Supplemental Material

Reactive Oxygen Species Regulate Osteopontin Expression in a Murine Model of Post-Ischemic Neo-vascularization

Alicia N. Lyle[1], Giji Joseph[1], Aaron Fan[1], Daiana Weiss[1], Natalia Landazuri[1], W. Robert Taylor[1,2,3]

Extended Materials and Methods

Animals

Male C57BL/6 mice were purchased from Jackson Laboratories (Maine, USA). Transgenic Catalase overexpressing ($Tg^{SMC\text{-}Cat}$) mice were bred in house in the Department of Animal Resources at Emory University. $Tg^{SMC\text{-}Cat}$ mice have increased expression of human catalase in vascular tissue through the SMC specific smooth muscle myosin heavy chain (MHC) promoter and were characterized previously.[1] These transgenic mice are on a C57Bl/6 background and for all experiments $Tg^{SMC\text{-}Cat}$ mice were compared to wild-type littermate controls. In some experiments, PEG-Catalase dissolved in sterile saline was administered intravenously by osmotic mini-pump at a rate of 10,000 U/kg/day. This concentration was previously reported in the literature, though administered by daily intravenous injection, and was determined to be the dose at which H_2O_2 was significantly decreased in the adductor muscle in this HLI model. Administration of PEG-Catalase began at the time of hind limb surgery and continued for 5 days post-surgery, unless otherwise noted. All animals used were male and between 8 and 10 weeks of age. The animals were housed and cared for according to the guidelines approved by the Emory University Institutional Animal Care and Use Committee.

Osmotic Mini-pump Implantation

Mice were pre-anesthetized using 3% isofluorane (oxygen delivered at 0.5L/min with 3% isofluorane for induction and 2.0% isofluorane for maintenance). For PEG-Catalase pumps, fur was removed using clippers and an incision was made in the neck to expose the jugular vein. A jugular catheter attached to a primed osmotic-mini pump (Alzet osmotic mini-pump, model 1007D, Durect Corporation, Cupertino, CA) was inserted and tied into the jugular vein and the pump inserted subcutaneously at the back of the neck. The neck wound was sutured closed and mice were administered Buprenex (0.01-0.1mg/kg, SQ) as needed.

Hind Limb Ischemia Surgery

Male mice between 8 and 10 weeks of age were pre-anesthetized with 3% isoflurane in a chamber and then anesthetized with 2% isoflurane through a nose cone. All hair was removed from the surgical site, the area was cleaned with sterile saline, and disinfected with Betadine. Aseptic technique was employed. A unilateral incision was made over the right medial thigh of the mouse. The superficial femoral artery and vein were ligated with 6-0 silk suture proximal to the deep femoral artery branch point, and then a second ligation was performed just proximal to the branching of the tibial arteries. The length of the artery and vein were excised between the two ligation points. The skin was closed with 6-0 silk suture. The animals received Buprenex (0.01-0.1mg/kg, SQ) post-operatively for analgesia as needed. The mice were then allowed to recover on a heated platform.

LASER Doppler Perfusion Imaging

LASER Doppler perfusion imaging (LDPI) was completed at 5 days after surgery for each genotype and treatment condition. Mice were anesthetized using inhalation of isofluorane and scanned with the LDPI system (PIM II Laser Doppler Perfusion Imager). Perfusion of the proximal region of the ischemic limb, just below the proximal ligation point, and non-ischemic limb was assessed. Perfusion of the ischemic limb proximal region was quantified and normalized to the same region of the non-surgical limb.

Micro-CT Imaging

Quantitative micro-CT was used for evaluation of collateral vessel formation in the ischemic limb at postoperative day 5 as described previously.[2, 3]. Briefly, mice were euthanized (n=6-9 for each group) and sequentially perfused with saline containing 4 mg/mL of papaverine, 10% formalin, followed by a lead chromate-based contrast agent (Flow Tech, Inc, South Windsor, CT). Bone was demineralized in formic acid based solution (Cal-Ex II, Fisher Scientific, Pittsburgh, PA) for 48 hours. Samples were then imaged at a 30-μm voxel size, and the tomograms were used to render binarized 3-D images. Stereological algorithms were used to quantify vascular volume to tissue volume ratio, connectivity, and vascular density, which were then normalized to the contralateral control limb.

Lentivirus

The viral vector was derived from the HIV-based lentivirus backbone pLV-CMV-GFP-U3Nhe, as described previously,[4-6] and was a kind gift of Dr. Kerry J. Ressler. The pLV-CMV-GFP-U3Nhe vector allows for virally mediated expression of green fluorescent protein (GFP) driven by a cytomegalovirus (CMV) promoter. pLV-CMV-GFP-U3Nhe control vector will hereafter be referred to as LV-GFP. We then generated a dual-tagged human Osteopontin (OPN) expressing an HA-tag at the N-terminus and a Myc-tag at the C-terminus. We then inserted a wild-type internal ribosome entry site (IRES),[7] followed by the dual-tagged full-length OPN downstream of GFP to generate a lentivirus that expresses both GFP and tagged OPN, hereafter referred to as LV-OPN. The wild-type IRES used has been shown previously to promote high translation of the downstream, compared to the upstream coding sequence in the same mRNA,[7] thereby allowing the final construct to produce high OPN and relatively low GFP protein.

Viral production procedures have been described in detail previously.[4, 8, 9] In brief, active viral particles were produced by co-transfecting these lentiviral packaging constructs with

plasmids coding for delta8.9 and VSV-G into HEK-293T cells. The packaged, unconcentrated virus was collected over a period of 5 days post-transfection, and then concentrated using ultracentrifugation and resuspension in sterile PBS/1% BSA. The resulting titer was assessed in HEK-293T cells, and the observed titer of the LV-GFP and LV-OPN expressing viruses used here were each ~$2x10^9$ infectious particles per mL. For *in vivo* use, both lentiviruses were diluted to a final concentration of ~$1x10^9$ infectious particles per mL. All animals received HLI. After the artery and vein were tied off and excised, the adductor muscle (inner thigh) was injected with 20 µL of either LV-GFP lentivius or LV-OPN lentivirus using a similar approach as described previously.[4]

Detection of ROS

To evaluate intracellular production of $O_2^{\bullet-}$, we used DHE-HPLC to measure the conversion of 2-hydroxyethidium from DHE using high-performance liquid chromatography (HPLC). Hydrogen peroxide measurements in intact tissues were made using the Amplex Red Assay in which we measured the oxidation of Amplex Red (100 µmol/L, Sigma-Aldrich, St. Louis, MO) in the presence of horseradish peroxidase using the Amplex Red Assay Kit (Molecular Probes, Carslbad, CA), as described previously (Dikalov et al., 2008). Briefly, Amplex Red and horseradish peroxidase type II (0.1 U/mL) in Krebs HEPES buffer were added to the tissue samples. Fluorescence readings were made in triplicate in a 96-well plate at Ex/Em=530/580 nm using 100-µL of Amplex/Krebs buffer in which the tissue was incubated. H_2O_2 production was calculated and normalized to tissue wet weight.

Immunofluoresence

Mice were sacrificed at indicated times and tissues were perfused in situ with saline and then fixed with 10% buffered formalin. Tissue sections from paraffin embedded proximal hindlimbs were paraffin embedded and cut into 5 µm sections. Antigen retrevial was performed in citrate buffer, pH 6.0 (Invitrogen, Carlsbad, CA), prior to incubating with primary antibodies.

Osteopontin antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) and R&D Systems (Minneapolis, MN) were used at a dilution of 1:200 in 3% BSA, followed by incubation with Horse anti-Mouse secondary antibody (Vector Labs, Burlingame, CA) or Donkey anti-Goat secondary antibody at a dilution of 1:400 in 3% BSA, and finally incubated in Streptavadin QDot 655 (Invitrogen, Carlsbad, CA) at a dilution of 1:200 in 3% BSA. For double staining experiments, Mac3 antibody (BD Pharmingen, San Diego, CA) was used at a dilution of 1:50, Lectin antibody (Vector Labs, Burlingame, CA) was used at 1:100, and smooth muscle alpha-actin antibody (Abcam, Cambridge, MA) was used at 1:200. Images of the sections were collected using the 20X and 40X Plan-Neo air objectives (Numerical aperture 0.50) on a Zeiss Axioskop Microscope equipped with an AxioCam camera.

Immunoblotting

Adductor muscle tissue were homogenized with glass mortar and pestle or VSMCs were homogenized in Hunter's Lysis Buffer (25 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10 mmol/L Na-pyrophosphate, 10 mmol/L NaF, 0.1 mmol/L Na-orthovanadate, 1% Na deoxycholate, 1% Triton X 100, 0.1% SDS, 10% Glycerol, and protease inhibitors) for all experiments.[10] Homogenates were rocked with end-over-end rotation at 4°C for 20 minutes to allow for complete tissue or cell lysis. Cell samples were sonicated on ice at 10 watts for 10 x 1 second pulses to further disrupt the cell membrane using a Microson Ultrasonic Cell Disruptor XL (Misonix, Inc., Farmingdale, NY). Whole cell or whole tissue lysates were utilized for Western Blot (WB) experiments. Protein concentrations were assessed using the Bradford assay (BioRad, Hercules, CA). For WB analysis, proteins were separated using SDS-PAGE and transferred to nitrocellulose membranes, blocked, and incubated with the appropriate primary antibodies. The Osteopontin antibodies used were from Santa Cruz Biotechnology (Santa Cruz, CA) and R&D Systems (Minneapolis, MN). GAPDH antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The β-Actin antibody was from Cell Signaling (Danvers, MA). HRP-conjugated secondary antibodies (BioRad, Hercules, CA) were used for

detection using enhanced chemiluminescence (ECL; GE Healthcare UK, Little Chalfont, Buckinghamshire, UK). Band intensity was quantified by densitometry using ImageJ 1.38 software.

Cell Culture

Vascular smooth muscle cells from mouse aorta (MASMs) were isolated (passages 4-10) and cultured in Dulbecco's Modified Eagle's Media (DMEM; Sigma Aldrich, Saint Louis, MO) supplemented with 10% Fetal Bovine Serum (FBS; Sigma Aldrich, Saint Louis, MO), 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin, all of which were obtained from Mediatech, Inc. (Manassas, VA). Cells were stimulated with H_2O_2 after 48 hours of quiescence in serum free DMEM for all experiments.

RNA Isolation and Quantitative RT-PCR

Total RNA was extracted from muscle tissue or VSMCs using the RNeasy kit (Qiagen). Superscipt II (Invitrogen) and random primers were used for RT. OPN (primer sequences: GTATGAGACGGGACAGCTATTTCTCCA and CTGACATAGTCCAAGCCTGGGATG) and 18S rRNA, were measured by amplification of cDNA from muscle tissue or VSMCs using the LightCycler real-time thermocycler and SYBR green dye. All are normalized to 18S rRNA.

Statistical Analysis

Results are expressed as mean ± S.E.M. from at least three independent experiments. Statistical significance for quantitative results was assessed using analysis of variance (ANOVA), followed by Bonferroni's Multiple Comparison post-hoc test. In some cases, a t-test was used to assess significance. A value of $p<0.05$ was considered statistically significant.

Supplemental References

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Supplemental Figure Legends

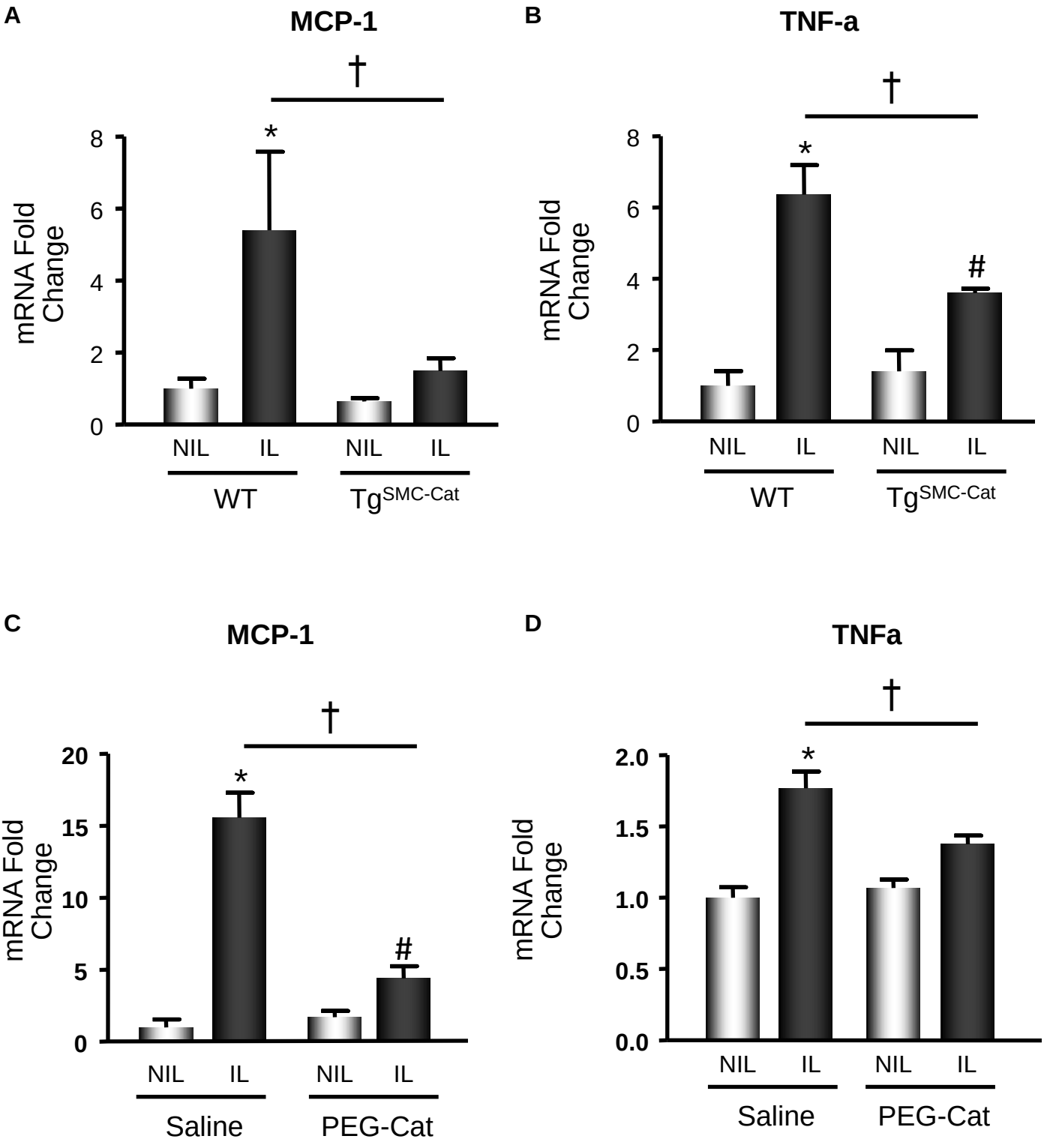
Supplemental Figure I. Expression of Inflammatory Markers in the Proximal Region of the Ischemic Limb is H_2O_2-dependent. mRNA levels of the inflammatory markers MCP-1 and TNF-α were measured in the adductor muscles of the non-ischemic (NIL) and ischemic limbs (IL) at 5 days after femoral artery ligation in $Tg^{SMC\text{-}Cat}$ mice or wild-type littermate controls (WT) and in C57Bl/6 mice infused with Saline or 10,000 U/kg/day PEG-Catalase (PEG-Cat). **A.** Ischemia significantly increased MCP-1 in the IL of WT animals ($p<0.05$ vs. WT NIL), which was blunted in the IL $Tg^{SMC\text{-}Cat}$ mice ($p<0.02$ vs. WT IL). **B.** TNF-α increased in the IL of WT animals ($p<0.01$ vs. WT NIL), which was blunted in the IL $Tg^{SMC\text{-}Cat}$ mice ($p<0.0001$ vs. WT IL). **C.** MCP-1 expression was increased in the IL of Saline control animals ($p<0.001$ vs. Saline NIL), which was blunted in the IL of PEG-Cat infused ($p<0.0001$ vs. Saline IL). **D.** TNF-α increased in the IL of WT animals ($p<0.01$ vs. WT NIL), which was blunted in the IL $Tg^{SMC\text{-}Cat}$ mice ($p<0.0001$ vs. WT IL). Data are expressed as fold change compared with WT NIL or Saline NIL. Bars are means ± S.E.M., n=5-6 per genotype/treatment.

Supplemental Figure II. H_2O_2-dependent OPN Mediates Collateral Formation. To determine the functional importance of PEG-Catalase mediated decreases in H_2O_2 and OPN on collateral formation, we used LASER Doppler Perfusion Imaging (LDPI) to evaluate reperfusion and Micro-CT to quantify collateral formation in the IL. Perfusion and collateral formation was assessed in the proximal regions of the IL and were normalized to the NIL. All measurements were performed at 5d post-surgery. IL were compared between treatment groups. **A.** Representative LDPI tracings from Saline and PEG-Catalase infused animals. **B.** Quantitative analysis of Saline and PEG-Catalase LDPI tracings (*$p<0.01$, n=5). **C.** Representative Micro-CT angiographs from Saline and PEG-Catalase mice.

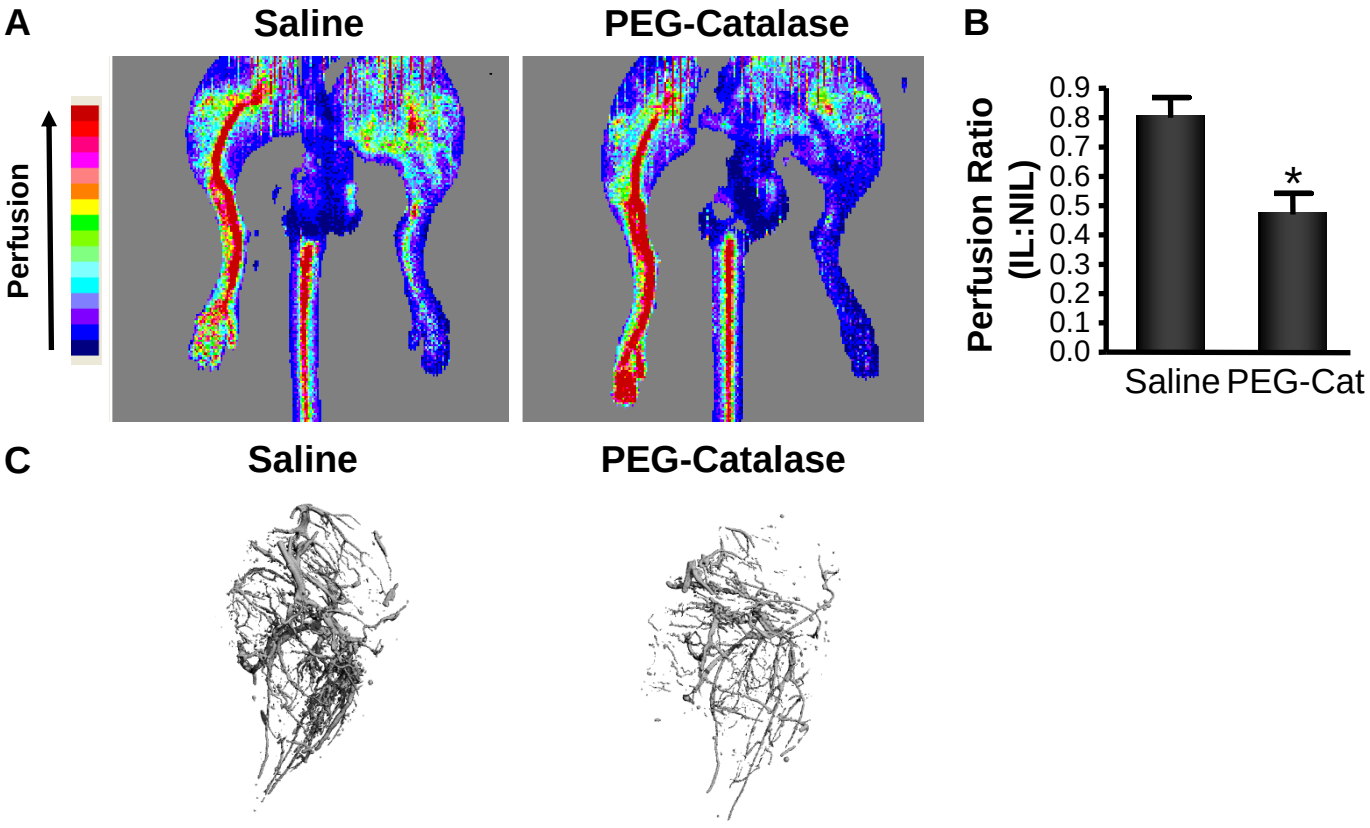
Supplemental Figure III. Lentivirus Delivery of Myc-tagged OPN or GFP in the Ischemic Leg. For *in vivo* use, both the LV-GFP and LV-OPN lentiviruses were diluted to a final

concentration of ~$1x10^9$ infectious particles per mL. All animals received HLI. After the artery and vein were tied off and excised, the adductor muscle was then injected with 20 µL of either LV-GFP or LV-OPN lentivirus. To verify that cells within the muscle were successfully transduced by lentivirus, we performed immunofluorescence staining. **A.** WT animals transduced with LV-GFP were positive for GFP (red) and negative for Myc-tagged OPN (green). **B.** We also show that $Tg^{SMC\text{-}Cat}$ adductor muscles were successfully transduced by LV-OPN lentivirus, as shown by Myc staining for Myc-tagged OPN (green) in LV-OPN animals, whereas $Tg^{SMC\text{-}Cat}$+LV-GFP show no Myc expression. Because TgSMC-Cat animals express GFP in all tissues, due to the nature of the transgene, these sections were only stained with Myc to detect successful expression of Myc-OPN and were not co-stained for GFP.

Supplemental Figure I

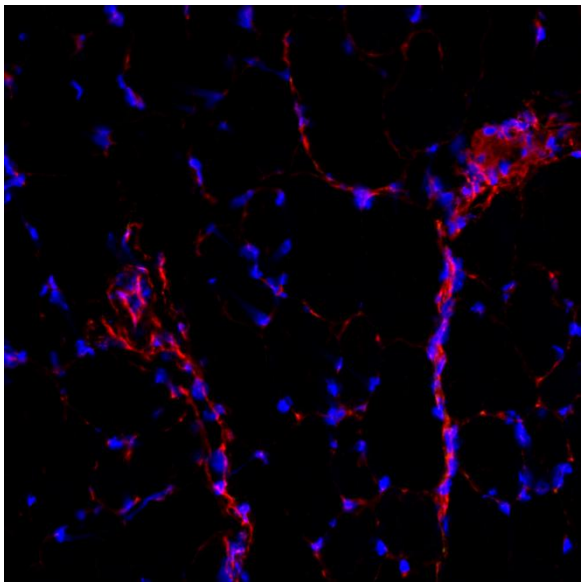


Supplemental Figure II

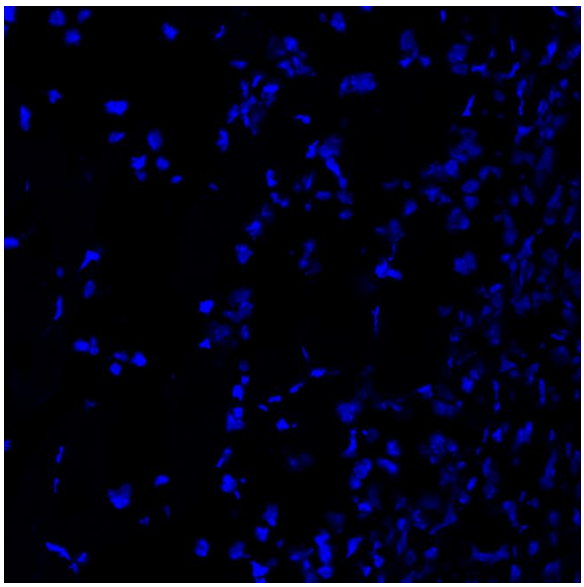


Supplemental Figure III

A WT + LV-GFP



B $Tg^{SMC\text{-}Cat}$ + LV-GFP



$Tg^{SMC\text{-}Cat}$ + LV-OPN