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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 accounts for over 90% of cancer patient 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 is critical for the development of effective cancer therapies. Here, we demonstrate a critical role for lysyl oxidase (LOX) in establishing 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 cross-linking 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 extracellular matrix homeostasis, fibrosis and cancer with important clinical implications for both the treatment of fibrotic disease and cancer.

Keywords

Metastasis; Lysyl Oxidase; Fibrosis; Breast Cancer; Extracellular Matrix

Introduction

Metastases are responsible for over 90% of cancer patients 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

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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 remodelling of the extracellular matrix (ECM) in promoting tumor growth and metastasis(5-13). At the same time, the diverse and more importantly, dynamic nature of the complex reciprocal signalling networks between both malignant and non-malignant 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 remodelling processes that continually reshapes the ECM.

The mechanisms contributing to primary tumor development, metastatic progression and tissue fibrosis share many commonalities including increased matrix deposition and remodelling(19-22). ECM remodelling is particularly prevalent during fibrosis leading to functional changes in biochemical and biomechanical properties of the extracellular matrix and resulting microenvironment, which in turn supports the activation of pathogenic signalling pathways and further tissue remodelling(6, 7, 23). Lysyl oxidase (LOX) is an extracellular amine oxidase whose primary function is to post-translationally modify collagens and elastin in the extracellular matrix, thereby catalysing the covalent crosslinking of fibres(24, 25). This crosslinking is essential for the stabilization of collagen fibrils and fibres, 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 *et al.* 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 ever-expanding 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 tumours” is generally excepted, 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). Whilst still the subject of much debate, surgical and adjuvant intervention 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 (IR) induced stimulation of myofibroblasts, have also been recently reviewed(39). However, to date, there exists limited clinical evidence regarding the presence of pre-existing 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 often hard to discern. Due to 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 pre-existing 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 favourable to colonization and growth of metastasising 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 ATCC 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. 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 H&E, or Picrosirius Red to assess fibrotic change 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/OCT post excision 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 30µm freshly cut sections.

Immunohistochemistry

4µm sections were stained for αLOX (8), αFN (Abcam), αSMA (Abcam), Ki67 (Novocastra), or SRC-P^[Tyr418] (Invitrogen) overnight and visualized with 3,3'-Diaminobenzidine. Picrosirius red analysis was undertaken using 4µm 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 performed 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-P^[Tyr418] (Invitrogen), SRC (Abcam) SMAD2-P^[S465/467] (Cell Signaling Technology), SMAD-2 (Cell Signaling Technology), p53 (Cell Signaling Technology) and beta-actin (Abcam). Conditioned media was prepared as previously described(8, 9). All primary antibody incubations steps were carried out overnight.

Determination of matrix stiffness

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

Statistical analyses

Error bars represent the standard error of the mean (SEM). Data were analysed using the Student t test unless otherwise stated, and considered significant when $p < 0.05$. All statistical tests were two-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 two models of pulmonary fibrosis in immune competent mice: chemically using single dose bleomycin instillation and radiologically, using single non-lethal low dose thoracic irradiation. Both models resulted in progressive pulmonary fibrosis as determined by significantly increased Ashcroft Score(40) (Fig 1 a). This was characterized by loss of alveolar structure, collapse of alveolar space and thickening of alveolar septae (Fig 1 b) as a result of abnormal ECM ultrastructure due to increased collagen deposition, as determined by SirCol Assay (Fig 1 c). We also found 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 1 d, e, f). Increased alpha smooth muscle actin (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 1 g and Supplementary Fig S1 a), which were detectable from as early as 3 days post bleomycin 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 S1 b)(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 2 a) and by Ashcroft Score (Fig 2 b). 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 2 c, e) when compared to lungs of control mice. Induction of fibrosis and/or treatment with the α LOX antibody showed no discernible differences in body weight compared to that of matched control IgG (Fig 2 d). Taken together, these findings suggest 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 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 S1 d) indicating that the presence of fibrotic tissue within the body did not affect primary tumor growth. In contrast, histological evaluation of lung tissue three weeks post-implantation showed a significant increase of approximately 2.5 fold in pulmonary metastatic burden (Fig 3 a, b) in the lungs of bleomycin treated and irradiated mice compared to control treated reflected in terms of frequency of metastases (Fig 3 c) and also marginally in relative size of metastases (Fig 3 d). This data is the first to show that tissue fibrosis is directly associated with a significant increase in metastatic lesions in orthotropic 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 pre-conditioning model was set up in which the establishment of pulmonary fibrosis was used as the pre-conditioning step prior to tail vein injection of 4T1 tumor cells. The presence of established pulmonary fibrosis enhanced metastatic colonization (Fig 3 e, 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 3 g, h) in fibrotic compared to 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 2 b), 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 4 a). However, we did observe a significant decrease, by approximately 50%, in pulmonary metastatic burden in antibody treated mice (Fig 4 b, c). These results suggest that therapeutic targeting of LOX can be used to block the generation of the pro-metastatic 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 utilized 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 pathophysiological alterations including significantly increased collagen deposition,

increased fibrillar and cross-linked collagen and formation of septa (Fig 5 a, b) manifesting as a significant increase in METAVIR state (Fig 5 c).

Liver fibrosis is characterized by an accumulation of ECM proteins secreted by activated hepatic stellate cells (HSCs). The proliferation and differentiation of HSCs into myofibroblast-like cells can be detected by the expression of alpha-smooth muscle actin (α SMA)(43). We noted high levels of expression in α SMA in DMN treated livers, with barely detectable levels in control (Fig 5 d) confirming the development of hepatic fibrosis. Concomitantly, we also observed tissue specific increases in LOX expression (Fig 5 d), in a manner similar to that observed during pulmonary fibrosis. Importantly, we noted a close co-localization in expression with α SMA both in the livers of DMN treated mice, but also the lungs of bleomycin treated and irradiated mice (Supplementary Fig S2 a) 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 5 c), however, it showed no effect on either expression of α SMA or LOX (Fig 5 d) 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 S2 b). 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) utilising a different model.

Following induction of hepatic fibrosis, orthotopic implant of 4T1 carcinoma cells showed no significant difference in primary tumor growth (Fig 5 e) but did show a significant increase in hepatic metastatic burden in terms of frequency of metastases (Fig 5 f, g) though not significantly in terms of average lesion size in DMN treated mice. Reduction of fibrosis with the function-blocking LOX antibody prior to tumor cell implant significantly reduced hepatic metastatic burden in these mice compared to matched IgG control (Fig 5 f, 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 cross-linking 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 6 a). We also noted an increase in LOX mRNA levels (Supplementary Fig S3 a) and expression of secreted LOX (Fig 6 a) in parallel with elevated α SMA expression following bleomycin exposure, and an induction of Collagen I expression (Supplementary Fig S6 b) 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 6 a), confirming that LOX expression, activity and subsequent matrix cross-linking 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), lead to a loss in the ability of bleomycin to induce subsequent LOX expression (Supplementary Fig S3 c, d, e). Hence both the activation of fibroblasts and subsequent secretion of LOX is key 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 S2 c, d, e)

LOX has been shown to increase matrix stiffness through its collagen cross-linking 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 non-enzymatically respectively. Treatment of collagen I matrices with both LOX and ribose led to significant increases in the complex (G^*) moduli (stiffness) of the matrices as measured by shear rheology (Fig 6 b). Increasing the stiffness of collagen I matrices subsequently led to a significant increase in the rate of cellular proliferation of seeded 4T1 cells at 7 days (Fig 6 c), 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 S3 f). 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 S3 g), indicating that observed changes are as 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 6 d). This data suggests 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 excised and the presence of GFP cells within the lung was assessed at 2hr, 6hr, 12hr, 24hr, 48hr and 72hr post inoculation by both Flow Cytometry (Fig 6 e) and immunofluorescence (Fig 6 g, Supplementary Fig S3 h). We observed no significant difference in tumor cell lodging within the lung at 2 hours post injection (Fig 6 e, g, Supplementary Fig S3 h). 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 6 g). This data supports 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 antibody. On matrices remodeled by activated fibroblasts, we observed an increase in SRC kinase activation (Supplementary Fig S3 i), which was not present in fibroblasts treated with our LOX antibody. This data suggests that the increased proliferation of 4T1 mammary carcinoma cells seeded onto fibroblast-remodelled 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 Ki67 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 Ki67 positive cells within lung metastases, (Fig 6 f, h) and also SRC-P^[Tyr418] positive staining (Supplementary Fig S3 j) 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 pro-metastatic 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 anti-metastatic 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 post-translationally modified in the extracellular space to create an insoluble fibrotic matrix of collagen fibres. We have shown that inhibition of LOX leads to a decrease in levels of post-translational modification (crosslinking) of collagens and subsequently a reduction in the generation of the insoluble fibrotic matrix manifesting as a decrease in severity of pulmonary fibrosis in our models.

We have further identified a previously uncharacterized role for LOX in establishing and mediating the microenvironmental milieu within fibrotic tissues that is favourable to colonization of metastasising tumor cells. We show that LOX is critical in establishing and mediating this microenvironmental milieu, through regulation of post-translational 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 inhibition of LOX also targets early stages of tumorigenesis including transformation events in pre-malignant 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 shows that targeting post-translational 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 where they showed LOXL-2 in male BALB/c mice increased with CCl₄-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 S3 k), 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 highlights 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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T.R.C. conceived the project, planned, designed and performed the experiments, analyzed the data, and wrote the paper, assisted by J.T.E., A.B., D.B., H.E.B., M.W.H and G.L.

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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 = approx 50 μ m). (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 = approx 50 μ m) (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 to control. (G) Rising pulmonary collagen deposition in bleomycin-treated pulmonary tissue over time as determined by SirCol quantification (n = 3 mice per group).

Figure 2. LOX inhibition decreases pulmonary fibrosis following injury
(A) Collagen deposition in bleomycin-treated and irradiated pulmonary tissue with or without treatment with the function blocking LOX antibody as determined by SirCol quantification (Biocolor Ltd.). **(B)** Ashcroft scores representing the fibrotic changes in lungs of control, bleomycin-exposed, and irradiated mice receiving either vehicle or α LOX antibody. Blocking LOX activity decreases the degree of fibrosis observed **(C)** Quantitative analysis of fibrillar collagen in control, bleomycin-treated and irradiated lung treated with vehicle or α LOX antibody. Inhibition of LOX activity leads to a decrease in higher order organisation of collagen during fibrosis **(D)** Changes in body weight of mice are consistent between all treatment groups. **(E)** Sirius Red staining (orthogonal light) for cross-linked fibrillar collagen in lungs of control, bleomycin-treated and irradiated mice treated with vehicle or α LOX antibody. (Scale bar = approx 50 μ m).

Figure 3.

Tissue fibrosis enhances primary breast cancer metastasis

(A) Examples of H&E staining of pulmonary tissue from control, bleomycin-treated and irradiated mice showing presence of pulmonary metastases (arrowheads). An increased metastatic burden is observed in fibrotic lung compared to control (B) Quantitative analysis of total tumor burden (% of Total Lung), (C) frequency of lung metastases and (D) relative average size of metastases. (E) Examples of H&E staining of pulmonary tissue from control, bleomycin-treated and irradiated mice following tail vein injection of wt 4T1 tumor cells and foci formation (14d [arrowheads]) (F) Quantitative analysis of total tumor burden (% of Total Lung), (G) frequency of lung foci and (H) relative average size of foci showing an increase in foci formation in both bleomycin-induced and irradiation-induced fibrotic lung. (Scale bars = approx 200 μ m).

Figure 4.

Therapeutic targeting of LOX prevents fibrosis-enhanced pulmonary metastases

(A) Effects on 4T1 wt primary tumor volume by pre-induced fibrosis shows no significant

changes in primary tumor growth (B) Examples of H&E staining of lung tissue showing

decrease in the presence of pulmonary metastases in antibody treated fibrotic models (C)

Relative quantitative analysis of total metastatic burden (% of Total Lung) shows decreases

metastases in α LOX treated fibrotic lung compared to control. (Scale bars = approx

200 μ m).

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 cross-linking as a result of fibrosis (B) Quantitative analysis of collagen cross-linking 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 alpha smooth muscle actin (α SMA) expression in liver samples showing increased levels during fibrosis (E) Effects on 4T1 wt primary tumor volume by pre-induced 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 = approx 200 μ m).

Figure 6.

Inhibition of LOX driven fibrosis reduces pulmonary colonization and proliferation of breast cancer metastases

(A) Immunoblot analysis of alpha smooth muscle actin (α SMA) and LOX expression in fibroblasts in response to bleomycin treatment at 48 hours. Inhibition of LOX activity does not affect activation of fibroblasts by bleomycin treatment nor LOX expression. (B) Relative stiffness of LOX- or ribose-modified collagen matrices as measured by shear rheology. Data is representative of two independent experiments. (C) Increasing stiffness of collagen I matrices leads to an increase in the rate of proliferation of 4T1 tumor cells. Data

representative for 7 days growth on pre-modified collagen I matrix **(D)** Activated fibroblast remodelling of collagen I matrices leads to an increase in proliferation of seeded 4T1 mammary carcinoma cells at 7 days **(E)** Flow Cytometry analysis of GFP positive 4T1 mammary carcinoma cells present in disaggregated lung tissue following tail vein injection, n=3 mice per group per time point. **(F)** Quantitative scoring of Ki67 positive nuclei in 4T1 mammary carcinoma pulmonary metastases. Bleomycin induced fibrosis increases proliferation within the lung, which can be attenuated by inhibiting LOX mediated fibrosis. **(G)** Representative confocal images of lung tissue following tail vein injection of GFP expressing 4T1 mammary carcinoma cells. The lodging of tumor cells in the lung is clearly visible at all stages. **(H)** Representative immunohistochemistry for Ki67 in pulmonary metastases. (Scale bars = approx 200 μ m).