

Supplemental Material

Materials and Methods

Human tissue

Atherosclerotic carotid artery samples were obtained from endarterectomy patients (Brigham and Women's Hospital Institutional Review Board protocol #1999P001348), embedded in optimum cutting temperature compound and stored at -80°C until use. Autopsy carotid artery samples were obtained from Brigham and Women's Hospital (Institutional Review Board protocol #2013P002517/BWH), cut and then stored at -80°C until use. Human aortic valve tissue was obtained from patients undergoing valve replacement (Institutional Review Board protocol #2011P001703), embedded and stored at -80°C until use.

Primary cell culture

Primary human coronary artery and aortic smooth muscle cells were obtained from Promocell (Heidelberg, Germany) and expanded in SMC Growth Medium 2 (Promocell) supplemented with epidermal growth factor (0.5 ng/mL), insulin (5 µg/mL) basic fibroblast growth factor- B (2ng/mL), and 5% fetal bovine serum. Cells were cultured at 37°C (5% CO₂, 90% humidity) and used between passages 3 and 10. Cells from at least three human donors were used. Primary C57Bl/6 mouse aorta SMCs (CellBiologics, Chicago, IL) were cultured using the same methods and media as primary human SMCs. Both human (PromoCell) and mouse (CellBiologics) primary SMCs were isolated via collagenase tissue digestion. SMC identity was confirmed by αSMA FACS analysis using a BDFACS Aria™ IIu flow cytometer (BD Biosciences, Franklin Lakes, NJ) and anti-actin, α-smooth muscle-cy3 antibody (#C6198, Sigma). Primary human VICs were obtained from isolated human aortic valve tissue in a similar manner as reported¹. Primary human osteoblasts isolated from femoral bone tissue were obtained from Promocell, cultured in a similar manner to SMCs, and used between passages 2 and 4.

PCSK9 gain-of-function AAV

PCSK9 gain-of-function AAV injections were performed as previously described^{2,3}. Briefly 10-week-old male mice were given a single tail vein injection of recombinant murine PCSK9 AAV (pAAV/D377Y-mPCSK9; 1X10¹¹ vector genome copies/injection diluted in 200µL sterile saline solution). AAV was produced at the University of North Carolina Vector Core (Chapel Hill, NC) using the murine PCSK9 gain-of-function plasmid generated by Bjørklund et al.² and obtained from Addgene (#58376; Cambridge, MA).

Mice

C57Bl/6;129 heterozygous *Drp1*^{+/-} mice have been previously described^{4,5}. Age-matched sibling male wild type (*Drp1*^{+/+}) and heterozygous (*Drp1*^{+/-}) mice were used for experiments. Male C57Bl/6 wild type mice (#000664; Jackson Laboratory), Apolipoprotein E-deficient (*ApoE*^{-/-}) mice (#002052; Jackson Laboratory, Bar Harbor, ME), *Ldlr*-deficient mice (#002207 ; Jackson Laboratory), and PCSK9 gain-of-function AAV injected mice were fed an atherogenic high-fat, high-cholesterol diet (21% fat and 1.25% cholesterol, Research Diets D12108C, New Brunswick, NJ, USA) for 18-20 weeks. All *Drp1*^{+/+} and *Drp1*^{+/-} mice used in experiments were maintained on atherogenic diet for 20 weeks. Following 18-20 weeks on the atherogenic diet, mice were carbon dioxide euthanized. Blood was collected by cardiac puncture, and serum was isolated by spinning samples at 9,000 RPM for 15 minutes, and stored at -80°C until use. Tissues for histology were embedded in optimum cutting temperature compound and stored at -

80°C until use. All animal procedures were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee (protocol #010-2013).

Immunohistochemistry and immunofluorescence

Tissues were cut into slices with 7 μm thickness, and cryosections were fixed in acetone or 4% paraformaldehyde. Following blocking in 4% serum, sections were incubated in primary antibodies: DRP1 for human IHC [1:50, Abcam #ab56788, Cambridge, MA]; as the DRP1 antibody used for human IHC was made in mouse, a different monoclonal antibody made against mouse DRP1 in rabbit was used for mouse DRP1 IHC [1:250, Abcam #ab184247]; DRP1 for IF [1:50, Cell Signaling Technology #8570, Danvers, MA]; CD68 for IHC [1:700, Dako #M0876, Carpinteria, CA]; CD68 for IF [1:200, Dako]; HHF-35 [1:35, Enzo Life Sciences #ENZ-C34931, Farmingdale, NY]; α -SMA [1:200, Dako #M0851; for IHC used with secondary antibody #B0287 from Sigma, St. Louis, MO]; PKC δ [1:50, Santa Cruz #sc-937, Dallas, TX], MAC3 [1:900, BD Biosciences #550292, San Jose, CA]), and then incubated with biotin-labeled secondary antibody (Vector Laboratories, Burlingame, CA), followed by incubation with streptavidin-peroxidase (KPL, Gaithersburg, MD), and then incubation in AEC solution (Dako). Oxidative stress immunofluorescence analysis was performed using the Cellular Reactive Oxygen Species Deep Red Detection Assay kit according to manufacturer's protocol (Abcam). Tartrate-resistant acid phosphatase (TRAP) was stained using the TRAP staining kit (B-Bridge International, Cupertino, CA). TNAP activity was stained using the VECTOR Red Alkaline Phosphatase Substrate Kit (Vector Laboratories, Burlingame, CA). Neutral lipids were detected by Oil Red O staining. Collagen was stained using 0.1% Picrosirius Red (pH=2; Polysciences, Warrington, PA). Stains were quantified according to the methods previously described in Yabusaki et al.⁶; all values were obtained in a blinded analysis. For immunofluorescence of smooth muscle cells, cells were grown on 0.1% gelatin coated Lab-Tek II chambered cover glass #1.5 borosilicate slides (Lab-Tek, Rochester, NY), washed with PBS and then fixed in 4% paraformaldehyde for 15 minutes, followed by permeabilization using 0.3% Triton X-100 for 5 minutes, washed, and then blocked with 1% BAS in PBS for 30 minutes. Calcification immunofluorescence staining was performed by incubating cells with Osteosense-680 EX [1:100; PerkinElmer, Boston, MA] overnight at 37°C. Collagen was stained with the CNA collagen probe (provided by Carlijn Bouten) similar to how we have previously described⁷, by incubating cells at 37°C with CNA probe (1:50) for 1 hour prior to fixation. Actin was stained using CytoPainter Phalloidin iFluor 555 Reagent [2 μl phalloidin in 2000 μl PBS with 1% BSA; Abcam], with a 60 minute incubation at room temperature. Tubulin was imaged by incubating slides with anti- α -tubulin [1:200, Abcam #ab15246] for 2 hours at room temperature, followed by incubation with Alexa Fluor 488-labelled secondary antibody [1:200; Life Technologies, Carlsbad, CA]. Nuclear staining was performed with DAPI (Life Technologies). Prior to visualization 200 μL PBS was added to each well. Slides were examined using the confocal microscope A1 (Nikon Instruments Inc., Melville, NY), and all images were processed with Elements 3.20 software (Nikon Instruments Inc.).

Osteogenic differentiation

Fully confluent control smooth muscle cells (SMCs) were cultured for a total of 21 days in DMEM containing 4.5 g/L glucose and L-glutamine (Lonza, Walkersville, MD), supplemented with 10% FBS and 1% penicillin-streptomycin (termed control media, CM). To induce osteogenic differentiation of SMCs, fully confluent cells were cultured for a total of 21 days in CM with the following additions: 10 nmol/L dexamethasone, 10 mmol/L β -glycerol phosphate, and 100 $\mu\text{mol/L}$ L-ascorbic acid 2-phosphate (termed osteogenic media, OM). Oxidative stress was induced by the addition of 0.3 mmol/L H_2O_2 with each media change. For SMC DRP1 inhibition experiments, Mdivi-1 (50 or 25 $\mu\text{mol/L}$) in dimethyl sulfoxide (DMSO; final DMSO concentration of 0.01%) or DMSO vehicle control (0.01%) was added to cell culture media. Mdivi-1 was solubilized in media prior to changing cell culture media by repeated hard vortexing for several minutes and warming media to 37°C until Mdivi-1 was fully solubilized

(visualized under a light microscope and examined for the absence of insolubilized Mdivi-1 crystals). Primary human VICs were cultured with some differences in the calcification media compared to that used for SMCs, based on the VIC calcification method used by Bouchareb et al¹. Briefly, VICs were cultured for a total of 21 days in CM (DMEM containing 4.5g/L glucose and L-glutamine, supplemented with 5% FBS and 1% penicillin-streptomycin). To induce osteogenic differentiation of VICs, fully confluent cells were cultured for a total of 21 days in CM with the following additions: 2 mmol/L NaH₂PO₄ and 50 µg/mL L-ascorbic, termed osteogenic media (OM). Media was changed every three days.

RNA analysis

TriZol (Life Technologies, Grand Island, NY) was used to isolate RNA from cultured cells. QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) was used to perform reverse transcription on total RNA. cDNA was generated using the Quanta qScript cDNA Synthesis Kit (Bioscience Inc, Gaithersburg, MD). mRNA expression was quantified by TaqMan real-time PCR (Life Technologies) with the following probes: Hs02758991_g1 (human *GAPDH*); Mm99999915_g1 (mouse *Gapdh*); Hs00247147_m1 (human *DRP1*); Mm01342903_m1 (mouse *Drp1*); Hs01029144 (human *TNAP*); Mm00475834_m1 (mouse *Tnap*); Hs00164004_m1 (human *COL1A1*); Hs01001343_g1 (human *SOX9*); Mm00448840_m1 (mouse *Sox9*); Hs00751239_s1 (human *MSX2*); Hs00231692_m1 (human *RUNX2*). Expression levels were normalized to *GAPDH*. Results were quantified using the $\Delta\Delta CT$ method.

RNA interference

RNA knockdown was performed by using 20 nmol/L validated DRP1 siRNA: 4390824 (DRP1; ThermoFisher), 4390843 (negative control; ThermoFisher). Transfection was performed using Lipofectamine RNAiMAX (ThermoFisher). siRNA was added at the beginning of each experiment, and with each media change every three days until sample collection.

Protein analysis

Cells and tissues were lysed in RIPA buffer (Thermo Scientific, Rockford, IL) containing Halt combined protease and phosphatase inhibitor cocktails (Thermo Scientific). Protein content was determined by the Pierce BCA assay (Thermo Scientific). Protein lysate was loaded onto 4-15% gels (Bio-Rad, Hercules, CA) in SDS loading buffer (Boston BioProducts, Ashland, MA). Primary antibodies used for Western blot analysis included: DRP1 (1:1,000; Abcam and Cell Signaling Technology), β -actin (1:5,000, Novus, Littleton, CO), CHOP (1:1000, Cell Signaling Technology), PERK (1:1000, Cell Signaling), α -tubulin (1:1000, Abcam), acetyl-alpha-tubulin (1:1000, Cell Signaling Technology), TOMM20 (1:1000, Abcam). DRP1 translocation to the mitochondria in response to H₂O₂ treatment was assessed by cell fraction and Western blot analysis. Five hours post-treatment cells were lysed in sucrose buffer (0.25mol/L sucrose, 1mmol/L EDTA, 10 mmol/L Tris-HCL, pH 7.4, and Halt inhibitor cocktail), and spun at 1,000g to remove nuclei and cell debris. Supernatant was then transfer to a new tube and spun at 15,000g for 15 minutes. The supernatant (containing the cytosolic and microsomal fraction) was collected. The pellet (containing the mitochondrial fraction) was suspended in lysis buffer and both fractions were then loaded on 4-15% gels and analyzed by Western blot. Oxidative stress was measured by use of the Oxyblot Kit (EMD Millipore). COL1A1 ELISAs (human SMC media 1:2000 dilution, human VIC media 1:1000 dilution, R&D Systems, Minneapolis, MN), MGP ELISA (Lifespan Bioscience, Inc,), OPG (R&D Systems) were all performed according to manufacturer's instructions.

Mitochondrial aspect ratio and TMRE

Following smooth muscle cell mitochondria staining with 50 nmol/L MitoTracker Red (ThermoFisher; 15 minute incubation at 37°C) images were acquired by confocal microscopy (Nikon Instruments Inc.) using a 40X immersion objective and processed for quantification using an in-house code in Matlab (Mathworks, Natick, MA) adapted from Lihavainen et al.⁸ First, a high pass filter and a median filter were

applied to remove background and noise. The preprocessed images were then binarized with a common threshold to allow segmentation of the mitochondria and the binarized images were used to select single cells. To obtain a measure of the shape each mitochondrion was approximated to an ellipse. The major and minor axes were used to calculate the aspect ratio. Mitochondrial aspect ratios were additionally assessed using MitoTracker Red images in conjunction with NIH ImageJ software. The orientation of the major axis was used to assess mitochondrial alignment and color code individual mitochondrion to assess mitochondrial alignment. Basic mitochondria function was assessed using the tetramethylrhodamine ethyl ester (TMRE) Mitochondria Membrane Potential Assay Kit (Abcam), with no TMRE added samples serving as negative controls. Smooth muscle cells were grown on 96-well plates and live cell analysis for TMRE was performed by a colorimetric method using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

Protein Kinase C activity

Protein kinase C activity was assessed by lysing fully confluent smooth muscle cells cultured for 14 days in RIPA buffer containing EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN). Cell lysate was analyzed using the PKC Kinase Enzyme Activity Kit (Enzo Life Sciences, Farmingdale, NY), with values made relative to the control media with vehicle (0.01% DMSO) group.

Intracellular calcium quantification

Cells were cultured in 96-well black walled plates for 14 days and live cell intracellular calcium was determined with the Fluo-4 Direct Calcium Assay (ThermoFisher) according to manufacturer's protocol using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

TNAP, Alizarin Red, PPI, and aorta Osteosense analysis

Tissue non-specific alkaline phosphatase (TNAP) activity was analyzed using whole cell lysates and the TNAP enzyme activity assay (BioVision, Inc., Milpitas, CA). Matrix calcium deposition was analyzed by the use of Alizarin Red staining. Briefly cells were washed in PBS then fixed in 4% paraformaldehyde for 30 minutes, and then washed with distilled water. Cells were stained with freshly prepared and filtered Alizarin Red S (Sigma), pH 4.2, with excess stain being removed with four subsequent distilled water washes. Images were taken using a Nikon camera. Alizarin Red stain was quantified by extracting the stain from cells using 100 mmol/L cetylpyridinium chloride with gentle shaking at room temperature for 30 minutes, and measuring the absorbance at 540nm. Pyrophosphate (PPI) was measured using conditioned media collected from cells after 14 days in culture using the High Sensitivity Pyrophosphate Assay Kit (Sigma Aldrich). Calcification in the mouse aorta was analyzed via Osteosense (100 μ L) tail vein injections 24 hours prior to euthanization and imaging. Aortas were perfused with saline, dissected and imaged using fluorescent reflection imaging (Image Station 4000 MM, Eastman Kodak, New Haven, CT). Imaging was performed using excitation/emission filter sets of 630 nm/700 nm with a f-stop setting of 2.4, the field of view set to 83.96 mm, the focal plane set at 7.61 mm, and an exposure time of 180 seconds. A custom MATLAB script was used to calculate the positive stained regions, as previously described in detail³.

Electron microscopy

Electron microscopy was performed at the Beth Israel Deaconess Medical Center Electron Microscopy Core and additionally at the Massachusetts General Hospital Program in Membrane Biology Electron Microscopy Core. Briefly, tissue was fixed in modified Karnovsky's fixative (2.5% glutaraldehyde, 2% paraformaldehyde, in 0.1 mol/L cacodylate buffer, pH 7.4), and post-fixed in 1% osmium tetroxide, dehydrated, and then embedded in Epon resin. Grids were imaged on a JEOL 1400 TEM equipped with a side mount Gatan Orius SC1000 digital camera. EM images were quantified using Nikon NIS-Elements

AR software. A region of interest was drawn around each mitochondrion using the software regions of interest (ROI) tool. The area of the ROI (μm^2) was calculated with the software ROI Statistics tool.

Serum analysis

Serum total cholesterol and triglyceride levels were quantified using the Total Cholesterol E Kit (Wako Chemicals, Richmond VA) and the LabAssay Triglyceride Kit (Wako Chemicals), with colorimetric analysis performed using a SpectraMax M5 plate reader. PCSK9 was quantified using the mouse PCSK9 Quantikine ELISA Kit (1:20,000 dilution used for PCSK9 gain-of-function AAV injected mice; R&D Systems) according to manufacturer's protocol.

Bone micro-CT and bone marrow cell isolation/differentiation

Femur bones were collected from male *Drp1^{+/+}* and *Drp1^{+/-}* mice following 20 weeks on atherogenic diet (1.25% cholesterol, D12108C, Research Diets Inc.). Bones were stored in 70% ethanol and kept at 4°C until processing. Blinded micro-CT analysis was performed by the Harvard School of Dental Medicine micro-CT Core facility using X-ray microtomography. Primary human femoral bone osteoblast cells were plated at a 100% confluency and cultured in control media (DMEM+10%FBS+1% pen strep), or the same osteogenic media (OM) used to drive mineralization in smooth muscle cells with the slight modification of using DMEM containing 1g/L glucose (ThermoFisher, Waltham, MA). DRP1 was inhibited by the addition of 50 $\mu\text{mol/L}$ Mdivi-1. Oxidative stress was induced by the addition of 0.3 mmol/L H_2O_2 with each media change. Media was changed every three days, with Alizarin Red staining analyzed after three weeks in culture in the same manner as performed with the SMCs.

Osteoclast differentiation and analysis

Human peripheral blood mononuclear cells (PBMCs) were isolated from commercially obtained buffy coat (Research Blood Components, Brighton, MA), and differentiated to osteoclasts using 50 ng/mL M-CSF for one day, followed by culturing with M-CSF and 50 ng/mL human RANKL (PeproTech, Rocky Hill, NJ) for 14 days. RANKL-treated cells were analyzed for TRAP activity and staining (B-Bridge International, Cupertino, CA). TRAP stained cells were imaged using an Eclipse TS100 microscope (Nikon) with SPOT idea digital camera and SPOT Basic Software (SPOT Imaging Solutions, Sterling Heights, MI). Bone resorption was analyzed by using Osteo Assay Surface 24-well plates (Corning, Corning, NY) plating PBMCs at 4×10^4 cells/well, and following the described osteoclast differentiation method. Pit formation was assessed under a light microscope and quantified based on binarized images using Matlab (Mathworks).

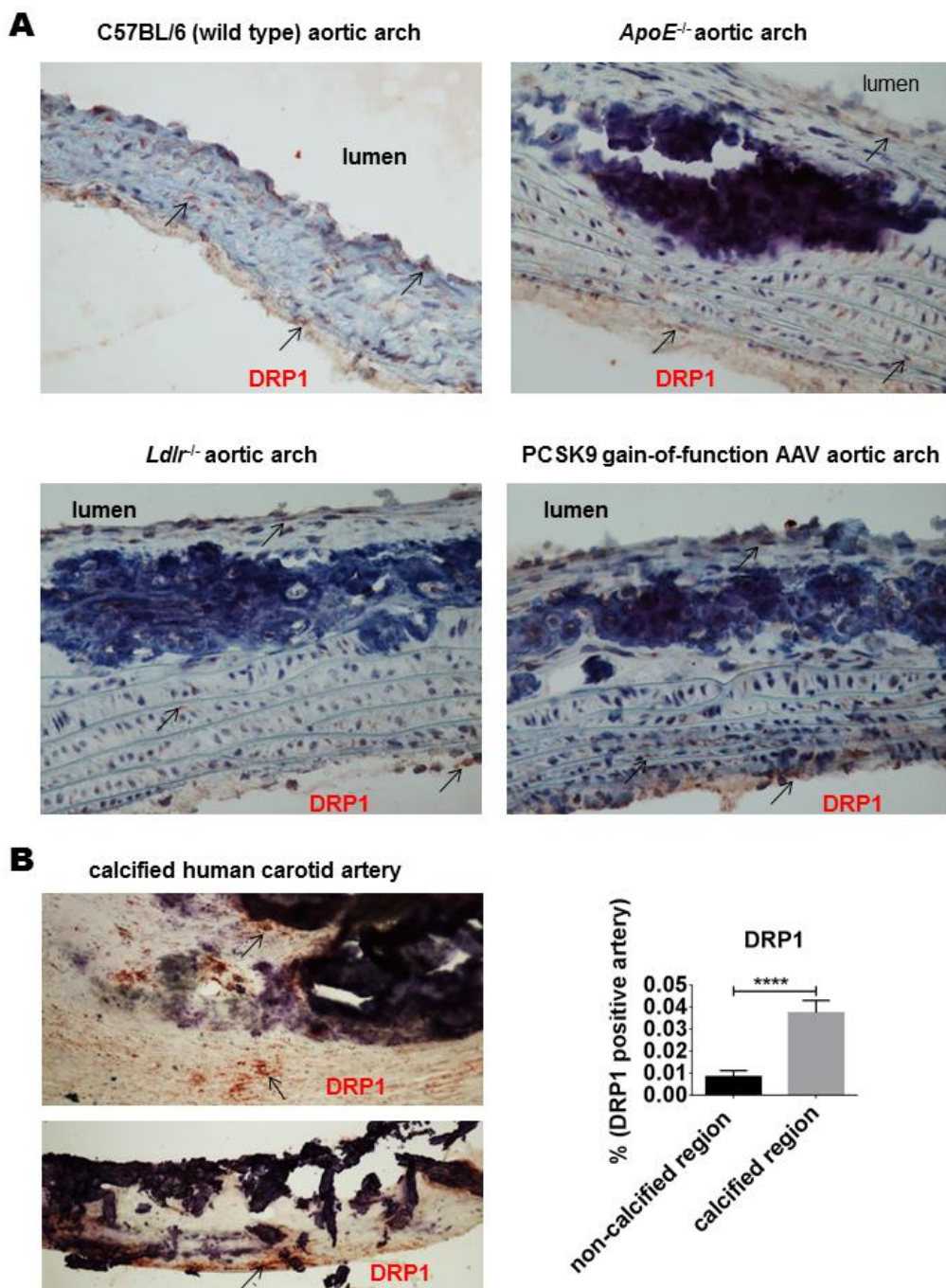
Statistical analysis

Data was analyzed using t-test or ANOVA with post hoc tests where appropriate, using PRISM software (GraphPad, San Diego, CA). P-values less than 0.05 were considered significant.

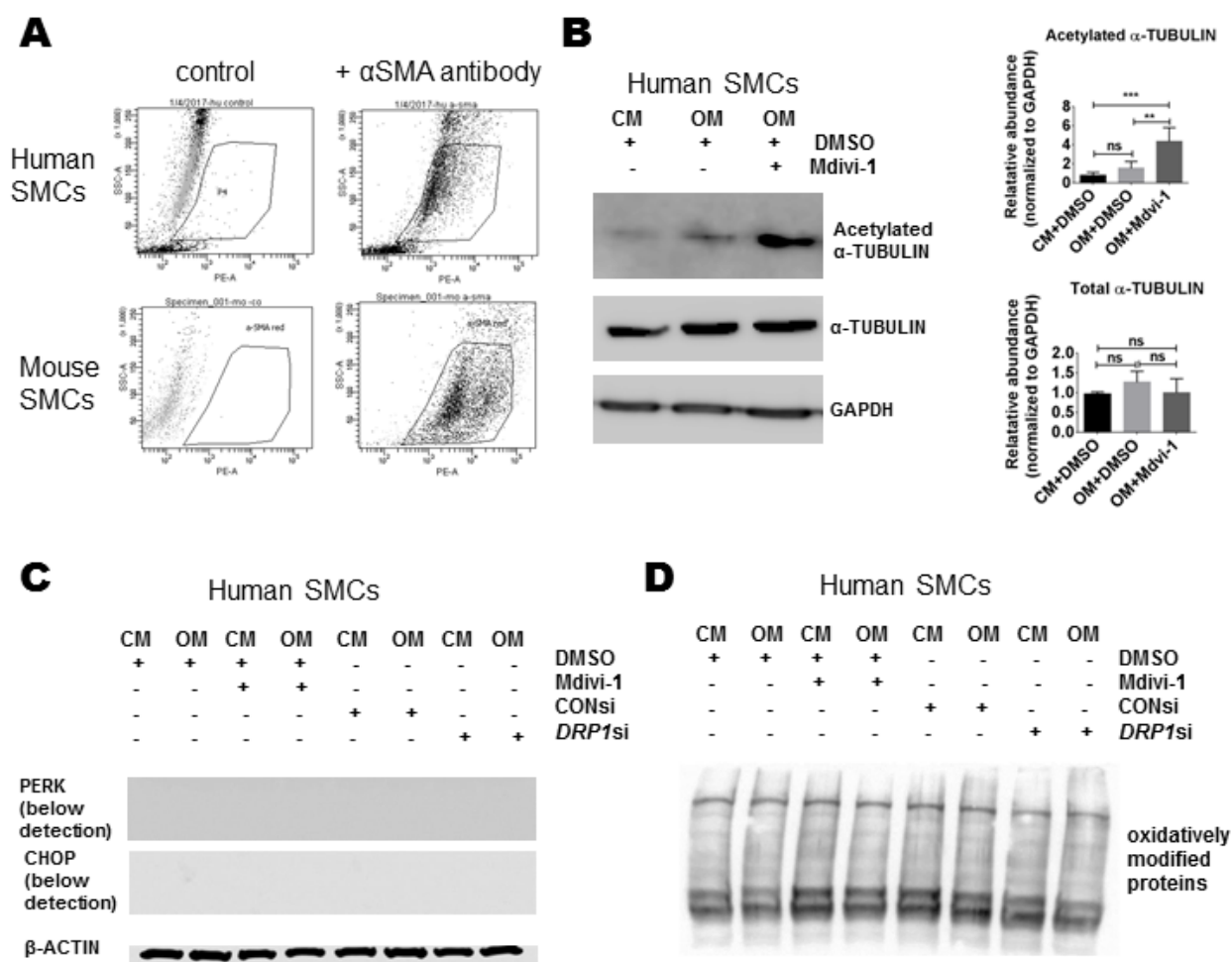
Supplementary References

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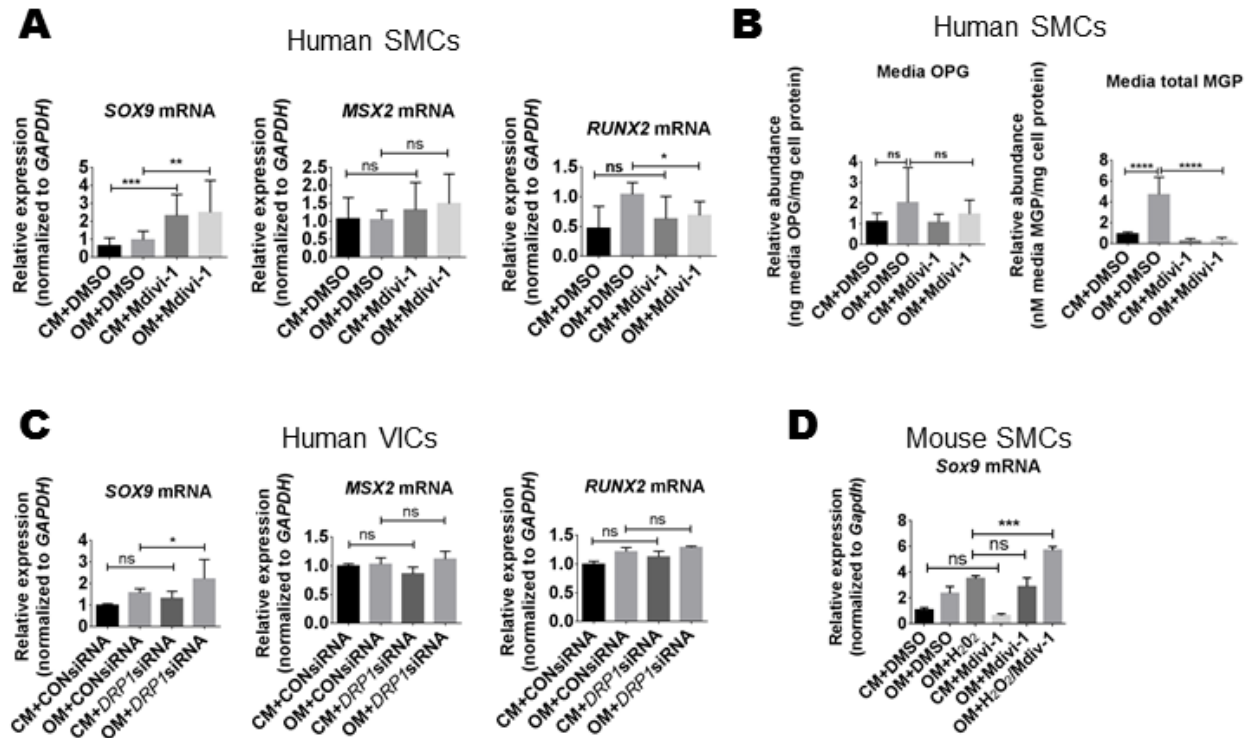
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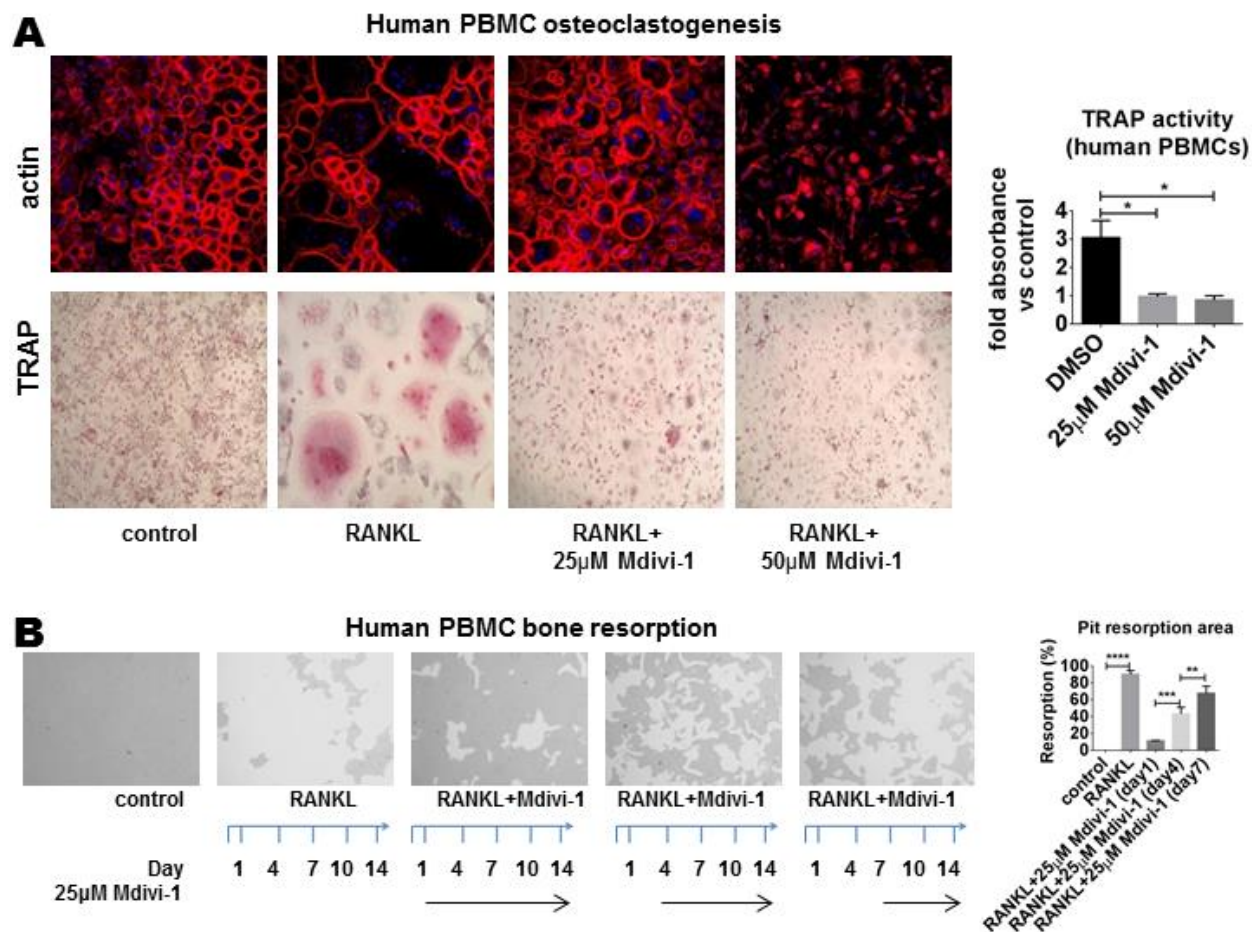
Online Figure I. DRP1 was not enriched in calcified aorta of commonly used atherosclerosis mouse models. (A) Representative DRP1 immunohistochemistry images of calcified aortic arches from wild type, *ApoE*^{-/-}, *Ldlr*^{-/-}, and PCSK9 gain-of-function AAV mice maintained on an atherogenic diet for 18-20 weeks; arrows indicate DRP1 in vascular wall cells (red reaction product) ($N=5$). (B) Representative DRP1 staining and quantification in non-calcified and calcified tissue regions of human carotid arteries; additional data shown in Figure 1A ($N=5$ donors). **** $P < 0.0001$; error bars indicate SEM.



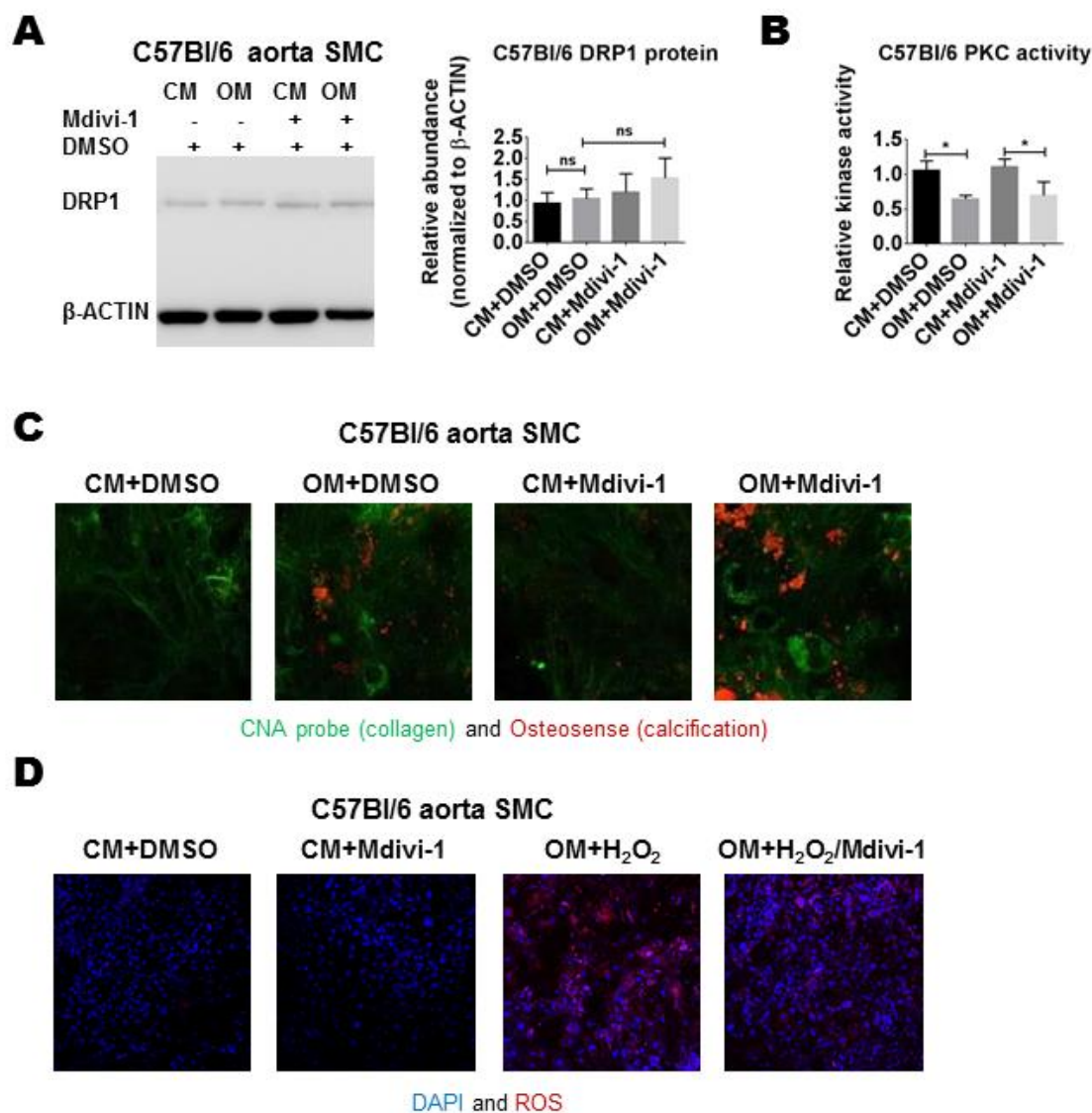
Online Figure II. Mdivi-1 increased acetylated α -TUBULIN in human SMCs. (A) Confirmation of human and mouse SMC identity by α SMA FACS analysis. (B) Acetylated α -TUBULIN protein from human SMCs treated with control (CM) or osteogenic media (OM) with Mdivi-1 (50 μ mol/L) or DMSO vehicle (0.01%) for two weeks. (C) Representative PERK and CHOP western blots (below detection) and (D) Oxyblot (oxidative stress) for human SMCs treated for two weeks with (+) or without (-) vehicle (0.01% DMSO), Mdivi-1 (50 μ mol/L), control siRNA (CONsi) or *DRP1* siRNA (*DRP1*si). $N=3$ donors for human SMCs, and 3 experiments for mouse SMCs; ns= not significant, ** $P < 0.01$, *** $P < 0.001$; error bars indicate STDEV.



Online Figure III. DRP1 inhibition increased *SOX9* expression in human SMCs, VICs and H₂O₂ treated mouse SMCs. (A) *SOX9*, *MSX2*, *RUNX2* mRNA, and (B) cell culture media OPG and MGP ELISA from human SMCs cultured for two weeks in control (CM) or osteogenic media (OM) with Mdivi-1 (50 μ mol/L) or DMSO vehicle (0.01%). (C) *SOX9*, *MSX2*, and *RUNX2* mRNA from human VICs treated with control siRNA (CONsiRNA) or *DRP1* siRNA (*DRP1*siRNA) for two weeks. (D) *Sox9* mRNA from mouse SMCs cultured for two weeks in CM or OM with H₂O₂ (0.3 mmol/L), with or without Mdivi-1 (50 μ mol/L), *N*=6 donors for human SMCs, 3 donors for VICs, and 3 experiments for mouse SMCs; ns= not significant, * *P* < 0.05, ** < 0.01, *** < 0.001; error bars indicate STDEV.



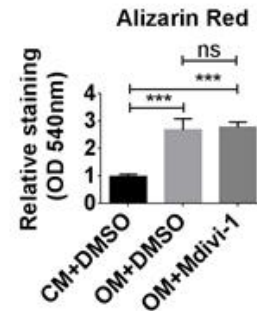
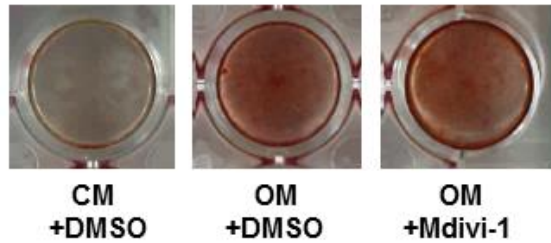
Online Figure IV. Mdivi-1 reduced human PBMC osteoclastogenesis. (A) Representative actin and TRAP staining images, and TRAP activity quantification for human peripheral blood mononuclear cells (PBMCs) from control (no RANKL), and cells differentiated into osteoclasts-like cells with the addition of RANKL or RANKL with 25 or 50 µmol/L Mdivi-1; $N=6$. (B) Representative bone resorption (light gray color) images of human PBMCs differentiated into osteoclasts with RANKL or RANKL with 25 µmol/L Mdivi-1 added at the beginning of the differentiation process or at days four or seven; $N=6$. * $P < 0.05$, ** < 0.01 , *** < 0.001 , **** < 0.0001 ; error bars indicate STDEV.



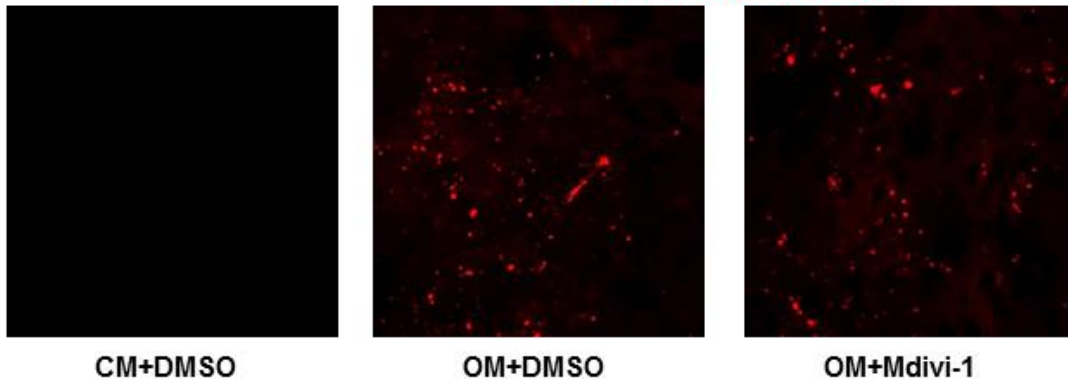
Online Figure V. Mdivi-1 did not alter primary C57Bl/6 mouse aortic SMC osteogenic media-induced calcification without the addition of oxidative stress. (A) DRP1 protein from C57Bl/6 aortic SMCs cultured for two weeks in control media (CM) or osteogenic media (OM) with vehicle (0.01% DMSO) or Mdivi-1 (50 μ mol/L). (B) PKC activity from C57Bl/6 SMCs treated for two weeks. (C) Representative Osteosense (red; calcification) and CNA probe (green; collagen) immunofluorescence of C57Bl/6 SMCs treated for three weeks. (D) Reactive oxygen species (ROS) immunofluorescence from C57Bl/6 SMCs treated for two weeks with or without H₂O₂ (0.3 mmol/L) and Mdivi-1 (50 μ mol/L). $N=3$; error bars indicate STDEV; ns= not significant, * $P < 0.05$.

A

Human bone osteoblasts Alizarin Red (calcification)

**B**

Human bone osteoblasts Osteosense (calcification)



Online Figure VI. Osteogenic media induced calcification in human bone osteoblasts. (A) Alizarin Red staining and (B) Osteosense staining from primary human bone osteoblasts treated in control media (CM) or osteogenic media (OM) with DMSO vehicle (0.01%) or Mdivi-1 (50 $\mu\text{mol/L}$) for two weeks; $N=3$; ns= not significant, *** $P < 0.001$; error bars indicate STDEV.