SUPPLEMENTAL MATERIAL:

Plasminogen activator inhibitor-1 can regulate myoendothelial junction formation

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SUPPLEMENTAL METHODS:

Mice: All wildtype C57Bl/6 mice or PAI-$1^{-/-}$ mice (Jackson) were males between 8-10 weeks of age and used according to the University of Virginia Animal Care and Use Committee guidelines. Mice used for high fat comparison were C57Bl/6 mice fed a caloric-rich diet (5.45 kcal/g, 0.2% cholesterol, 35.5% fat; Bio-Serv). Mice were euthanized with an intraperitoneal injection of 60-90 mg/kg pentobarbital.

Vascular cell-co-culture: A VCCC composed of EC and VSMC was assembled as originally described[1]. For these experiments, VSMC and EC were derived from human umbilical vein (both Cell Applications, Inc, San Diego). Endothelial cells were grown in M199 (Gibco) supplemented with 10% FBS (Gibco), 1% glutamine (Gibco), 1% penicillin/streptomycin (Gibco), and endothelial cell growth supplement (5 ug/mL, BD Biosciences); VSMC were grown in M199 supplemented with 10% FBS, 1% penicillin/streptomycin, 1% glutamine. Endothelial cells and VSMC were cultured on opposite sides of 24 mm diameter polyester Transwell inserts (0.4 μm pore width) at a seeding density of $7.5x10^4$ VSMC and $3.6x10^5$ EC for 6 days. All of these conditions induced MEJ formation similar to that seen in the resistance vasculature. So as to limit MEJ formation (e.g, as seen in aorta/carotid) using the VCCC, we coated Transwells with type I collagen. Additional cell lines were derived from human coronary artery (Lonza, Walkersville). Endothelial cells were grown in EBM-2 MV (Lonza) supplemented with Lonza bullet kits as per manufacturers instructions (Lonza), VSMC were grown in SmBM (Lonza) supplemented with Lonza bullet kits as per manufacturers instructions (Lonza). Seeding densities of $7.5x10^4$ VSMC and $3.6x10^5$ EC were used.

Recombinant PAI-1 (rPAI-1; 0.1 μg/mL) and PAI-1 mAbs (10 μg/mL) were added every 24 hours to the EC monolayer after the initial 24 hour EC incubation for a total of 48 hours. Biotin-conjugated rPAI-1 was added to the EC monolayer 30 minutes prior to isolation. For Ca^{2+} imaging experiments, cells were cultured on 12 mm diameter polyester Transwell inserts (0.4 μm pore width) at a seeding density of $1.9x10^4$ VSMC and $9.0x10^4$ EC, cells were treated with mAb to PAI-1 or rPAI-1 as described above.

Isolation of MEJ fractions: Following 6 days in culture, the VSMC monolayer was scraped with PBS into lysis buffer (PBS with 100 mmoles/L NaF, 0.5% NP-40, and 1% protease inhibitor cocktail (Sigma)) and repeated separately for the EC monolayer. The MEJ fractions were collected by removing the denuded Transwell membranes from the plastic insert, placing the membrane in lysis buffer, and vortexing for 5 minutes. All fractions were sonicated for 10 seconds, spun at 2500 rpm for 5 minutes, and the supernatant collected. All steps were performed at 4^oC.

Immunoblots. Protein fractions in lysis buffer were mixed with 5X lamelli buffer and run on a 10% SDS-PAGE Gels, transferred to nitrocellulose and imaged on a Li-Cor Odyssey Imager[25]. Silver stains and GelCode Blue stains (both Thermo Scientific) were used as per manufacturer's instructions.

Antibodies and Protein: Phalloidin conjugated to Alexa 488 or Alexa 594, donkey anti-rabbit or donkey anti-mouse Alexa 488 or Alexa 594 were all obtained from Invitrogen. Goat anti-rabbit or anti-mouse IRDye 680 or 800CW was used for immunoblots (Li-cor Biosciences). Primary antibodies used were: SMα-actin (monoclonal, Sigma); VE-cadherin (polyclonal, Santa Cruz Biotechnologies), GAPDH (monoclonal, Zymed), PAI-1 polyclonal (Abcam), PAI-1 monoclonal (used for inhibition of PAI-1 activity, Technoclone), PAI-1 monoclonal (used for immunoblot analysis, BD Biosciences), Cx37 and Cx43 (polyclonal, ADI), Cx43 (polyclonal, Sigma) Cx45 (polyclonal[26]), uPA and tPA (polyclonal, Santa Cruz Biotechnologies). Anti-rabbit 10 nm gold beads were obtained from Jackson Labs. Recombinant PAI-1 (rPAI-1) was obtained from Technoclone and biotin-conjugated rPAI-1 was obtained from Cell Sciences.

2D-DIGE Analysis: 2D-DIGE was performed by Applied Biomics (Hayward, California). In brief, pellets containing proteins from MEJ, VSMC and EC fractions were prepared and washed with washing buffer (10 mmoles/L Tris-HCl, 5 mmoles/L magnesium acetate, pH8.0). Cell pellets were resuspended in 200 μl 2-D cell lysis buffer (30 mmoles/L Tris-HCl, pH 8.8, containing 7 moles/L urea, 2 moles/L thiourea and 4% CHAPS). Cells were sonicated at 4^oC, and incubated with shaking for 30 minutes at

room temperature. Samples were spun for 30 min at 14,000 rpm and the supernatant collected. Protein samples were loaded at 3-8 mg/mL per sample.

Thirty µg of each cell lysate sample were labeled with 1.0 µl of a diluted CyDye, Cy2, Cy3 or Cy5 (1:5 diluted with DMF from 1 nmoles/µl stock), vortexed and incubated on ice for 30 minutes. One µl of 10 mmole/L lysine was added to each sample, vortexed and incubated on ice for 15 minutes. Samples were labeled with Cy2, Cy3 and Cy5 and mixed with 2X 2-D sample buffer (8 moles/L urea, 4% CHAPS, 20 mg/ml DTT, 2% pharmalytes), 100 µl destreak solution and rehydration buffer (7 moles/L urea, 2 moles/L thiourea, 4% CHAPS, 20 mg/ml DTT, 1% pharmalytes) for a total volume of 250 µl for the 13 cm IPG strip. Samples were mixed and spun before loading.

Samples were run using the protocol provided (Amersham BioSciences) at 20^{o}C. Following isoelectric focusing, IPG strips were incubated in fresh equilibration buffer 1 (50 mmoles/L Tris-HCl, pH 8.8, containing 6 moles/L urea, 30% glycerol, 2% SDS and 10 mg/ml DTT) for 15 minutes with slow shaking. The strips were rinsed in fresh equilibration buffer 2 (50 mmoles/L Tris-HCl, pH 8.8, containing 6 moles/L urea, 30% glycerol, 2% SDS and 45 mg/ml DTT) for 10 minutes with slow shaking. The IPG strips were rinsed once in SDS-gel running buffer and transferred into gradient SDS-Gel (9-12% SDS-gel prepared using low florescent glass plates) and sealed with 0.5% (w/v) agarose solution (in SDS-gel running buffer). The SDS-gels were run at 15^{o}C.

Immediately following the SDS-PAGE, image scans were made using Typhoon TRIO (Amersham BioSciences). The scanned images were analyzed by Image QuantTL software (GE-Healthcare), and subjected to in-gel analysis and cross-gel analysis using DeCyder software version 6.5 (GE-Healthcare). The ratio change of the protein differential expression was obtained from in-gel DeCyder software analysis.

Mass Spectrometry: Mass spectrometry was performed by Applied Biomics (Hayward, CA). Proteins of interest were digested in-gel with modified porcine trypsin protease (Trypsin Gold, Promega). The digested tryptic peptides were desalted by Zip-tip C_{18} (Millipore). Peptides were eluted from the Zip-tip with 0.5 µl of matrix solution (cyano-4-hydroxycinnamic acid (5 mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid, 25 mmoles/L ammonium bicarbonate)) and spotted on the MALDI plate (model ABI 01-192-6-AB).

The MALDI-TOF MS and TOF/TOF tandem MS/MS were performed on an ABI 4700 mass spectrometer (Applied Biosystems, Framingham, MA). MALDI-TOF mass spectra were acquired in reflectron positive ion mode, averaging 4000 laser shots per spectrum. TOF/TOF tandem MS fragmentation spectra were acquired for each protein, averaging 4000 laser shots per fragmentation spectrum on each of the 10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions).

Both the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine (Matrix science) to search the National Center for Biotechnology Information non-redundant (NCBInr) database. Searches were performed without constraining protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues, and with one missed cleavage allowed in the search parameters. Candidates with either protein score confidence interval percent (C.I.%) or Ion C.I.% greater than 95 were considered significant.

Immunostaining: Immunohistochemistry on the VCCC was performed as previously described on the VCCC[1].

Quantification of MEJs using the VCCC: The number of F-actin filled pores per micrometer was quantified using Metamorph (Universal Imaging Corps, version 7.5.6.0). Phalloidin staining was used to visualize F-actin from EC and VSMC extensions (i.e., in vitro MEJs) within the pores of the Transwell, viewed transverse to the monolayers.

Immunolabeling on TEM Sections: Visualization of proteins on TEM sections was performed as described[2]. Quantification for the number of electron-dense gold-beads was initially performed by immunolabeling for PAI-1 (as described above) with a minimum of 5 TEM images per coronary arteriole, 10 µm apart, from a minimum of three mice. The area of the EC monolayer, MEJ, or VSMC monolayer

was quantified using Metamorph software calibrated for measuring area (μm^2). EC and VSMC monolayers were traced from apical to basal lateral membrane. To quantify MEJ area, cellular extensions that penetrated the IEL were defined by a straight line was drawn across the basal lateral membrane from which the MEJ originated, dissecting the cellular extension from the monolayer above, tracing the entire length of the cellular extension through the IEL to the base of the adjacent monolayer's basal lateral membrane and the area inside these lines was defined as the area of the MEJ. The number of gold beads in each set area (EC, MEJ or VSMC) was counted, being careful not to include electron-dense ribosomes. Measurements represent the average number of beads per micron squared ± SE.

Quantification of PAI-1 on actin bridges in vivo: Quantification of PAI-1 on actin bridges that form between EC and VSMC in vivo was performed as previously described[2].

Ultrastructure Electron Microscopy: Coronary arterioles (diameter 50-100 μm) obtained from each mouse were fixed in 4% paraformaldehyde and 2% gluteraldahyde at 4ºC and ultrastructural TEM images were obtained as described in[3]. We quantified the total number of MEJs within a vessel using a minimum of 5 TEM images per coronary arteriole, using standard TEM protocol as described above. To quantify the radial length of a vessel, a single line was traced along the EC basal lateral membrane with this distance measured using calibrated Metamorph software. Cellular extensions that penetrated both the traced EC basal lateral membrane and IEL and came within <250 nm of membranous contact between EC and VSMC was counted as a single MEJ. The numbers given represent the average number of MEJs per 10 μm radial length ± SE. A minimum of three mice, with a minimum radial diameter of 150 mm total per mouse, and 10 μm longitudely between each TEM section, were used.

Heart Transplants: Heart transplants were performed as described[4]. In brief, the donor heart was removed by excision of the ascending aorta below the brachiocephalic artery and pulmonary artery proximal to its bifurcation. The heart was transplanted into the recipient mouse through an abdominal midline incision and the donor ascending aorta was anastomosed to the recipient abdominal aorta. The donor pulmonary artery was anastomosed to the recipient inferior vena cava. Once adequate haemostasis was achieved, the donor heart was allowed to fill with blood and the wound closed. Mice were injected with 0.1 mL Buprenix + NaCl for a total of four times 48 hours post-surgery. Five days post-surgery, the donor hearts were harvested for TEM analysis.

Recombinant PAI-1 Tail Vein Injections: Fifty microliter (μL) of rPAI-1 (1000 ng/μg) was injected into PAI-$1^{-/-}$ via lateral tail vein every 12 hours for five days. Tissue was harvested for TEM analysis.

Calcium Imaging: Fura-2 AM (16.6 μmoles/L in 0.1% DMSO mixed in MOPS-buffered saline) was loaded into EC and VSMC monolayers as previously described for Fluo-4 AM[5]. A converted Olympus IX-71 with a Bioptek Transwell chamber and inflow and outflow pipettes was used to perfuse 37ºC MOPS-buffered saline over the EC and VSMC monolayers of the VCCC (see Supplementary Fig I). Ultra-rapid rapid image acquisition at 340 nm and 380 nm utilized a water-cooled Hamamatsu EM-CCD coupled to Slidebook imaging software running on a Dell Precision T5400 with dual 3 GHz processors and 8 GB of RAM. The VSMC were stimulated with 10 μmoles/L phenylephrine (PE) and the EC intracellular calcium concentrations $[Ca^{2+}]_i$ were recorded (as previously described[5]).

SUPPLEMENTAL FIGURES

Supplementary Figure I: Gross anatomical of heterotypic heart transplant. " a" indicates anterior and "p" indicates posterior anatomical alignment, bold arrow indicates donor heart

Supplementary Figure II: Schematic of setup for measuring heterocellular calcium communication in the VCCC. Buffer flow rate of 1 mL/min was applied to the EC monolayer (top), 0.5 mL/min was applied to the VSMC monolayer (bottom) and 10 μmole/L of PE was applied to the VSMC monolayer. All components are maintained at 37^{o}C.

Supplementary Figure III: Incidence of Connexins in VSMC, MEJ and EC fractions from the vascular cell co-culture. Immunoblots for Cx37, Cx40 and Cx43 in isolated VSMC, MEJ and EC fractions. In all images, black arrows represents 37 kDa.

Supplementary Figure IV: 2D-DIGE analysis of isolated MEJ protein fractions from coronary vascular cell co-culture. 2D-DIGE blots for isolated coronary VSMC, MEJ and EC fractions, comparing VSMC (red) to EC (green) (A, top), SMC (green) to MEJ (red) (A, middle) and EC (green) to MEJ (red) (A, bottom). Arrows labeled 1-3 represent three spots with increased protein expression in the MEJ fraction (greater than 2.5-fold). Using Quantitative DeCyder analysis, representative protein fluorescent intensity peaks for spots 1-3 in VSMC, MEJ and EC fractions are indicated by a magenta tracer (B). Mass spectrum of spots 1 (C, top), 2 (C, middle) and 3 (C, bottom) identify each spot as plasminogen activator inhibitor-1.

Supplementary Figure V: Incidence of tPA and uPA in VSMC, MEJ and EC fractions from vascular cell co-culture. Quantification of immunoblots for tPA (A), active uPA (B) and inactive uPA (C) in VSMC, MEJ and EC fractions isolated from the VCCC. In all images values are expressed as protein/GAPDH, * $p<0.05$.

Supplementary Figure VI: Localization of PAI-1 to cremasteric and mesenteric MEJs. Transmission electron microscopy images of mouse arterioles labeled for PAI-1 using 10 nm gold particles. Conditions include coronary arterioles treated with normal rabbit serum (A), cremaster arterioles (B) and mesenteric arterioles (C). Bar in A is 0.5μm; bar in B is 0.5μm and is representative for B and C. In all images VSMC and EC monolayers are labeled and IEL is indicated (*).

Supplementary Figure VII: Analysis of PAI-1 localization and effects on MEJ formation in coronary and umbilical vein vascular cell co-cultures. Quantification of immunoblots for PAI-1 in human microvascular coronary VSMC, MEJ and EC fractions (A), protein values are expressed as PAI-1/GAPDH. Representative transverse images of Transwells stained with phalloidin for control (top) and following inhibition of PAI-1 by application of 10 μg/mL PAI-1 mAb to the EC monolayer (- PAI-1, middle) and increased PAI-1 by application of 0.1 μg/mL rPAI-1 to the EC monolayer (+ PAI-1, bottom) are shown for umbilical vein VCCCs in B (quantified in Figure 4). The changes in MEJ numbers in experiments with umbilical vein cells were identical to experiments performed on recapitulated VCCC using human microvascular coronary EC and VSMC (C). Representative transverse images of Transwells plated with human microvascular coronary EC and VSMC and stained with phalloidin are shown in D. For all images, only the EC of the VCCC were treated for the final 48 hours of culture in 24 hr increments. Bars in B and C are 10μm and representative for all accompanying images.

Supplementary Figure VIII: Ultrastructure analysis of mesenteric and cremasteric vessels in C57Bl/6 and PAI-1$^{-/-}$ mice. Transmission electron microscopy images of mouse mesenteric arterioles isolated from C57Bl/6 mice (A) and PAI-1$^{-/-}$ mice (B). In (C) TEM images of mouse cremaster arterioles isolated from C57Bl/6 mice are shown and in (D), TEM images from

PAI-$1^{-/-}$ mouse cremaster arterioles are shown. Bar in A is 2 μm and is representative for all images. In all images (*) indicates IEL, "L" indicates vessel lumen and is oriented above the EC monolayer.

Supplementary Figure IX: Body weight, blood glucose levels and PAI-1 expression in high fat C57Bl/6 mice compared to normal C57Bl/6 mice. Body weight (grams) and blood glucose levels (mg/dL) measurements from normal C57Bl/6 (A, top, n=2) and C57Bl/6 + high fat (A, bottom). Quantification of total PAI-1expression from C57Bl/6 mice and C57Bl/6 + high fat mice (B), image values are expressed as protein expression/GAPDH, *$p<0.05$

Supplementary Figure X: PAI-1 expression in saline and rPAI-1 injected PAI-$1^{-/-}$ mice. Quantification of total PAI-1 expression from PAI-$1^{-/-}$ mice injected with saline (PAI-$1^{-/-}$ + saline, n=2) and rPAI-1 (PAI-$1^{-/-}$ + rPAI-1). Image values are expressed as average protein expression/GAPDH, *$p<0.05$.

Supplementary Figure XI: In vitro MEJ formation using the vascular cell co-culture with collagen or fibronectin coated Transwells. Immunocytochemistry using phalloidin staining of F-actin (green) on transverse sections of fibronectin-coated VCCCs (A) or transverse sections of collagen-coated VCCCs (B). Arrows in A indicate actin bridges (i.e. in vitro MEJs); bar in A is 10 μm and representative for both images.

Supplementary Figure XII: Connexin expression at the MEJ in vitro, following treatments with rPAI-1 or mAb to PAI-1. Immunocytochemistry on transverse sections of the VCCC labeled for Cx37 (A), Cx40 (B), Cx43 (C) and Cx45 (D); conditions include application of 0.1 μg rPAI-1 (left), control (center) and application of 10 μg mAb to PAI-1 (right). Bar in A is 10 μm and is representative for all images.

Reference List

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2. Isakson BE, Best AK, Duling BR. Incidence of protein on actin bridges between endothelium and smooth muscle in arterioles demonstrates heterogeneous connexin expression and phosphorylation. *Am J Physiol Heart Circ Physiol.* 2008;294:H2898-H2904.

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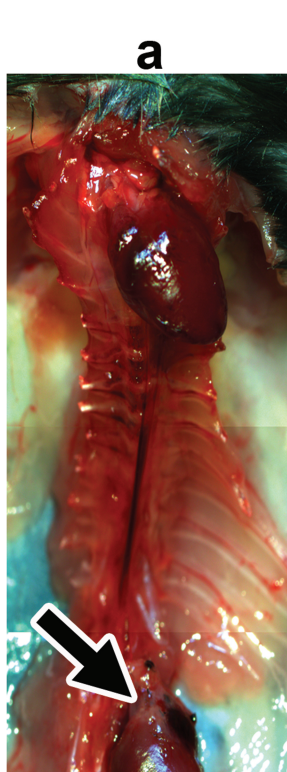
4. Niimi M. The technique for heterotopic cardiac transplantation in mice: experience of 3000 operations by one surgeon. *J Heart Lung Transplant.* 2001;20:1123-1128.

5. Isakson BE, Ramos SI, Duling BR. Ca2+ and inositol 1,4,5-trisphosphate-mediated signaling across the myoendothelial junction. *Circ Res.* 2007;100:246-254.

Supp Fig I



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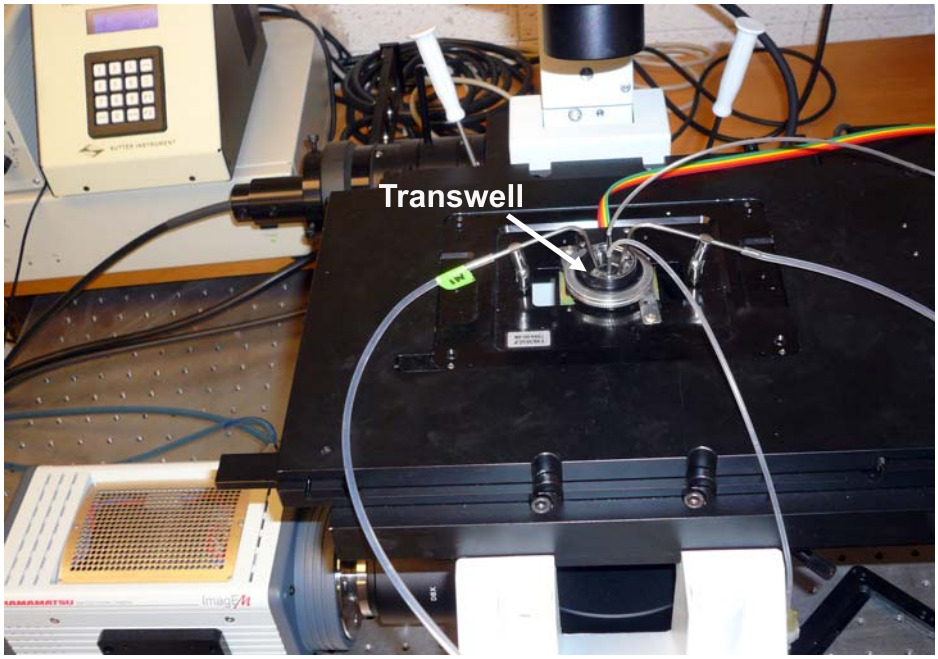

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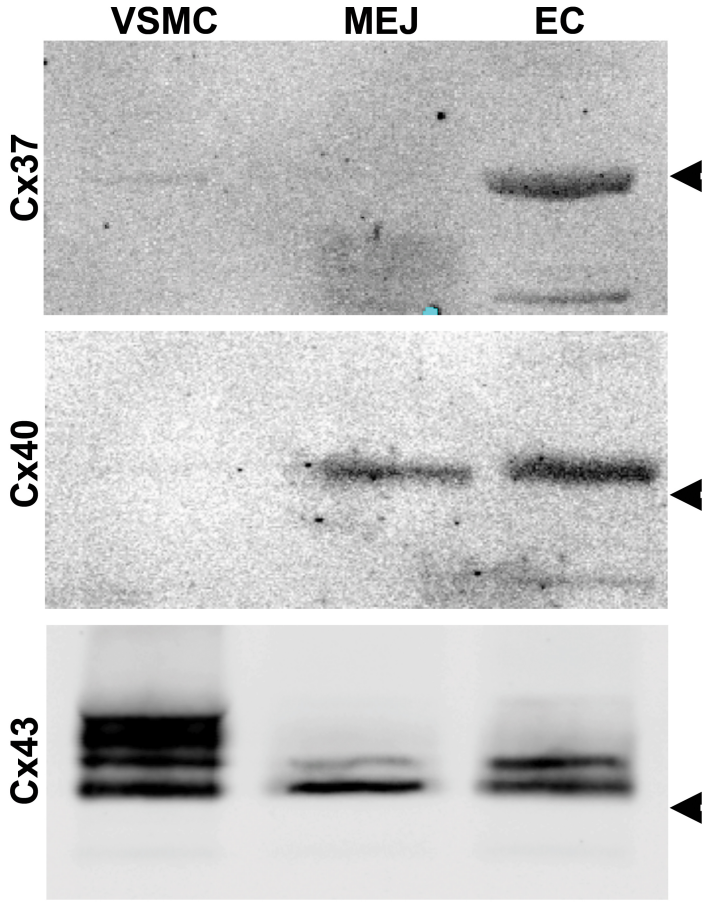

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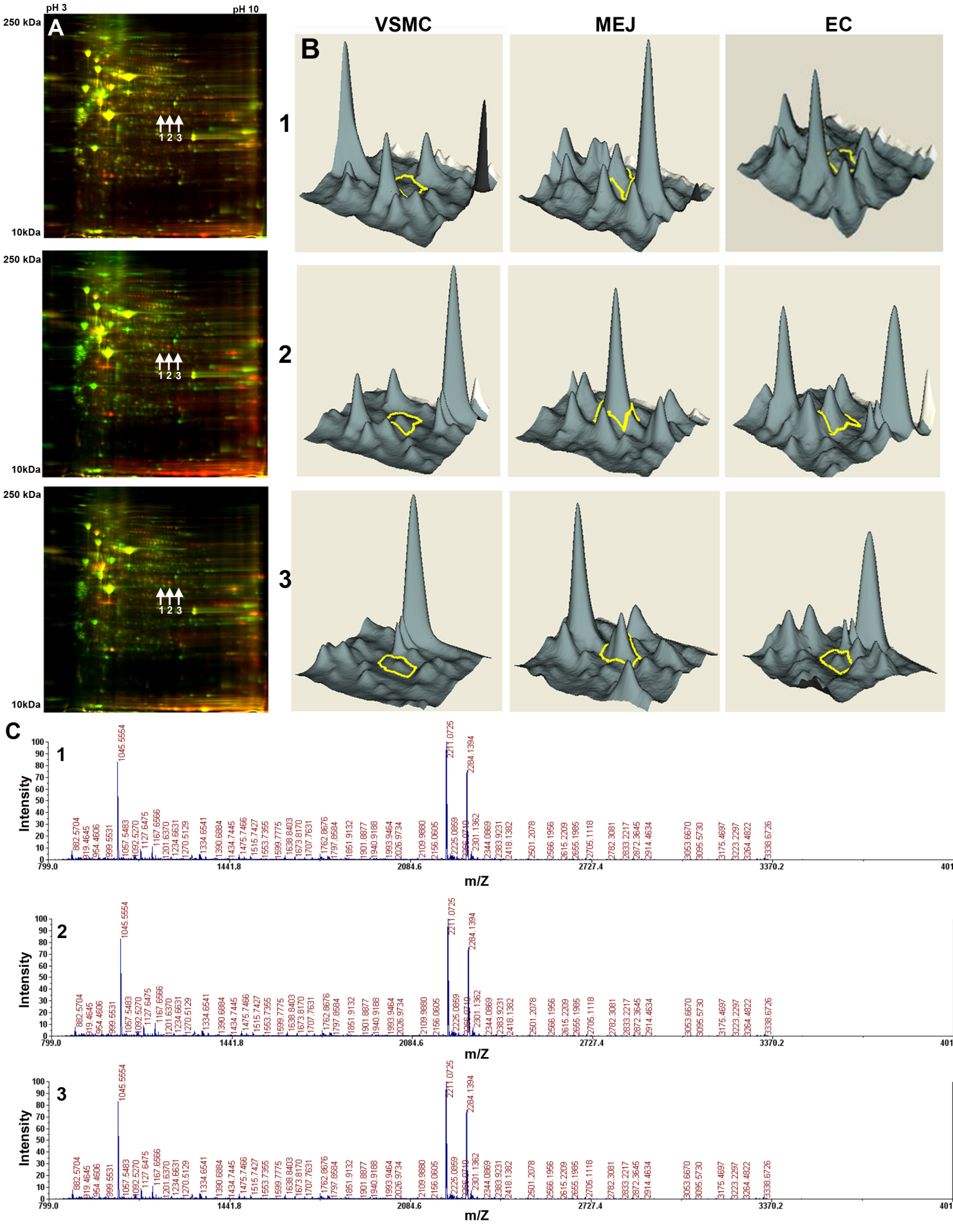
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10kDa
1 2 3
B
VSMC
MEJ
EC
1
2
3
C
Intensity
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1441.8
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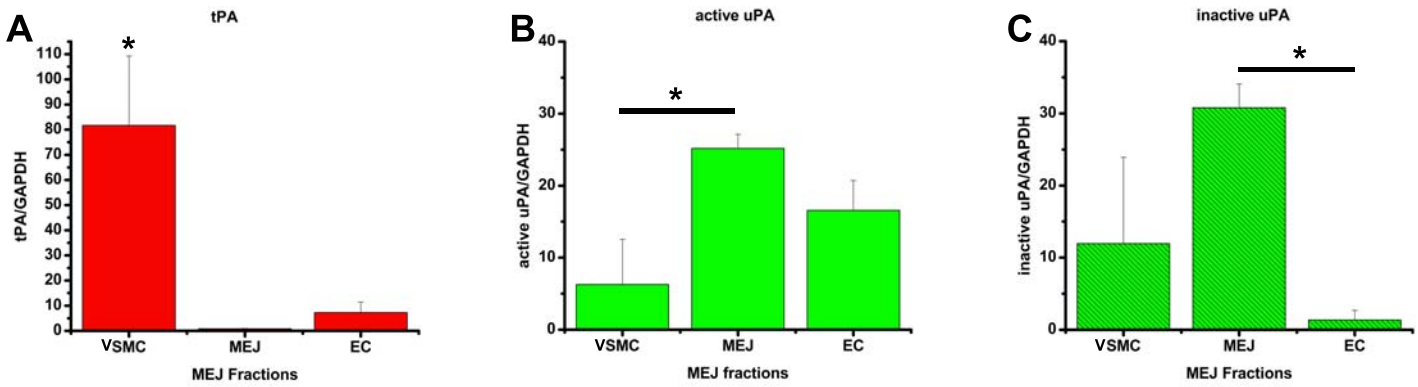
2284.1394

Supp Fig V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MEJ
EC
MEJ Fractions
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active uPA/GAPDH
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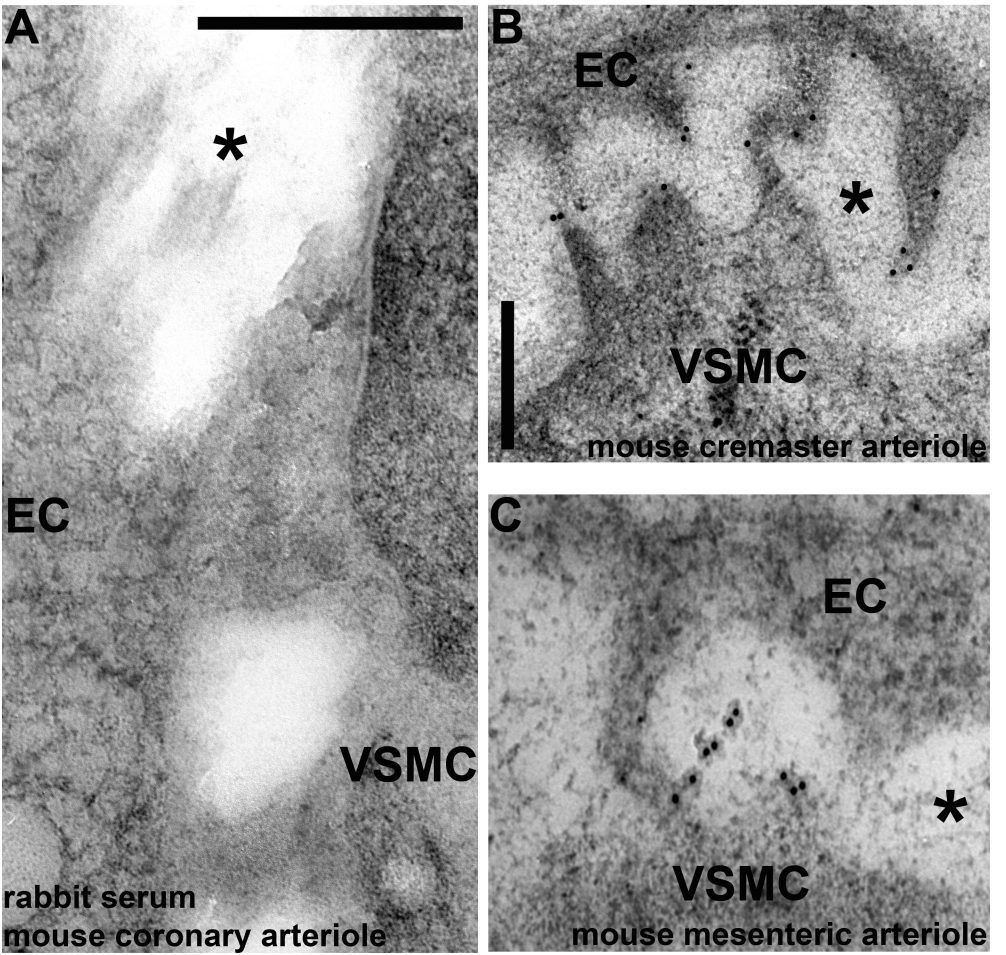
inactive uPA/GAPDH
VSMC
MEJ
EC
MEJ Fractions

Supp Fig VI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*
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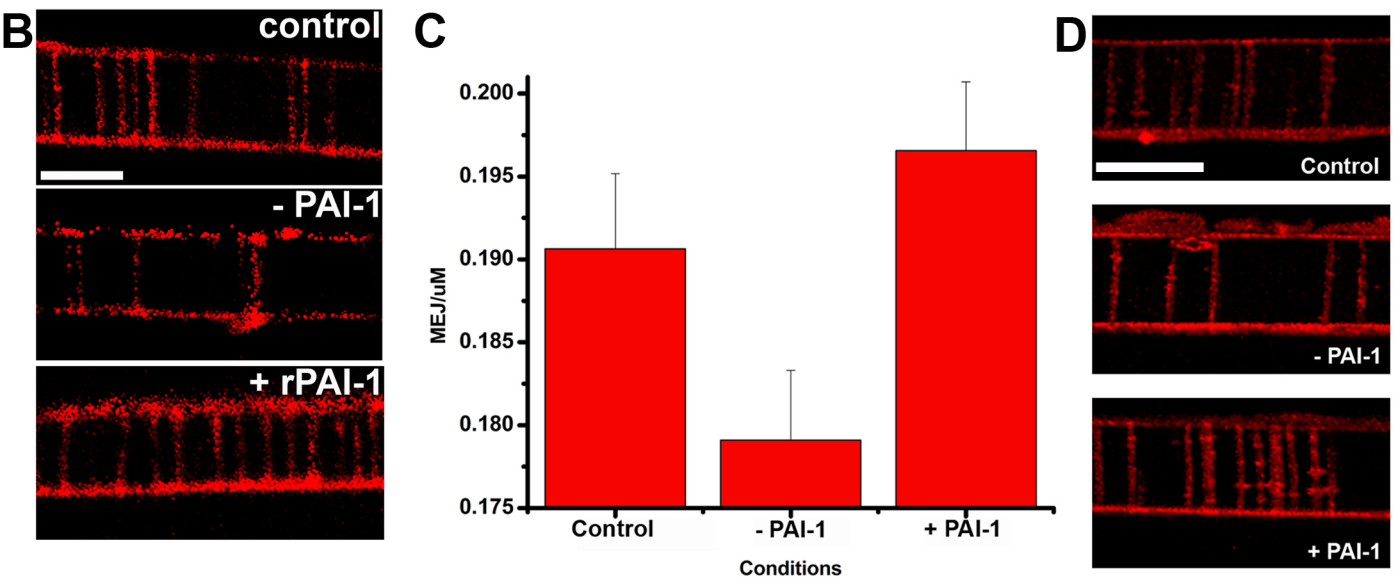
EC
*
VSMC
mouse cremaster arteriole
C
EC
*
VSMC
mouse mesenteric arteriole

Supp Fig VII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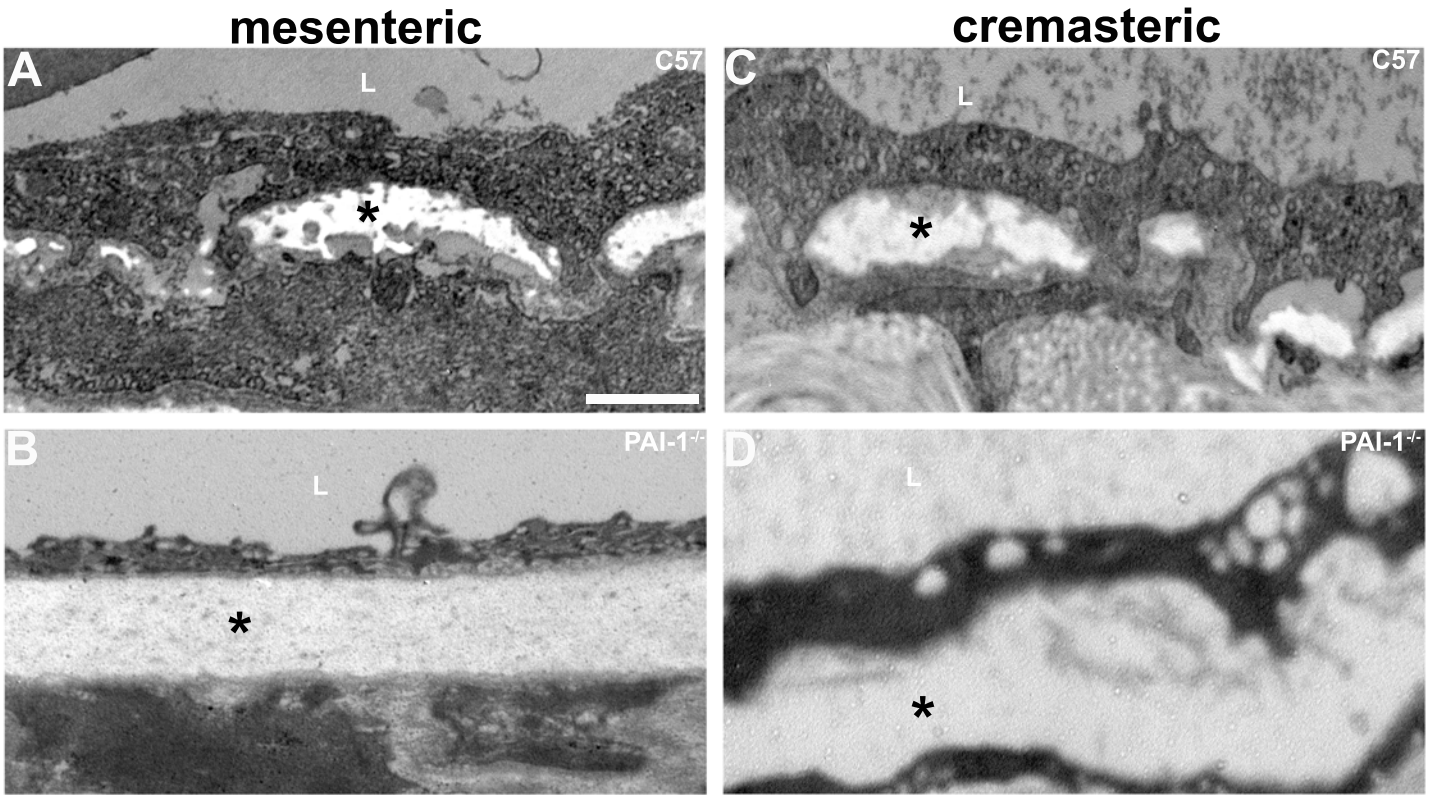
0.05
0.00
PAI-1/GAPDH
VSMC
MEJ
EC
Cell Lysates
PAI-1
GAPDH
B
control
- PAI-1
+ rPAI-1
C
0.200
0.195
0.190
0.185
0.180
0.175
MEJ/uM
Control
- PAI-1
+ PAI-1
Conditions
D
Control
- PAI-1
+ PAI-1

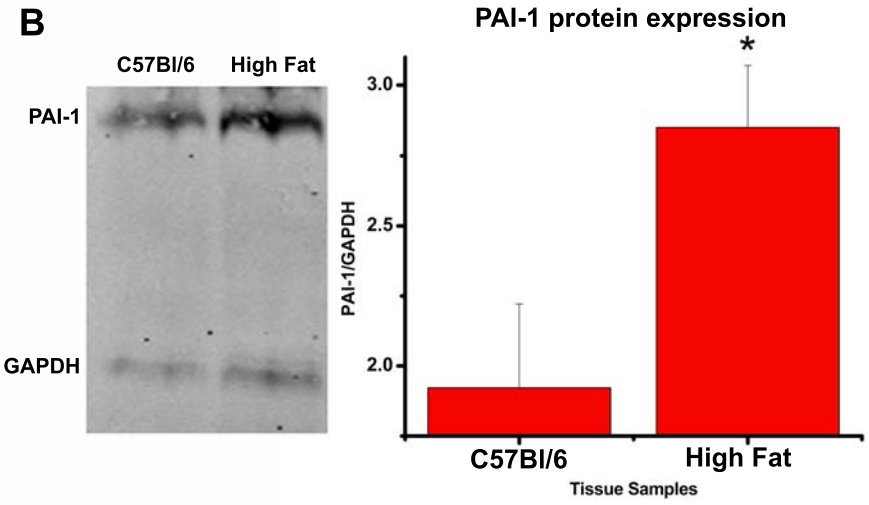
Supp Fig VIII



Supp Fig IX

A	n	Weight (g)	Blood glucose (mg/dL)
C57Bl/6:	3	29.1 +/- 1.2	132.8 +/- 12.5
C57Bl/6+High Fat:	4	45.9 +/- 2.3*	178.8 +/- 9.8*

B



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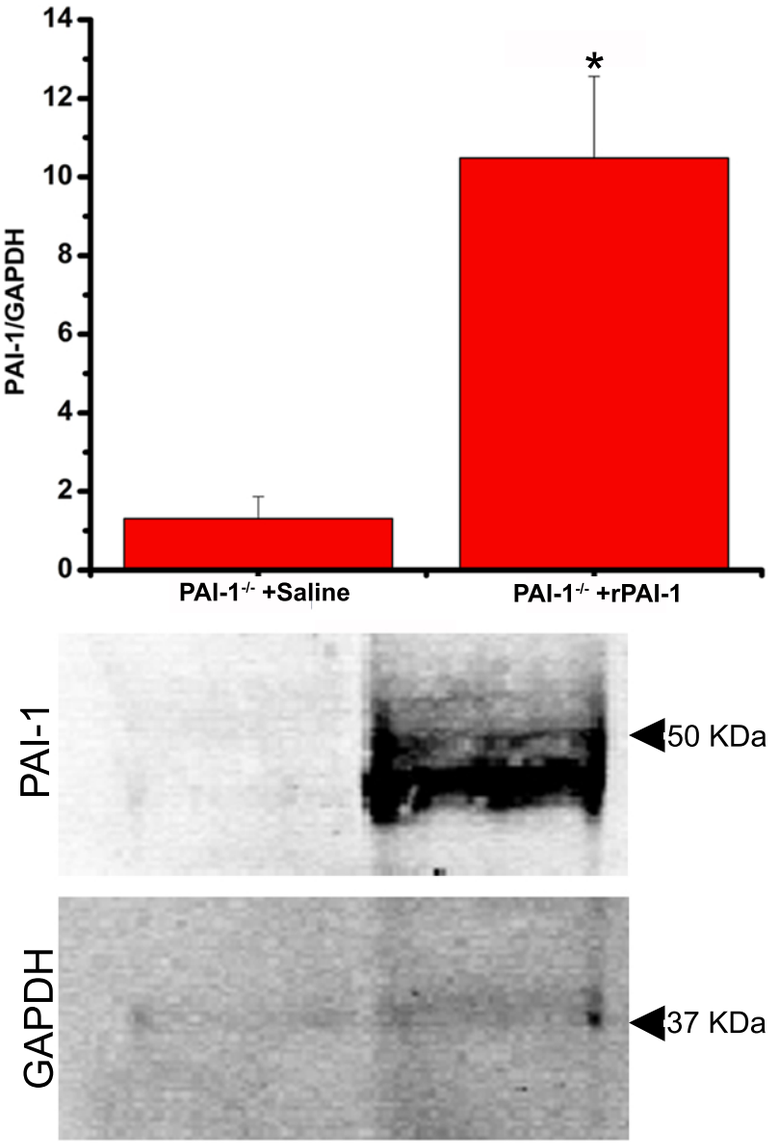
14
12
10
8
6
4
2
0
PAI-1/GAPDH
*
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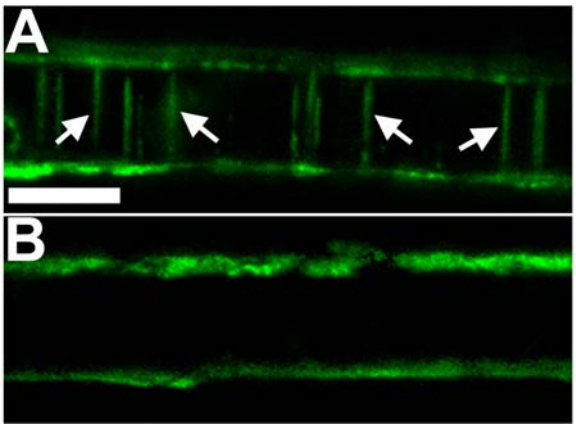
PAI-1-/- +rPAI-1
PAI-1
50 KDa
GAPDH
37 KDa

Supp Fig XI



Supp Fig XII