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Hypoxia-Induced Lysyl Oxidase Is a Critical Mediator of Bone Marrow Cell Recruitment to Form the Premetastic Niche

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SUMMARY

Tumor cell metastasis is facilitated by "premetastatic niches" formed in destination organs by invading bone marrow-derived cells (BMDCs). Lysyl oxidase (LOX) is critical for premetastatic niche formation. LOX secreted by hypoxic breast tumor cells accumulates at premetastatic sites, crosslinks collagen IV in the basement membrane, and is essential for CD11b+ myeloid cell recruitment. CD11b+ cells adhere to crosslinked collagen IV and produce matrix metalloproteinase-2, which cleaves collagen, enhancing the invasion and recruitment of BMDCs and metastasizing tumor cells. LOX inhibition prevents CD11b+ cell recruitment and metastatic growth. CD11b+ cells and LOX also colocalize in biopsies of human metastases. Our findings demonstrate a critical role for LOX in premetastatic niche formation and support targeting LOX for the treatment and prevention of metastatic disease.

INTRODUCTION

During tumor progression, cells can acquire the capability for invasion and metastasis to escape the primary tumor mass and colonize nutrient-rich new organs (Gupta and Massague, 2006; Hanahan and Weinberg, 2000). There are few effective treatment options for patients with metastatic disease (Steeg, 2006), and over 90% of cancer-related deaths can be attributed to tumor metastases (Gupta and Massague, 2006). Increased metastases, enhanced tumor progression, and decreased patient survival have been associated with primary tumors that contain large numbers of poorly oxygenated (hypoxic) tumor cells (Cairns et al., 2003; Hockel and Vaupel, 2001; Pouyssegur et al., 2006). Improved understanding of the role of tumor hypoxia in the metastatic process is clearly needed so that more effective therapeutic strategies can be devised to treat metastatic cancer.

Tumor cell metastasis is facilitated by formation of "premetastatic niches" in destination organs (Kaplan et al., 2005) that consist of clusters of bone marrow-derived cells (BMDCs). These BMDCs are thought to create an environment that is permissive for the subsequent invasion and growth of tumor cells (Condeelis and Pollard, 2006; Coussens and Werb, 2002). The main BMDCs identified at premetastatic sites are hematopoietic progenitor cells that express vascular endothelial growth factor receptor-1 (VEGFR-1), along with BMDCs expressing CD133, CD34, and c-Kit (Kaplan et al., 2005). CD11b+ (Mac-1+) cells have also been identified in metastatic target organs (Hiratsuka et al., 2006), and primary tumors are known to recruit CD11b+Gr-1+ myeloid cells (Yang et al., 2008) and CD45+ monocytic lineage cells (including VEGFR-1+ and CD11b+ cells; Du et al., 2008). CD11b+ cells have a variety of functions that may enhance metastatic tumor growth. CD11b+Gr-1+ cells are known to be myeloid-derived suppressor cells that are capable of inhibiting

SIGNIFICANCE

Understanding the metastatic process is central to the development of improved therapies to treat cancer patients. Premetastatic niches are comprised of largely unidentified proteins and bone marrow-derived cells and are thought to prepare target organs for the subsequent arrival of metastatic tumor cells. We have found that lysyl oxidase (LOX) secreted by hypoxic tumor cells is a critical mediator of premetastatic niche formation. LOX modifies the extracellular matrix and induces the recruitment and invasion of CD11b+ myeloid cells to premetastatic sites, thereby promoting a cascade of events that increases metastatic tumor growth. The central role of LOX in premetastatic niche formation identifies LOX as a viable therapeutic target for the treatment and prevention of metastatic disease.

T cell- and NK cell-mediated immune responses (Liu et al., 2007; Serafini et al., 2006). CD11b+Gr-1+ cells also incorporate into tumor endothelium and enhance angiogenesis (Yang et al., 2004), while CD11b+ myeloid cells enhance tumor growth through vasculogenesis (Ahn and Brown, 2008). The presence of CD11b+ cells at premetastatic sites may have important implications for using anti-VEGF therapy to disrupt the premetastatic niche (Kaplan et al., 2005), since tumors containing CD11b+Gr-1+ cells show decreased response to anti-VEGF therapy (Shojaei and Ferrara, 2008). Thus, myeloid lineage cells may be important components of the premetastatic niche.

The mechanism by which BMDCs are recruited to premetastatic sites is poorly understood. Unidentified tumor-secreted factors are thought to induce elevated fibronectin expression at premetastatic sites and increase the recruitment of VEGFR-1+ cells (Kaplan et al., 2005). The recruitment of CD11b+ myeloid cells to premetastatic sites may be influenced by VEGF-A and by the TGF-β and/or TNF-α pathways (Hiratsuka et al., 2006). However, tumor-secreted proteins that are essential for formation of the premetastatic niche and that could potentially be targeted therapeutically are still largely unknown.

Lysyl oxidase (LOX) is an amine oxidase that crosslinks collagens and elastins in the extracellular matrix (Kagan and Li, 2003). *LOX* expression is increased in tumor cells exposed to physiologically relevant levels of hypoxia (Denko et al., 2003), and LOX is associated with metastasis and poor survival in patients with breast cancer or head and neck cancer (Erler et al., 2006). LOX has been shown to enhance tumor cell invasion in vitro (Erler et al., 2006; Kirschmann et al., 2002), and inhibition of the expression or the enzymatic activity of secreted LOX eliminates metastases in an orthotopic model of breast cancer (Erler et al., 2006). Based on the marked decreases in metastatic growth that we observed previously with therapeutic LOX inhibition and the ability of LOX to remodel the extracellular matrix, we hypothesized that LOX may influence multiple steps in the metastatic process. We therefore studied the role of LOX in the recruitment and invasion of BMDCs to premetastatic sites and in formation of the premetastatic niche.

RESULTS AND DISCUSSION

LOX Is Required for BMDC Recruitment to Premetastatic Sites

To investigate the role of LOX in formation of the premetastatic niche, we orthotopically implanted mice with either wild-type (WT) MDA-MB-231 human breast tumor cells or MDA-MB-231 cells expressing a *LOX*-targeting shRNA with significantly reduced LOX protein expression and secretion (see Figure S1A available online). Analysis of lungs harvested 6 weeks after tumor implantation indicated that mice bearing *LOX* shRNA tumors had significantly reduced numbers of pulmonary metastatic lesions compared to WT tumor-bearing mice (Figure 1A, hematoxylin and eosin [H&E] panels; Figure S1B). These data are in agreement with our previous results indicating that inhibition of LOX decreases tumor cell invasion and metastasis (Erler et al., 2006). Interestingly, when lungs from these mice were analyzed for the presence of BMDCs by flow cytometry (Figure S1C), we found that the lungs of WT tumor-bearing mice had significantly more CD11b+ myeloid cells and c-Kit+ (CD117+) myeloid pro-

genitor cells than mice with *LOX* shRNA tumors (Figure 1B). We did not observe a significant increase in the numbers of F4/80+ mature macrophages, and 95% of the CD11b+ cells found in the lungs of WT tumor-bearing mice were negative for F4/80. Since monocytes have the potential to mature into macrophages once in tissues, the increased number of CD11b+F4/80− cells indicates that CD11b+ BMDCs recruited to metastatic sites are largely immature progenitor cells from the myeloid lineage. These data are in agreement with the increased number of immature myeloid cells relative to mature myeloid cells observed during tumor progression in mouse tumor models and in cancer patients (Kusmartsev et al., 2008). We also found that CD11b+ cells colocalized with tumor cells in pulmonary foci of WT tumor-bearing mice (Figure 1A, immunofluorescence panels), while clusters of CD11b+ cells and tumor cells were not observed in lungs of mice with *LOX* shRNA tumors. The bone marrow-derived origin of cells in pulmonary premetastatic sites was confirmed by transplanting male bone marrow cells into lethally irradiated female mice prior to tumor implant and staining the excised lungs with a Y chromosome-specific fluorescent DNA probe (Figure S1D). Taken together, these data demonstrate a role for LOX in the development of pulmonary foci in tumor-bearing mice that contain tumor cells and CD11b+ myeloid cells.

We then determined how LOX integrates into the kinetics of pulmonary foci formation by excising lungs from mice bearing orthotopic WT MDA-MB-231 tumors at various times after tumor implant. Consistent with previous reports (Kaplan et al., 2005), we found focal areas of fibronectin (FN) staining 3 days after tumor implantation surrounding terminal bronchioles and distal alveoli in the lungs (Figure S1E), which are common areas of pulmonary metastasis. We used a human-specific FN antibody, indicating that at least some of the pulmonary FN was secreted from the primary tumor. Interestingly, LOX colocalized exclusively with FN at these sites within 7 days of tumor implantation, and both FN and LOX staining intensified over the next week. We found recruitment of CD11b+ cells to areas of LOX staining by 14 days after tumor implantation and observed increased numbers of CD11b+ cells over the next few weeks. Tumor cells were observed in regions of CD11b+ cell accumulation by 3–5 weeks after tumor implant. Importantly, we did not observe pulmonary LOX staining, CD11b+ cell clusters, or tumor cells in mice bearing *LOX* shRNA-expressing tumors. These data indicate that LOX secreted by the primary tumor binds to regions of FN accumulation in the lungs of WT tumor-bearing mice before the recruitment of CD11b+ cells or tumor cells.

We then wanted to validate the role of LOX in BMDC recruitment in a model system without tumor cells present. LOX is known to be secreted by hypoxic tumor cells (Erler et al., 2006), and we therefore used WT or *LOX* shRNA-expressing MDA-MB-231 cells exposed to hypoxia in vitro (2% O$_2$ for 24 hr) to produce conditioned media (CM) with or without LOX. A fluorescence-based enzymatic activity assay (Palamakumbura and Trackman, 2002) was used to verify the presence of enzymatically active LOX in CM from WT cells (Figure S2A). We injected LOX-containing CM into tumor-free mice daily for 3 weeks, which is consistent with the time frame of BMDC recruitment to premetastatic sites (Kaplan et al., 2005). Interestingly, tumor-free mice injected with CM from hypoxic WT cells (high LOX activity) had increased pulmonary accumulation of
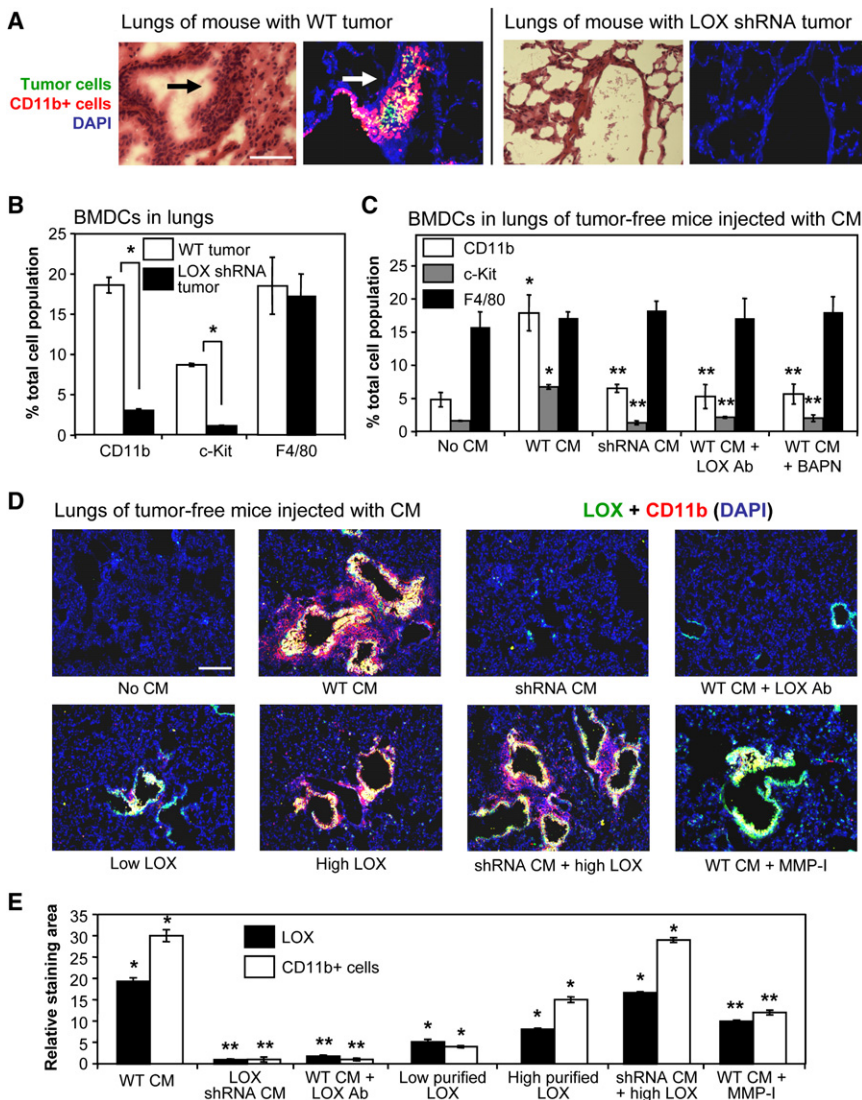
Figure 1. LOX Secreted from Hypoxic Tumor Cells Colocalizes with CD11b+ Cells in the Lungs and Increases CD11b+ Cell Recruitment and Invasion

(A) Nude mice were orthotopically implanted with 10^7 wild-type (WT) or *LOX* shRNA-expressing MDA-MB-231 human breast tumor cells. Lungs were excised 6 weeks later, and frozen serial sections were stained either with hematoxylin and eosin (H&E) or with pan-cytokeratin (green) to identify tumor cells and CD11b (red) to identify myeloid cells. Arrows indicate pulmonary cell clusters (foci). Scale bar = 75 μm.

(B) Lungs from mice bearing WT or *LOX* shRNA-expressing tumors were homogenized and analyzed by flow cytometry for numbers of CD11b+ myeloid cells, c-Kit+ (CD117) myeloid progenitor cells, and F4/80+ mature macrophages. Data are mean ± SEM. *p < 0.05.

(C) Tumor-free mice were injected with the indicated conditioned media (CM) daily for 3 weeks. Homogenized lungs were analyzed for CD11b+, c-Kit+, and F4/80+ cells by flow cytometry. LOX Ab, LOX-specific antibody; BAPN, small-molecule inhibitor of LOX. Data are mean ± SEM. *p < 0.05 relative to control; **p < 0.05 relative to mice injected with WT CM.

(D) Merged immunofluorescence staining of LOX (green) and CD11b+ cells (red) in representative lung sections from tumor-free mice injected daily for 3 weeks with the indicated CM. Colocalization is indicated by yellow. Low/high LOX, relative concentration of purified LOX; MMP-I, matrix metalloproteinase inhibitor. Scale bar = 150 μm.

(E) Image analysis of lung sections from mice in (D). Data indicate the relative area of LOX (green in [D]) or CD11b (red in [D]) staining relative to control mice. Data are mean ± SEM. *p < 0.05 relative to control; **p < 0.05 relative to mice injected with WT CM.

CD11b+ myeloid cells and c-Kit+ myeloid progenitor cells (Figure 1C). These data are in agreement with previous reports suggesting involvement of progenitor cells and myeloid lineage cells in the premetastatic niche (Hiratsuka et al., 2006; Kaplan et al., 2005). Importantly, the numbers of pulmonary BMDCs were not increased by injection of CM derived from hypoxic cells expressing *LOX* shRNA, or when LOX-containing CM was combined with either an antibody that binds to the active site of LOX and blocks enzymatic function (Erler et al., 2006) or a small-molecule inhibitor of LOX (β-aminopropionitrile; BAPN). These data indicate that LOX secreted by hypoxic tumor cells significantly increases the number of myeloid lineage cells in the lungs without requiring the presence of metastatic tumor cells.

We also analyzed sections of lung tissue excised after 3 weeks of LOX-containing CM or purified LOX protein injections. Tumor-free mice injected with LOX-containing CM had intense LOX staining around terminal bronchioles and distal alveoli in the lungs. Interestingly, CD11b+ cells were found colocalized with LOX at these sites and were also observed in lung tissue directly adjacent to areas of LOX staining (Figure 1D). This pattern of

CD11b staining suggests CD11b+ cell invasion into lung tissue surrounding areas of LOX staining, which is consistent with reports describing a role for LOX in enhancing monocyte migration (Denholm et al., 1989; Lazarus et al., 1995). Total LOX or CD11b staining relative to naive mouse lungs is quantified in Figure 1E; the proportion of CD11b+ cells that invaded into lung tissue surrounding areas of LOX staining is quantified in Figure S2B. Invading BMDCs were observed as quantifiable clusters of densely stained cells surrounding bronchioles in H&E-stained lung tissue (Figures S2C and S2D). In agreement with the flow cytometry data in Figure 1C, CD11b+ cell accumulation was dramatically reduced in the lungs of mice injected with CM from *LOX* shRNA-expressing cells or when a LOX-inhibitory antibody was administered with the WT CM (Figures 1D and 1E). Taken together, these data indicate that LOX secreted by hypoxic tumor cells accumulates in the lungs and is essential for the recruitment of CD11b+ cells.

We also purified LOX from the CM of hypoxic WT cells for subsequent administration to tumor-free mice. The LOX purity was verified and LOX functional activity was validated by inducing

migration of *LOX* shRNA-expressing cells in vitro (Figure S2E). We used two concentrations of LOX protein, with the higher LOX concentration having about half the enzymatic activity of LOX typically found in hypoxic WT CM (Figure S2A). Importantly, the recruitment of CD11b+ cells induced by injection of LOX-containing CM could be replicated in tumor-free mice injected only with purified LOX (Figure 1D), but the overall levels of LOX and CD11b+ cell staining in the lungs were less than in mice injected with hypoxic WT CM (Figure 1E). Interestingly, addition of CM from hypoxic *LOX* shRNA-expressing cells to injections of purified LOX significantly increased the levels of pulmonary LOX and CD11b+ cell accumulation (Figures 1D and 1E) and also stimulated CD11b+ cell invasion into the surrounding lung tissue to levels similar to those observed with hypoxic WT CM (Figure 1D; Figure S2B). These data indicate that while accumulation of LOX secreted by hypoxic tumor cells is essential for pulmonary recruitment of CD11b+ cells, one or more other factors present in CM from hypoxic WT or shRNA-expressing tumor cells are capable of enhancing the effects of LOX. It is worth noting that fibronectin is a hypoxia-induced secreted protein (Caniggia et al., 2000) that is known to be elevated at premetastatic sites (Kaplan et al., 2005) and has been reported to increase LOX enzymatic activity (Fogelgren et al., 2005). Indeed, we have found that LOX colocalizes with fibronectin at premetastatic sites (Figure S1E) prior to recruitment of CD11b+ cells. The precise role of fibronectin in LOX-mediated formation of the premetastatic niche is currently under investigation.

Since invasion of BMDCs is increased by activation of matrix metalloproteinases (Coussens and Werb, 2002), we added a chemical matrix metalloproteinase (MMP) inhibitor (Koivunen et al., 1999) to LOX-containing WT CM prior to injection. The MMP inhibitor (MMP-I) induced modest decreases in relative LOX and CD11b+ cell staining in the lungs (Figure 1E) but dramatically changed the pattern of CD11b+ cell accumulation. CD11b+ cells were recruited to areas of LOX staining but did not invade into the surrounding lung tissue to form cell clusters as had occurred with injection of hypoxic WT CM alone (Figure 1D; Figures S2B and S2D). These data indicate that the ability of CD11b+ cells to invade into lung tissue adjacent to regions of LOX accumulation is dependent on MMP activity.

LOX Promotes BMDC Adhesion and Invasion by Crosslinking Collagen IV in Basement Membrane

To establish a mechanistic role for LOX in the recruitment and MMP-dependent invasion of BMDCs, we first pretreated growth factor-reduced Matrigel (reconstituted basement membrane) with LOX prior to contact with CD11b+ cells isolated from whole bone marrow by magnetic bead-assisted cell sorting (Liu et al., 2007). We found increased adhesion of CD11b+ cells to Matrigel preincubated with LOX that could be inhibited with BAPN during the preincubation step (Figure 2A). LOX is known to crosslink collagens and elastins in the extracellular matrix (Kagan and Li, 2003), thereby increasing the tensile strength of basement membranes (Maki et al., 2002). Chemically crosslinking Matrigel by preincubation with a high concentration of glucose (Kent et al., 1985) recapitulated the increased CD11b+ cell adhesion observed on Matrigel preincubated with LOX. We also found increased adhesion of CD11b+ cells (Figure 2B) and c-Kit+ cells (Figure S3A) to naive mouse lung tissue preincubated with LOX

ex vivo. CD11b+ cells adhered to the ex vivo lung tissue in areas that stained positively for LOX (Figure 2C). These data indicate that CD11b+ cells and c-Kit+ cells readily adhere to basement membrane and lung tissue that has been crosslinked by LOX.

To determine how the increased adhesion of BMDCs to matrices crosslinked by LOX would affect the MMP-dependent invasion of BMDCs observed in Figures 1D and 1E, we assayed the MMP activity of BMDCs and monocytes after contact with matrices preincubated with LOX. Since collagen IV is a major constituent of Matrigel and LOX is known to crosslink collagen IV, we incorporated collagen IV matrices into our studies. We chose to focus on MMP-2 and MMP-9 because these MMPs are selectively inhibited by the MMP-I used in Figures 1D and 1E (Koivunen et al., 1999). Interestingly, monocytes in contact with Matrigel (or collagen IV) preincubated with LOX-containing WT CM (but not CM from *LOX* shRNA-expressing cells) had elevated MMP-2 activity (Figure 2D). Monocyte MMP-2 activity was also increased by contact with chemically crosslinked Matrigel. Increases in MMP-9 activity were observed in monocytes in contact with Matrigel or collagen IV preincubated with hypoxic CM from either WT or shRNA-expressing tumor cells (Figure S3B), indicating that the increase in MMP-9 activity was LOX independent. Consistently, MMP-9 activity was also not increased in monocytes in contact with chemically crosslinked Matrigel. We found that MMP-2 activity was increased in freshly isolated BMDCs in contact with collagen IV matrices preincubated with LOX-containing WT CM, purified LOX, or with matrix that was chemically crosslinked (Figure 2E). BMDC MMP-2 activity was reduced by the presence of LOX antibody or BAPN during matrix preincubation, or by MMP inhibition. Thus, preincubation of Matrigel or collagen IV with enzymatically active LOX increases the MMP-2 activity of BMDCs and monocytes that are subsequently in contact with the modified matrices.

BMDC Invasion through Basement Membrane Crosslinked by LOX Requires MMP Activity

To further define the role of LOX and MMPs in BMDC invasion, we performed in vitro transwell invasion assays using freshly harvested murine bone marrow, the established RAW monocyte cell line, and freshly isolated CD11b+ cells or c-Kit+ cells. We placed CM from hypoxic WT or *LOX* shRNA-expressing tumor cells into transwell chambers containing filters coated with Matrigel. The CM was removed after 24 hr and replaced with freshly harvested murine BMDCs, and the numbers of BMDCs that invaded through the "modified" Matrigel were quantified 24 hr later (Figure 2F). Preincubation of Matrigel with LOX-containing WT CM dramatically increased the subsequent invasion of BMDCs compared to preincubation with *LOX* shRNA CM or WT CM containing the LOX-targeting antibody. We also stained the bone marrow cells that invaded through the modified Matrigel and found that 94% ± 1% were CD11b+ and 47% ± 7% were c-Kit+. Increased BMDC invasion was also observed when the Matrigel was preincubated with purified LOX protein alone or in combination with hypoxic shRNA CM. The MMP dependence of BMDC invasion was confirmed by addition of the MMP inhibitor to the cells during invasion (Figure 2F). These data are consistent with the decreased CD11b+ cell invasion observed in the lungs of mice injected with LOX-containing CM and the MMP-I (Figure 1D; Figure S2B). Chemical crosslinking of
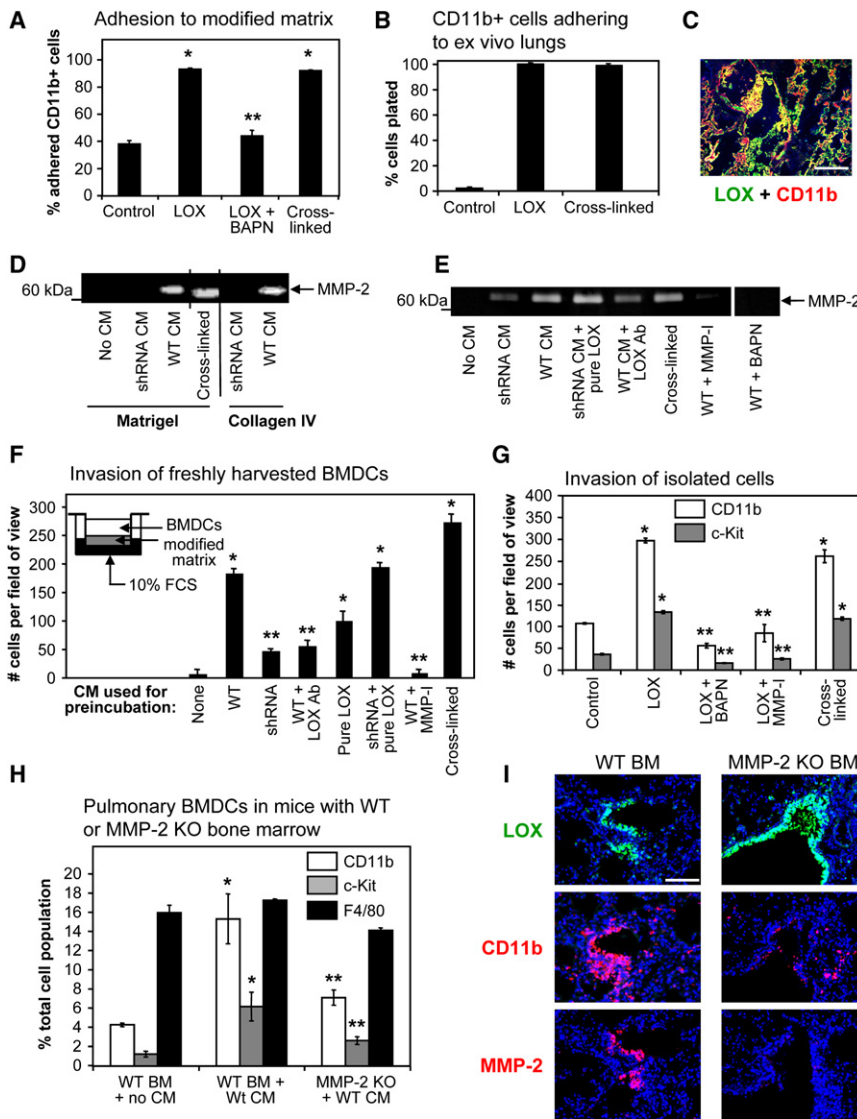
Figure 2. LOX Secreted from Hypoxic Tumor Cells Promotes BMDC Invasion by Crosslinking Collagen IV, Increasing BMDC Adhesion, and Enhancing MMP-2 Activity of the Invading BMDCs

(A) Matrigel-coated wells were incubated with the indicated additives for 24 hr; Matrigel crosslinked with glucose was included for comparison. Solutions were removed, and CD11b+ cells isolated from murine whole bone marrow were added. Numbers of CD11b+ cells remaining in solution were quantified after 2.5 hr. Data are mean ± SEM. *p < 0.05 relative to control; **p < 0.05 relative to matrix preincubated with LOX.

(B) Naive mouse lung tissue was excised, and a 2 cm^3 piece was incubated in serum-free media containing either LOX or glucose for 6 hr. Media were changed, CD11b+ cells were added, and the numbers of cells remaining in solution after 12 hr were quantified. Data are mean ± SEM.

(C) Lung tissue from (B) was frozen, sectioned, and stained for LOX (green) and CD11b+ cells (red). Scale bar = 300 μm.

(D) Gelatin zymography showing MMP-2 activity of monocytes in contact with Matrigel or collagen IV preincubated with the indicated CM.

(E) Gelatin zymography showing MMP-2 activity of freshly harvested bone marrow-derived cells (BMDCs) in contact with collagen IV preincubated with the indicated CM.

(F) Matrigel filters were incubated with the indicated CM or purified protein for 24 hr. The CM was then removed, and freshly harvested whole murine bone marrow cells were allowed to invade through the "modified" Matrigel. BMDCs that invaded through the modified Matrigel were also stained for CD11b and c-Kit. Data are mean ± SEM. *p < 0.05 relative to control; **p < 0.05 relative to matrices preincubated with WT CM.

(G) Matrigel filters were preincubated as in (F), and invasion of isolated CD11b+ cells or c-Kit+ cells through the modified matrix was quantified. Data are mean ± SEM. *p < 0.05 relative to control; **p < 0.05 relative to matrices preincubated with LOX.

(H) Mice with WT bone marrow or MMP-2 knockout (KO) bone marrow were injected daily with the indicated CM for 3 weeks prior to flow cytometric analysis of lungs for CD11b+, c-Kit+, or F4/80+ cells. Data are mean ± SEM. *p < 0.05 relative to "no CM" mice; **p < 0.05 relative to mice with WT bone marrow injected with WT CM.

(I) Immunofluorescence staining for LOX, CD11b+ cells, and MMP-2 in representative frozen serial sections of lungs from mice with WT or MMP-2 KO bone marrow injected with WT CM. Scale bar = 75 μm.

Matrigel with glucose also increased the subsequent invasion of BMDCs. Similar increases in the invasion of freshly harvested BMDCs were observed through collagen IV matrices preincubated with LOX-containing CM (Figure S3C), but not with matrices composed of laminin (the other main component of Matrigel). We obtained similar results using isolated CD11b+ cells, c-Kit+ cells (Figure 2G), or an established monocyte cell line (Figure S3D). Taken together, these data indicate that enzymatically active LOX modifies the collagen IV component of basement membrane (Matrigel) in a manner that is functionally similar to chemical cross-linking. CD11b+ cells adhere more readily to matrices crosslinked by LOX (Figures 2A–2C) and respond with increased MMP-2 expression (Figures 2D and 2E). The actions of LOX and MMP-2 remodel the matrix such that it is more permissive for subsequent invasion of CD11b+ cells and c-Kit+ cells (Figures 2F and 2G).

To further validate the role of BMDC MMP-2 activity in LOX-mediated recruitment and invasion of CD11b+ cells in vivo, we injected LOX-containing CM into female mice transplanted with bone marrow from either male WT mice or MMP-2 knockout (KO) mice. As expected, LOX-containing CM induced pulmonary recruitment of CD11b+ and c-Kit+ cells in mice with WT bone marrow. However, mice with MMP-2 KO bone marrow injected with WT CM had significantly decreased numbers of CD11b+ cells and c-Kit+ cells in the lungs relative to mice with WT bone marrow injected with WT CM (Figure 2H). We also found that CD11b+ cells in areas of pulmonary LOX staining expressed MMP-2 (Figure 2I), consistent with the increased MMP-2 activity observed in BMDCs in contact with LOX-modified matrices in vitro. Fewer CD11b+ cells were observed in lungs of MMP-2 KO mice injected with WT CM, and the CD11b+ cells colocalized
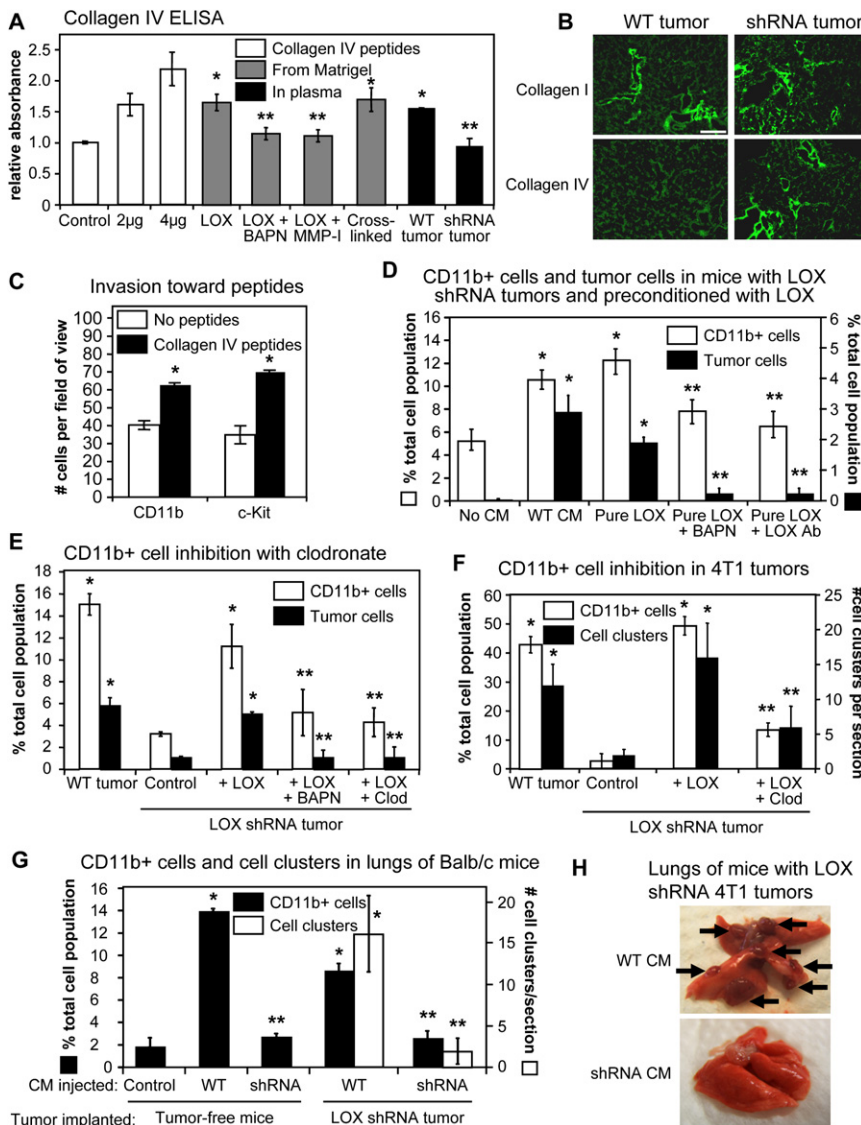
Figure 3. LOX and BMDC MMP-2 Activity Remodel Collagen IV and Promote Pulmonary Metastatic Growth

(A) ELISA to detect collagen IV remodeling via peptide formation. Collagen IV peptides exogenously added to media (white bars) are provided for comparison. Matrigel preincubated for 24 hr with the indicated additives (gray bars) was subsequently contacted with CD11b+ cells for 24 hr. Peptides released into the surrounding media were quantified by ELISA. Plasma samples from the indicated tumor-bearing mice (black bars) were also analyzed. Data are mean ± SEM. *$p < 0.05$ relative to control; **$p < 0.05$ relative to matrices preincubated with LOX (gray) or relative to WT tumor-bearing mice (black).

(B) Sections of lungs from WT or *LOX* shRNA tumor-bearing mice illustrating loss of collagen IV antibody epitope in some areas through LOX-mediated collagen IV remodeling. Collagen I staining was not affected. Scale bar = 150 μm.

(C) Numbers of isolated CD11b+ cells or c-Kit+ cells invading through naive Matrigel toward collagen IV peptides in the bottom of the transwell. Data are mean ± SEM. *$p < 0.05$ relative to control (no peptides).

(D) Flow cytometric quantification of CD11b+ cells and tumor cells (human pan-cytokeratin positive) in lungs of mice bearing *LOX* shRNA-expressing tumors. Mice were "preconditioned" by injection of WT CM or purified LOX protein for 2 weeks after tumor implant. Lungs were harvested 6 weeks after tumor implant. Data are mean ± SEM. *$p < 0.05$ relative to control mice; **$p < 0.05$ relative to mice preconditioned with LOX protein.

(E) Flow cytometric quantification of CD11b+ cells and tumor cells in lungs of mice with *LOX* shRNA-expressing tumors treated with the indicated solutions. Clod, clodronate. Data are mean ± SEM. *$p < 0.05$ relative to control shRNA tumor-bearing mice; **$p < 0.05$ relative to shRNA tumor-bearing mice injected with LOX protein.

(F) Same experiment as in (E), using 4T1 murine mammary tumor cells in BALB/c mice. Pulmonary cell foci (clusters) were quantified from H&E-stained lung tissue. Data are mean ± SEM. *$p < 0.05$ relative to control 4T1 shRNA tumor-bearing mice; **$p < 0.05$ relative to 4T1 shRNA tumor-bearing mice injected with LOX protein.

(G) Flow cytometric analysis for CD11b+ cells in lungs of tumor-free BALB/c mice or BALB/c mice bearing *Lox* shRNA-expressing 4T1 tumors injected with the indicated CM daily for 3 weeks. Pulmonary cell foci were quantified from H&E-stained lung tissue. Data are mean ± SEM. *$p < 0.05$ relative to control mice; **$p < 0.05$ relative to mice injected with WT CM.

(H) Lungs of BALB/c mice implanted with *Lox* shRNA-expressing 4T1 tumors and injected with the indicated CM daily for 3 weeks. Arrows indicate macroscopic lung metastases.

only partially with LOX staining. Taken together, these data indicate that MMP-2 activity in BMDCs is required for the invasion and maximal LOX-mediated recruitment of CD11b+ cells to areas of pulmonary LOX accumulation for formation of the premetastatic niche. These results are consistent with recent identification of *MMP-2* as a tumor progression gene associated with breast cancer metastasis to the lung (Gupta and Massague, 2006), with our previous findings that *LOX* is strongly associated with *MMP-2* expression in breast cancer patients (Erler et al., 2006), and with the MMP-I data in Figures 1D and 1E. We hypothesized that while LOX-mediated crosslinking of the basement membrane is required for adhesion of CD11b+ cells and initiation of the premetastatic niche, an additional MMP-dependent mechanism may increase CD11b+ cell recruitment to premetastatic sites.

LOX and BMDC MMP-2 Activity Remodel Collagen IV in Basement Membrane and Promote Pulmonary Metastatic Growth

MMP-2 is known to cleave collagen IV into peptides (Egeblad and Werb, 2002), and some collagen IV peptides have chemoattractant properties (Cameron et al., 1991; Shahan et al., 2000). Using a collagen IV ELISA, we observed the release of collagen IV peptides during invasion of CD11b+ cells through Matrigel preincubated with purified LOX (Figure 3A). Peptide release was decreased when LOX enzymatic activity was inhibited

with BAPN during the preincubation step or when MMP-2 activity from the CD11b+ cells was inhibited. We obtained similar findings using collagen IV matrices (data not shown) but did not detect the release of laminin peptides from laminin matrices preincubated with LOX-containing CM and contacted with BMDCs (Figure S3E). Taken together, these data indicate that collagen IV peptides are released by the MMP-2 activity of CD11b+ cells in contact with Matrigel crosslinked by LOX.

We also observed increased collagen IV peptides in the plasma of mice bearing WT tumors (Figure 3A) and a decrease in collagen IV staining in some lung regions from WT tumor-bearing mice relative to *LOX* shRNA tumor-bearing mice (Figure 3B). These data are indicative of collagen remodeling and a breakdown of antibody-recognizable triple-helical collagen IV in the basement membrane (Harrison et al., 2006; Jemal et al., 2008; Liu et al., 2007) in lungs of mice bearing tumors that express *LOX*. Pulmonary collagen I staining was similar in both WT and *LOX* shRNA tumor-bearing mice.

To determine the ability of collagen IV peptides to attract BMDCs, we quantified the numbers of isolated CD11b+ cells and c-Kit+ cells that invaded through naive Matrigel toward exogenously added collagen IV peptides (Figure 3C). We found that collagen IV peptides enhanced the invasion of myeloid lineage cells. Thus, the generation of chemoattractive collagen IV peptides in lung regions that have CD11b+ cells in contact with LOX-crosslinked basement membrane will induce further recruitment and invasion of BMDCs to these sites.

Increased MMP-2 activity in BMDCs also increases the invasion of tumor cells (Hagemann et al., 2004), and collagen crosslinking leads to increased stiffness of the extracellular matrix, which enhances growth of tumor foci (Paszek et al., 2005). Taken together with our data, these observations suggest that LOX-mediated increases in BMDC MMP-2 activity may increase the subsequent invasion of metastatic tumor cells to the premetastatic niche and enhance metastatic growth. Indeed, we have previously observed a role for LOX in enhancing the metastatic growth of breast tumors (Erler et al., 2006), but we wanted to distinguish between the role of LOX in the dissemination of metastatic tumor cells and the role of LOX in formation of the premetastatic niche. We therefore "preconditioned" lungs with LOX for a fixed period of time before the arrival of metastatic tumor cells. It is worth noting that preconditioning lungs with LOX in vivo is analogous to preincubating Matrigel (or collagen IV) matrices with LOX in vitro (Figure 2). MDA-MB-231 tumor cell metastases are detectable in lungs from 3–4 weeks after primary tumor implantation (Erler et al., 2006), and BMDCs are recruited to premetastatic sites within 2 weeks of primary tumor implantation (Figure S1E). We therefore preconditioned the lungs of mice bearing orthotopic *LOX* shRNA-expressing tumors by injecting LOX-containing CM or purified LOX for only the first 2 weeks after tumor implantation. Lungs were analyzed for CD11b+ cells and tumor cells 4 weeks later. We observed increased CD11b+ cell recruitment in the lungs of shRNA tumor-bearing mice preconditioned with LOX or with LOX-containing CM (Figure 3D), despite the fact that *LOX* shRNA-expressing tumors are largely nonmetastatic (Figure S1B). Importantly, CD11b+ cell recruitment was diminished by inhibition of LOX with BAPN or a LOX-targeting antibody. The presence of densely stained pulmonary cell clusters was verified and quantified in H&E-stained lung sec-

tions (Figure S4A). These data indicate that the action of enzymatically active LOX secreted by hypoxic tumor cells is a critical prerequisite to pulmonary CD11b+ cell recruitment, premetastatic niche formation, and the enhanced development of lung metastases.

## Myeloid Cell Depletion Decreases Metastatic Growth

We then wanted to determine whether LOX was sufficient to increase metastases of breast tumors to the lungs in the absence of myeloid cells. We therefore used clodronate to deplete myeloid cells, monocytes, and macrophages (Van Rooijen and Sanders, 1994; Zeisberger et al., 2006) from mice bearing shRNA tumors and supplemented with LOX injections. We found that clodronate significantly decreased the numbers of CD11b+ cells and the numbers of metastatic tumor cells in the lungs of MDA-MB-231 tumor-bearing mice (Figure 3E).

We also investigated CD11b+ cell recruitment in mice bearing highly aggressive 4T1 murine mammary tumors. 4T1 cells expressing *Lox* shRNA secreted minimal detectable enzymatically active LOX compared to WT 4T1 cells (Figure S4B). Clodronate decreased CD11b+ cell recruitment and metastatic tumor cell foci formation in the lungs of 4T1 tumor-bearing mice (Figure 3F). These data indicate that depletion of BMDCs decreases the metastatic growth of breast tumor cells in the lung. However, clodronate is not specific for CD11b+ cells, and pleiotropic effects associated with BMDC depletion may preclude therapeutic CD11b+ cell depletion to target the premetastatic niche. These data highlight the utility of inhibiting a tumor-secreted protein such as LOX to target the premetastatic niche and metastatic growth.

LOX Increases CD11b+ Cell Recruitment and Metastatic Growth of 4T1 Murine Mammary Tumors

We also investigated the influence of LOX-mediated CD11b+ cell recruitment and premetastatic niche formation in the growth of pulmonary metastatic foci from 4T1 tumors. CD11b+ cell recruitment was observed in the lungs of tumor-free BALB/c mice injected with hypoxic WT 4T1 CM, but not with CM from hypoxic *Lox* shRNA-expressing 4T1 cells (Figure 3G). BALB/c mice were also orthotopically implanted with WT or shRNA-expressing 4T1 tumor cells and given daily injections of CM from hypoxic WT or shRNA-expressing 4T1 cells for 3 weeks. Increased CD11b+ cells and tumor cell foci were observed in the lungs of mice bearing WT 4T1 tumors regardless of the daily injections of LOX-containing CM (Figures S4C and S4D). Mice with *Lox* shRNA-expressing 4T1 tumors exhibited increased pulmonary CD11b+ cells when LOX-containing WT CM was provided (Figure 3G). Similar to our observations in mice with MDA-MB-231 tumors expressing *LOX* shRNA, 4T1 tumors that expressed *Lox* shRNA were virtually nonmetastatic unless LOX-containing CM was provided, which increased the numbers of microscopic metastatic foci (Figure 3G; Figure S4E) and macroscopic metastatic tumors (Figure 3H). These data demonstrate a role for LOX secreted by hypoxic tumor cells in pulmonary CD11b+ cell recruitment and metastatic growth of 4T1 tumors in an immunocompetent mouse strain.

We also studied the role of LOX in CD11b+ cell recruitment to tissues other than lung. We found LOX colocalized with fibronectin and BMDCs in the livers of WT tumor-bearing mice
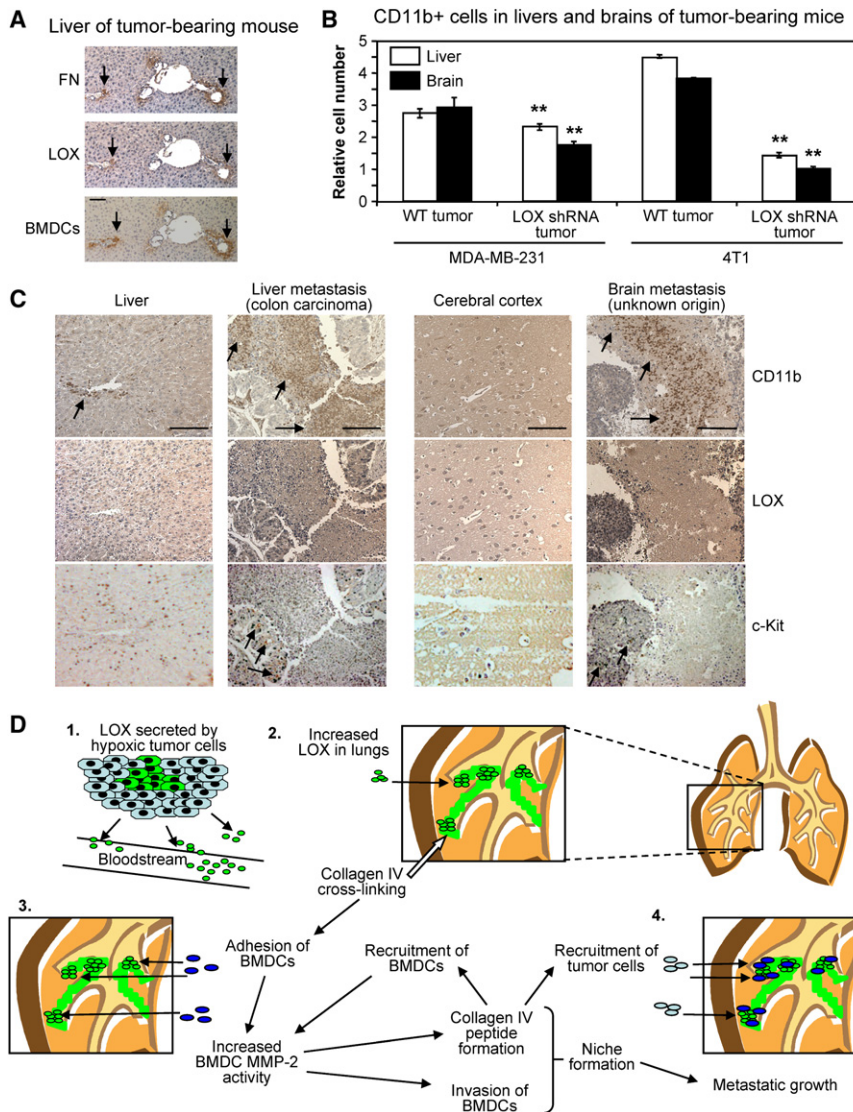
Figure 4. CD11b+ Cells and LOX Are Associated with Liver and Brain Metastases in Patients

(A) Serial sections from liver tissue of WT tumor-bearing mouse stained for fibronectin (FN), LOX, and BMDCs. Arrows indicate FN, LOX, and BMDC staining, respectively. Scale bar = 50 μm.

(B) Flow cytometric analysis for CD11b+ cells in livers and brains of nude mice bearing MDA-MB-231 human breast tumors or BALB/c mice with 4T1 murine mammary tumors. Data are mean ± SEM. **p < 0.05 relative to WT tumor-bearing mice.

(C) Tissue microarrays (TMAs) of clinical metastases stained for CD11b+ cells, LOX, or c-Kit+ cells. Samples from normal cerebral cortex and liver are provided as negative controls. Metastatic and normal TMAs were stained simultaneously and were photographed with identical microscope and camera settings. Arrows indicate regions of CD11b+ cells or c-Kit+ cells. Scale bars = 150 μm.

(D) Model for the role of LOX in premetastatic niche formation. (1) Hypoxic primary tumor cells secrete LOX into the bloodstream. (2) LOX accumulates in the lungs of tumor-bearing mice and crosslinks collagen IV. (3) Adhesion of CD11b+ cells to cross-linked matrix increases BMDC MMP-2 activity. Collagen IV remodeling by LOX and MMP-2 leads to peptide formation, invasion of CD11b+ cells, and increased recruitment of BMDCs. (4) LOX-dependent formation of the premetastatic niche enhances metastatic growth.

cluding metastases sampled from the brain, liver, neck, ovary, greater omentum, and lymph nodes. The primary tumors that gave rise to metastases on the TMA varied, with carcinomas of the breast, colon, stomach, thyroid, esophagus, or nasopharynx producing metastases that were associated with CD11b+ cells. Importantly, CD11b+ cells were not found in significant numbers in most normal tissues apart from the spleen, indicating that the presence of large clusters of CD11b+ cells in metastatic target organs such as the liver or brain is stimulated by tumor-derived factors (Figure 4C). We also found that CD11b+ cells in human metastases were typically found in areas that stained positively for LOX. These data establish that myeloid lineage cells are associated with tumor metastases in a wide variety of cancer patients and also suggest that targeting LOX-mediated recruitment of CD11b+ cells to metastatic sites represents a viable therapeutic strategy for the clinic.

(Figure 4A), consistent with our lung data. We observed modest increases in CD11b+ cell recruitment to the livers and brains of WT MDA-MB-231 tumor-bearing mice relative to mice with *LOX* shRNA tumors (Figure 4B) and found large LOX-dependent increases in CD11b+ cell recruitment to the livers and brains of 4T1 tumor-bearing BALB/c mice. Importantly, 4T1 tumors readily metastasize to the liver and brain, and we have observed metastatic MDA-MB-231 tumor cells in the liver 10 weeks after tumor implant. These data indicate that LOX also affects the recruitment of CD11b+ cells to the livers and brains of tumor-bearing mice.

LOX and BMDCs in Human Metastatic Tumor Samples
In order to assess the relevance of LOX and CD11b+ cell recruitment to human metastases, we stained tissue microarrays (TMAs) containing samples of clinical metastatic nodules from a variety of different sites for CD11b+ cells, c-Kit+ cells, or LOX (Figure 4C; Figure S4F). Interestingly, we found that 51 out of 95 human metastatic lesions contained CD11b+ cells, in-

Implications for the Role of LOX in Metastasis
Elucidating the microenvironmental influences on metastatic growth is paramount to understanding how to inhibit this lethal multistep process in cancer patients (Steeg, 2006). Formation of the premetastatic niche has been shown to enhance the establishment and growth of metastatic foci (Kaplan et al., 2005), and we have identified LOX as a tumor-secreted protein that is

critically involved in premetastatic niche formation (Figure 4D). Our data show that LOX secreted by hypoxic primary tumor cells accumulates with fibronectin at sites of future metastasis, crosslinks collagen IV in the basement membrane, and increases adhesion of CD11b+ cells. Adherent CD11b+ cells produce MMP-2, which degrades collagen IV, increasing CD11b+ cell invasion into the lung tissue and releasing chemoattractive collagen IV peptides. The collagen IV peptides enhance further recruitment of CD11b+ cells, generating a positive feed-forward loop for increased accumulation of BMDCs, increased extracellular matrix remodeling, and creation of the premetastatic niche. Importantly, formation of the premetastatic niche is critically dependent on the accumulation of enzymatically active LOX. Taken together, our data demonstrate a crucial role for LOX secreted by hypoxic tumor cells in formation of the premetastatic niche and in the enhancement of metastatic tumor growth. These data support targeting hypoxia-induced secreted LOX for the treatment and prevention of metastatic cancer.

EXPERIMENTAL PROCEDURES

Cell Lines and Tumor Implants

MDA-MB-231 WT and *LOX* shRNA-expressing cells have been described previously (Erler et al., 2006). 10^7 tumor cells were implanted orthotopically in the mammary fat pad for in vivo experiments. 4T1 murine mammary cells (American Type Culture Collection) were infected with retrovirus to stably express murine *Lox* shRNA (5′-TCTCTCCTCCTCCTTCTAC-3′). All animal studies were approved by and conformed to the regulatory standards of the Stanford University Administrative Panel on Laboratory Animal Care in accordance with US federal law.

Immunological Studies

For immunofluorescence studies and selected H&E-stained sections, lungs were perfused with a 1:1 mixture of PBS/OCT postexcision before embedding in OCT (Tissue-Tek). For paraffin-embedded samples, lungs were perfused with formalin prior to formalin fixation. Western blots were performed as described previously (Erler et al., 2006). Collagen IV ELISAs were performed with the DELPHIA assay (PerkinElmer) according to the manufacturer's instructions.

Antibodies used included CD11b (eBioscience), F4/80 (Abcam), c-Kit/CD117 (ACK2; eBioscience), collagen IV (Millipore), laminin (Chemicon), pan-cytokeratin (ICN), and a LOX antibody that recognizes a peptide sequence from the active site of both human and murine LOX (Erler et al., 2006). Alexa 488 and 594 fluorescent secondary antibodies were used to visualize immunofluorescence staining. Images were photographed using a Nikon 360 microscope camera and analyzed using QCapture software. Flow cytometry analysis for BMDCs was performed as described previously (Kaplan et al., 2005).

Conditioned Media Assays

Conditioned media (CM) consisted of serum-free, phenol red-free modified Eagle's medium cultured on WT or *LOX* shRNA-expressing MDA-MB-231 cells incubated in hypoxia (2% O_2) for 24 hr. CM was passed through a 0.2 μm filter, and 300 μl was intraperitoneally injected daily into mice (Kaplan et al., 2005). For LOX inhibition, β-aminopropionitrile (BAPN; 100 mg/kg) was added daily to CM, and the LOX-targeting antibody (purified; 1 mg/kg) was added twice weekly (Erler et al., 2006). CTT gelatinase inhibitor of MMP-2 and MMP-9 activity (BIOMOL International) was added to CM twice weekly and dosed at 50 μg/mouse (Koivunen et al., 1999). Purified LOX protein was obtained by nickel agarose extraction from WT hypoxic CM and injected twice weekly at either 2 μg/mouse (low dose) or 5 μg/mouse (high dose). A fluorescence-based assay was used to assess LOX enzymatic activity as described previously (Palamakumbura and Trackman, 2002).

Adhesion Assay, Invasion Assays, and MMP Gelatin Zymography

CD11b+ cells and c-Kit+ cells were isolated from whole bone marrow using magnetic bead-assisted cell sorting according to the manufacturer's instructions (Miltenyi Biotec). Matrices were incubated with CM or LOX for 24 hr prior to removal of the CM and addition of BMDCs for adhesion or invasion assays. BAPN was used at 200 μM, and 200 mg/ml glucose for 24 hr was used to chemically crosslink matrices (Kent et al., 1985). In vitro invasion of whole bone marrow cells, RAW monocytes, CD11b+ cells, and c-Kit+ cells was measured in a transwell assay (BD Biosciences) as described previously (Erler et al., 2006). Cell migration ("scratch") assays were performed as described previously (Erler et al., 2006). Gelatin zymography was performed to assess MMP activity as described previously (Hagemann et al., 2004).

Ex Vivo Assays and Clodronate Encapsulation

A 2 cm^3 piece of lung tissue was maintained in 0.5 ml serum-free medium (Hiratsuka et al., 2006) and crosslinked by incubating with LOX or glucose (Kent et al., 1985) for 6 hr. Isolated CD11b+ cells or c-Kit+ cells were added, and the number of cells remaining in the medium was counted.

Clodronate was encapsulated in liposomes of cholesterol and phosphatidylcholine (Sigma) prepared under nitrogen (Van Rooijen and Sanders, 1994; van Rooijen and van Kesteren-Hendrikx, 2003).

Human Samples

Tissue microarrays (TMAs) were purchased from Pantomics, Inc. or from Tissue Array Networks. The TMAs contained human tissues obtained with informed consent according to US federal law and are exempt from consideration by the Stanford Administrative Panel on Human Subjects in Medical Research. TMAs were stained with rabbit monoclonal anti-human CD11b (AbCam) or anti-LOX (Erler et al., 2006) antibody. Metastatic and normal TMAs were stained simultaneously, and images were captured with identical settings using a Nikon 360 microscope camera.

Statistical Analyses

Data were analyzed by Student's t test; $p < 0.05$ was considered significant. Error bars represent standard error of the mean (SEM).

SUPPLEMENTAL DATA

The Supplemental Data include four figures and can be found with this article online at http://www.cancercell.org/supplemental/S1535-6108(08)00378-4.

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