Colorectal cancer (CRC) is the third most common cancer worldwide, causing approximately 529,000 deaths annually (1). CRC tumors are commonly classified based on TNM staging system developed by the American Joint Committee on Cancer, with stage 0 representing carcinoma in situ and stage IV representing advanced metastatic disease (2). TNM staging is widely used for preoperative evaluation of patients and is now recommended over the pathologically based Duke classification (3). Treatment of primary CRC usually involves surgical resection, but greater than 50% CRC-specific deaths occur as a result of metastatic disease, with advanced-stage CRC having a median survival of only 6–9 months from diagnosis (4). Therefore, identification of patients at high risk of metastatic dissemination is required to improve survival rates.

Metastasis is a complex multistep process (5–7), and to a large extent is driven by the tumor microenvironment (8). The mechanisms by which tumor cells can modify their environment have become novel therapeutic targets for drug development (9–11), and one such target is lysyl oxidase (LOX). LOX is part of a multigene family, consisting of five members (LOX, lysyl oxidase-like...
**CONTEXT AND CAVEATS**

**Prior knowledge**
Lysyl oxidase (LOX) is known to play an important role in metastasis of many cancers. Whether it plays an important role in invasion and metastasis of colorectal cancer (CRC) is not known.

**Study design**
LOX expression was checked in a tissue microarray of patient CRC tissue samples, and the expression was manipulated in CRC cell lines to investigate a role for LOX in invasion and proliferation of CRC cells, as well as the mechanism involved, both in vitro and in vivo. Subcutaneous tumor models and intrasplenic metastatic models were developed in mice for in vivo studies.

**Contribution**
LOX was overexpressed in primary tumor tissues of CRC patients, and the highest expression was observed in metastatic tissues. LOX overexpression increased CRC cell proliferation and invasion in vitro, and increased subcutaneous tumor growth and metastasis in vivo, whereas results were contrary in case of knockdown of LOX expression. LOX-mediated functions were dependent on the activation of SRC kinase, and the use of dasatinib, an SRC kinase inhibitor, inhibited the functions.

**Implications**
The results may help identify CRC patients who are likely to benefit from dasatinib treatment.

**Limitations**
Results need validation in other patient cohorts.

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From the Editors

**Patients, Materials, and Methods**

**Human CRC Tissue Microarray**
To determine the clinical relevance of LOX in CRC, a tissue microarray (TMA) consisting of normal colon mucosa (n = 49), primary (n = 510), and metastatic (n = 198) CRC samples was obtained from the University of Aberdeen, UK (34–37) (Supplementary Table 1, available online). The primary tumor samples represented disease stages I (n = 90), II (n = 201), and III (n = 219). Each normal sample was acquired from a distance of at least 10 cm from the tumor margin. All tumor samples were obtained and handled in accordance with the approved guidelines of the Institutional Review Board of University of Aberdeen.

**Human CRC Whole-Tissue Samples**
Samples were collected from patients (n = 45) with primary colorectal adenocarcinoma who underwent surgical resection at Norrköping and Linköping University Hospitals, Sweden. The samples included the corresponding normal mucosa specimens taken from the margin of distant resection, which were histologically free from pretumor and tumor (38). These samples were used to verify results obtained from the TMA. All tumor samples were obtained and handled in accordance with the approved guidelines of the Institutional Review Board of the university hospitals.
Cell Lines, Cell Culture, and Reagents

A panel of human cell lines representing normal colon mucosa and a range of CRC disease stages was obtained to assay for LOX expression. An early-stage colon adenocarcinoma (Duke stage B) cell line, SW480, and a lymph node metastasis cell line, SW620, were developed from the same patient (39). Luciferase-expressing SW480 and SW620 cell lines were a kind gift from Dr Xiao-Fan Wang at the Duke University Medical Centre (40). These lines were authenticated by using short tandem repeat repeat analysis. Additional cell lines used to analyze LOX protein expression included CRC cell lines LS-174T (Duke stage B), DLD-1 (Duke stage C), and HCT116 (Duke stage D), which were obtained from the American Type Culture Collection (ATCC) (distributed by LGC Standards, Middlesex, UK), where cell lines are authenticated on a regular basis (www.lgcstandards-atcc.org/ATCC). Science/AuthenticationandPreservation/Authentication Technology/tabid/1020/Default.aspx, last accessed 17/01/2011).

A derivative of the HCT116 cell line, HKe3, was generated by disrupting the mutant v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) allele (41) and obtained as a kind gift from Isaac Garcia-Murillas, Institute of Cancer Research, UK. All tumor-derived cell lines mentioned above were grown at 37°C and 5% CO2 in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.5% penicillin, and 0.5% streptomycin, as previously described (17). Medium and supplements were obtained from Invitrogen Ltd, Paisley, UK. A normal human colonic epithelial cell line, NCM460 (42), was obtained from INCELL Corporation (San Antonio, TX) and grown at 37°C and 5% CO2 in M3:10 medium (INCELL), supplemented with 10% FBS, 0.5% penicillin, and 0.5% streptomycin. The Phoenix Retroviral Expression System was obtained from ATCC (distributed by LGC standards) and grown at 37°C and 5% CO2 in DMEM supplemented with 10% FBS, 0.5% penicillin, and 0.5% streptomycin. All cell lines were regularly tested to confirm the absence of mycoplasma using the e-Myco polymerase chain reaction (PCR) detection kit (Chembio Ltd, Hertfordshire, UK).

Monoclonal and Polyclonal Antibodies

Mouse anti-human LOX monoclonal antibody, which was raised against LOX protein corresponding to amino acids 188-202 (sequence: Asp-Thr-Tyr-Glu-Arg-Pro-Arg-Pro-Gly-Gly-Arg-Tyr-Arg-Pro-Gly), was obtained from Open Biosystems (Huntsville, AL). Rabbit anti-human LOX polyclonal antibody, known to block LOX function in vitro and in vivo (17,18,25), and mouse anti-human LOX monoclonal antibody, which was raised against LOX protein corresponding to amino acids 188–202 were purchased from Abcam plc (Cambridge, UK). Other polyclonal antibodies were rabbit anti-human Ki67 antibody (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK), rabbit anti-human phosphorylated SRC(Tyr418) antibody (Invitrogen Ltd), and rabbit anti-human phosphorylated FAK(Tyr397) (Abcam plc). Horseradish peroxidase–conjugated rabbit anti-mouse and goat anti-rabbit secondary antibodies, and biotinylated goat anti-rabbit secondary antibody were purchased from Dako UK Ltd (Cambridgeshire, UK).

Immunohistochemical Analysis

To investigate LOX expression in tumor sections, immunohistochemical analysis was performed on human CRC TMA, human CRC patient whole-tissue samples (n = 45), or subcutaneous tumor samples from mice (n = 3 tumors per group of mice per experiment, described later in the “In Vivo Studies”). All tissue specimens were formalin fixed and paraffin embedded, and 5-μm tissue sections were mounted onto SuperFrost Plus microscope slides (VWR International, Lutterworth, UK). Sections were deparaffinized by immersing the slides twice in 100% xylene at room temperature for 10 minutes each. This was followed by incubating twice in 100% ethanol for 10 minutes each and rehydrating with decreasing concentrations of ethanol (90% and 70%; vol/vol in water, 10 minutes each) before a final 5-minute incubation in water. Sections were then incubated with 3% hydrogen peroxide in methanol at room temperature for 15 minutes to quench endogenous peroxidase activity. Antigen retrieval was carried out in a target retrieval citrate buffer (pH 6.0) (Dako UK Ltd) at 95°C for 15 minutes. Sections were allowed to cool for 15 minutes and rinsed with room temperature phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4). This was followed by blocking of nonspecific binding by incubation with 5% goat serum (Invitrogen Ltd) at room temperature for 1 hour. Sections were incubated with the following primary antibodies—rabbit anti-human LOX, rabbit anti-human Ki67, rabbit anti-human SRC, rabbit anti-human phosphorylated SRC(Tyr418), and rabbit anti-human phosphorylated FAK(Tyr397). All antibodies were used at 1:100 dilution in PBS with 1% goat serum at 4°C overnight. As negative staining controls, separate slides of tissue sections were incubated in PBS containing 1% goat serum in absence of primary antibodies. After washing with PBS, all sections were incubated with a biotinylated goat anti-rabbit secondary antibody (1:400 dilution) at room temperature for 45 minutes, followed by incubation with streptavidin–biotin peroxidase solution at 1:500 dilution (Vector Laboratories, Inc, Burlingame, CA) at room temperature for 30 minutes. Visualization of antibody binding was carried out using 3,3′-diaminobenzene (BioGenex Laboratories, Inc, San Ramon, CA), and sections were lightly counterstained using Mayer haemalum (Sigma-Aldrich). Sections were dehydrated by immersing in increasing concentrations of ethanol (70%, 90%, 100%; vol/vol in water) for 5 minutes each, followed by immersing twice in 100% xylene for 5 minutes each. For each experimental condition, at least three sections were stained for the protein of interest. Ki67 immunoreactivity was determined by calculating the percentage of Ki67-positive nuclei across 10 fields of view (representing at least three subcutaneous tumors grown in nude mice) at ×40 magnification.
The images of immunohistochemical staining were taken on an Olympus BX51 research microscope (Olympus UK Ltd, Southend-on-Sea, UK) with a DP72 camera (Olympus UK Ltd) or C-4040 camera (Olympus UK Ltd). Immunohistochemical staining of the TMA was scored by a highly experienced gastrointestinal pathologist who was unaware of patient outcome data. The intensity of cytoplasmatic staining was graded by semiquantitative analysis—“negative” (−) when no staining was detected; “weak” (+) when cytoplasmatic staining was weak; “moderate” (++) when cytoplasmatic staining was moderate, and “strong” (+++) when cytoplasmatic staining was strong and homogenous. The experiment was performed one time.

**Immunoblot Analysis**

For detection of secreted LOX, 4 × 10^6 cells of CRC cell lines (HKe-3, SW480, SW620, LS-174T, DLD-1, and HCT116) or NCM460 cells were plated into T75 cm² flasks (BD Falcon, Oxford, UK) and incubated in serum-free DMEM medium for 16 hours. The conditioned medium was then collected from cells in culture, passed through a 0.45-µm filter (Sartorius Stedim, Epsom, UK), and concentrated in a 10 000 Da molecular weight cutoff column (Sartorius Stedim) by centrifuging at 3000g for 20 minutes. Levels of phosphorylated SRC or phosphorylated FAK proteins were determined by coating six-well plates (BD Falcon) with 1.5 mg/mL rat-tail type I collagen (BD Biosciences, Oxford, UK) (diluted with DMEM supplemented with 10% FBS, 0.5% penicillin, and 0.5% streptomycin). SW480 and SW620 cells were treated with 0.25% trypsin and 1 mM EDTA (both reagents from Invitrogen) and approximately 3 × 10^6 cells of each cell line were suspended in 2 mL DMEM supplemented with 10% FBS, 0.5% penicillin, and 0.5% streptomycin. Cells were seeded onto the collagen-coated wells and incubated at 37°C for 16 hours before preparing lysates, as described below. To determine the effect of chemical inhibition of SRC, SW480 and SW620 CRC cells were treated with a small-molecule SRC inhibitor dasatinib (LC Laboratories, Woburn, MA) at 1 µM concentration, at 37°C for 16 hours, before preparing lysates. Because dasatinib inhibits multiple kinases, we also treated the cells with an SRC inhibitor with the same kinases as dasatinib, but not SRC kinase, at 1 µM concentra-

*Construction of LTQ LOX mutant*

To generate a catalytically inactive mutant form of LOX, primers were designed to mutate amino acid lysine 320 to alanine in human full-length LOX (IMAGE clone 30915536; BioScience Lifesciences, Nottingham UK) (25). This residue has been shown to form a covalent cross-link with tyrosine 355 to create the LTQ cofactor, which is essential for catalytic activity (45). A standard PCR reaction was performed using a Quickchange site–directed mutagenesis kit (Stratagene, La Jolla, CA). The following primers were used:

**LOX, Forward:** 5′-GAGATGGGCAGAGCCCACGAGTTTTCTGTCTTG AAG-3′

**LOX, Reverse:** 5′-CTTCAAGAGAAGACTTGTGCTG GTGGCCCTCCAGCACTC TC-3′

The mutated bases are underlined (wild-type nucleotide sequence was AA). Parent vector was removed by digestion with DpnI (New England Biolabs, Beverly, MA), and 5 µL of the PCR product was used to transform chemically competent DH5alpha cells (Invitrogen). Plasmid DNA was extracted using the Qiagen Miniprep kit (Qiagen, Crawley, UK). The presence of the introduced mutation was confirmed by DNA sequencing (GATC Biotech, London, UK). Lack of enzymatic activity was confirmed by LOX activity assay, described below.

**LOX activity assay**

To investigate the activity of LOX in conditioned medium, activity assays that detect the level of hydrogen peroxide were performed as previously described (25). Briefly, conditioned media from SW480 cells incubated in phenol red-free, serum-free DMEM medium (Invitrogen) for 16 hours was collected, as described before, and 50 µL was added to 150 µL of reaction mixture (described later in “In Vivo Studies”) through a cell strainer (BD Falcon) in 1 mL cold PBS, followed by centrifuging at 500g at 4°C for 5 minutes. The cell pellet was then treated with 100 µL NP40 lysis buffer (described above) and centrifuged at 16000g at 4°C for 15 minutes to remove cell debris. The supernatant was removed and used for immunoblot analysis. Samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis then transferred to polyvinylidene difluoride membrane of 0.45-µm pore size (GE Healthcare, Buckinghamshire, UK), as previously described (17). Membranes were blocked with 5% bovine serum albumin (Sigma-Aldrich) or 5% nonfat dry milk in Tris-buffered saline (TBS; 25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4) with 0.1% Tween-20 (Sigma-Aldrich), referred to as TBST, at room temperature for 2 hours, followed by incubation at 4°C overnight with primary antibodies (mouse anti-human LOX, rabbit anti-human SRC, rabbit anti-human phosphorylated SRC [Tyr418], and rabbit anti-human phosphorylated FAK [Tyr397]) at 1:1000 dilution or mouse anti-human ACTB antibody at 1:5000 dilution. Membranes were washed three times, 15 minutes each, with TBST before incubation at room temperature for 45 minutes with peroxidase-conjugated secondary antibodies (Dako UK Ltd) at 1:4000 dilution. All antibody dilutions were done in TBST with 1% bovine serum albumin or 1% nonfat dry milk. After washing the membranes with TBST three times, 15 minutes each, bound antibodies were visualized using Western Blotting Luminal Reagent (Santa Cruz Biotechnology, Santa Cruz, CA).

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containing 1 M urea (Sigma-Aldrich), 0.2 U/mL horseradish peroxidase (Sigma-Aldrich), 10.6 mM cadaverine (Sigma-Aldrich), and 20 µM amplex red (Molecular Probes, Invitrogen) in a 96-well clear-bottom black plate (Perkin Elmer, Waltham, MA). A chemical inhibitor of LOX, BAPN (Sigma-Aldrich), was added to some of the wells at 10 µM concentration to determine LOX-specific activity. Plates were incubated at 37°C for 1 hour then read at 544 nm excitation and 590 nm emission (46) in a SpectraMax M5 plate reader (Molecular Devices, Wokingham, UK). Assays were performed three independent times.

### Retroviral Vectors

Four specific sequences of short hairpin RNA targeting different regions of human LOX mRNA sequence were designed to rule out the possibility of nonspecific effect and were constructed into pSIREN vector (Clontech, Mountain View, CA). A noncoding retroviral vector (pSIREN) was used as a control. The retrovirus was packaged and amplified in Phoenix cells (ATCC). The short hairpin RNA targeting sequences were: shLOX-1: 5′-GTTCCTGTCTCTCAGTAACC-3′ (17); shLOX-2: 5′-GACACATCTGTGACTATGT-3′; shLOX-3: 5′-CCTGCCGTGTATGATACCTAT-3′; and shLOX-4, 5′-CGACGGACATCAGATTCTT-3′.

### Stable Transfection and Infection

SW480 or SW620 cells were grown to 60% confluence in T25 cm² flasks (BD Falcon) and transfected with 10 µg DNA of full-length LOX vector, LTOQ mutant LOX vector, or a mammalian nonviral empty vector control (pEGFP-N1; Clontech), using 20 µL of Lipofectamine 2000 (Invitrogen, following manufacturer’s instructions). A polyclonal population of stably transfected cells was selected using 1 mg/mL geneticin (G418) (Sigma-Aldrich).

Phoenix cells were grown to 60% confluence in T75 cm² flasks (BD Falcon) and transfected with 30 µg of the retroviral constructs (described above), in antibiotic-free DMEM containing 10% FBS, using Lipofectamine 2000, according to the manufacturer’s instructions. Approximately 24 hours after transfection, the media was changed to fresh DMEM (containing 10% FBS, 0.5% penicillin, and 0.5% streptomycin), and after an additional 24 hours incubation at 37°C, this media was collected, passed through a 45-µm filter (Sartorius Stedim), and frozen in aliquots at −80°C. SW620 cells were infected with this retroviral supernatant (multiplicity of infection = 20; G. Lang et al., The Institute of Cancer Research, London, UK, unpublished data) containing retroviral constructs in the presence of 4 µg/mL polybrene (Millipore, Watford, UK) to increase the efficiency of infection. Polyclonal populations of stably infected cells were selected using 30 µg/mL puromycin or 10 µg/mL blastocidin where appropriate.

### Quantitative Reverse Transcription–PCR

To detect the levels of LOX mRNA in CRC cell lines, quantitative reverse transcription–PCR was performed. SW480 and SW620 cells (4 × 10⁶) were plated into separate T75-cm² flasks and incubated in serum-free DMEM medium for 16 hours. Cells were treated with 0.25% trypsin and 1 mM EDTA (both reagents from Invitrogen) and resuspended in DMEM. Total RNA was isolated from 1 × 10⁶ cells using Trizol (Invitrogen), and purified RNA was treated with DNaseI (New England Biolabs), according to the manufacturer’s instructions. Template cDNA was generated using Omniscript reverse transcriptase (Qiagen) and subsequently used for amplification. 18S rRNA was used as an internal control. The primer sequences are listed below:

- **LOX, Forward:** 5′-CGTCCACGTACGAGCAAG-3′
- **LOX, Reverse:** 5′-CCTGTGACGTGACTATGTG-3′
- **18S rRNA, Forward:** 5′-AATTCCGATAACGACAGAGCTCT-3′
- **18S rRNA, Reverse:** 5′-CGGACATCTAAGGCGATCAC-3′

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 performed using an Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA), and analysis was carried out using sequence detection system software v2.2.1 (Applied Biosystems). The experiment was performed three independent times in triplicate.

### In Vitro Growth Assay

To investigate the role of LOX in CRC proliferation, SW480 and SW620 cells were treated with 0.25% trypsin and 1 mM EDTA and resuspended in DMEM (supplemented with 10% FBS, 0.5% penicillin, and 0.5% streptomycin). Approximately 3 × 10⁶ cells were seeded onto the plastic of six-well plates (BD Falcon) and incubated at 37°C. To examine three-dimensional growth in a collagen matrix, 48-well plates (BD Falcon) were coated with 100 µL of 50% type I collagen (BD Biosciences) and incubated at 37°C for 30 minutes. Cells were diluted to 4 × 10⁴ per milliliter of DMEM (supplemented with 10% FBS, 0.5% penicillin, and 0.5% streptomycin), and 100 µL cells was mixed with an equal volume of type I collagen. This was added to the layer of collagen and allowed to set at 37°C for 4 hours. Then, 1 mL of prewarmed (37°C) DMEM (supplemented with 10% FBS, 0.5% penicillin, and 0.5% streptomycin) was added to each well, and the plates were incubated at 37°C for 10 days. Chemical inhibitors dasatinib (1 µM), SrcI (0.3 µM), and imatinib (1 µM), or blocking antibodies against human LOX and human ITGB1, ITGB3, and ITGB4 (2 µg/mL each) were added to the collagen and medium. After incubation at 37°C for 10 days, the three-dimensional cultures were treated with 1 mg/mL collagenase, type I (Invitrogen) (dissolved in PBS) and 0.25% trypsin and 1 mM EDTA to enable counting of cells. Viable cell counts were taken every 24 hours (for two-dimensional growth) or after 10 days (for three-dimensional growth) using a hemocytometer (Hawksley, Lancung, UK) that indicated cell growth, and trypan blue (Sigma-Aldrich) staining was used for exclusion of dead cells (47). The results were expressed as total number of cells or as a percentage inhibition of growth, using the number of living cells incubated with dimethyl sulfoxide (DMSO) as a 100% reference. Three independent experiments in quadruplicate were performed.

### Cell Invasion Assay

SW480 and SW620 cells were grown in serum-free DMEM medium for 24 hours, and 1 × 10⁶ cells/mL were seeded into a collagen matrix, as described above. Aliquots of 100 µL cells (resuspended in 50% collagen in serum-free DMEM) were added
to wells of a 96-well clear-bottom black plate (Perkin Elmer), as previously described (48). Plates were centrifuged at 300g at 4°C for 5 minutes. After incubation at 37°C for 3 hours, DMEM medium containing 10% FBS was added to the wells to promote invasion of the cells upward into the collagen, and the plates were incubated at 37°C for 24 hours. Cells were fixed with 4% formaldehyde (Sigma-Aldrich) and stained using 50 µg/mL Hoechst (Invitrogen). Plates were then sealed with black tape to prevent evaporation and exposure to light. Analysis was performed using an INCell 3000 Analyzer (GE Healthcare) by collecting confocal z slices at a height of 40 µm from the bottom of the wells for counting invading cells and a height of 3 µm from the bottom of the wells. Nuclear staining of each slice was quantified automatically by the INCell 3000 software with the Object Intensity module for the determination of the percentage of invasive cells. Three independent experiments in quadruplicate were performed.

In Vivo Studies

Primary Subcutaneous Tumor Model. Adult female immunodeficient MF1 nude mice (Harlan, Derby, UK), 6 weeks old and weighing 19–24 g, were injected subcutaneously into the left and right flanks with SW480 cells (4 × 10^6 cells per mouse for SW480 control and SW480+LOX groups; n = 3 mice per group) or SW620 cells (1 × 10^6 per mouse for SW620 control and SW620+shLOX groups; n = 3 mice per group), resuspended in 100 µL PBS, using a 1-mL insulin syringe (Kendall Healthcare, Mansfield, MA), and 30-gauge needle (BD Biosciences). These mice were left untreated to allow analysis of the effect of LOX manipulation on primary tumor growth.

For analysis of the effect of LOX-targeting antibody or dasatinib treatment on primary tumor growth, cells were implanted in mice (n = 24 mice per cell line) as above. On day 1, mice injected with CRC cells were randomly divided into four groups—one group of mice (n = 6) was injected into the peritoneum with a rabbit anti-human LOX polyclonal antibody (25 µg per injection) and the second group of mice (n = 6) was injected with the control rabbit IgG (Sigma-Aldrich) (25 µg per injection) as a control. Treatment of these mice with control IgG or rabbit anti-human LOX polyclonal antibody began on day 7 and was continued weekly for 4 weeks. SRC inhibitor dasatinib (375 µg per injection), and the fourth group of mice (n = 6) was injected with 2% DMSO in citrate/citric acid buffer as a control. Treatment of these with DMSO or dasatinib mice began on day 1 and was continued daily until the experimental endpoint (4–5 weeks from surgery).

Once weekly, mice were injected with 120 mg/kg luciferin and metastatic dissemination of the cells was monitored using IVIS Lumina II (Caliper Lifesciences, Runcorn, UK). Mice were killed by CO2 asphyxiation 5 weeks after tumor cell injection. Metastasis was quantified by measuring luminescent signal from each organ at the experimental endpoint. The technician and the research fellow who injected the drugs and antibodies were blinded to the specificity of the treatments. The research fellow who analyzed the IVIS data was blinded to the type of treatment received by the mice. All in vivo experiments were approved by the Home Office and performed following United Kingdom Coordinating Committee on Cancer Research Guidelines for the welfare and use of animals in cancer research.

Statistical Analysis. Data were analyzed using the Student t test or Mann–Whitney U test, unless otherwise stated, and considered statistically significant when the P value was less than .05. Means and 95% confidence intervals are provided where appropriate. Bar graphs represent the mean, and error bars represent 95% confidence intervals across independent experiments, unless otherwise stated. Statistical analyses were performed using Office Excel 2004 (Microsoft Corporation, Reading, UK). All statistical tests were two-sided.

Results

Analysis of LOX Expression in CRC Patients and Cell Lines. To determine the expression of LOX protein in CRC tumors, immunohistochemical staining was performed on a TMA from

Metastatic Model by Intraperitoneal Injection of CRC Cells. Adult female immunodeficient MF1 nude mice (Harlan), 6 weeks old and weighing 19–24 g, were anaesthetized (1:1:3 Hypnorm [VetaPharma Limited, Leeds, UK]:Hypnovel [Roche, Welwyn Garden City, UK];water); dose, 10 mL/ kg). An incision was made on the left side of the abdomen, and the spleen was exposed. Mice were then injected into the spleen with luciferase-expressing SW480 and SW620 cells (40) (2 × 10^6 cells per mouse per cell line; n = 24 mice per cell line), resuspended in 50 µL PBS, using a 1 mL insulin syringe (Kendall Healthcare), and 30-gauge needle (BD Biosciences). On day 1, mice injected with each CRC cell line were randomly divided into four groups—one group of mice (n = 6) was injected into the peritoneum with a rabbit anti-human LOX polyclonal antibody (25 µg per injection) and the second group of mice (n = 6) was injected with the control rabbit IgG (25 µg per injection) as a control. Treatment of these mice with control IgG or rabbit anti-human LOX polyclonal antibody began on day 7 and was continued biweekly for 4 weeks. The third group of mice (n = 6) was injected through oral gavage with dasatinib (375 µg per injection), and the fourth group of mice (n = 6) was injected with 2% DMSO in citrate/citric acid buffer as a control. Treatment of these with DMSO or dasatinib mice began on day 1 and was continued daily until the experimental endpoint (4–5 weeks from surgery).
CRC patients, which has been described in previous studies (34–37). We observed an increase in LOX staining in CRC primary tumor tissues (stages I–III) compared with normal colon tissue (Figure 1, A). To assess the relative expression levels of LOX in the various stages of CRC, the immunohistochemical staining of LOX was scored in a semiquantitative manner by examination of the cytoplasmic staining intensity by a highly experienced gastrointestinal pathologist. In normal colon tissue, moderate or strong expression of LOX was not observed, but in primary tumor and metastatic tissues, moderate and strong LOX expression was noted in 166 (33%) of the 510 and 95 (48%) of the 198 tissue samples, respectively. The observed increases in LOX staining for normal vs tumor tissue, and tumor vs metastatic tissue, were statistically significant ($P < .001$) (Figure 1, B). An increase in LOX expression in primary tumor tissue, compared with normal tissue, suggested a possible association with CRC disease stage. We observed a statistically significant increase in LOX expression between stage I and stage II tissue samples ($P < .001$) (Figure 1, C). To further confirm this association, whole-tissue samples from a second patient cohort, collected at Norrköping and Linköping university hospitals in Sweden (38), were also stained for LOX expression, and the same trend was observed (Supplementary Figure 1, available online).

Next, we assessed the level of secreted LOX protein by immunoblot analysis in a panel of CRC cell lines representing normal colon mucosa and a range of CRC disease stages (Figure 1, D). The level of secreted LOX protein was undetectable in the normal colon cell line, NCM460, low in the poorly tumorigenic and nonmetastatic cell lines, HKe3 and SW480, respectively, and high in the tumorigenic LS-174T and DLD-1 cell lines, as well

![Figure 1](image-url)

**Figure 1.** Analysis of lysyl oxidase (LOX) expression in human colorectal cancer cells. **A)** Immunohistochemical staining of cell nuclei (blue) and LOX protein (brown) in representative images from normal tissue and colorectal cancer (CRC) stages I–III, from a patient tissue microarray (TMA). It can be seen that LOX expression is restricted to the tumor cells. Magnification x20; inset magnification x40. Scale bar = 50 µm. Images are representative of one experiment. **B)** Distribution of LOX immunoreactivity scores in normal colon, primary tumors, and metastatic CRC tumors from the patient TMA. ***$P < .001$ (Mann–Whitney $U$ test). **C)** Distribution of LOX immunoreactivity scores in primary tumors, stages I–III, from the patient TMA. ***$P < .001$ (Mann–Whitney $U$ test). **D)** Immunoblot analysis of secreted LOX from a panel of cell lines derived from normal colon mucosa or CRC tumor tissues. The blot is representative of one independent experiment.

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as the metastatic cell lines HCT116 and SW620. Taken together, these findings suggest that LOX may play a role in CRC progression.

**Effect of LOX Expression on CRC Cell Proliferation**

To investigate a role for LOX in CRC progression, first we manipulated LOX expression in the patient-matched cell lines, SW480 and SW620, as they expressed low and high levels of LOX protein, respectively (Figure 1, D). To determine the effect of LOX overexpression on the behavior of the nonmetastatic SW480 cell line, we transfected SW480 cells with a mammalian nonviral plasmid construct containing the full-length LOX (SW480+LOX) and observed a substantial increase in LOX protein and mRNA expression (Figure 2, A, top and bottom panels). To knockdown LOX expression in the metastatic cell line SW620, we infected cells with a LOX-specific short hairpin RNA (shLOX-3) to generate stably transfected SW620+shLOX-3 cells (referred to as SW620+shLOX in this study) and observed a substantial reduction in LOX protein and mRNA levels (Figure 2, A). Overexpression or knockdown of LOX did not affect levels of the related proteins LOXL1 and LOXL2 (Supplementary Figure 2, available online). LOXL3 and LOXL4 proteins were not detectable in these cell lines. To rule out the possibility of off-target effects, three additional stably transfected SW620+shLOX cell lines were generated (SW620+shLOX-1, SW620+shLOX-2, and SW620+shLOX-4) using different short hairpin constructs designed to specifically target different regions of LOX. A substantial knockdown of LOX expression was noted in all short hairpin RNA infected cell lines (Supplementary Figure 3, A, available online), and these additional cell lines were used for in vitro and in vivo assays to validate findings.

To investigate the role of LOX in CRC proliferation, SW480, SW480+LOX, SW620, and SW620+shLOX cells were seeded in collagen gels and counted after 10 days of incubation. The control cells were transfected with a noncoding vector. We observed that LOX overexpression statistically significantly increased three-dimensional growth of the SW480 cell line in collagen compared with the control (SW480 control vs SW480+LOX, mean total number = 3.58 × 10^3 cells, 95% confidence interval [CI] = 3.39 × 10^3 to 3.77 × 10^3 cells, vs mean total number = 5.72 × 10^3 cells, 95% CI = 5.26 × 10^3 to 6.19 × 10^3 cells, **P = .037**) (Figure 2, B). LOX knockdown statistically significantly suppressed three-dimensional growth of the SW620 cell line in collagen compared with the control (SW620 control vs SW620+shLOX, mean total number = 7.93 × 10^3 cells, 95% CI = 7.69 × 10^3 to 8.17 × 10^3 cells, vs mean total number = 5.52 × 10^3 cells, 95% CI = 4.44 × 10^3 to 6.60 × 10^3 cells, **P = .011**) (Figure 2, B). Reduced cell proliferation was also noted in the three additional stable cell lines with LOX knockdown (shLOX-1, shLOX-2, and shLOX-4) (Supplementary Figure 3, B, available online). Furthermore, cell growth in collagen was reduced when the cell lines with high LOX expression, SW480+LOX and SW620, were treated with a function-inhibiting LOX antibody (17, 25) (Figure 2, B). In contrast, the two-dimensional growth of these cells (SW480, SW480+LOX, SW620, and SW620+shLOX) on tissue culture plastic was not affected by the overexpression or knockdown of LOX (Supplementary Figure 4, available online).

Effect of LOX on CRC Tumor Growth

To investigate the role of LOX in CRC growth in vivo, the CRC cell lines with manipulated LOX expression were grown as subcutaneous tumors in nude mice. Tumors in mice (n = 3) implanted with SW480+LOX cells showed a statistically significantly increased tumor volume at the experimental endpoint compared with tumors in mice (n = 3) implanted with SW480 control cells.
that were transfected with empty vector (for SW480 control vs SW480+LOX, mean tumor volume = 0.29 cm³, 95% CI = 0.10 to 0.48 cm³; vs SW480+LOX, mean tumor volume = 0.62 cm³, 95% CI = 0.55 to 0.69 cm³; P = .036) (Figure 3, A and C). Tumors in mice (n = 3) implanted with SW620+shLOX cells showed statistically significantly reduced tumor volume at the experimental endpoint compared with tumors in mice (n = 3) implanted with SW620 control cells that were transfected with noncoding vector (for SW620 control vs SW620+shLOX, mean tumor volume = 0.27 cm³, 95% CI = 0.18 to 0.36 cm³; vs mean tumor volume = 0.05 cm³, 95% CI = 0 to 0.10 cm³; P = .048) (Figure 3, B and C). These findings were validated using two further stable LOX knockdown cell lines (SW620+shLOX-1 and SW620+shLOX-2) (Supplementary Figure 3, C, available online). Immunohistochemical staining for LOX protein in subcutaneous tumors at the experimental endpoint showed that the overexpression and knockdown of LOX protein were stable in vivo (Figure 3, D). To understand the effect of LOX on tumor growth, the subcutaneous tumor sections were stained for expression of the Ki67 antigen (MKI67) (Figure 3, E), which is a nuclear protein associated with cell proliferation. Tumors in mice (n = 3) implanted with SW480+LOX cells expressed statistically significantly higher levels of Ki67 compared with tumors in mice (n = 3) implanted with SW480 control cells (SW480 control vs SW480+LOX, mean percentage of Ki67-positive cells = 1.18%, 95% CI = 0.80% to 1.56%, vs mean percentage of Ki67-positive cells = 3.86%, 95% CI = 3.13% to 4.59%, P < .001) (Figure 3, F). Similarly, tumors in mice (n = 3) implanted with SW620+shLOX cells showed statistically significantly reduced levels of Ki67 compared with tumors in mice (n = 3) implanted with SW620 control cells (for SW620 control vs SW620+shLOX, mean percentage of Ki67-positive cells = 7.31%, 95% CI = 5.98% to 8.64%; vs mean percentage of Ki67-positive cells = 3.28%, 95% CI = 2.31% to 4.25%; P < .001).

**Effect of SRC Activation, Loss of LOX Activity, and Integrin Activation on CRC Proliferation**

**Effect of SRC Activation on Cell Proliferation.** As inhibition of LOX has been reported to reduce SRC kinase phosphorylation in vitro (20), and SRC plays a key role in CRC progression (28), we analyzed the levels of phosphorylated SRC(Tyr418) in the CRC cell lines with manipulated LOX expression (SW480+LOX and SW620+shLOX) by immunoblot analysis. SW480+LOX cells showed an increased level of phosphorylated SRC(Tyr418) compared with SW480 control cells transfected with the empty vector (Figure 4, A). Conversely, SW620+shLOX cells showed reduced level of phosphorylated SRC(Tyr418) compared with the control cells (Figure 4, A). When cells overexpressing LOX were treated with the SRC inhibitor dasatinib (1 µM) (32), the level of phosphorylated SRC(Tyr418) was not detectable (Figure 4, A).

To investigate if dasatinib showed an effect on three-dimensional cell proliferation, the aforementioned CRC lines were seeded into collagen gels, and treated with DMSO (control) or dasatinib (1 µM). LOX-associated increase in three-dimensional cell growth in collagen was completely abrogated by dasatinib treatment (SW480+LOX control vs dasatinib treated, mean total number = 5.72 x 10⁶ cells, 95% CI = 4.38 x 10⁶ to 7.06 x 10⁶ cells, vs mean total number = 3.66 x 10⁵ cells, 95% CI = 2.61 x 10⁵ to 4.71 x 10⁶, P = .044) (Figure 4, B). Importantly, treatment with dasatinib did not affect the expression of LOX in CRC cell lines (Supplementary Figure 5, A, top panel, available online) or in subcutaneous tumors grown in nude mice (Supplementary Figure 5, A, bottom panel, and Supplementary Figure 5, B, available online). SW620 cells that were stably transfected to overexpress a very high level of LOX (SW620+LOX) (Supplementary Figure 6, A, available online), needed a higher concentration of dasatinib (0.5 µM), compared with SW620 control transfected cells, to reduce phosphorylated SRC(Tyr418) to an undetectable level (Supplementary Figure 6, B, available online). In addition, the three-dimensional growth inhibitory effects of dasatinib were suppressed in the SW620+LOX cell line (Supplementary Figure 6, C, available online).

As dasatinib inhibits a number of kinases, we tested a second SRC inhibitor, SrcI (43), which was developed from a completely different chemical scaffold. We also tested imatinib (44), which inhibits a number of the same kinases as dasatinib (50), but did not inhibit phosphorylated SRC(Tyr418), as observed in the immunoblot analysis (Supplementary Figure 7, A, available online). When the effects of SrcI and imatinib were tested on three-dimensional cell proliferation in collagen, we found that like dasatinib, SrcI treatment resulted in statistically significantly inhibited growth of cells with high levels of phosphorylated SRC(Tyr418) (SW480+LOX, control vs SrcI-treated, P < .001; SW620 control, control vs SrcI-treated, P = .014), whereas imatinib had no statistically significant effect (Supplementary Figure 7, B, available online). The results suggested that the inhibition of three-dimensional growth in collagen observed with dasatinib treatment was because of inhibition of SRC phosphorylation. Taken together, these findings suggested that LOX increased CRC proliferation through SRC activation.

**Effect of LOX LTQ Mutant on Phosphorylated SRC(Tyr418) Level and Cell Proliferation.** To determine whether LOX catalytic activity was required for LOX-dependent changes on SRC phosphorylation and three-dimensional growth in collagen, we generated an LTQ mutant LOX (Lys320Ala) and expressed it in SW480 cells. The LTQ cofactor is essential for LOX catalytic activity (14). We observed that mutant LOX was overexpressed in SW480 cells (Supplementary Figure 8, A, available online) but showed no LOX-specific activity (Supplementary Figure 8, B, available online). The catalytically inactive mutant LOX showed no increase in the level of phosphorylated SRC(Tyr418) (Figure 4, C). The mutant LOX was also unable to promote three-dimensional growth of SW480 cells in collagen (SW480 control vs SW480+LOX, mean total number = 2.80 x 10⁶ cells, 95% CI = 2.61 x 10⁶ to 2.99 x 10⁶ cells, vs mean total number = 4.91 x 10⁶ cells, 95% CI = 4.76 x 10⁶ to 5.06 x 10⁶ cells, P < .001; SW480+LOX vs SW480+mutant LOX cells, mean total number = 4.91 x 10⁶ cells, 95% CI = 4.76 x 10⁶ to 5.06 x 10⁶ cells, vs mean total number = 2.95 x 10⁵ cells, 95% CI = 2.82 x 10⁵ to 3.08 x 10⁵ cells, P < .001) (Figure 4, D).

**Effect of Integrins on Phosphorylated SRC(Tyr418) Level and Cell Proliferation.** Integrins are known to mediate interactions between the cell and ECM via an “outside-in” signaling mechanism (51). Therefore, we investigated whether integrins were
Figure 3. Effect of lysyl oxidase (LOX) on proliferation of subcutaneous CRC tumors in mice. A) Effect of LOX overexpression on tumor proliferation. SW480 and SW480+LOX cells were implanted subcutaneously into the left and right flanks of 6- to 8-week-old female nude mice (n = 3 mice per group), and tumor volume was measured on days 5, 13, 19, and 22 after implantation. B) Effect of knockdown of LOX expression on tumor proliferation. SW620 and SW620+shLOX cells were implanted subcutaneously into mice (n = 3 mice per group) as above, and tumor volume was measured on days 5, 13, 19, 22, and 24 after implantation. In both (A) and (B), mean tumor volumes and error bars representing 95% confidence intervals of three replicates from one representative experiment are shown. C) Average normalized tumor volume from mice (n = 3 per group) at the experimental endpoint. Mean volumes and error bars representing 95% confidence
involved in transducing the effects of extracellular LOX activity to intracellular SRC phosphorylation using blocking antibodies against integrin, beta 1 (ITGB1), integrin, beta 3 (ITGB3), and integrin, beta 4 (ITGB4) subunits. These particular integrin subunits were tested as they have been shown to be involved in mechanosensing (52,53) and therefore were good candidates as transducers of LOX-mediated collagen crosslinking in the ECM. When compared to treatment with an IgG control, function blocking antibodies for the integrin subunits tested showed a reduced level of phosphorylated SRC(Tyr418) in SW620 cells seeded onto collagen-coated wells (Figure 4, E). However, when blocking antibodies were added to the collagen matrix in a three-dimensional growth assay, only blocking of ITGB3 or ITGB4 function showed statistically significantly reduced three-dimensional proliferation of SW480+LOX cells (for control IgG, mean total number = 4.05 × 10⁰ cells, 95% CI = 3.73 × 10⁰ to 4.37 × 10⁰ cells; for ITGB1 blocking antibody, mean total number = 4.75 × 10⁰ cells, 95% CI = 4.50 × 10⁰ to 5.00 × 10⁰ cells; for ITGB3 blocking antibody, mean total number = 3.17 × 10⁰ cells, 95% CI = 2.81 × 10⁰ to 3.53 × 10⁰ cells; for ITGB4 blocking antibody, mean total number = 3.07 × 10⁰ cells, 95% CI = 2.93 × 10⁰ to 3.21 × 10⁰ cells; P = .037 IgG control vs ITGB3 blocking antibody; P < .01 IgG control vs ITGB4 blocking antibody) (Figure 4, F). Treatment of SW620+shLOX with integrin blocking antibodies did not show a statistically significant inhibition of three-dimensional growth in collagen (P > .05, IgG control vs treated).

Effect of SRC Activation on In Vivo Tumor Growth in Mice and Human

Effect of SRC Activation on Subcutaneous Tumor Growth in Nude Mice. We investigated the association between LOX and phosphorylated SRC(Tyr418) in vivo. Lysates were prepared from subcutaneous tumors grown in nude mice (n = 3) and removed at the experimental endpoint, and phosphorylated SRC(Tyr418) levels were analyzed by immunoblot. Levels of phosphorylated SRC(Tyr418) were found to be associated with LOX expression levels, consistent with our in vitro observations (Figure 5, A). Because SRC phosphorylation was found to promote threedimensional cell proliferation in collagen, we tested whether it also increased tumor proliferation in vivo, by treating mice with dasatinib at 15 mg/kg daily by oral gavage. Immunohistochemical staining of subcutaneous tumors grown from SW480 and SW620 cells and removed at the experimental endpoint confirmed that dasatinib treatment (49) was effective at reducing the level of phosphorylated SRC(Tyr418) in nude mice (Figure 5, B). Dasatinib treatment showed a statistically significantly reduced growth of high LOX-expressing tumors (SW620 DMSO vs dasatinib treated, mean tumor volume = 0.21 cm³, 95% CI = 0.13 to 0.29 cm³, vs mean tumor volume = 0.06 cm³, 95% CI = 0 to 0.12 cm³; P = .043) (Figure 5, C and D). In contrast, dasatinib did not show a statistically significantly reduced growth of tumors expressing a low level of LOX (SW620+shLOX DMSO vs dasatinib treated, mean tumor volume = 0.05 cm³, 95% CI = 0.02 to 0.08 cm³, vs mean tumor volume = 0.08 cm³, 95% CI = 0.02 to 0.14 cm³; P > .05). The same trend was observed for the SW480 control and SW480+LOX cell lines (Figure 5, C and D). Taken together, this data suggested that LOX functionality was associated with phosphorylated SRC(Tyr418) levels and could be used to identify tumors that will respond to dasatinib treatment.

Association of LOX and SRC Activation in Patient TMA. We performed immunohistochemical staining to determine the levels of LOX, SRC, and phosphorylated SRC(Tyr418) in human CRC patient TMA. We noted that tumors with high LOX levels also showed high SRC and phosphorylated SRC(Tyr418) levels (Figure 5, E). Semi-quantitative scoring of the staining revealed this association was not statistically significant across all disease stages; however, a statistically significant association was found between LOX and SRC protein levels in patients with lymph node–positive (stage III) disease (P = .043), suggesting that an association between LOX and SRC may exist in a subset of CRC patients.

Effect of LOX and SRC Activation on FAK Activation

As FAK can be a downstream effector of integrin activation and SRC signaling, influencing cell proliferation, and invasion (54), we investigated the levels of phosphorylated FAK(Tyr397) in the CRC cell lines and subcutaneous tumors in mice. The phosphorylation of FAK is important for FAK-mediated signaling and has previously been associated with LOX expression (15,17). Lysates from cells expressing high levels of LOX (SW480+LOX and SW620) grown on collagen-coated wells were prepared, and immunoblot analysis showed a reduced phosphorylated FAK(Tyr397) level in response to LOX inhibition (antibody-treated) or phosphorylated SRC(Tyr418) inhibition (dasatinib-treated) in vitro (Figure 5, C and D). Taken together, this data suggested that FAK may be a downstream effector of LOX-mediated SRC activation.

Effect of LOX and Phosphorylated SRC(Tyr418) on Invasion and Metastasis of CRC Cells

As LOX expression was elevated in metastatic CRC patient samples, and both LOX and SRC have been implicated in invasion and...
Figure 4. Effects of SRC activation, loss of lysyl oxidase (LOX) catalytic activity, and integrin activation on CRC proliferation. 

A) Immunoblot analysis of phosphorylated SRC(Tyr418) levels in SW480 and SW620 cells overexpressing (SW480+LOX) or underexpressing (SW620+shLOX) LOX cultured on collagen-coated wells. The levels of phosphorylated SRC(Tyr418) are shown. Actin, beta (ACTB) was used as the loading control. The effect of inhibition of SRC phosphorylation was studied by treating the cells with 1 µM dasatinib or control dimethyl sulfoxide (DMSO). p = phosphorylation.

B) Effect of inhibition of SRC activation on three-dimensional cell growth in collagen. Quantitative analysis was done after 10 days of three-dimensional growth in collagen in the presence of DMSO control or dasatinib-treated. The mean total number of cells and error bars representing 95% confidence intervals are shown. ***P < .001, *P = .044, SW480+LOX, DMSO vs dasatinib-treated. Data are representative of three independent experiments.

C) Immunoblot analysis of phosphorylated SRC(Tyr418) levels in SW480 cells overexpressing LOX LTQ mutant. Cells were cultured on collagen-coated wells for 16 hours for the analysis. Actin, beta (ACTB) was used as the loading control. p = phosphorylation.

D) Effect of overexpression of LOX LTQ mutant in SW480 cells on three-dimensional cell growth in collagen. Cells were grown for 10 days. The mean total number of cells and error bars representing 95% confidence intervals are shown. ***P < .001. Data are representative of three replicates from a representative experiment.

E) Effect of inhibition of integrin activation on phosphorylated SRC(Tyr418) level. Immunoblot analysis of SW620 cells cultured on collagen-coated well. Cells were treated with control IgG from mouse serum, or blocking antibodies against ITGB1, ITGB3, and ITGB4, each at a concentration of 2 µg/mL, for 16 hours and then assayed for phosphorylated SRC(Tyr418) level. Actin, beta (ACTB) was used as the loading control. p = phosphorylation. Blot is representative of three independent experiments.

F) Effect of blocking integrin activation on three-dimensional cell growth in collagen. A control IgG or blocking antibodies against ITGB1, ITGB3, and ITGB4 were added to the collagen and to the cell media at 2 µg/mL and grown for 10 days. The mean total number of cells and error bars representing 95% confidence intervals are shown. Data are representative of three replicates from a representative experiment. **P < .01, *P = .037. All P values were calculated using the two-sided Student t test.
metastasis (16,20), we investigated a role for LOX in CRC metastasis and whether any effects were mediated through SRC activation. As invasion is the first step of metastasis, we subjected cell lines with manipulated LOX expression (SW480+LOX and SW620+shLOX) and their respective controls to an invasion assay through collagen (48). We observed statistically significantly increased invasion of SW480+LOX cells compared with control cells (SW480 control vs SW480+LOX, mean percentage invasion = 43.7%, 95% CI = 38.6% to 48.8%, vs mean percentage invasion = 60.4%, 95% CI = 55.2% to 65.6%; P = .015) (Figure 7, A). This increased invasion was abrogated by the addition of the LOX-targeting antibody (antibody-treated SW480+LOX control cells, mean percentage invasion = 33.8%, 95% CI = 19.8% to 47.8%, P = .018, antibody-treated vs control) or dasatinib (1 μM) (dasatinib-treated SW480+LOX cells, mean percentage invasion = 45.9%, 95% CI = 35.5% to 56.3%, P = .046, dasatinib-treated vs control) (Figure 7, A). Consistently, we observed statistically significantly decreased invasion of the SW620+shLOX cells compared with control cells (SW620 control vs SW620+shLOX, mean percentage invasion = 57.6%, 95% CI = 52.9% to 62.3%, vs mean percentage invasion = 37.0%, 95% CI = 31.6% to 42.4%; P = .013). Addition of the LOX-targeting antibody (antibody-treated SW620 control cells, mean percentage invasion = 35.9%, 95% CI = 21.4% to 50.4%, P = .029 antibody-treated vs control) or dasatinib (1 μM) (for dasatinib-treated SW620 control cells, mean percentage invasion = 30.4%, 95% CI = 20.8% to 40.0%, P = .021, dasatinib-treated vs control) also showed a statistically significant decrease in invasion (Figure 7, A). These findings suggested that LOX enhanced the invasion of SW480 and SW620 CRC cells in collagen via SRC activation.

To investigate whether LOX plays a role in promoting metastasis in vivo, luciferase-expressing SW480 and SW620 CRC cells (40) were surgically implanted into the spleen of nude mice. Metastasis was monitored by weekly noninvasive IVIS imaging of luciferase activity, and bioluminescent signal from metastatic organs was quantified at the experimental endpoint (4–5 weeks from surgery). Consistent with previous reports (55,56), the SW480 cell line did not metastasize, whereas the SW620 cell line metastasized to distant organs including the colon and the liver (Figure 7, B and C). However, overexpression of LOX in the SW480 cell line showed a statistically significant increase in the relative average normalized metastatic tumor burden, calculated by adding the luminescent signal from the colon, liver, and stomach (SW480 control vs SW480+LOX, mean = 1.0 luminescent signal, 95% CI = 0.3 to 1.7 luminescent signal, vs mean = 2.2 luminescent signal; P = .044) (Figure 7, B and C). Consistently, knockdown of LOX in the SW620 cell line showed a statistically significant decrease in the relative average normalized metastatic tumor burden (SW620 control vs SW620+shLOX, mean = 1.0 luminescent signal, 95% CI = 0.3 to 1.7 luminescent signal, vs mean = 0.3 luminescent signal, 95% CI = 0.1 to 0.5 luminescent signal; P = .035) (Figure 7, B and C). To confirm a role for LOX in metastasis, mice were treated with a LOX-targeting antibody biweekly from day 7 postsurgery. Using the LOX-targeting antibody resulted in statistically significantly reduced relative average normalized metastatic tumor burden in mice implanted with cells expressing high levels of LOX (antibody-treated SW480+LOX, mean = 0.3 luminescent signal, 95% CI = -0.2 to 0.8 luminescent signal, P = .011, antibody-treated vs control; for antibody-treated SW620, mean = 0.1 luminescent signal, 95% CI = 0.0 to 0.2 luminescent signal, P = .031, antibody-treated vs control) (Figure 7, B and C). Dasatinib treatment showed a statistically significantly reduced relative average normalized metastatic tumor burden of SW620 cells (dasatinib-treated SW620 cells, mean = 0.03 luminescent signal, 95% CI = 0 to 0.06 luminescent signal, P = .031, dasatinib-treated vs control) (Figure 7, B and C). The LOX-targeting antibody and dasatinib had little effect on metastasis of cells expressing low levels of LOX (SW480 and SW620+shLOX) (Figure 7, B and C). Taken together, these data suggested a role for LOX in metastasis of CRC through activation of SRC and suggested that LOX expression could be used to identify tumors that will respond to dasatinib treatment.

**Discussion**

In this study, we demonstrated a key role for LOX in CRC progression by manipulating LOX expression levels in SW480 and SW620 human CRC cell lines. We also examined LOX expression in a TMA from CRC patient tumors and found an increased level of LOX in patient tumor tissues compared with normal colon tissue. This level was further increased in metastatic tumors, associated with increasing stages of CRC disease progression. In support of this, we showed that LOX expression was undetectable in the normal colon cell line (NCM460) and that LOX expression was elevated in the metastatic CRC cell line (SW620), compared with the matched nonmetastatic CRC cell line (SW480). Genetic knockdown of LOX expression or immunological inhibition of LOX in SW620 cells with high LOX expression showed a statistically significantly reduced cell proliferation in vitro and in vivo. Overexpression of LOX in SW480 cells with low LOX expression showed the converse, further supporting these data. We also presented preclinical evidence that targeting LOX in CRC statistically significantly reduces tumor growth and metastasis in a mouse model.

These observations led us to investigate the potential mechanisms underlying LOX-mediated effects on CRC proliferation. An association between LOX inhibition and decreased SRC signaling had previously been reported (20,33), and SRC is known to play a major role in CRC progression (28), so we investigated a role for SRC phosphorylation in this model. We found that LOX expression was associated with phosphorylated SRC(Tyr418) levels in CRC cell lines both in vitro and in tumors grown in mice in vivo. We found that LOX-mediated increase in SRC phosphorylation was dependent on LOX catalytic activity as overexpression of a catalytically inactive form of LOX failed to increase the level of phosphorylated SRC(Tyr418) and promote three-dimensional growth in collagen. Chemical inhibition of phosphorylated SRC(Tyr418) using dasatinib inhibited LOX-driven proliferation in vitro and in vivo suggesting that the SRC signaling pathway plays a role in LOX-associated changes during tumorigenesis. Although dasatinib inhibits a number of kinases, we showed that...
Figure 5. Association of SRC activation with lysyl oxidase (LOX) levels and effect of inhibition of SRC activation on LOX-mediated tumor growth in vivo. A) Analysis of LOX and phosphorylated SRC(Tyr418) levels in subcutaneous tumors in mice (n = 3 per group). Immunoblot analysis of subcutaneous tumors grown from SW480 and SW620 cell lines and then assayed for LOX and phosphorylated SRC(Tyr418) levels. Actin, beta (ACTB) was used as the loading control. p = phosphorylation. Three independent experiments were performed. Blot is representative of one independent experiment. B) Analysis of phosphorylated SRC(Tyr418) in subcutaneous tumors expressing increased or decreased LOX levels and treated with dasatinib. Representative immunohistochemical analysis of tumor sections shows cell nuclei stained in blue and phosphorylated SRC(Tyr418) in brown confirms dasatinib treatment effectively inhibits phosphorylation of SRC(Tyr418) in subcutaneous tumors grown in nude mice. Magnification ×20. Scale bar = 20 µm. Data are representative of three independent experiments. C) Effect of dasatinib treatment (15 mg/kg) on subcutaneous tumor growth. Cells were implanted subcutaneously into both flanks of 6- to 8-week-old nude mice and were then treated daily with dimethyl sulfoxide (DMSO) control or dasatinib. Error bars represent the 95% confidence intervals of three replicates within a representative experiment (n = 3 mice per group). Three independent experiments were performed. D) Average normalized tumor volume from mice (n =3 mice per group) treated with DMSO or dasatinib. Bars represent average normalized tumor volume at experimental endpoint (**P < .01, *P = .043). Error bars represent 95% confidence intervals. Data are representative of at least three independent experiments. All P values were calculated using the two-sided Student t test.

(continued)
Figure 6. Effect of lysyl oxidase (LOX) and phosphorylation of SRC(Tyr418) on phosphorylation of focal adhesion kinase (FAK) (Tyr397). A) Immunoblot analysis of phosphorylated FAK(Tyr397) levels in SW480+LOX and SW620 cells. Cells were cultured on collagen-coated wells and treated with dimethyl sulfoxide (DMSO) control or dasatinib (1 µM), or rabbit IgG control or rabbit anti-human LOX polyclonal antibody (2 µg/mL) for 16 hours. Actin, beta (ACTB) was used as the loading control. p = phosphorylation. Blot is representative of three independent experiments. B) Analysis of phosphorylated FAK(Tyr397) in subcutaneous tumors (n = 3 mice per group). Immunohistochemical staining shows cell nuclei in blue and phosphorylated FAK(Tyr397) in brown. Magnification x40. Scale bar = 20 µm. Data are representative of three independent experiments.

Because LOX has been shown to be an integral player in controlling breast cancer metastasis (17,21), we investigated a role for LOX in CRC metastasis. High LOX expression levels were associated with increased in vitro invasion and in vivo metastasis. Importantly, the inhibition of LOX or phosphorylated SRC(Tyr418) statistically significantly reduced distant metastatic growth of the highly metastatic SW620 cell line in mice and often showed complete elimination of metastatic dissemination.

The role for LOX in promoting invasion and metastasis of CRC cells was consistent with our previous findings in breast cancer mouse models (17,21) and other reports suggesting a key role for LOX in metastatic progression (16,18,20,24). However, our results are not in agreement with a previous report that showed LOX was a tumor suppressor in colon cancer (26). In this study (26), LOX mRNA levels were shown to be reduced in 75% of CRC patient samples tested. However, it should be noted that the sample size used in this study was rather small (n = 66), and only two patients had metastatic disease. Thus, no conclusions could be drawn about the role of LOX in metastasis. We observed low LOX expression levels in CRC patients with early-stage disease, and...
Elevated levels in patients with late-stage disease, consistent with studies in other types of cancer (16, 17). Our findings support a role for SRC kinase as a major player in LOX-mediated progression of CRC, as previously suggested (20). We investigated the effect of chemical inhibition of SRC activation in CRC cells by using dasatinib. Dasatinib is a small-molecule inhibitor that has already been approved for treatment of patients with chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia (33). There are a number of ongoing clinical trials involving dasatinib and metastatic CRC.

Figure 7. Effect of lysyl oxidase (LOX) and SRC activation on in vitro invasion and in vivo metastasis. A) Invasion assay of SW480 and SW620 cells in collagen. Function-inhibiting rabbit anti-human LOX polyclonal antibody (2 µg/mL) or dasatinib (1 µM) were added before incubation at 37°C for 24 hours. Data are representative of three independent experiments. *P = .015, SW480 control vs SW480+LOX; *P = .018, SW480+LOX control vs antibody-treated; *P = .013, SW620 control vs SW620+shLOX; *P = .028, SW620 control vs dasatinib-treated. All P values were calculated using the two-sided Student t test.

B) Analysis of metastatic burden in an intrasplenic model. Luciferase-expressing SW480 or SW620 cells (2 × 10⁶) were implanted into the spleen of nude mice and metastatic burden was measured as total normalized luminescent signal from metastatic organs (liver, colon, and stomach) at the experimental endpoint (4–5 weeks from surgery). Mice were treated with a function-inhibiting rabbit anti-human LOX polyclonal antibody (2 µg/mL) or dasatinib (1 µM). Data are representative of three independent experiments. *P = .044, SW480 control vs SW480+LOX; *P = .011, SW480+LOX control vs antibody-treated; *P = .031, SW620 control vs antibody-treated; *P = .049, SW620 control vs dasatinib-treated. All P values were calculated using the two-sided Student t test.

C) Representative images of mice surgically implanted with luminescent cells into the spleen. The images were taken at the experimental endpoint (4-5 weeks from surgery). Data are representative of three independent experiments.
However, determining which patients will benefit from the treatment is a major challenge. In our CRC patient samples, LOX expression was observed to increase with disease progression, and furthermore, dasatinib treatment statistically significantly decreased tumor growth and metastatic dissemination only in high LOX-expressing tumors. We propose that LOX expression may serve as a useful indicator of SRC activation and may enable identification of patients who are likely to benefit from dasatinib treatment. We have also demonstrated that dasatinib treatment can reduce CRC tumor progression in vitro and in vivo in mouse models of subcutaneous tumor growth and intrasplenic metastasis. These effects were statistically significant only in tumors with high LOX levels, providing the first in vivo evidence of a relationship between LOX expression and SRC activation.

Our study has a few limitations. The SW480 and SW620 cell lines were used for most of the in vitro assays; however, further investigation in nonmetastatic and metastatic matched cell lines are needed to support our results. Tumor growth and metastasis were studied in immunodeficient mice, and although we confirmed our findings by repeating the experiments independently, study on a larger number of mice may enhance the importance of the findings. Although the intrasplenic model (57) is widely used and accepted as a model for CRC metastasis, the use of an orthotopic model would capture all stages of metastasis and may further strengthen our findings. The results of our study have provided an understanding of the mechanism by which LOX and SRC act to promote proliferation and invasion of CRC, but future studies will be important to carry out further investigations into the nature of the integrin signaling and downstream effectors of SRC. Although we have noted a statistically significant relationship between LOX expression and SRC activation in stage III patients, validation of our findings in other patient cohorts is required to confirm the association.

In summary, we propose that targeting LOX or key events associated with LOX-mediated CRC disease progression, such as SRC activation, may provide a viable therapeutic treatment strategy against metastatic CRC. Additionally, we have shown that assessing LOX protein expression in patient tumors can provide insight into disease stage. Furthermore, we have provided evidence in support of a key role for SRC kinase as a key mediator of LOX effects on CRC tumor growth and metastasis. Finally, because we have observed an in vivo association between LOX expression and SRC activation, we propose that LOX expression may serve as a valuable indicator to aid selection of CRC patients that will benefit from dasatinib treatment.

This study, to our knowledge, is the first to demonstrate a role for LOX in promoting colorectal metastasis and to present preclinical evidence that targeting LOX in CRC statistically significantly reduces tumor growth and metastasis in a mouse model. Furthermore, we provided novel evidence that LOX mediated these effects through activation of SRC in vivo.

References


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### Notes

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