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Supplemental Information

Blocking Surgically Induced Lysyl Oxidase

Activity Reduces the Risk of Lung Metastases

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Supplemental Online Materials

Supplemental Experimental Procedures

Flow cytometry

Blood was drawn from anaesthetized mice by cardiac puncture, collected in EDTA tubes and red blood cells were lysed. Lung samples or Matrigel plugs were prepared as single cell suspensions as previously described (Adini et al., 2009; Gingis-Velitski et al., 2011). Viable circulating endothelial cells (CEC), endothelial progenitor cells (CEPs), and endothelial cells were quantified by flow cytometry using the following surface markers: for CECs, CD45-/VEGFR2+; for CEPs, CD13+/VEGFR2+/CD117+/CD45-; and for endothelial cells, CD45-/VEGFR2+/CD31+. All antibodies were purchased from BioLegend or BD biosciences, using the following clones: CD45 (30-F11); CD13 (R3-242); VEGFR2 (89B3A5); CD117 (ACK2); and CD31 (390). In some experiments GFP+ EMT/6 or mCherry+ 4T1 cells colonizing the lungs were quantified. Analyses were performed using CyAn Flow cytometer. At least 100,000 cells per sample were acquired. Analyses were considered informative when an adequate number of events (ie >25, typically 50 -150) were collected in the appropriate enumeration gates of samples from untreated control mice. Percentages of stained cells were determined and compared to appropriate negative controls. Positive staining was defined as being greater than non-specific background staining, and 7-aminoactinomycin D (7AAD) was used to distinguish apoptotic and dead cells from viable cells (Philpott et al., 1996).

Immunostaining

Frozen lung sections or Matrigel plugs were immunostained as previously described (Gingis-Velitski et al., 2011). Briefly, endothelial cells in Matrigel plugs were stained with anti-CD31 antibody (1:100, BD Biosciences). LOX expression in lung sections was evaluated using rabbit anti-LOX antibody as previously described (Erlor et al., 2006). Cy3-conjugated and DyLight-488-conjugated secondary antibodies were used (1:200, Jackson ImmunoResearch). In some experiments, lungs were embedded in paraffin. Lung sections were immunostained with LOX (as above) followed by histidine peroxidase anti-mouse and anti-rabbit secondary antibodies (Nichirei, Japan). Staining was developed using AEC simple stain solution (Nichirei). Hematoxylin (Sigma) was used as a counterstain. Polyclonal rabbit anti-Phospho-Paxillin (1:100, Cell signaling, 2541), and a secondary mouse-anti rabbit Cy3-conjugated antibody (1:200, Jackson ImmunoResearch) were used. Nuclei were stained with 6-diamidino-2-phenylindole (DAPI). Immunostaining for hypoxia was performed using Hypoxyprobe-1 (Chemicon International, Temecula, CA) according to the manufacturer's instructions, and as previously described in (Shaked et al., 2006). Briefly, mice received an IP injection of pimonidazole hydrochloride (60mg/kg) 90

min before euthanasia. Peritoneum cryosection immunostaining was performed using anti-pimonidazole antibody (1:200) and its secondary Cy3-conjugated rat anti-mouse antibody (1:200, Jackson ImmunoResearch Laboratories Inc.). Images were captured using the Leica CTR 6000 system unless otherwise indicated.

Depletion of LOX from plasma

Neutralizing antibodies for LOX (10 µg/ml) were generated by GenScript against the EDTSCDYGYHRRFA peptide. Anti-LOX antibodies (1 µg) were added to 500 µl plasma obtained from BALB/c control mice, mice that underwent surgery, or pooled plasma samples from patients (n=6) at baseline or 24 hours post-surgery. The mix was incubated for 1 hour with rotation at 4°C. Antibodies were then depleted from the plasma using a mix of protein A/G sepharose beads (Abcam, ab193262). LOX depletion was verified by ELISA (Cloud-Clon-Corp., SEC580Mu), in accordance with the manufacturer's instructions. Subsequently, plasma was injected into naive BALB/c mice as described in the text.

Ex vivo pulmonary metastatic assay (PuMA)

The assay was performed as previously described (Mendoza et al., 2010). Briefly, EMT/6-GFP+ cells (2.5×10^4) were injected to mice via the tail vein. Fifteen minutes later, mice were anesthetized, and the trachea was cannulated with a 21G intravenous catheter and attached to a gravity perfusion apparatus. The lungs were filled in the vertical position with heated agarose medium solution containing M-199 media, sodium bicarbonate, hydrocortisone, bovine insulin, penicillin/streptomycin and agarose. The ratio of agarose to medium was 1:1 (w/v). The lungs were then removed and placed in cold PBS. Transverse serial sections (1-2mm in thickness) were gently sliced from each lobe with a scalpel and incubated on Matrigel covered plates for 6 days at 37°C. The lung slices were then analyzed for GFP+ cells using Olympus SZX9 fluorescence stereo microscope or Leica CTR 6000 microscope system.

Collagen assay

Collagen production in lung lysates was quantified using the SIRCOL Collagen Assay Kit (Biocolor, Belfast, UK) in accordance with the manufacturer's instructions. Briefly, SIRCOL dye reagent was added to lung extracts followed by agitation in a mechanical shaker for 30 minutes. The collagen-dye complexes were pelleted by centrifugation at 12,000g for 10 minutes. The pellet was washed with acid-salt wash-reagent and centrifuged again at 12,000g for 10 minutes. Alkali reagent was then added to the samples, standards and blanks. Absorption at 555 nm was measured using a spectrophotometer, and collagen

concentration was calculated according to the standard curve. Results were normalized according to the protein concentration in lung extracts. The experiment was performed in triplicate.

Aortic ring assay

The aortic ring assay was performed by dissecting 1mm long aortic rings from non-tumor bearing BALB/c mice, as previously described (Gingis-Velitski et al., 2011). The aortic ring was embedded in Matrigel (BD Bioscience), and then cultured for 10 days in DMEM supplemented with 10% plasma from control mice or 24 hours after mice underwent surgery. Plasma-containing medium was replaced every 3 days. Sprouting microvessels in the Matrigel-embedded aortic ring were visualized by light-microscopy using the Leica CTR 6000 system.

Tube forming assay

Tube forming assay was carried out in 48 well plates pre-coated with 150µl of Matrigel. HUVEC cells (2×10^5 , purchased from LONZA, Switzerland) were seeded in each well and incubated for 12hr with M-199 medium containing 10% plasma from control mice or mice that underwent surgery. Microtubes formed by the HUVECs were analyzed using Time Lapse Microscopy by Zeiss Axio observer system. The number of bifurcations was counted per field, and then plotted. The experiment was performed in triplicate.

Matrigel plug assay

The evaluation of host cells colonizing Matrigel plugs was performed as previously described (Gingis-Velitski et al., 2011). Briefly, 50 µl plasma from control mice or from mice 24 hours after they underwent surgery were added to 0.5ml Matrigel (ratio of 1:10). Subsequently, Matrigel was implanted into both flanks of 8-10 week-old BALB/c mice to create plugs. After 10 days, plugs were removed and either sectioned or prepared as single cell suspensions. Frozen sections were immunostained for endothelial cells (as described below). Images were captured using Leica CTR 6000 system.

Evans Blue permeabilization assay

Lung vascular permeability was performed using Evans blue assay as previously described (Hiratsuka et al., 2011). Briefly, control or post-surgery (24 hours) mice were intravenously injected with Evans blue solution (100µl/mouse, 4mg/ml PBS). After 2 hours, mice were perfused with PBS and subsequently lungs were removed and imaged. Lungs were homogenized in PBS (100mg tissue/ml) and incubated in formamide (2mg/100mg tissue) at 60°C overnight. Evans blue absorption in tissue was evaluated using

ELISA reader (TECAN infinite M200Pro) with absorbance at 620nm and 740nm wavelengths, as previously described (Hiratsuka et al., 2011).

Western blot

MCF7 cell lysates were subjected to SDS-PAGE. Proteins were electro-transferred to nitrocellulose membranes, which were then probed with polyclonal rabbit anti-paxillin (1:1000; Cell Signaling, 2542) and anti-phospho-paxillin (1:1000; Cell Signaling, 2541), or anti- α tubulin (1:1000, Abcam, Ab4074). For the detection of p-pax, cells were lysed in Hepes 50 mM PH-7.5, EDTA 4 mM, Triton 1%, 0.5 mg/ml Na₃VO₄, 4.5 mg/ml Na₂P₂O₇.

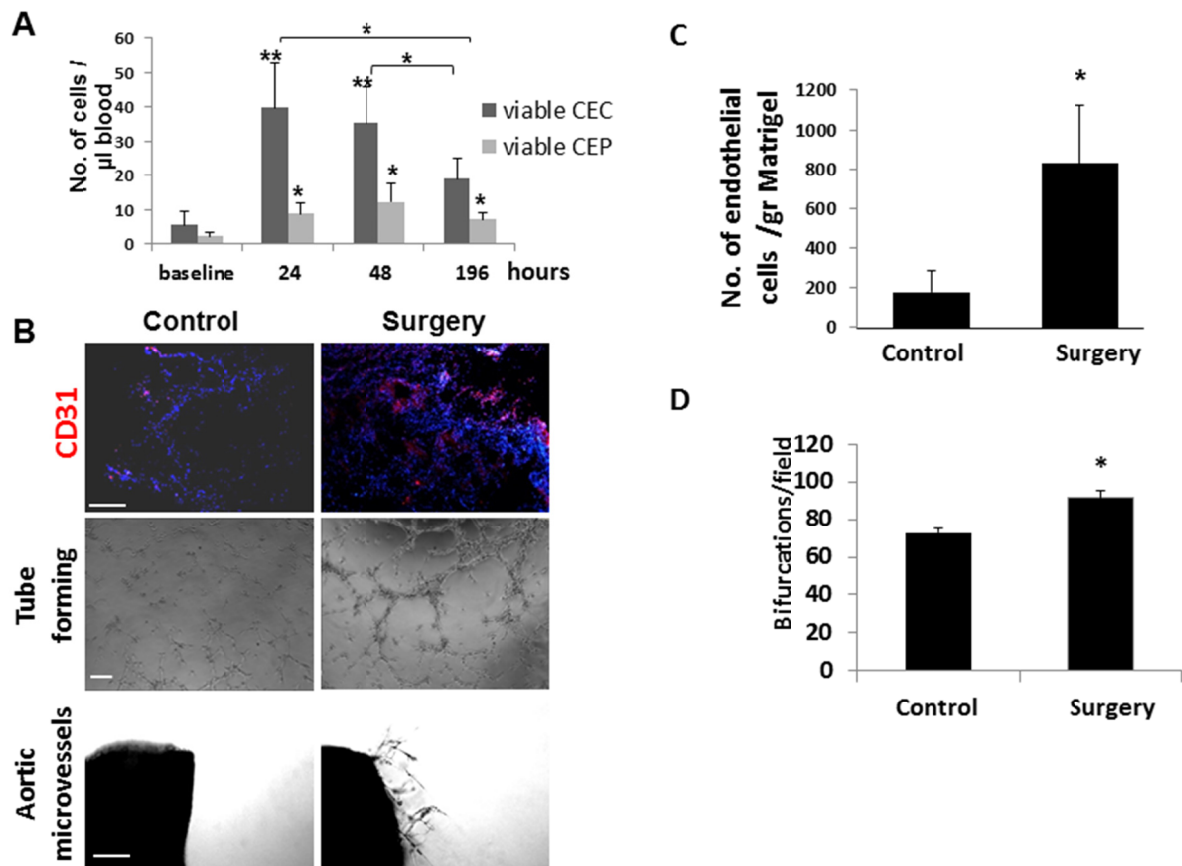


Figure S1 (related to Figure 1): Increased systemic and local angiogenesis in mice that undergo surgery. (A) Abdominal surgery was performed on 8-10 week old non-tumor bearing BALB/c mice (n=5). Blood was drawn from the retro-orbital sinus at several time points following surgery as indicated, and subsequently assessed for levels of viable CECs and viable CEPs. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ using one way ANOVA followed by Tukey post-hoc test. (B) Upper panel: Plasma obtained from control or post-surgery mice was added to Matrigel in a 1:10 ratio. The Matrigel was implanted into the flanks of mice (n=5 mice/group), and plugs were removed after 10 days and sectioned. Sections were stained for endothelial cells using anti-CD31 antibodies (red). Nuclei were stained with DAPI (blue). Middle panel: HUVECs cultured on Matrigel-coated plates for 12 hours in the presence of 10% plasma drawn from control and post-surgery mice. Microvessel tube formation (n=3/group) was assessed. Lower panel: Matrigel-embedded aortic rings (n=4/group) were incubated with medium containing 10% plasma from control and post-surgery mice. Microvessel sprouting was analyzed using Leica CTR 6000 system. Images were captured with the Leica CTR 6000 system. Representative images are shown. Scale bar=200 μ m. (C) The number of endothelial cells in Matrigel (B) was quantified by preparing single cell suspensions that were immunostained with CD31 (endothelial cell marker) and assessed by flow cytometry. (D) The number of bifurcations of HUVEC tube forming assay (B) were quantified. * $p < 0.05$, as assessed by two-tailed Student's t-test.

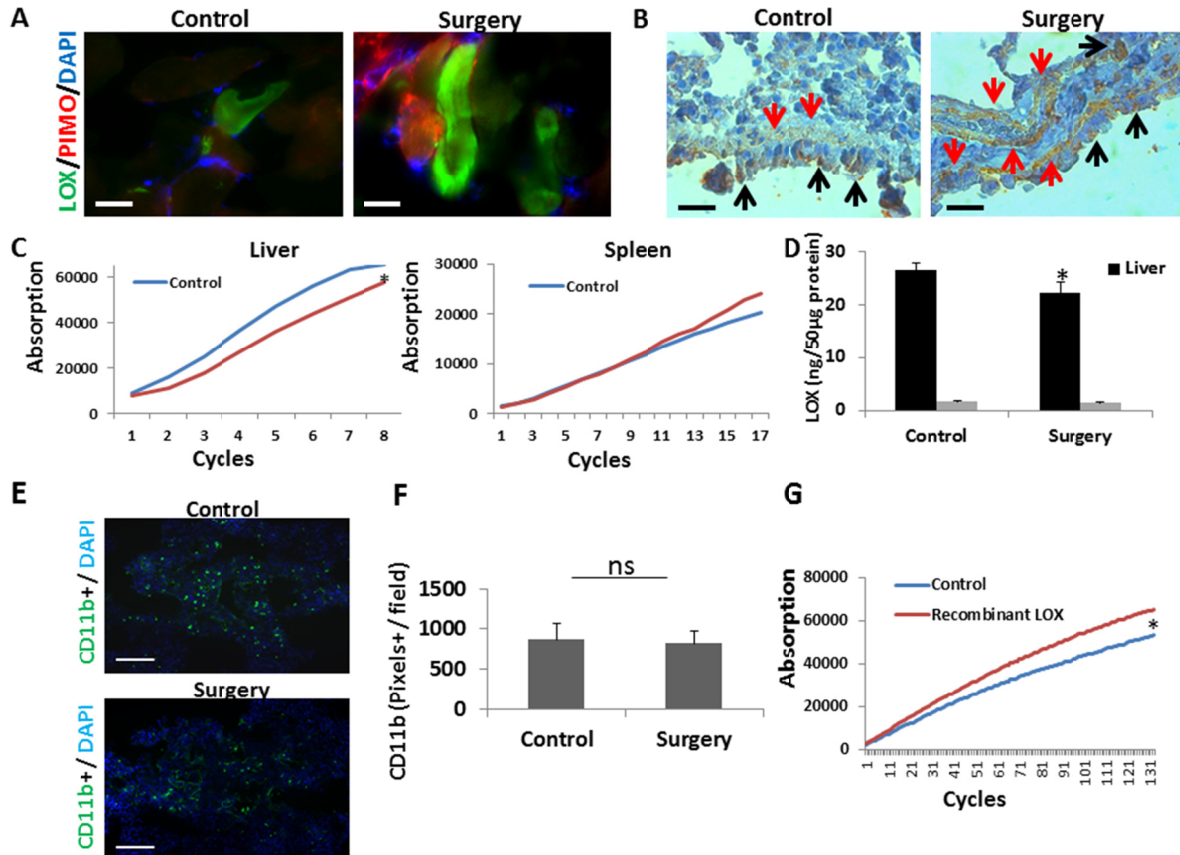


Figure S2 (related to Figure 2): LOX activity and expression in organs as well as CD11b colonization of lungs from mice that undergo surgery. (A-B) A 1 cm incision in the abdomen of non-tumor bearing 8-10 week old BALB/c mice was performed. Control mice have not undergone any surgical procedure (n=3-5 mice /group). After 24 hours, mice were sacrificed, and peritoneum and lungs were removed. (A) High magnification images of immunostained peritoneum from control or post-surgery mice. LOX and pimonidazole marking hypoxia are designated in green and red, respectively. Nuclei were stained with DAPI (blue). Scale bar=20µm. (B) Lung paraffin sections were immunostained for LOX (brown). Counterstaining was performed using hematoxylin. Images were taken in x1000 magnification. Extracellular and intracellular LOX immunostainings are indicated in red and black arrows, respectively. Scale bar=20µm. (C-D) Eight-to-ten week old BALB/c control or 24 hours post-surgery mice (n=3 mice/group) were sacrificed and organs such as liver and spleen were harvested. Lysates of liver and spleen were assessed for LOX activity (C) or LOX expression (D). LOX activity was assessed by paired Student's t-test. A representative graph from three biological replicates is provided. (E-F) Eight-to-ten week old BALB/c mice underwent surgery or were left as controls (n=4 mice/group). Three days later, the mice were sacrificed and the lungs were removed. Lungs sections were immunostained for CD11b (green). DAPI was used for counterstaining (blue). (E) Representative images of lung sections stained with CD11b are provided. Scale bar= 200µm. (F) The number of positive pixels for CD11b from each field was quantified using Photoshop 6 (n=20 fields/group). (G) Lysates of control lungs were assessed for LOX activity after the mice were treated with recombinant LOX or PBS (Control). *p<0.05, or non-significant (ns) as assessed by two-tailed Student's t-test.

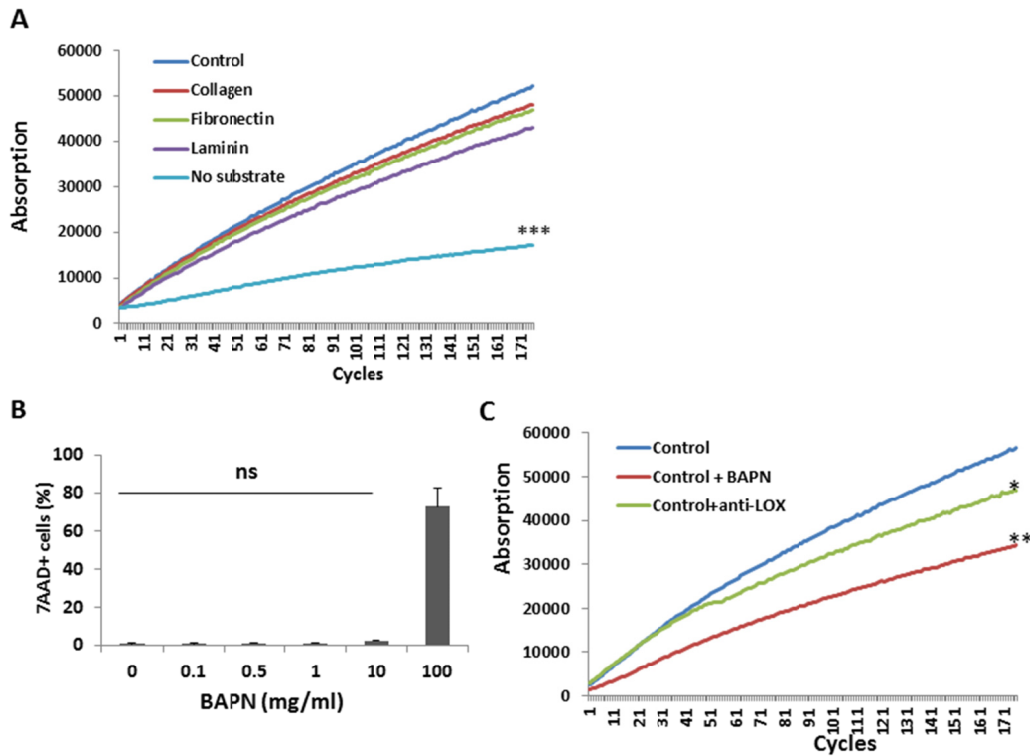


Figure S3 (related to Figures 3-4): LOX activity in different conditions. (A) Lysates of control lungs were used in an oxidation assay in which the substrate was replaced by collagen, fibronectin or laminin. 1,5-diaminopentane was used as a positive control (Control), while no substrate was used as a negative control. (B) EMT/6 cells were cultured in the presence of escalating doses of BAPN as indicated. After 24 hours in culture, cells were analyzed by flow cytometry for the percentage of 7AAD+ cells. Three biological replicates were carried out and results were plotted. Results did not reach statistical significance (ns) at a dose range of 0-10mg/ml, as assessed by one way ANOVA followed by Tukey post-hoc test. (C) Lysates of lungs from post-surgery mice treated with BAPN (100mg/kg) or anti-LOX antibodies (20mg/kg) were assessed for LOX activity. LOX activity in all experiments was assessed by two-tailed Student's t-test. A representative graph from three biological replicates is provided. * $p < 0.05$; ** $p < 0.01$; using Student's t-test.

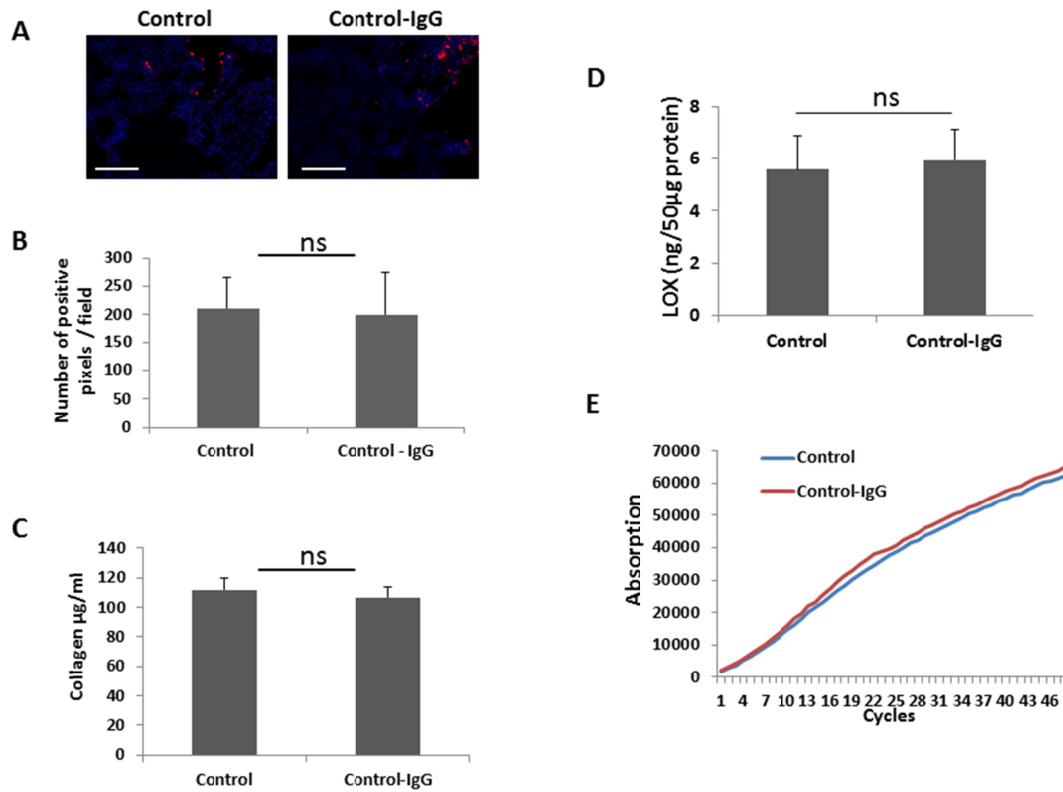


Figure S4 (related to Figure 4): LOX expression and activity in the lungs of mice treated with rabbit IgG. Eight-to-ten week old BALB/c mice ($n=3$ mice/group) were left untreated (control) or injected with 20mg/kg rabbit IgG. After 3 days, lungs were removed. (A) Lung sections were immunostained for LOX (red). Representative images are provided. Scale bar=200 μm . (B) LOX positive pixels (from A) were calculated per field ($n=15$ fields/group). (C-E) Lung lysates were assessed for newly synthesized collagen (C) LOX expression (D) and LOX activity (E). Representative graph for LOX activity is provided from 3 biological replicates. Non-significant (ns) changes were found as assessed by two-tailed Student's t-test.

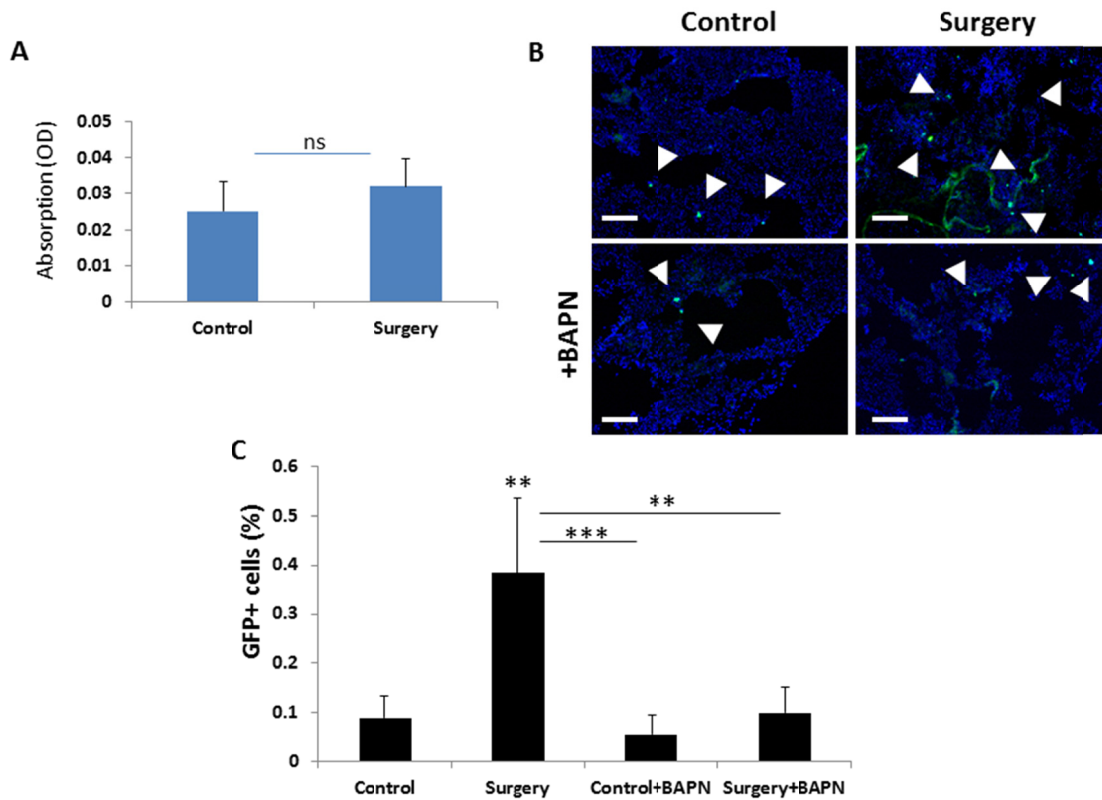


Figure S5 (related to Figure 4): Lung permeability and tumor cell seeding in control and post-surgery mice. (A) Eight-to-ten week old BALB/c mice (n=3/group) were left untreated or underwent abdominal surgery. After 24 hours, mice were injected with Evans blue to evaluate lung permeability, as described in Experimental procedures. Lung images were captured, and Evans blue absorption was quantified in lung lysates. ns – not significant. (B-C) Eight-to-ten week old BALB/c mice were treated with BAPN or PBS for 3 sequential days. After 24 hours, mice either underwent surgery or were left as controls (n=4 mice/group). After an additional 24 hours EMT/6-GFP+ cells (2×10^5 /mouse) were injected through the tail vein. Twenty minutes later, mice were perfused with PBS and lungs were removed. (B) Lung cryosections were imaged under fluorescent microscope to evaluate the number of GFP+ cells (green) in the lungs. White arrows indicate GFP+ cells. Scale bar=200 μ m. (C) In a parallel experiment, lungs were removed and prepared as single cell suspensions. The percentage of GFP+ cells in the lungs was quantified by flow cytometry. **p<0.01; *** p<0.001 as assessed by one way ANOVA followed by Tukey post-hoc test.

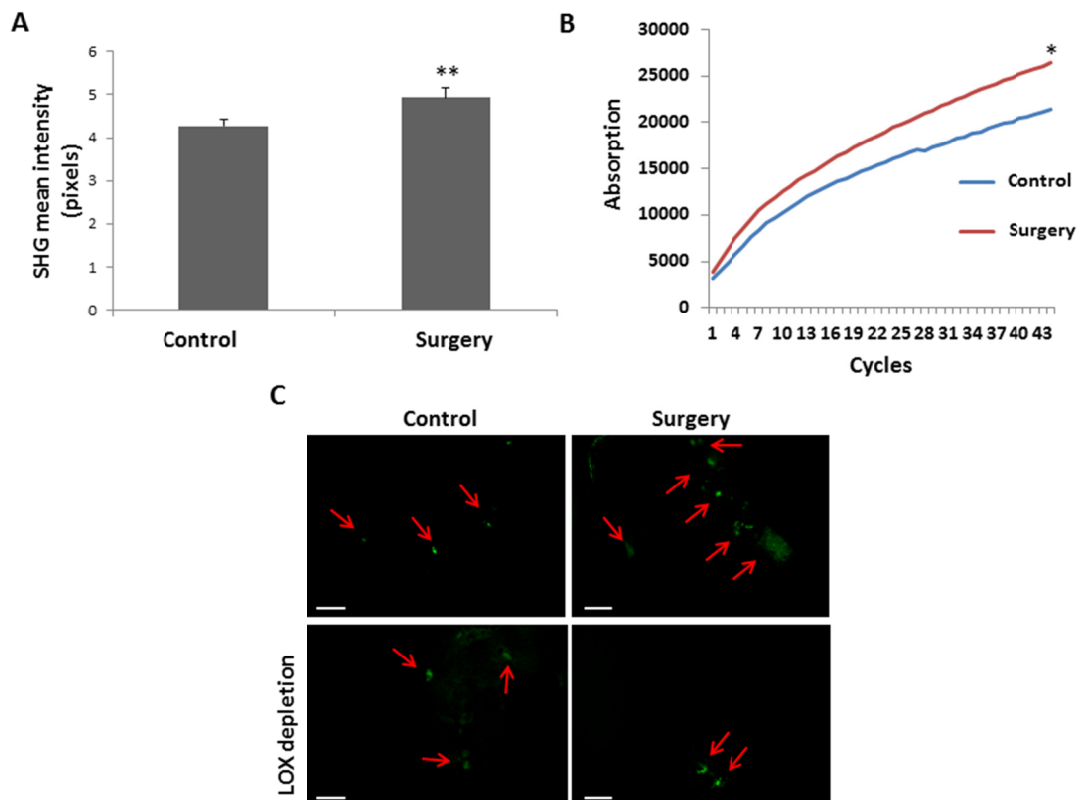


Figure S6 (related to Figure 6): Increased fibrillary collagen, LOX activity, and tumor cell seeding in the lungs of mice injected with post-surgery plasma from patients. (A) Relative collagen intensity in the lungs of mice was measured by two-photon second harmonic generation (SHG) analysis from images presented in Fig. 6G. The fluorescent signal was determined using densitometric analysis (ImageJ). ** $p < 0.01$ as assessed by two-tailed Student's t-test. (B) Lungs from 8-10 week old CB.17 SCID mice injected with plasma from colorectal cancer patients at baseline or 24 hours after they underwent surgery ($n=3$ patients; plasma specimen for each mouse) were lysed. The lysates were assessed for LOX activity. LOX activity was assessed by paired Student's t-test. A representative graph from three biological replicates is provided. * $p < 0.05$ as assessed by two-tailed Student t-test. (C) Plasma (pooled) from colorectal cancer patients ($n=6$) at baseline or 24 hours post-surgery was depleted for LOX as described in Experimental Procedures. Subsequently, the plasma was injected into naïve SCID CB.17 mice ($n=3$ mice/group) and 24 hours later EMT/6-GFP+ cells (2.5×10^4) were intravenously injected for PuMA. Lungs were sliced and subsequently cultured in medium for 6 days. Images of lung sections were obtained on day 6 in culture using Leica CTR 6000 system. Red arrows indicate GFP+ cells. Scale bar= $200 \mu\text{m}$.

REFERENCES

- Adini, A., Fainaru, O., Udagawa, T., Connor, K.M., Folkman, J., and D'Amato, R.J. (2009). Matrigel cytometry: a novel method for quantifying angiogenesis in vivo. *J ImmunolMethods* *342*, 78-81.
- Erler, J.T., Bennewith, K.L., Nicolau, M., Dornhofer, N., Kong, C., Le, Q.T., Chi, J.T., Jeffrey, S.S., and Giaccia, A.J. (2006). Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature* *440*, 1222-1226.
- Gingis-Velitski, S., Loven, D., Benayoun, L., Munster, M., Bril, R., Voloshin, T., Alishekevitz, D., Bertolini, F., and Shaked, Y. (2011). Host response to short-term, single-agent chemotherapy induces matrix metalloproteinase-9 expression and accelerates metastasis in mice. *Cancer Res* *71*, 6986-6996.
- Hiratsuka, S., Goel, S., Kamoun, W.S., Maru, Y., Fukumura, D., Duda, D.G., and Jain, R.K. (2011). Endothelial focal adhesion kinase mediates cancer cell homing to discrete regions of the lungs via E-selectin up-regulation. *Proc Natl Acad Sci U S A* *108*, 3725-3730.
- Mendoza, A., Hong, S.H., Osborne, T., Khan, M.A., Campbell, K., Briggs, J., Eleswarapu, A., Buquo, L., Ren, L., Hewitt, S.M., *et al.* (2010). Modeling metastasis biology and therapy in real time in the mouse lung. *J Clin Invest* *120*, 2979-2988.
- Philpott, N.J., Turner, A.J., Scopes, J., Westby, M., Marsh, J.C., Gordon-Smith, E.C., Dalglish, A.G., and Gibson, F.M. (1996). The use of 7-amino actinomycin D in identifying apoptosis: simplicity of use and broad spectrum of application compared with other techniques. *Blood* *87*, 2244-2251.
- Shaked, Y., Ciarrocchi, A., Franco, M., Lee, C.R., Man, S., Cheung, A.M., Hicklin, D.J., Chaplin, D., Foster, F.S., Benezra, R., *et al.* (2006). Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors. *Science* *313*, 1785-1787.