Lysyl Oxidase Plays a Critical Role in Endothelial Cell Stimulation to Drive Tumor Angiogenesis

Ann-Marie Baker, Demelza Bird, Jonathan C. Welt, Morgane Gourlaouen, Georgina Lang, Graeme I. Murray, Andrew R. Reynolds, Thomas R. Cox, and Janine T. Erler

Abstract

Identification of key molecules that drive angiogenesis is critical for the development of new modalities for the prevention of solid tumor progression. Using multiple models of colorectal cancer, we show that activity of the extracellular matrix-modifying enzyme lysyl oxidase (LOX) is essential for stimulating endothelial cells in vitro and angiogenesis in vivo. We show that LOX activates Akt through platelet-derived growth factor receptor β (PDGFRβ) stimulation, resulting in increased VEGF expression. LOX-driven angiogenesis can be abrogated through targeting LOX directly or using inhibitors of PDGFRβ, Akt, and VEGF signaling. Furthermore, we show that LOX is clinically correlated with VEGF expression and blood vessel formation in 515 colorectal cancer patient samples. Finally, we validate our findings in a breast cancer model, showing the universality of these observations. Taken together, our findings have broad clinical and therapeutic implications for a wide variety of solid tumor types.

Introduction

The tumor microenvironment has emerged as a key mediator of tumor progression (1) and an important target for drug development (2). Lysyl oxidase (LOX) is a secreted amine oxidase that plays a key role in modifying the primary tumor microenvironment by crosslinking collagens and elastin in the extracellular matrix (ECM; refs. 3, 4), thereby causing stiffening of the matrix and enhancing invasive and metastatic properties of the tumor (5–9). The local environment at a metastatic site (pre-metastatic niche) also plays an important role in the development of metastases (10). We have previously shown that tumor-derived LOX promotes metastasis by modulating the recruitment of bone marrow–derived cells to premetastatic niches (11).

Formation of new blood vessels, a process known as angiogenesis, is essential for tumor growth and progression (12, 13). Angiogenesis has been described as one of the hallmarks of cancer (14, 15) and is the subject of extensive study in the context of tumorigenesis (16). The VEGF signaling pathway plays a pivotal role in promoting angiogenesis and has become a major target for pharmaceutical intervention (17–19).

We have previously shown that LOX promotes tumor growth and metastasis in colorectal cancer (CRC; ref. 20). Here, we investigate for the first time a role for LOX in tumor angiogenesis and use clinically relevant inhibitors to abrogate LOX-mediated effects.

Materials and Methods

Human CRC tissue microarray

A CRC tissue microarray (TMA; n = 515) was obtained from the University of Aberdeen (Aberdeen, United Kingdom; refs. 20–23). Expression of LOX, VEGF, and CD31 in the patient TMA was examined by immunohistochemical staining and analyzed as previously reported (20). Immunohistochemical staining of the TMA was scored by a highly experienced gastrointestinal pathologist who was unaware of patient outcome data.

Immunohistochemical analysis

Primary antibodies used for immunohistochemical analysis are detailed in the Supplementary Methods. The protocol for detection and visualization was as previously described (20).

Cell lines

Luciferase-expressing SW480 and SW620 CRC cell lines were a kind gift from Dr. Xiao-Fan Wang at the Duke University Medical Center (Durham, NC; ref. 24). These cells have been validated by short tandem repeat (STR) analysis (20). HT29 and LS174T CRC cells were obtained from the American Type Culture Collection, and 4T1 murine mammary cells were a kind gift from Fred Miller. SW480, HT29, and LS174T cells were transfected with an empty vector (control) or a vector containing full-length LOX (+LOX), and SW620 cells were infected...
with retroviral supernatant containing a scrambled control construct (5'-CAGATTTGACGACATGAC-3'; SW620 control) or a short-hairpin human LOX-targeting construct (5'-CCTGGCTATTGATACCTAT-3'; SW620 + shLOX) as previously described (9, 20). 4T1 cells underwent mock infection (4T1 control) or infection with retroviral supernatant containing a short-hairpin murine LOX-targeting construct (5'-TCTCTCTTCTCTTCTAC-3'; 4T1 + shLOX) as previously described (11). Normal human dermal fibroblasts were obtained from TCS Cell Works. The fibroblasts and tumor-derived cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen Ltd.) supplemented with 10% FBS (Invitrogen) and 0.5% penicillin/streptomycin at 37°C and 5% CO₂. Human umbilical vein endothelial cell (HUVEC; TCS Cell Works) were cultured as previously described (25). All cell lines were routinely checked for mycoplasma.

**Collection of conditioned media**

Concentrated conditioned media (CM) was prepared as previously described (20), checked for equal total protein content, aliquoted, and stored at −80°C for use in in vitro and in vivo assays. For cell experiments, fresh media were added to the cells and supplemented with concentrated CM at 1:20 dilution for 16 hours.

**Mice**

CD1 nude mice (6- to 8-week-old for subcutaneous tumor implantation and 5-week-old for in vivo sponge assay), Balb/c (6- to 8-week-old), and C57BL/6 mice (5-week-old) were obtained from Harlan Laboratories.

**Tumor implantation**

SW480 and SW620 were implanted as subcutaneous tumors in 6- to 8-week-old female nude mice as previously described (20). Treatment with LOX-targeting antibody (αLOX) or control rabbit IgG involved twice weekly injection into the peritoneum at a dose of 1 mg/kg. The technician who injected the antibodies was blinded to the specificity of the treatments. HT29 and LS174T cells were implanted subcutaneously in each flank of 6- to 8-week-old female nude mice (2.5 × 10⁶ cells per injection; n = 4 mice per condition). Mice were culled when tumors reached a maximum volume of 0.90 cm³, and excised tumors were fixed in 4% paraformaldehyde (PFA) in PBS for 24 hours before processing. 4T1 cells were implanted as orthotopic tumors into the mammary fat pad as previously described (11).

**HUVEC wound closure assay**

Endothelial cell migration was measured using a scratch wound assay. Detailed description is provided in the Supplementary Methods.

**Angiogenic sprouting assay**

Assays were conducted as previously described (25), with the gel supplemented with concentrated CM at a dilution of 1:20 where indicated. After setting of the gel, 2 × 10⁵ fibroblasts were seeded on top of each well. Gels were incubated for 7 days at 37°C with EGM2 media containing growth factors (10 ng/mL VEGF and/or 50 ng/mL FGF), human IgG or bevacizumab (50 μg/mL), sunitinib (100 nmol/L), or vehicle [dimethyl sulfoxide (DMSO)] where indicated. After 7-day incubation, beads were fixed in 4% PFA in PBS for 20 minutes. The number of sprout tips per bead was counted under an inverted light microscope.

**Immunoblotting**

Cell lysates, CM, and tumor lysates were prepared for immunoblot analysis as previously described (20). Details of antibodies and treatments used are presented in the Supplementary Methods.

**LOX activity assay**

To investigate LOX enzymatic activity, activity assays were conducted as previously described (26). This assay was used to validate the activity of commercially available recombinant human LOX (huLOX; Origene), and also to validate the function blocking effect of the LOX-targeting antibody (αLOX; ref. 20).

**ELISA**

Media was collected from cells after 16-hour incubation at 37°C and centrifuged for 5 minutes at 12,000 × g to remove debris. A human VEGF Quantikine ELISA kit was purchased from R&D Systems (Catalog #DVE00), and the cell media were analyzed according to the manufacturer’s instructions.

**Quantitative reverse transcription PCR**

To detect the levels of LOX or VEGF mRNA in CRC cell lines, quantitative reverse transcription PCR (qRT-PCR) was carried out as previously described (20), with β-actin as an internal control. The primer sequences are listed in the Supplementary Methods. The PCR conditions were: 50°C for 2 minutes, 94°C for 15 minutes, followed by 40 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The PCR was carried out using an Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems), and analysis was conducted using sequence detection system software v2.2.1 (Applied Biosystems).

**Angiogenesis array**

Filtered, unconcentrated CM was collected from CRC cell lines as previously described (20). A Proteome Profiler Human Angiogenesis Antibody Array was purchased from R&D Systems (Catalog #ARY007), and the content of the CM was analyzed according to the manufacturer’s instructions.

**In vivo sponge assay**

Sterile sponges (Caligen Foam Ltd.) of approximately 1 cm³ volume were subcutaneously implanted (27) into anesthetized female 5-week-old mice. Details of treatments are provided in the Supplementary Methods.

**Statistical analysis**

Data are presented as mean ± SEM. Unless stated otherwise, data were analyzed using the Student t-test, and considered statistically significant when the P value was less than 0.05. All statistical tests were 2-sided.
LOX Promotes Tumor Angiogenesis

Study approval

All in vivo experiments were approved by the Home Office and carried out following United Kingdom Coordinating Committee on Cancer Research guidelines for the welfare and use of animals in cancer research. The development and use of the CRC TMA had the approval of The North of Scotland Research Ethics Service (ref. no. 08/S0801/81).

Results

Tumor-derived LOX promotes establishment of blood vessels in vivo and stimulates endothelial cell migration and angiogenic sprouting in vitro

To investigate the role of LOX in angiogenesis, we used the non-metastatic SW480 CRC cell line and the patient-matched metastatic SW620 cell line (28). We previously showed that the growth of these cells is positively regulated by secreted LOX (20). SW480 and SW620 cell lines with manipulated LOX expression were grown as subcutaneous tumors in nude mice, and sections from size-matched tumors were examined for the endothelial marker CD31 by immunohistochemistry (Fig. 1A). We noted a significant increase in CD31-positive blood vessels in LOX-overexpressing (SW480 + LOX) tumors compared with control tumors (Fig. 1B; \( P = 0.046 \)). Treatment with a LOX-targeting antibody (20) that blocks enzymatic function (ref. 9; \( \alpha \)LOX) abrogated this increase (Fig. 1B; \( P = 0.047 \)). Consistently, knockdown of LOX (SW620 + shLOX) or treatment with \( \alpha \)LOX in the SW620 tumors reduced the density of CD31-positive blood vessels (Fig. 1B; \( P = 0.021 \) for control vs. shLOX, \( P = 0.039 \) for control vs. \( \alpha \)LOX). To validate these results, full-length LOX was stably overexpressed in 2 additional human CRC cell lines, HT29 and LS174T (29). These cell lines were implanted as subcutaneous tumors in nude mice, and sections from size-matched tumors were examined for blood vessel density. Consistently, we found that tumors overexpressing LOX displayed a significant increase in blood vessel density (Supplementary Fig. S1A and S1B; \( P = 0.011 \) for LS174T control vs. +LOX, \( P = 0.011 \) for LS174T control vs. +LOX). Taken together, these results suggest a role for LOX in promoting angiogenesis in these mouse models.

We tested whether secreted LOX had an effect on endothelial cells in vitro using HUVEC migration and angiogenic sprouting assays. CM containing secreted LOX was collected from the CRC cell lines and used to supplement the basal media of the HUVEC migration assay. We observed a significant increase in HUVEC migration when CM with elevated LOX levels was added (Fig. 1C; \( P = 0.007 \)), and a significant decrease when CM with LOX knockdown was added (Fig. 1C; \( P = 0.048 \)). However, the addition of \( \alpha \)LOX had no significant effect on

with control IgG or LOX-targeting antibody (\( \alpha \)LOX) at 0 hours, \( n = 4 \) per condition. D, representative images of the angiogenic sprouting assay. Arrows, sprouts of endothelial cells extending from the HUVEC-coated bead. E, quantification of in vitro sprouting from HUVEC-coated beads treated with negative control (no growth factors), positive control (VEGF only), or CM collected from CRC cell lines. \( n = 2 \) wells per condition; 15 beads per well. Bars in B, C, and E represent mean ± SEM. *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \) using the 2-sided Student t test.

Figure 1. LOX promotes establishment of CD31-positive blood vessels in subcutaneous tumors and endothelial cell migration and angiogenic sprouting in vitro. A, representative immunohistochemical staining of CD31 in sections of SW480 and SW620 tumors grown in nude mice treated with a control IgG or LOX-targeting antibody (\( \alpha \)LOX). CD31 is shown in brown and cell nuclei in blue. Scale bar, 100 µm. B, normalized density of CD31-positive blood vessels in SW480 and SW620 subcutaneous tumors grown in nude mice treated with a control IgG or LOX-targeting antibody (\( \alpha \)LOX), \( n \geq 4 \) tumors per condition. C, quantification of HUVEC migration in the presence of conditioned media collected from SW480 or SW620 CRC cells with manipulated LOX expression. Measurements of wound area were taken at 0 and 8 hours and used to calculate percentage wound closure. HUVECs were treated

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Tumor-derived LOX promotes secretion of VEGF in vitro and in subcutaneous tumors

To investigate which angiogenic factors are secreted from SW480 and SW620 CRC cell lines and which are affected by LOX expression, a human angiogenesis antibody array was used. We noted increased VEGF levels in the CM collected from the SW480 + LOX cells compared with the SW480 control cells (Fig. 2A) and decreased VEGF levels in the SW620 + shLOX line compared with the SW620 control. We also noted substantial changes in the levels of three other proteins tested in the array [platelet-derived growth factor-AA (PDGF-AA), insulin-like growth factor–binding protein-2 (IGFBP2), and tissue inhibitor of metalloproteinase-1 (TIMP-1)]. Immunoblot analysis confirmed an association between secreted LOX and secreted VEGF-A (herein referred to as VEGF) protein in the

Figure 2. LOX promotes VEGF expression in tumor cells. A, secreted VEGF levels in CM collected from SW480 control cells or LOX-overexpressing cells (SW480 + LOX) in vitro, detected using a human angiogenesis antibody array. B, immunoblot of secreted VEGF and LOX levels in CM collected from SW480 control or LOX-overexpressing (SW480 + LOX) cells and SW620 control or LOX-knockdown (SW620 + shLOX) cells in vitro. kDa, kilodalton. C, representative immunohistochemical staining of VEGF in sections of SW480 and SW620 subcutaneous tumors grown in nude mice. VEGF is shown in brown and cell nuclei in blue. Scale bar, 200 μm. D, effect of addition of recombinant human LOX (huLOX) or LOX function inhibiting antibody (αLOX) on secretion of VEGF protein in SW480 cells as determined by ELISA. n = 2 wells per condition. E, effect of addition of huLOX or αLOX on VEGF mRNA levels in SW480 cells as determined by qRT-PCR. n = 3 wells per condition. F, effect of extracellular LOX levels on VEGF mRNA in SW480 (top) and SW620 (bottom) cell lines. CM collected from cells with manipulated LOX levels were added to cells with manipulated LOX levels as indicated. Cells were incubated for 16 hours before the collection of mRNA and analysis by qRT-PCR. n = 3 wells per condition. Bars in D–F represent mean ± SEM. *P < 0.05; **P < 0.01 using the 2-sided Student t test.

HUVEC migration (Fig. 1C; P > 0.05), suggesting that LOX itself does not directly affect HUVEC migration.

To further characterize the effect of the CM on the HUVECs behavior, angiogenic sprouting assays were conducted (Fig. 1D and E). We noted that addition of CM with high LOX levels from SW480 + LOX cells) resulted in significantly more angiogenic sprouts than control CM (from SW480 control cells; Fig. 1E; P < 0.001). Consistently, addition of CM with LOX knockdown (from SW620 + shLOX cells; Fig. 1E; P < 0.001). These results suggest that CRC cells secrete pro-angiogenic factors capable of promoting HUVEC migration and sprouting and that levels of these factors are associated with secretion of LOX from the tumor cells.
SW480 and SW620 cell lines (Fig. 2B). To investigate whether this relationship was evident in vivo, we stained sections from subcutaneous tumors for VEGF protein (Fig. 2C) and observed a similar association. To validate LOX-mediated upregulation of VEGF in CRC, the LOX-overexpressing HT29 and LS174T human CRC cell lines were examined for VEGF expression. Consistent with the SW480 cell line, LOX overexpression in HT29 and LS174T resulted in an increase in VEGF secretion in vitro (Supplementary Fig. S2A) and elevated VEGF immunoreactivity in subcutaneous tumors (Supplementary Fig. S2B).

To further confirm LOX-mediated upregulation of VEGF, SW480 cells were treated with purified huLOX protein (Ori-gene), or a LOX function-blocking antibody (αLOX) for 16 hours before analysis. The huLOX was shown to be active in an assay for LOX-specific enzymatic activity, and this activity could be blocked in a dose-dependent manner by the addition of αLOX (Supplementary Fig. S2C). Addition of huLOX protein to CRC cells resulted in a significant increase in VEGF secretion, as measured by ELISA (Fig. 2D; \( P = 0.049 \)). Conversely, inhibition of LOX activity by treatment with αLOX significantly reduced VEGF protein secretion as measured by ELISA (Fig. 2D; \( P = 0.005 \)). To test whether this LOX-mediated upregulation of VEGF occurred at the transcriptional level, qRT-PCR was carried out on huLOX and αLOX-treated SW480 cells and their respective controls. We found that VEGF was significantly increased at the transcriptional level by addition of huLOX (Fig. 2E; \( P = 0.020 \)), and VEGF mRNA levels were significantly decreased upon treatment with αLOX (Fig. 2E; \( P = 0.036 \)). Consistent results were obtained with the LOX-overexpressing HT29 and LS174T cell lines (Supplementary Fig. S2D and S2E).

Furthermore, addition of CM collected from SW480 LOX-overexpressing cells in culture to SW480 control cells led to a significant increase in VEGF mRNA as measured by qRT-PCR (Fig. 2F, top; \( P = 0.009 \)). Consistently addition of CM collected from SW480 control cells to LOX-overexpressing cells resulted in significantly lower VEGF mRNA levels (Fig. 2F, top; \( P = 0.031 \)). Moreover, addition of high LOX containing CM to SW620 cells with knockdown of LOX expression resulted in a significant increase in VEGF mRNA (Fig. 2F, bottom; \( P = 0.006 \)). Conversely, addition of CM containing knockdown of LOX to cells expressing high LOX levels did not result in an increase in VEGF mRNA levels (Fig. 2F, bottom; \( P = 0.044 \)). These data show that extracellular LOX secreted from CRC cells promotes VEGF transcription and secretion in CRC tumor cells.

**Activation of Akt is required for LOX-mediated upregulation of VEGF**

As LOX has recently been shown to promote phosphorylation of Akt at serine 473 (30) and Akt signaling has been shown to promote VEGF transcription (31, 32), we investigated LOX-mediated Akt phosphorylation in our cell lines. In the case of the SW480 control and SW480 + LOX cells, fresh media were added and supplemented with CM from either the SW480 control or the SW480 + LOX cells. Interestingly, when the control CM was added to LOX-overexpressing cells, phosphorylation of Akt was reduced (Fig. 3A). Conversely, CM taken from SW480 + LOX cells was added to SW480 control cells, an increase in phosphorylation of Akt was observed (Fig. 3A). This trend was also evident in the SW620 control and SW620 + shLOX cells (Supplementary Fig. S3A) and displayed the same trend as the observed changes in VEGF mRNA (Fig. 2F).

To further confirm that LOX is responsible for the increase in activation of Akt, SW480 cells were treated with huLOX or αLOX. Addition of huLOX to SW480 cells resulted in an increase in phospho-Akt (Ser473) and treatment with αLOX led to a decrease (Fig. 3B). Consistent results were obtained with the HT29 and LS174T CRC cells (Supplementary Fig. S3B and S3C).

To investigate the relationship between LOX and Akt activation in vivo, lysates from subcutaneous tumors (Fig. 1A) were examined for phospho-Akt(Ser473) by immunoblotting. Consistent with in vitro observations, we noted an increase in phosphorylated Akt in SW480 tumors overexpressing LOX, which could be inhibited by treating with αLOX (Fig. 3C). Consistently, we observed a decrease in phosphorylated Akt in SW620 tumors with a LOX knockdown or treated systemically with αLOX (Supplementary Fig. S3D). Immunohistochemical staining for phosphorylated Akt in subcutaneous tumor sections was used to validate the results of the tumor lysate immunoblots (Fig. 3D and Supplementary Fig. S3E).

To confirm that LOX-mediated changes in phosphorylation of Akt are responsible for the changes in VEGF transcription, cells were treated with the specific Akt inhibitor MK-2206 (ref. 33; Fig. 3E and Supplementary Fig. S3F). The increase in phosphorylation of Akt induced by addition of huLOX could be abrogated by addition of MK-2206 or αLOX (Supplementary Fig. S3G).

ELISA analysis of VEGF protein secreted from these cells revealed that significantly less VEGF is secreted when Akt phosphorylation is inhibited (Fig. 3F; \( P = 0.027 \) for control vs. 250 nmol/L; \( P = 0.0018 \) for control vs. 500 nmol/L; \( P < 0.001 \) for control vs. 1,000 nmol/L). This was confirmed in the SW620 cell line (Supplementary Fig. S3H). Furthermore, inhibition of Akt phosphorylation significantly inhibited VEGF transcription in both the SW480 (Fig. 3G; \( P = 0.015 \) for control vs. 250 nmol/L; \( P = 0.022 \) for control vs. 500 nmol/L) and SW620 lines (Supplementary Fig. S3I). These results show that the activity of extracellular LOX drives phosphorylation of Akt, which is required for LOX-mediated upregulation of VEGF transcription and secretion.

**LOX-dependent PDGFRβ activation upregulates Akt phosphorylation and VEGF secretion**

It has previously been reported that LOX activity can activate cell surface receptor proteins, in particular PDGFRβ (34). Furthermore, PDGFRβ signaling is known to activate Akt (35) and elevate VEGF secretion (36). To investigate whether LOX activity can induce activation of PDGFRβ in CRC cell lines, we supplemented the cell media with 150 ng/mL huLOX for a duration of 16 hours before cell lysis and analysis by immunoblotting. In the SW480 cell line, we observed an increase in phospho-PDGFRβ(Tyr751) after addition of huLOX, consistent...
with the observed upregulation of Akt phosphorylation (Fig. 4A). We were able to validate this LOX-dependent activation of PDGFRβ in the SW620, HT29, and LS174T cell lines (Supplementary Fig. S4A).

To confirm that phosphorylation of PDGFRβ is essential for LOX-dependent activation of Akt and secretion of VEGF, we stimulated PDGFRβ phosphorylation using 25 ng/mL PDGFB-BB ligand, then applied increasing doses of the PDGFRβ inhibitor JNJ-10198409 (37) before cell lysis and analysis by immunoblotting. Stimulation with PDGFB-BB resulted in elevated phospho-Akt(Ser473), which could be abrogated by treating with the PDGFRβ inhibitor (Fig. 4B). This PDGFRβ-dependent Akt phosphorylation was validated in 3 additional LOX-expressing CRC cell lines (Supplementary Fig. S4B). Furthermore, after prolonged inhibition of PDGFRβ, we analyzed secreted VEGF protein and VEGF mRNA expression. We found that in the SW480 cell line, stimulation of PDGFRβ with PDGFB-BB increased VEGF protein secretion from the SW480 cells, as measured by ELISA (Fig. 4C), and this could be abrogated by treating with increasing doses of PDGFRβ inhibitor (Fig. 4C). The changes in VEGF mRNA were consistent with the observed levels of secreted VEGF protein (Fig. 4D). Furthermore, VEGF mRNA was found to be dependent on PDGFRβ activation in 3 further CRC cell lines (Supplementary Fig. S4C). Taken together, our data suggest that LOX activity activates PDGFRβ signaling, resulting in an increase in Akt phosphorylation and VEGF secretion.
Treatment with bevacizumab or sunitinib can abrogate LOX-mediated effects on endothelial cell migration and angiogenic sprouting in vitro

To confirm that tumor-derived VEGF is responsible for the increased migration and sprouting of the HUVECs, we treated HUVECs with CM collected from the CRC cell lines and then collected lysates for analysis of signaling pathway activation. When CM from SW480 LOX-overexpressing cells (SW480 + LOX) was added to HUVECs, we saw an increase in phosphorylation of VEGF receptor 2 (VEGFR2) and the downstream signaling molecule PLC-γ when compared with SW480 control CM (Fig. 5A). Conversely, CM collected from SW620 LOX knockdown cells (SW620 + shLOX) failed to induce VEGF signaling to the extent of the SW620 control CM (Fig. 5A). CM were also collected from the HT29 and LS174T LOX-overexpressing cell lines and their respective controls. Again upon adding to HUVECs, CM, LOX-overexpressing CM were able to stimulate VEGFR2 and PLC-γ phosphorylation to a greater extent (Supplementary Fig. S5A).

To further confirm that LOX-mediated changes in VEGF secretion are responsible for in vitro observations, we treated HUVECs with the VEGF signaling pathway inhibitors sunitinib (ref. 38; Sutent, Pfizer) and bevacizumab (ref. 39; Avastin, Genentech/Roche), both of which are currently in use in the clinic, with good efficacy in a number of tumor types (40, 41). Treatment with 100 nmol/L sunitinib or 50 μg/mL bevacizumab was sufficient to inhibit VEGFR2 phosphorylation in the HUVECs (Fig. 5B). Using these inhibitors in a HUVEC migration assay, we found that inhibition of VEGF signaling suppressed migration of HUVECs where a LOX-overexpressing (and therefore high VEGF) CM had been added (Fig. 5C; P = 0.020 for vehicle vs. sunitinib; P = 0.027 for IgG vs. bevacizumab). However, where HUVECs had been treated with low LOX (and therefore low VEGF) CM, the inhibitory effect was not significant, suggesting that tumor-derived VEGF is responsible for the changes in HUVEC migration. This was also confirmed using CM collected from the SW620 cell line (Supplementary Fig. S5B). Bevacizumab and sunitinib were also able to abrogate LOX-dependent increases in HUVEC migration induced by CM collected from HT29 and LS174T cells (Supplementary Fig. S5C and S5D).

Inhibition of VEGF was additionally tested in the angiogenic sprouting assay. Sunitinib or bevacizumab treatment almost completely eliminated sprouting (Fig. 5D; P < 0.001), even in the presence of CM collected from high LOX-expressing cells, suggesting that VEGF in the CRC CM is primarily responsible for promoting angiogenic sprouting in vitro. This was confirmed in the SW620 cell line (Supplementary Fig. S5E). Taken together, these results show that VEGF production as stimulated in a LOX-dependent manner can promote HUVEC migration and angiogenic sprouting in vitro, and this can be abrogated by inhibiting VEGF signaling using clinically relevant agents.

CM secreted by LOX-expressing tumor cells promotes VEGF-mediated angiogenesis in vivo

To investigate whether tumor-derived VEGF promotes angiogenesis in vivo in a LOX-dependent manner, sponges...
were implanted subcutaneously into mice and injected in situ with CM collected from CRC cell lines with manipulated LOX levels. Consistent with our in vitro findings, CM with high LOX (and therefore high VEGF) levels promoted formation of blood vessels in the sponge (Fig. 6A and B; \( P = 0.006 \)), as shown by scoring of immunohistochemical staining for the endothelial marker endomucin. Injection of CM from SW620 cells with a LOX knockdown resulted in significantly fewer blood vessels than control CM (Supplementary Fig. S6A and S6B; \( P = 0.046 \)). Addition of human VEGF to the low LOX-expressing SW480
control CM significantly increased blood vessel formation (Fig. 6B, \( P = 0.009 \)), confirming a role for VEGF. Mice receiving injections of SW480 CM containing high LOX were treated systemically with sunitinib or bevacizumab, both of which resulted in a significant reduction of endomucin-positive vessels (Fig. 6B; \( P = 0.009 \) for vehicle vs. sunitinib treatment; \( P = 0.017 \) for IgG vs. bevacizumab treatment). These results show that VEGF produced by LOX-expressing CRC tumor cells...
can induce angiogenesis in vivo, and the effects can be inhibited by sunitinib or bevacizumab treatment.

**LOX is clinically correlated with VEGF expression and blood vessel formation in patient samples**

To investigate the clinical relevance of our findings, we examined a CRC patient TMA (refs. 20–23; n = 515 patients). We have previously examined LOX expression in this TMA and found that LOX levels are significantly higher in tumor tissue than normal colon, and expression is associated with increasing tumor stage (20). Analysis of VEGF immunohistochemical staining (Supplementary Fig. S6C) revealed that this trend is also true of VEGF expression (Fig. 6C and Supplementary Fig. S6D; P < 0.001). Furthermore, we noted a significant correlation between LOX and VEGF expression in the CRC patient samples (Fig. 6D and E; P = 0.004 by the Pearson χ² test), with high LOX levels correlating with high VEGF levels. Moreover, we observed a significant correlation between LOX expression and blood vessel formation as determined by CD31 staining (Supplementary Fig. S6E and Supplementary Table S1; P = 0.045 by the Pearson χ² test). These findings provide strong evidence in support of a role for LOX in enhancing secretion of VEGF and thereby promoting angiogenesis in CRCs.

**LOX promotes VEGF secretion via Akt phosphorylation in vitro and angiogenesis in vivo in a mouse model of breast cancer**

To investigate the generalizability of our findings, we used the 4T1 breast cancer model. LOX expression was significantly reduced in the 4T1 cell line through shRNA expression (4T1 shLOX; Supplementary Fig. S7A), resulting in significantly reduced VEGF expression (Supplementary Fig. S7B), consistent with our observations in the CRC models. Furthermore, the addition of human recombinant LOX (huLOX) to the shLOX 4T1 cells significantly increased VEGF mRNA and phosphorylation of Akt (Supplementary Fig. S7B and S7C). Consistently, inhibition of LOX using the blocking antibody significantly decreased VEGF mRNA and phosphorylation of Akt (Supplementary Fig. S7B and S7C).

With in vitro results consistent with the CRC models, we implanted the 4T1 control and 4T1 shLOX cells as orthotopic tumors in syngeneic Balb/c mice. The knockdown of LOX expression was stable in vivo (Supplementary Fig. S7D) and resulted in a decrease in VEGF expression (Supplementary Fig. S7E). To determine the effect on angiogenesis, sections of the 4T1 tumors were stained with endomucin (Supplementary Fig. S7F) and the number of blood vessels scored across each section. Consistent with the findings in the CRC model, the knockdown of LOX resulted in a significant decrease in endomucin-positive blood vessels (Supplementary Fig. S7G). These results show that LOX plays a critical role in promoting tumor angiogenesis in multiple tumor types.

**Discussion**

LOX is emerging as a key mediator of tumor growth and metastasis in a number of human solid cancers (8, 9, 20, 42, 43). A relationship between LOX and angiogenesis has not been previously reported. Here, we show a novel role for LOX in tumor progression, in which LOX upregulates VEGF transcription and secretion, via PDGFRβ-mediated Akt activation, leading to enhanced angiogenesis in mouse models of colorectal and breast cancer. This is the first time a direct link between LOX and VEGF-mediated angiogenesis has been shown.

We observed a significant association between LOX and blood vessel density in the SW480, SW620, HT29, and LS174T human CRC cell lines grown as subcutaneous tumors in nude mice, leading us to investigate a role for LOX in CRC angiogenesis. We found that LOX itself was not responsible for promoting angiogenesis but instead upregulated VEGF secretion. We confirmed an association between LOX and Akt activation in 4 CRC cell lines in vitro and in vivo, and furthermore, provide novel evidence that this activation event is required for LOX-mediated increases in VEGF transcription.

We sought to determine the mechanism by which extracellular LOX activity can be transduced to Akt activation inside the cell. While a role for hypoxia-inducible factor-1 (HIF-1) in activating Akt has been shown (30), we were unable to detect any HIF-1 in cell lysates collected from the cell lines used to produce CM, likely as these were collected in normoxic conditions when the HIF-1α subunit is rapidly degraded. We therefore investigated alternative mechanisms. It has previously been reported that LOX enzymatic activity can activate PDGFRβ in vascular smooth muscle cells (34), and furthermore PDGFRβ activation can lead to enhanced phosphorylation of Akt (35) and elevated VEGF secretion (36). By using 4 human CRC cell lines, we show an induction of PDGFRβ phosphorylation in response to addition of active human LOX protein. Furthermore, stimulation of the receptor with PDGF-BB consistently induced Akt phosphorylation and VEGF secretion in each of the CRC cell lines tested, and this could be abrogated by treating with a PDGFRβ inhibitor. This suggests that PDGFRβ on CRC cells can be activated by extracellular LOX activity, thereby inducing Akt phosphorylation and VEGF secretion.

Notably, a previous report has suggested that LOX promotes PDGFRβ signaling in vascular smooth muscle cells by increasing receptor affinity and capacity for the PDGF-BB ligand and by reducing turnover of pathway components (34); however further work is required to confirm if this is also the case in cancer cells. LOX-mediated matrix modifications have been shown to modulate tumor cell signaling through integrins (7, 20), and it is certainly possible that such signaling events act to promote PDGFRβ pathway activation via receptor crosstalk (44). The relative contribution of LOX to PDGFRβ-associated disease remains to be determined; however, we postulate that elevated LOX levels may indicate enhanced sensitivity to PDGFRβ inhibitors. It is noteworthy that although our data suggest an important role for PDGFRβ in transducing LOX-dependent signals, it is likely that this is not the only receptor that extracellular LOX can act upon.

In our study, we used both bevacizumab (Avastin, Genentech/Roche) and sunitinib (Sutent, Pfizer), which are inhibitors of VEGF and VEGFR2, respectively, and already approved for clinical use (45). The increases in HUVEC migration and angiogenic sprouting induced by LOX were completely abrogated by bevacizumab or sunitinib treatment, confirming that
Vascular endothelial growth factor (VEGF) is primarily responsible for the observed effects of tumor cell-derived CM on HUVECs in vitro.

These findings were confirmed by our in vitro studies, whereby both inhibitors prevented LOX-associated increases in vessel formation. Bevacizumab is of particular interest as it does not interact significantly with murine VEGF (46), and as a result it will not inhibit angiogenesis induced by host-derived VEGF, and hence specifically inhibits the human CRC-derived VEGF injected into the sponge. Our results provide strong evidence that LOX-mediated upregulation of VEGF is responsible for the LOX-dependent changes in angiogenesis in vivo. Importantly, immunohistochemical staining of a CRC TMA revealed that LOX expression is clinically correlated with VEGF expression and blood vessel formation in patients, validating the findings in vitro and in mouse models. Therapeutic targeting of LOX may therefore provide a novel way to prevent VEGF-mediated angiogenesis in CRCs.

Of note, one of the LOX family members, lysyl oxidase-like-2 (LOXL2), has recently been linked with the regulation of sprouting angiogenesis in the zebrafish embryo (47). It will thus be of great interest to further explore the role of the LOX family members in both fundamental and disease-specific biologic functions.

In summary, our study has shown that LOX, an extracellular matrix–modifying enzyme known to have a pivotal role in cancer progression, promotes angiogenesis in in vitro and in vivo models of CRCs. In support of this, we found that LOX was significantly associated with blood vessel density in patient samples. We have provided evidence of a novel link between LOX expression and VEGF secretion in vitro, in vivo, and in patients and shown that this occurs through PDGFBR-mediated activation of Akt. Our results suggest that inhibition of LOX in a therapeutic setting has potential to slow cancer progression not only by inhibiting invasion and metastasis but also by reducing tumor angiogenesis. These findings have important clinical implications for the development of novel strategies for the treatment of patients with cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A.-M. Baker, A.R. Reynolds, T.R. Cox, J.T. Erler
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.-M. Baker, J.C. Welti, M. Gourlaouen, G. Lang, G.I. Murray, A.R. Reynolds, J.T. Erler
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): A.-M. Baker, G.I. Murray, T.R. Cox, J.T. Erler
Writing, review, and/or revision of the manuscript: A.-M. Baker, A.R. Reynolds, T.R. Cox, J.T. Erler
Study supervision: G.I. Murray, A.R. Reynolds, T.R. Cox, J.T. Erler

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Bird, G. Lang

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References

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