no one has yet investigated the effects of collagen I crosslinking on metastatic tumor growth.

There is an everexpanding body of evidence emerging, implicating the microenvironmental changes associated with tissue fibrosis in enhancing tumor progression (10). At present, however, the precise mechanisms are yet to be fully elucidated and the role of fibrosis in metastatic dissemination of unrelated primary tumors remains unclear. The contribution of fibrosis in cancer progression and metastasis has been previously reviewed (37). The existence of so-called primary “scar tumors” is generally accepted, a clear example being in the case of hepatic fibrosis, which can lead to portal hypertension and liver failure and is associated with an increased risk of liver cancer (38). Although still the subject of much debate, surgical and adjuvant treatment in cancer, which leads to local scarring, is also thought to lead to the generation of supportive niches for growth and invasion of cancer cells that escape the operation field. The potential effects of radiotherapy on cancer invasion and metastasis, including the role of ionizing radiation-induced stimulation of myofibroblasts, have also been recently reviewed (39). However, to date, there exists limited clinical evidence regarding the presence of preexisting fibrosis in influencing progression of unrelated primary tumors. Indeed, cases are often anecdotal, and links between the initial etiopathogenetic factors of organ fibrosis and final cancer diagnosis are hard to discern. Because of high degrees of heterogeneity in patient cohorts (age, race and gender, tumor subsite, staging, treatments, and so on), we were unable to find convincing evidence in favor or against the presence of preexisting fibrosis in enhancing metastatic colonization of tissues.

Here, we investigate the role LOX plays in creating a pro-metastatic tissue microenvironment during fibrosis. We identify a novel critical role for LOX in establishing and mediating the microenvironment within fibrotic tissues that is subsequently favorable to colonization and growth of metastasizing tumor cells. We show that LOX-dependent collagen crosslinking is key to the development of collagen architecture that is responsible for creating a growth permissive microenvironment capable of enhancing metastatic colonization. This is the first study to functionally show that organ fibrosis and collagen organization at the metastatic site preferentially supports tumor cell colonization and growth leading to metastatic disease.

Materials and Methods

In depth, detailed Materials and Methods are provided in the Supplementary Material.

Cell lines

The 4T1 cells (obtained from American Type Culture Collection Cell Biology Collection) used have been previously described (8, 9). 4T1-GFP cells were generated by stable transfection with a pBOS-H2BGFP vector (BD Pharmingen). Immortalized dermal fibroblasts were a kind gift from Johanna Myllyharju and Joni Mäki, Biocenter Oulu, University of Oulu, Finland. All cell lines regularly tested negative for mycoplasma and murine pathogens by IMPACT testing (IDEXX Laboratories, Inc.)

Tumor histology

Tissue samples were perfused (lung only) with 4% paraformaldehyde following excision, fixed and embedded in paraffin, sectioned and stained for hematoxylin and eosin (H&E), or picrosirius red to assess histocytic changes following standard histopathology operating protocols, and images captured using both parallel and orthogonal light.

Immunofluorescence

Lungs were perfused with a 1:1 mixture of PBS/optimun cutting temperature (OCT) postexcision before embedding in OCT (Tissue-Tek) and immediately freezing on dry ice. Staining for presence of LOX was carried out using the LOX-specific antibody synthesized by OpenBiosystems as previously described (8). For lung colonization studies, the presence of tumor cells was assessed by detection of GFP signal in 30 μm freshly cut sections.

Immunohistochemistry

Four micrometer sections were stained for α-LOX (8), αFN (Abcam), α-smooth muscle actin (α-SMA; Abcam), Ki-67 (Novocastra), or SRC-0.3 (Invitrogen) overnight and visualized with 3,3′-diaminobenzidine. Picrosirius red analysis was undertaken using 4 μm paraffin sections stained with 0.1% picrosirius red (Direct Red 80, Sigma) for fibrillar collagen. Quantitative intensity measurements of fibrillar collagen signal were carried out using ImageJ.

Immunoblotting

Immunoblotting was conducted as previously described (8, 9). The antibodies used included α-SMA (Abcam), LOX (synthesized by OpenBiosystems) to target a conserved peptide sequence from the active site of both human and mouse proteins, as previously described (8), LOXL-1 (Abcam), LOXL-2 (Santa Cruz), LOXL-3 (Santa Cruz), LOXL-4 (Santa Cruz), SRC (Invitrogen), SRC (Abcam) SMAD2 (Invitrogen), SMAD2 (Abcam).
(Cell Signaling Technology), SMAD-2 (Cell Signaling Technology), p53 (Cell Signaling Technology), and β-actin (Abcam). Conditioned media was prepared as previously described (8, 9). All primary antibody incubations steps were carried out overnight.

Determinant of matrix stiffness

Relative stiffness of LOX-, ribose-, or fibroblast-modified collagen gels was measured by shear rheology.

Statistical analyses

Error bars represent the SEM. Data were analyzed using the Student t test unless otherwise stated, and considered significant when \( P < 0.05 \). All statistical tests were 2-sided.

Results

LOX is critical for the development of a fibrotic microenvironment following injury

We investigated the role of LOX in creating a fibrotic microenvironment by establishing 2 models of pulmonary fibrosis in immunocompetent mice: chemically using single-dose bleomycin instillation and radiologically using single nonlethal low-dose thoracic irradiation. Both models resulted in progressive pulmonary fibrosis as determined by significantly increased Ashcroft Score (ref. 40; Fig. 1A). This was characterized by loss of alveolar structure, collapse of alveolar space, and thickening of alveolar septae (Fig. 1B) as a result of abnormal ECM ultrastructure due to increased collagen deposition, as determined by SirCol Assay (Fig. 1C). We also found

Figure 1. LOX is critical for the development of a fibrotic microenvironment following injury. A, Ashcroft scores representing the extent of fibrosis in lungs of control, bleomycin-treated, and irradiated mice. B, Sirius Red staining (parallel light) of pulmonary tissue from control, bleomycin-treated, and irradiated lung tissue. Fibrosis is highlighted by increased collagen deposition, significant disorganized thickening of alveolar septae, loss of normal alveolar architecture, and considerable obstruction of alveoli due to collapse of alveolar space (scale bar, ~50 μm). C, increased collagen deposition in bleomycin-treated and irradiated pulmonary tissue as determined by SirCol quantification. D, immunofluorescence of control, bleomycin-treated, and irradiated pulmonary tissue for LOX (AlexaFluor, green) shows increased levels of expression during fibrosis (scale bar, ~50 μm). E, quantitative analysis of LOX expression from immunofluorescence based on signal intensity measured using ImageJ. F, immunoblot analysis of LOX protein levels confirms elevated expression in both bleomycin-treated and irradiated pulmonary tissue following exposure compared with control. G, rising pulmonary collagen deposition in bleomycin-treated pulmonary tissue over time as determined by SirCol quantification (n = 3 mice/group).
that LOX expression increased in fibrotic lungs induced by both irradiation and bleomycin exposure with LOX signal being associated with regions of fibrosis and in particular increased collagen I expression (Fig. 1D–F). Increased α-SMA expression, a marker for activated fibroblast differentiation in pulmonary fibrosis, and increased collagen I and fibronectin deposition, as a result of fibroblast activation, have also been shown during pulmonary fibrosis (41). In our model of bleomycin-induced pulmonary fibrosis, we observed increased expression of LOX correlating with activation of fibroblasts (α-SMA) and secretion of ECM components (collagen I and fibronectin; Fig. 1G and Supplementary Fig. S1A), which were detectable from as early as 3 days postbleomycin treatment. We observed no discernible differences in the expression levels of the other LOX-like family members, LOXL-1, LOXL-3, and LOXL-4 in either bleomycin-treated or irradiated fibrotic tissue. We did, however, note a small increase in LOXL-2 expression in bleomycin-induced fibrotic lung, consistent with a previously published report (Supplementary Fig. S1B; ref. 42).

To determine the role of LOX in injury-induced pulmonary fibrosis, we inhibited LOX activity using our function-blocking antibody, previously described in (8, 9). Treatment with our LOX-specific antibody (α-LOX) led to a significant reduction in the degree of fibrosis observed. Histologically, α-LOX treatment resulted in a significant reduction in fibrosis showing decreased levels of alveolar thickening and maintenance of alveolar structure as assessed by detection of collagen deposition (Fig. 2A) and by Ashcroft score (Fig. 2B). Notably, treatment with the α-LOX antibody showed a decreased higher order assembly of collagens as determined by decreased fibrillar collagen presence evaluated by picrosirius red staining and orthogonal light interrogation (Fig. 2C and E) when compared with lungs of control mice. Induction of fibrosis and/or treatment with the α-LOX antibody showed no discernible differences in body weight compared with that of matched control immunoglobulin G (IgG; Fig. 2D). Taken together, these findings suggest that LOX is playing a critical role in the development of a fibrotic microenvironment in terms of deposition of mature collagen fibrils contributing to altered ECM ultrastructure.

The fibrotic milieu supports primary breast cancer metastasis through enhanced colonization and outgrowth at secondary sites

It has long been known that fibrosis is closely associated with enhanced primary tumor growth although the mechanisms are unclear. Hence, we investigated whether the generation of a fibrotic milieu would enhance metastatic spread in a xenograft model of primary breast cancer metastasis (43, 44). We used the same treatment groups as above to inhibit LOX activity and examined primary lung metastases as well as bone lesions. The results are shown in Fig. 3. Blocking LOX activity decreases the degree of fibrosis observed (Fig. 3A) and decreases the number of lung metastases when compared with those in the control groups (Fig. 3B and C). Additionally, treatment with α-LOX antibody showed decreased primary tumor size (Fig. 3D) with no discernible differences in body weight or change in distance between the lung metastases and bone lesions compared with those in the control groups (Fig. 3E and F). Taken together, these findings suggest a role for LOX in the development of a fibrotic microenvironment contributing to metastasis.
colonization of tumor cells from a primary tumor. Orthotopic implant of 4T1 mammary carcinomas into the mammary fat-pad of mice following establishment of pulmonary fibrosis either chemically (bleomycin) or radiologically showed no differences in primary tumor growth (Supplementary Fig. S1D), indicating that the presence of fibrotic tissue within the body did not affect primary tumor growth. In contrast, histologic evaluation of lung tissue 3 weeks postimplantation showed a significant increase of approximately 2.5-fold in pulmonary metastatic burden (Fig. 3A and B) in the lungs of bleomycin-treated and irradiated mice compared with control treated reflected in terms of frequency of metastases (Fig. 3C) and also marginally in relative size of metastases (Fig. 3D). These data are the first to show that tissue fibrosis is directly associated with a significant increase in metastatic lesions in orthotopic breast cancer models.

We hypothesized that the effect of a fibrotic microenvironment was affecting metastasis at the late stage, specifically metastatic colonization and outgrowth. A preconditioning model was set up in which the establishment of pulmonary fibrosis was used as the preconditioning step before tail vein injection of 4T1 tumor cells. The presence of established pulmonary fibrosis enhanced metastatic colonization (Fig. 3E and F) of the lung by tail vein injected wt 4T1 mammary carcinoma cells. We observed increases in frequency and average size of metastatic foci contributing to total metastatic burden (Fig. 3G and H) in fibrotic compared with control lungs. Together these findings show that the fibrotic milieu significantly increases metastasis by enhancing metastatic tumor cell colonization and outgrowth.

Therapeutic targeting of LOX prevents fibrosis-enhanced pulmonary metastases

Having noted a reduction in the extent to which fibrosis develops when LOX activity is inhibited (Fig. 2B), we investigated the effect of blocking LOX activity following induction of pulmonary fibrosis on the observed increase in metastatic colonization. Importantly, we saw no discernible effects of α-LOX antibody treatment on the growth of primary tumors subsequently implanted into the mammary fat pad (Fig. 4A). However, we did observe a significant decrease, by approximately 50%, in pulmonary metastatic burden in antibody-treated mice (Fig. 4B and C). These results suggest that therapeutic targeting of LOX can be used to block the generation of the prometastatic fibrotic environment that enhances metastatic colonization of tumor cells.

LOX-mediated hepatic fibrosis enhances metastatic colonization

To determine whether LOX-mediated appropriation of tissue for metastatic tumor cell colonization is limited to pulmonary fibrosis, we used a second model of hepatic fibrosis. Treatment with dimethylnitrosamine (DMN) induces liver fibrosis in a highly reproducible manner and in our model led to the induction of hepatic fibrosis characterized by pathophysiologic alterations including significantly increased collagen deposition, increased fibrillar and crosslinked collagen, and formation of septa (Fig. 5A and B) manifesting as a significant increase in METAVIR state (Fig. 5C).

Liver fibrosis is characterized by an accumulation of ECM proteins secreted by activated hepatic stellate cells (HSC). The proliferation and differentiation of HSCs into myofibroblast-like cells can be detected by the expression of α-SMA (43). We noted high levels of expression in α-SMA in DMN-treated livers, with barely detectable levels in control (Fig. 5D) confirming the development of hepatic fibrosis. Concomitantly, we also observed tissue specific increases in LOX expression (Fig. 5D), in a manner similar to that observed during pulmonary fibrosis. Importantly, we noted a close colocalization in expression with α-SMA not only in the livers of DMN-treated mice, but also the lungs of bleomycin-treated and irradiated mice (Supplementary Fig. S2A), suggesting that α-SMA⁺ cells are the primary producers of LOX. Treatment with the LOX function-blocking antibody led to a significant decrease in the METAVIR state indicating a decrease in the fibrotic reaction (Fig. 5C), however, it showed no effect on either expression of α-SMA or LOX (Fig. 5D) indicating that HSC activation was unaffected. There were no detectable differences in expression of the LOX-like family members LOXL-1, LOXL-3, and LOXL-4 (Supplementary Fig. S2B). However, we did note a slight increase in the levels of LOXL-2 in the liver of DMN-treated mice in support of a previous report (42) using a different model.

Following induction of hepatic fibrosis, orthotopic implant of 4T1 carcinoma cells showed no significant difference in primary tumor growth (Fig. 5E) but did show a significant increase in hepatic metastatic burden in terms of frequency of metastases (Fig. 5F and G) though not significantly in terms of average lesion size in DMN-treated mice. Reduction of fibrosis with the function-blocking LOX antibody before tumor cell implant significantly reduced hepatic metastatic burden in these mice compared with matched IgG control (Fig. 5F and G). These data show that LOX-dependent fibrosis-enhanced metastasis is not restricted to lung tissue but is also observed in the liver suggesting a commonality in the underlying mechanisms of LOX-mediated metastatic dissemination.

LOX-mediated collagen crosslinking during fibrosis enhances tumor cell proliferation to promote metastasis

As the primary responder cells in fibrosis, fibroblasts are activated and are the key producers of matrix materials such as collagen and fibronectin (26–28). α-SMA has been shown to be a marker of fibroblast activation in pulmonary fibrosis (41). To dissect the role LOX plays in fibrosis, fibroblasts plated onto collagen I matrices of physiologically relevant stiffness, were exposed to bleomycin. Following exposure, we observed an increase in α-SMA expression indicating an activated phenotype (Fig. 6A). We also noted an increase in LOX mRNA levels (Supplementary Fig. S3A) and expression of secreted LOX (Fig. 6A) in parallel with elevated α-SMA expression following bleomycin exposure, and an induction of collagen I expression (Supplementary Fig. S6B) confirming an elevated level of ECM production consistent with an activated phenotype. Treatment with our α-LOX antibody did not prevent activation of fibroblasts (as measured by α-SMA expression) in response to bleomycin (Fig. 6A), confirming that LOX expression, activity,
and subsequent matrix crosslinking are downstream of fibroblast activation. Furthermore, treatment with SD-208, a potent, selective ATP-competitive inhibitor of TGF-β receptor kinase I, which has been shown to block myofibroblast differentiation and profibrotic gene expression (44), leads to a loss in the ability of bleomycin to induce subsequent LOX expression.
(Supplementary Fig. S3C–S3E). Hence, both the activation of fibroblasts and subsequent secretion of LOX are critical to the progression of pulmonary fibrosis. Finally, we evaluated the effect of knocking down p53 in fibroblasts using a previously validated siRNA construct (45) on the expression of LOX. We observed no differences in the expression of LOX at either the mRNA or protein level upon knockdown of p53 post-bleomycin treatment (Supplementary Fig. S2C–S2E).

LOX has been shown to increase matrix stiffness through its collagen crosslinking activity. To determine the effects of LOX-mediated matrix stiffness on tumor cell behavior, 4T1 murine mammary carcinoma cells were plated onto type I collagen matrices, which had been modified with purified recombinant LOX or ribose to induce crosslinking (and hence stiffening) either enzymatically or nonenzymatically, respectively. Treatment of collagen I matrices with both LOX and ribose led to significant increases in the complex (G'/C3) moduli (stiffness) of the matrices as measured by shear rheology (Fig. 6B). Increasing the stiffness of collagen I matrices subsequently led to a significant increase in the rate of proliferation of seeded 4T1 cells at 7 days (Fig. 6C), suggesting that the LOX-mediated changes in biomechanical properties (stiffness) of the cellular environment directly influence cellular behavior.

We sought to confirm that the direct remodeling of matrices by activated fibroblasts was responsible for the increased metastatic potential of fibrotic tissue. Fibroblasts were plated onto thin collagen I matrices, and treated with bleomycin to activate them in the presence or absence of our α-LOX antibody. After being given time to remodel the collagen I matrices, fibroblasts were removed leaving the modified matrices intact. Shear rheology, to measure the macroscopic stiffness of the fibroblast remodeled matrices, showed an increase in matrix stiffness in bleomycin-treated but not control or antibody-treated matrices (Supplementary Fig. S3F). Importantly, treatment of identical collagen I matrices with bleomycin, LOX antibody or in combination, in the absence of cells led to no changes in matrix stiffness (Supplementary Fig. S3G), indicating that observed changes are a direct result of cellular interaction with the matrix. The plating of 4T1 mammary carcinoma cells onto these modified matrices led to a significant increase in the rate of proliferation on the collagen I matrices remodeled by activated fibroblasts, which was reduced by addition of the α-LOX antibody to the fibroblasts following treatment with bleomycin (Fig. 6D). These data suggest that following LOX-mediated fibroblast-driven remodeling, the resulting ECM is capable of promoting proliferation of tumor cells.

Fibrosis enhances seeding tumor cell persistence and survival in vivo

We then set out to determine the extent to which the fibrotic environment enhances the early stage of metastasis, namely survival and colonization of secondary organs. Following the establishment of pulmonary fibrosis, 4T1-GFP mammary carcinoma cells were injected into the tail vein of mice. Lungs were assessed at 2, 6, 12, 24, 48, and 72 hours post-inoculation by both flow cytometry (Fig. 6E) and immunofluorescence (Fig. 6G and Supplementary Fig. S3H). We observed no significant difference in tumor cell lodging within the lung at 2 hours after injection (Fig. 6E and G and Supplementary Fig. S3H). We observed clear differences in the persistence of the 4T1
mammary carcinoma cells present within the lung from 6 hours until 72 hours indicating that the fibrotic microenvironment is important in supporting tumor cell survival and subsequent outgrowth. Indeed, at 72 hours, we began to see the emergence of tumor cell clusters within fibrotic lungs (2–3 cells), whereas we only observed the presence of single cells in control lungs at the same time point (Fig. 6G). These data support our hypothesis that the presence of a fibrotic microenvironment supports initial survival and persistence of tumor cells leading to enhanced outgrowth and metastatic burden.

LOX-mediated matrix changes lead to activation of SRC kinase and enhanced proliferation

We investigated SRC activation as this has been previously linked to stiffness and LOX activity (6, 46, 47) and is known to enhance tumor cell proliferation. To determine whether SRC kinase activation may be important in driving the increases in proliferation observed in our in vitro model, we seeded 4T1 mammary carcinoma cells onto fibroblast-remodeled matrices and treated with bleomycin in the presence or absence of our LOX antibody. On matrices remodeled by activated fibroblasts,

Figure 5. Liver fibrosis enhances colonization of breast cancer cells in a LOX-dependent manner. A, Sirius Red staining of control and DMN-treated livers showing increased collagen deposition and crosslinking as a result of fibrosis. B, quantitative analysis of collagen crosslinking as measured by signal intensity. C, METAVIR hepatic fibrosis scores for control, DMN-treated, and DMN-treated with α-LOX antibody or matched IgG control. D, immunoblot analysis for LOX and α-SMA expression in liver samples showing increased levels during fibrosis. E, effects on 4T1 wt primary tumor volume by preinduced hepatic fibrosis shows no significant changes. F, quantitative analysis of hepatic micrometastases in tumor-bearing mice with or without DMN-induced fibrosis and/or α-LOX therapy. G, examples of H&E staining of liver tissue showing micrometastatic lesions (scale bars, ~200 μm).
we observed an increase in SRC kinase activation (Supplementary Fig. S3I), which was not present in fibroblasts treated with our LOX antibody. These data suggest that the increased proliferation of 4T1 mammary carcinoma cells seeded onto fibroblast-remodeled matrices may be mediated through SRC kinase activation.

Finally, to confirm this in our in vivo model, we stained sections of metastases from bleomycin-treated animals with and without α-LOX therapy for the proliferation marker Ki-67 and SRC-P[Tyr418]. As discussed previously, we noted a decreased frequency and size of pulmonary metastatic burden in our α-LOX–treated animals, but more interestingly, we observed a significant decrease in the presence of Ki-67–positive cells within lung metastases, (Fig. 6F and H) and also SRC-P[Tyr418]–positive staining (Supplementary Fig. S3J) in mice treated with α-LOX therapy supporting our in vitro data and strengthening our hypothesis that LOX activity during fibrosis is critical in the remodeling process responsible for generating a prometastatic environment that leads to enhanced metastatic tumor cell survival and proliferation.
Discussion

It has long been known that fibrotic signals enhance metastatic progression, though the underlying molecular mechanisms have remained unclear. The identification of key molecules and processes responsible for creating a milieu that promotes metastatic growth is critical for the development of urgently needed effective antimetastatic therapies. LOX is a potent mediator of metastasis and promising novel therapeutic target. However, the molecular mechanisms by which LOX promotes metastatic tumor cell colonization and growth are largely unknown and remain the subject of intense investigation.

Fibrosis is the exaggerated response to a traumatic event, which leads to the excessive deposition of connective tissue matrix. Type I collagen is the major fibrous collagen synthesized by activated fibroblasts during fibrosis. Typically fibrotic tissue contains increased concentrations of collagen, a rich blood supply and activated fibroblasts, as identified by α-SMA. Several groups have shown the attenuation of collagen production and deposition leads to reduced levels of pulmonary fibrosis (48–52). Following transcription, translation, and secretion, procollagen I assembles and is posttranslationally modified in the extracellular space to create an insoluble fibrotic matrix of collagen fibers. We have shown that inhibition of LOX leads to a decrease in levels of posttranslational modification (crosslinking) of collagen and subsequently a reduction in the generation of the insoluble fibrotic matrix manifesting as a decrease in severity of pulmonary fibrosis in our models. Our data is strongly supported by previous work by Wong and colleagues (53), where targeting LOX directly or indirectly through HIF1-α leads to reduced collagen cross-linking and CD11b(+) bone marrow derived cell recruitment in the lungs of mice during premetastatic niche formation.

We have further identified a previously uncharacterized role for LOX in establishing and mediating the microenvironmental milieu within fibrotic tissues that is favorable to colonization of metastasizing tumor cells. We show that LOX is critical in establishing and mediating this microenvironmental milieu, through regulation of posttranslational modification of the ECM, which enhances tumor cell survival and proliferation, and that therapeutic targeting of LOX prevents the development of this permissive microenvironment and the associated fibrosis-enhanced metastasis. Thus, blocking LOX activity in situations of fibrosis leads to beneficial alterations in the fibrotic matrix, which are no longer preferentially supportive to tumor cell colonization and growth.

Therapeutic targeting of LOX was efficacious in reducing both the extent of tissue fibrosis following injury and consequently the generation of growth-supportive environments for metastasis. Previous work has shown that inhibition of LOX also targets early stages of tumorigenesis including transformation events in premalignant tissue (7) and primary tumor cell invasion in vivo (8). Thus, inhibition of LOX through antibody treatment offers benefits, which extend beyond that of the primary site. Importantly, our data show that targeting posttranslational modification of ECM components by LOX is efficacious and a preferential approach to targeting collagen synthesis or fibroblast activation.

Our analysis of other LOX-Like family members revealed only LOXL-2 slightly changed as a result of tissue fibrosis, consistent with a previous report in which they showed LOXL-2 in male BALB/c mice increased with CCH-induced liver fibrosis, and also in C57BL/6 mice with bleomycin-induced lung fibrosis (42), both of which could be attenuated using a LOXL-2 function blocking antibody. Although the authors showed that using an antibody against LOX could not block these effects, it is important to note that the antibody used did not block enzymatic function (42). Our LOX antibody specifically detects LOX and not other family members, binds to recombinant LOX and not recombinant LOXL2 (Supplementary Fig. S3K), and blocks LOX enzymatic function to the same extent as a mutant version of LOX lacking catalytic function (46). Thus, the effects we observe in this study are attributed to LOX function and not the other family members.

Our findings provide an important link between extracellular matrix homeostasis, fibrosis and cancer, and place LOX as a central mediator combining these processes and a critical modulator of fibrosis-enhanced metastatic growth. Our data highlight the important clinical implications for both the treatment of fibrotic disease and cancer, and is the first to connect LOX-mediated microenvironmental changes in organs to metastatic dissemination of unrelated primary tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: T.R. Cox, J.T. Erler
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