SUPPLEMENT MATERIAL

DETAILED MATERIALS AND METHODS

Reagents, antibodies, growth factors, and tissue culture supplies – All biochemicals were purchased from Fisher, Sigma, or Pierce. Real-time fluorescent PCR and Dye Terminator Sequencing reagents were purchased from Applied Biosystems. RNAater Tissue Collection:RNA Stabilization Solution (#AM7020) and RNAase-free DNAse (DNA Free Kit; cat #AM1906) were purchased from Ambion. Total RNA purification kits were purchased from Qiagen (RNeasy MiniKit, #74104). Other molecular biology reagents and restriction enzymes were purchased from Invitrogen or Promega. Protease inhibitor cocktails and buffer constituents for protein analyses were purchased from Sigma. Reagents and standards for Bradford protein assays were purchase from Biorad (#500-0002). Sircol Collagen Assay kits from Biocolor Life Sciences were purchased via Accurate Chemical and Scientific, Westbury, NY (cat # CLR S1000). Slides, fixatives, and stains for histological assessment were purchased from Fisher Scientific. Tissue-Tek O.C.T. compound was purchased from Sakura Finetek U.S.A. Custom synthetic oligodeoxynucleotides, pre-cast SDS polyacrylamide gels, and Zymogram pre-cast zymography gels were purchased from Invitrogen. Reagents for protein preparation were obtained from Pierce, Sigma, and Fisher. Fetal bovine serum, tissue cultures media, media supplements, and antibiotics were purchased from Sigma and Invitrogen. Corning disposable tissue culture plasticware was purchased from Fisher. Human PTH(1-34) was obtained from Bachem (#H-4835). Recombinant mouse Wnt3a protein (#1324-WN) was purchased from R&D Systems. Mouse anti-PTH1R (sc-12722; 3D1.1; lot E0903) tubulin (sc-8035; Tu-02; lot D231), β-catenin (sc-7199; H-102; lot D038), and e-IF2 α (sc-133227;G-12; lot B1209) were purchased from Santa Cruz. Immobilon-P membranes for western blot were purchased from Millipore. I-block, alkaline phosphatase conjugated secondary antibodies (goat anti-mouse IgG+IgM #T2192; goat anti-rabbit #AC31RL) and chemiluminescent alkaline phosphatase substrate (CSPD) were purchased from Applied Biosystems / Tropix. Lucigenin (#B49203) and Krebs-Ringer bicarbonate buffer (#K4002) was purchased from Sigma-Aldrich. Dihydroethidium (DHE) was obtained from Molecular Probes. The colorimetric BrdU uptake and DNA incorporation ELISA for assaying cellular DNA synthesis was purchased from Roche Diagnostics (cat # 11647229001). Urinary calcium and creatinine concentrations were determined using the BioAssay Systems Quantichrom kit DICA-500 (Hayward, CA) and the Cayman Chemical Company creatinine assay kit 500701 (Ann Arbor, MI), respectively.

Tissue culture -- All cells were cultured at 37 ºC with humidified air supplemented with 5% CO2. A7r5 rat aortic VSMCs[1] were obtained from American Type Culture Collection (ATCC CRL-1444), and were maintained in Dulbecco's Modified Eagle's Media (DMEM) with 4.5 g/liter glucose, 4 mM glutamine, and 10% fetal bovine serum (FBS) with penicillin-streptomycin supplementation. C3H10T1/2 cells[2] were obtained from the American Type Cell Culture and cultured in Basal Eagle's medium containing 10% FBS. Primary mouse aortic VSMCs were obtained using a modification of our previously published method for obtaining adventitial myofibroblasts[1,3], passaged in the same media composition described above for A7r5 cells. Briefly, 6 to 8 male C57BL/6 mice, aged 2 to 3 months, were euthanized by exsanguination under ketamine-xylazine general anesthesia following protocols reviewed and approved by the institutional Animal Studies Committee. Subsequently, under sterile conditions, aortic segments from ascending aorta to the diaphragm were resected en bloc, rinsed twice with DMEM supplemented with antibiotics (200 IU/ml penicillin and 200 ug/ml of streptomycin) at room temperature, and placed into two 10 cm Corning tissue culture dishes containing fresh sterile

DMEM/penicillin/streptomycin. The adventitial cell layer was exhaustively dissected away, and the remaining aortic tissue sectioned into rings of ca. 2 mm in length using a sterile forceps and No. 11 scalpel. Aortic rings were then processed by transient digested with 2 mg/ml collagenase (Worthington, 243 U/mg) in serum – free DMEM for 4 hours at 37 ºC, followed by brief centrifugation with aspiration of the supernatant. The processed aortic rings were then placed into two separate sterile 10 cm tissue culture dishes containing 3 mL of growth media (10% fetal calf serum in Dulbecco's Modified Eagle's Medium, high glucose, supplemented with 100 IU / ml of penicillin and 100 ug/ml of streptomycin), and cultured at 37 ºC in a humidified incubator under 5% CO2. This amount of media is just sufficient to provide nutrition and hydration, and permits adherence of the aortic rings to the underlying tissue culture plastic. Two days later, 3 ml of additional growth media was added and changed every three days with daily monitoring. Two weeks after the initial plating, a sterile Pasteur pipette was used to aspirate the residual elastin-rich aortic rings, the outgrowth of adherent smooth muscle cells released by trypsinization, and re-plated at a 1:3 split onto 10 cm Corning tissue culture plates. Cells were amplified over the next 2 passages, and then used for transduction, gene expression, and collagen protein assays as indicated.

Expression plasmids, reporter plasmids, transient transfections, and luciferase assays – The vascular smooth muscle cell- specific murine *SM22* promoter[4] fragment - 441 to + 44 was generated by PCR from mouse genomic DNA, introducing 5'-XhoI and 3'-KpnI restriction sites. As we've previously detailed[5], the SM22 promoter fragment was ligated into the eukaryotic expression plasmid pTRE2 (Clontech; Cat. #631008), following removal of the vector's tetracycline response element and *CMV* promoter via XhoI-KpnI digestion. The resulting plasmid, pSM22/DT58.11, contains the *SM22* promoter placed upstream of the polylinker, rabbit β-globin 3'-UTR, and polyA sequences of pTRE2. The human Jansen receptor PTH1R(H223R) cDNA[6] (kind gift of H. Juppner) was used as a template and amplified by PCR to introduce (a) a unique 5'- BamHI restriction site just upstream of the initiator Met in good Kozak context; and (b) a unique 3'- KpnI restriction site just downstream of the stop codon of PTH1R(H223R). After digestion, this 1.8 kb PCR fragment was vectorially subcloned into the BamHI – KpnI of DT58.11, downstream of the 0.5 kb SM22 promoter, to create the SM-caPTH1R expression vector. The cDNA insert in SM-caPTH1R was re- sequenced to ensure fidelity and integrity of the open reading frame. The TOP (#21-204) and FOP (#21-205; control) luciferase reporter plasmids for activated Wnt/β-catenin signaling were purchased from Millipore. The cyclic AMP – responsive reporter pCRE-LUC (#219076) was purchased from Stratagene. The CMV-promoter driven eukaryotic expression vector pcDNA3 was purchased from Invitrogen. Mouse Wnt7a and Wnt7b cDNAs were obtained by RT-PCR amplification from C3H10T1/2 cells and subcloned into the KpnI-BamHI sites of pcDNA3 (Invitrogen, Carlsbad, CA) using techniques previously detailed[7]. The 3.6 Col1A1 promoter – luciferase reporter construct 3.6Col1A1LUC was generated by PCR to amplify the 3.6 kb mouse Col1A1 promoter fragment using mouse genomic DNA as template, followed by vectorial 2-step assembly of the full 3.6 kb fragment upstream of the luciferase reporter gene in the XhoI / Bgl II sites of pGL2 (Promega). The promoter insert in 3.6Col1A1LUC was sequenced to ensure fidelity following amplification and ligation. Empty expression vectors (pcDNA3 or pSM22/DT58.11) were used to maintain constant DNA concentrations in all transient transfections. Transient transfections and luciferase assays were carried out precisely as we've previously detailed[1,7].

Generation of SFG retroviruses expressing Wnt7a, Wnt7b, and caPTH1R -- Recombinant retrovirus pseudotyped with vesicular stomatitis virus G glycoprotein (VSV-G)[8] was utilized to introduce human PTH1R(H223R), mouse Wnt7a, or mouse Wnt7b cDNAs to aortic smooth muscle cells or C3H10T1/2 cell line using methods we've previously detailed[3,7]. The SFG

retroviral vector, SFG-LacZ control, and 293GPG packaging cell line were kindly provided by Dr. Dan Ory[8] (Washington University in St. Louis). PCR was used to introduce 5'- Nco I and 3'-BamHI sites onto the PTH1R(H223R) cDNA, and was vectorially cloned into the NcoI/BamHI site of SFG to create SFG-caPTH1R. However, because of the presence of 2 NcoI sites near the 3' end of the open reading frame, a single internal KpnI site upstream of this region was used to divide PTH1R(H223R) cDNA into 2 fragments by PCR to facilitate cloning. Following 3-way ligation, the cDNA insert was re-sequenced to ensure fidelity of the open reading frame. SFG-caPTH1R plasmid and pMDHygro were co-transfected using LipofectAMINE Plus reagent (Invitrogen) into the 293GPG packaging cell line, which stably expresses MuLV gag-pol and tetracycline-suppressed vesicular stomatitis virus G glycoprotein. After hygromycin selection, the stable packaging cells expressing SFG-caPTH1R were expanded. To generate the pseudotyped retrovirus, packaging cells were incubated in regular growth medium without tetracycline and hygromycin to upregulate expression of VSV-G protein. The conditioned medium was harvested daily and the media harvested from day 3 to day 7 containing the highest titer of viral particles (≥ 5 x 10^6 colony-forming units/ml) were combined, centrifuged to remove cell debris, and used for transduction. For negative control, the SFG-LacZ pseudotyped retrovirus was also prepared. The SFG-Wnt7b plasmid[9] was kindly provided by Dr. F. Long[9] (Washington University in St. Louis). Due to the presence of internal NcoI sites in the Wnt7a cDNA, we generated the SFG-Wnt7a plasmid from our pcDNA3-Wnt7a construct [7] using a strategy similar to that for generation of SFG-caPTH1R. A unique internal SacI site was used to divide Wnt7a cDNA into 2 fragments which were then cloned into the NcoI and BamHI sites of SFG vector. Of note, the introduction of NcoI primer to the 5' end of Wnt7a resulted in a non-synonymous Thr → Ala change at codon 2. Thus, site-directed mutagenesis (Stratagene) was employed to revert to the wild-type codon, and the Wnt7a cDNA insert was re-sequenced to ensure fidelity of the open reading frame (ABI Prism Dye Terminator kit, Foster City, CA).

Transduction of cultured cells with pseudotyped retroviruses – Retrovirus preparation and transduction was carried out using methods we've previously detailed[3,7]. Briefly, the day before transduction, cells (either primary aortic myofibroblasts or C3H10T1/2 cells) were seeded in 10 cm culture dishes at a density of 0.5 × 10^6/dish. The next day, 10 mL of 50% conditioned medium containing pseudotyped retrovirus and 8 μg/mL polybrene was added to each dish. Virus infection was allowed to proceed for 24 h. At that time point, the virus medium was removed, and fresh medium was added. Cells were grown to confluence in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS. Cells were passages and cultured for 7 – 14 days as outlined below. Media was changed every 2 – 3 days. For mineralization, then cells were maintained in 10% FBS in DMEM supplemented with 10 mM β-glycerol phosphate and 50 ug/ml ascorbic acid.

Analysis of transduced cell cultures for collagen gene expression and protein accumulation, alkaline phosphatase enzyme activity, and matrix calcification -- Assays of gene expression (RT-qPCR) and collagen protein accumulation (Sircol Assay) in transduced cell cultures closely followed the protocols as detailed for analysis of aortic tissue (vide infra). Alizarin red staining for calcium was carried out essential as described. Briefly, the cell monolayers grown as above in 6 well cluster dishes were rinsed with Tris-buffered saline (TBS; 50 mM Tris pH 7.4 / 0.15 M NaCl) and fixed in ice cold 70% ethanol for 1 hour. Following 3 washes in water, 1 mL of filtered 0.4% Alizarin red S in distilled deionized H20 was added per well, and plates incubated at room temperature for 10 minutes with gentle shaking. After aspiration, monolayers were then washed five times x 5 minutes in 3 - 4 mL of water, followed by one wash in PBS for 5 minutes. Following aspiration, stained monolayers were imaged by digital photography as previously detailed[10]. For alkaline phosphatase assays, cell monolayers

in 24 well cluster dishes were washed three times with TBS, then harvested by scraping into 500 uL of 10 mM Tris pH / 0.5 mM MgCl2 / 0.1% Triton X-100, and cells further disrupted by probe sonication (3 serial 20 second bursts at 40% maximum). Aliquots were then assayed for cellular protein (10 uL; Bradford protein assay) and alkaline phosphatase enzyme activity (40 uL aliquots). Assays for alkaline phosphatase activity implemented 3.3 mM para-nitrophenyl phosphate as substrate in 6.7 mM MgCl2 buffered with 0.07 M 2-amino-2-methyl-1-propanol at pH 10.3. Enzyme activity was monitored by measuring absorbance at 405 nm with a BioTek microQuant plate spectrophotometer, and specific activity expressed as nmol of para-nitrophenol produced per min / mg cellular protein.

Generation of SM-caPTH1R;LDLR+/- and SM-caPTH1R;LDLR+/-;TOPGAL transgenic mice -- Colonies were maintained following procedures reviewed and approved by the Washington University Animal Studies Committee. SM-caPTH1R transgenic mice (C57Bl/6 background) were generated using methods described[5,11]. Briefly, the vascular smooth muscle cell- specific murine SM22 promoter[4] fragment - 441 to + 44 (relative to start site of transcription) was generated by PCR from mouse genomic DNA, introducing 5'-XhoI and 3'-KpnI restriction sites. The 0.5 kb SM22 promoter fragment was ligated into the eukaryotic expression plasmid pTRE2 (Clontech; Cat. #631008), following removal of the vector's tetracycline response element and CMV promoter via XhoI-KpnI digestion. The resulting plasmid, pSM22/DT58.11[5], contains the SM22 promoter placed upstream of the polylinker, rabbit β-globin 3'-UTR, and polyA sequences of pTRE2. A 1.8 kb cDNA encoding the open reading frame of human Jansen variant PTH1R(H223R)[6] (GenBank # NM_000316.2; kind gift of H. Juppner) was amplified by PCR, introducing 5'- BamHI and 3'- KpnI restriction sites with adjacent, interposed initiator methionine and stop codon sequences, respectively (see above). Transient co-transfection of A7r5 aortic vascular smooth muscle cells with SM-caPTH1R expression vector and the pCRE –LUC reporter confirmed expression plasmid activity. After sequencing the insert to validate integrity of the open reading frame, the plasmid was digested with Acl I and Nde I to release the 3.6 kb fragment containing the SM22 promoter, human PTH1R(H223R) cDNA, rabbit β-globin 3'-UTR, and the polyA signal. This fragment was gel purified, bound and eluted from Qiagen silica resin, acetate-ethanol precipitated, re-solubilized in transgene buffer (10 mM Tris pH 7.4 / 0.1 mM EDTA), and transgenic (Tg) mice made via male C57BL/6 pronuclear injection (Washington University Mouse Genetics Core, Mia Wallace, Director). PCR genotyping for the SM-caPTH1R transgene was directed toward uniquely juxtaposed human PTH1R cDNA and rabbit β-hemogolobin 3'- UTR sequences following the protocol of Stratman[12]. Briefly, 5 mm tail segments were extracted for DNA using methods previously described[7,13]. SM-caPTH1R Tg genotyping amplimers were 5'-CAC TAC ATT GTC TTC ATG GCC ACA CCA TAC-3' and 5'-GAG GAG ACA ATG GTT GTC AAC AGA GTA G-3'. The PCR cycling conditions were as follows: initiation / polymerase activation at 95 ºC for 5 min, followed by 30 cycles of denaturation at 94 ºC for 30 seconds, annealing at 65 ºC for 30 seconds, and extension at 72 ºC for 1 min. The LDLR-/- mice were purchased from The Jackson Laboratory (stock #002207; C57BL/6 background), and stocks maintained using amplimer pairs directed toward the neomycin cassette (5'-AGG ATC TCG TCG TGA CCC ATG GCG A-3' and 5'-GAG CGG CGA TAC CGT AAA GCA CGA GG-3') and the genomic site of insertion in the LDLR gene (5'-CGC AGT GCT CCT CAT CTG ACT TGT C-3' and 5'-ACC CCA AGA CGT GCT CCC AGG ATG-3') to ensure genotype fidelity. SM-caPTH1R transgenic mice (LDLR+/+, C57BL/6) were crossed with LDLR-/- mice[14] (C57BL/6 background) to generate SM-caPTH1R;LDLR+/- experimental animals and LDLR+/- sibling controls. Further backcross onto the LDLR-/- parental background generated SM-caPTH1R;LDLR-/- mice. SM-caPTH1R;LDLR-/- mice were bred with TOPGAL reporter mice[15] to generate SM-caPTH1R;LDLR+/-;TOPGAL+ and LDLR+/-;TOPGAL+ littermates on a mixed C57BL/6:CD1 background. The TOPGAL reporter mice

(TCF/LEF Optimal Promoter / galactosidase reporter transgenic mice) were purchased from Jackson Labs (stock #004623; CD1 background) The TOPGAL genotyping amplimers were: 5'-GAG TGA CGG CAG TTA TCT GGA AGA TCA GGA-3' and 5'-GGA AAC CGA CAT CGC AGG CTT CTG CTT CAA TCA-3'.

Body composition and serum biochemical analyses —After overnight fasting, mice were anesthetized and body composition was determined by dual energy x-ray absorptiometry (DEXA) with a GE Lunar PixiMus using techniques previously detailed[10,16]. Subsequently, blood was withdrawn from the inferior vena cava in heparinized syringes. Blood samples were layered on top of Microtainer Serum Separator tubes (BD Biosciences #365956) and incubated overnight at 4 °C. Sera were obtained after centrifugation at 2,000 × *g* for 20 min and processed for analysis as previously described[5,7,10]. Serum concentration of osteoclast-derived tartrate-resistant acid phosphatase form 5b was determined by using MouseTRAP assay kit (Code SB-TR103; IDS Inc., Fountain Hills, AZ). Commercial EIAs and ELISAs for CTX (RatLaps, 1RTL4000, Nordic Bioscience Diagnostics via IDS Inc.) collagen N-terminal telopeptide (Rat/Mouse P1NP Assay; AC-33F1, IDS Inc.), and Dkk1 (DuoSet DY1765;R&D Systems) were carried out per the manufacturer's instructions. The mouse intact PTH ELISA kit (#60-2300) was purchased from Immutopics International. The rodent osteopontin RIA (#900-090A) was purchased from Assay Designs. Biochemical assays for serum cholesterol (Thermo Scientific #2350-400H), glucose (Thermo Scientific #1524-400H), triglycerides (Sigma #TR0100) and free fatty acids (Wako #994-75409) were performed using commercially available assay kit, following the manufacturer's instructions.

Western blot analysis of aortic PTH1R and β-catenin protein accumulation – Western blot analysis of extracted aortic protein was carried out using methods we've previously detailed.[11] Mouse aortas were rinsed with twice with ice cold PBS, then extracted at 4°C with a hand-held Tissue-Tearor rotary homogenizer (BioSpec Products Inc.) in 5 volumes of 10 mM Hepes, pH 7.4, 0.5% Triton X-100, 0.15 M NaCl, 2 mM EDTA, 2 mM EGTA, 0.5 mM DTT, and 0.02% sodium azide supplemented with comprehensive protease (P8340) and phosphatase (P2850 and P5726) inhibitor cocktails from Sigma. After centrifugation, 30 ug of tissue protein extract (Bradford) was resolved by SDS-PAGE then electotransferred to Immobilon-P membranes (Millipore Corp.); proteins were immunovisualized by Western blot as previously detailed, using anti-PTH1R (sc-12722; 3D1.1; lot E0903) and β-catenin (sc-7199; H-102; lot D038), tubulin (sc-8035; Tu-02; lot D231), and e-IF2α (sc-133227;G-12; lot B1209) antibodies at 1:1000. Alkaline phosphatase conjugated secondary antibodies (1:5000; goat anti-mouse IgG+IgM #T2192; goat anti-rabbit #AC31RL) and chemiluminescent alkaline phosphatase substrate (CSPD) were used to visualize immune complexes by exposure to x-ray film in autoradiography cassettes as previously detailed[7]. After digital capture of developed film images using a Hewlett-Packard ScanJet 5370C bed scanner, image analysis was performed by importing scanned JPEG images into Kodak 1D image analysis software. Absolute net pixel intensity of the CSPD-visualized bands with uniform rectangular region of interest were used to quantify western blot signals[17]. Data are expressed as net pixel intensity of the band, normalized to housekeeping protein control.

Quantitative assessment of gene expression by fluorescence RT-PCR – Messenger RNA accumulation was quantified using techniques we've previously detailed.[3,5,11] Fluorescence RT-PCR was performed to quantify relative mRNA levels of aortic osteogenic signaling molecules; RNA extraction techniques, methods, and validated amplimer pairs for these genes have been previously detailed[7]. Briefly, a 2 step reverse transcription – PCR was carried with use of TaqMan FAM dye fluorescence to quantify specific messages in cDNA aliquots arising from

reverse transcription of 75 ng of total RNA. Following the overnight fast, experimental animals are euthanized by exsanguination under ketamine-xylazine general anesthesia following protocols approved by the institutional Animal Studies Committee. Subsequently, aortic segments are resected en bloc from ascending aorta to the diaphragm, rinsed twice with sterile PBS (phosphate buffered normal saline), and each aorta then placed in 1.5 mL of RNAlater Tissue Collection:RNA Stabilization Solution (Ambion Cat. # AM7020), minced with a scalpel, and stored at 4 ºC. The next day, aortic tissue is processed with an Omni International TH tissue homogenizer using a 5 mm x 95 mm generator (Fisher cat.# 15-338-203) in 600 uL of Qiagen RNeasy MiniKit (Qiagen cat. #74104), to which 10 uL / mL fresh β-mercaptoethanol is added just prior to use. Total RNA is then isolated by spin column chromatography as per the manufacturer's instructions, eluted in 35 uL of nuclease-free sterile water. Total RNA is then treated with RNAse-free DNAse to remove any small amount of contaminating genomic DNA (Ambion DNA Free Kit; cat #1906), and quantified by spectrophotometry. Subsequently, reverse-transcription (RT) reaction (0 uL) were carried out with Superscript II Reverse Transcriptase (Invitrogen) as per the manufacturer's protocol, using oligo dT15 + random hexamer (1.25 uM final of each) primed total RNA (1.5 ug – 2 ug). After heat inactivation of RT (95 ºC for 15 minutes then snap cooled on ice), cDNA arising from 75 ng or100 ng (per assay) reverse transcribed total RNA was analyzed by biphasic fluorescence PCR (total volume 20 uL; 2 minutes at 50 ºC, 10 minutes at 95 ºC, then 40 cycles, 15 sec at 95 ºC, 1 min at 60 ºC). Expression of the indicated collagen, metalloproteinase, and osteogenic signaling genes was quantified by FAM dye-labeled fluorescence with an ABI 7300 Sequence Detection System in duplicate, implementing validated TaqMan probes (cat. #4331182) purchased from Applied Biosystems. Expression was normalized to the signal arising from 18S rRNA assayed in parallel duplicate aliquots, to control for efficiency in all RT-PCR studies. Relative mRNA accumulation was then expressed as the percent of control values. All analyses were repeated at least twice for each individual aortic specimen, with $\geq$ 5 animals per group per genotype.

Quantitative assessment of aortic collagen content by Sircol assay[18]-- Following the overnight fast, experimental animals were euthanized by exsanguination under ketamine-xylazine general anesthesia following protocols approved by the institutional Animal Studies Committee. Subsequently, aortic segments were resected en bloc from ascending aorta to just below the diaphragm (level of renal arteries), very quickly rinsed twice with sterile deionized water then 5 times with fresh water and blotted x 5 on a Kimwipe tissue wipe to remove adherent moisture, and stored in a sterile microfuge tube on ice until weighed in a tared, small polystyrene weighing dish (Fisher Cat. #02-294A) on a Mettler Toledo XS105 microbalance. Total aortic collagen was then extracted from murine aortas and quantified using the Sirius red dye binding assay[18] (Sircol Collagen Assay, Biocolor Life Sciences, US supplier Accurate Chemical and Scientific, Westbury, NY, cat # CLR S1000). Pre-weighed samples of insoluble cross-linked collagen processed identically in parallel were used to generate the standard curve for this assay. Briefly, aortic specimens were minced into 3 pieces and total collagen extracted by adding 500 uL of extraction reagent (10 mg of pepsin / ml in 0.5M acetic acid). Collagen was solubilized for 7 days at 25 ºC, After equilibration of all reagents to 25□ºC, duplicate aliquots or standards (50 uL / sample) were treated with 1 mL of Sircol dye reagent in 1.5 ml microcentrifuge tubes, mixed thrice by gentle inversion, then further treated by gentle agitation at room temperature for 60 minutes. The insoluble collagen-dye pellet was then collected by centrifugation (12,500 g x 10 minutes), the supernatant decanted, and collagen-bound Sirius red solubilized by addition of 1 ml of Sircol alkali reagent and vigorous vortexing for 5 minutes. After a brief centrifugation (1 minute x 12,500g), 200 uL of sample or standard was transferred to 96 microwell plates, and OD measured at 540 nm with a BioTek microQuant plate spectrophotometer, with correction for path length monitored at 900 nm.

LacZ / β-galactosidase histochemistry and quantitative assessment of aortic sections -- Aortic β-galactosidase staining was carried using methods we've previously detailed.[5,11,19] Comparisons of LacZ activity elaborated by *SM-caPTH1R;LDLR+/-;TOPGAL* mice vs. *LDLR+/-;TOPGAL* littermate controls were made in aortic frozen sections. A Microm HM 550 cryostat was used to prepare 16 micro frozen sections of ascending aortas from these TOPGAL reporter cohorts that had been previously imbedded in O.C.T. Compound (Tissue-Tek, Sakura Finetek). After thawing slides to room temperature, sections were immediately fixed in 0.25% glutaraldehyde in PBS for 10 minutes. After washing once with water and once with PBS, approximately 200 uL of X-gal staining solution[15] (1 mg/ml X-gal in 2 mM PBS supplemented with 2 mM MgCl2, 5 mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$) was layered over each section on the slide, and LacZ enzyme activity revealed by overnight development at 37 C in a sealed humidified chamber. The next morning, slides were rinsed once with PBS, twice with deionized water, and counterstained with nuclear fast read. After 2 x 2 minute washes in deionized water, sections were rapidly dehydrated by 30 second serial incubations in graded alcohol solutions (50%, 70%, 80%, 90% and 100%), immersed in xylene, transiently air dried, then covered in Permount slide mounting solution and sealed with a coverslip. Digital photomicrographs were obtained on either (a) a Nikon CoolPix 5000 camera mounted on a Nikon Eclipse TS100 microscope or (b) Leica DFC 420 Digital Camera mounted on a Leica 4000 DM digital microscope. After importing JPEG files into Adobe Photoshop, digital image analysis was performed by counting the numbers of vessel wall cells and aortic valve leaflet cells that stained blue with X-gal.

Quantitative assessment of aortic collagen content by picrosirius red histochemistry – Quantitative histochemical analysis of collagen accumulation by digital image analysis of picrosirius red stained specimens was performed using a modification of previously published dark field imaging protocols.[20,21] Following the overnight fast, experimental animals were euthanized by exsanguination under ketamine-xylazine general anesthesia following protocols approved by the institutional Animal Studies Committee. Subsequently, the heart and ascending aortic segment to the take-off of the innominate artery was resected en bloc, the heart transected with a sharp razor midway between the base and apex, rinsed thoroughly with sterile PBS to remove adherent blood and coagulum. The upper heart and ascending aorta were then fixed overnight in 10% neutral buffered formalin with gentle agitation at room temperature. Two days later, the upper heart and ascending aorta were placed in cassettes with the transected cardiac "flat" surface faced downward, and imbedded in paraffin. Subsequently, 6 micron paraffin sections were prepared cutting from the transected surface towards the base and aortic outflow tract through the aortic sinus into the ascending aorta. Sections were selected for evaluation of collagen by picrosirius red staining at the level of the ascending aortas distal to the aortic sinus and just proximal to the innominate artery. Inner aortic diameters at this level were equivalent for both LDLR+/- and SM-caPTH1R;LDLR+/- animals. Sections were de-paraffinized in xylene and rehydrated via graded alcoholic solutions to water, nuclei stained with Weigert's hematoxylin for 5 minutes, then rinsed in water for 10 minutes. Sections were subsequently stained for 1 hour in 0.1% picrosirius red F3B prepared in a saturated aqueous solution of picric acid, and then rinsed twice with 0.5% (v/v) glacial acetic acid in distilled deionized water. After shaking to encourage egress of adherent water, slides were dehydrated by treated three times with 100% ethanol, cleared in xylene, and covered with Permount underneath a cover slip. Digital photomicrographs were obtained on a Leica DFC 420 Digital Camera mounted on a Leica 4000 DM digital microscope using a 10X objective lens with dark field illumination with the following settings: dark field illumination intensity of 2X, exposure time of 1s, gain of 1X and color saturation of 1.5X, with gamma curve settings of gamma = 0.1, black = 8 and white =21. Image analysis was performed by importing digital JPEG images into Kodak 1D image analysis

software. Absolute net pixel intensity of the pircosirius – stained collagen visible with darkfield illumination was quantified in each aortic cross-section. For each animal, three adjacent sections were analyzed and averaged to provide the aortic fibrosis net intensity value for that animal. Graphed data are presented as the mean ± S.E. net pixel intensity obtained from analyses of aortas from n = 4 LDLR+/- and n = 5 SM-caPTH1R;LDLR+/- male mice challenged with HFD for 3 months. Digital micrometry was implemented to quantify aortic thickness, measured from the outer elastic lamina to the luminal / endothelial surface in these same animals. Digital images were captured on a Leica 4000 DM digital microscope equipped with a DFC 420 Digital Camera using FW 4000 Software, and mural thickness measured with Leica LAS Image Analysis Software. Wall thickness was measured approximately every 30 degrees on the 360 degree aortic circumference at a level just distal to the aortic sinus. These 11 – 12 measurements were then averaged to provide the mean ascending aorta wall thickness for each individual animal. Data are presented as the mean +/- S.E. wall thickness measured for n = 4 animals of each genotype.

Analysis of aortic calcium content – Aortic calcium content was measured using techniques we've previously detailed[5,19]. Following the overnight fast, experimental animals were euthanized by exsanguination under ketamine-xylazine general anesthesia following protocols approved by the institutional Animal Studies Committee. Subsequently, aortic segments were resected en bloc from ascending aorta to just below the diaphragm (level of renal arteries), very quickly rinsed twice with sterile deionized water, blotted x 4 on a Kimwipe tissue wipe to remove adherent moisture, weighed in a tared, small polystyrene weighing dish (Fisher Cat.# 02-294A) on a Mettler Toledo XS105 microbalance, and then transferred to a 2.0 ml polypropylene screw cap tube with o-ring seals. After heating 20 minutes at 70 ºC, samples are speed evaporated under 1000 mTorr vacuum at 70 ºC with centrifugation for 60 minutes, and the tared tubes re-weighed with the dried aortic tissue inside. Subsequently, 20 volumes (20 uL per ug dry aortic mass) of 10% formic acid in deionized water was added to each sample, a new screw cap tightly fixed, and aortic calcium extracted overnight at 37 ºC. The following morning, samples are cooled to room temperature for 30 minutes, centrifuged for 5 minutes at 13,200 rpm, and the supernate transferred by pipetting to another tube. Two 10 uL aliquots for each specimen are added to 50 uL of deproteinization buffer (0.3 ml of glacial acetic acid and 3.8 ml of 1 N KOH diluted to 50 mL with deionized water, pH 5.2). After heating for 5 minutes at 95 ºC in a screw cap centrifuge tube, samples are immediately centrifuged 5 minutes at 13,200 rpm, snap cooled on ice at 4 ºC for 2 minutes, and 10 uL aliquots mixed with 100 ul of freshly prepared OCPC color reagent (ortho-cresolphthalein complexone; Sigma phthalein purple cat. #P5631) as described by Connerty and Briggs[22]. The purple OCPC calcium complex is spectrophotometrically determined by absorbance at 570 nm using a uQuant BIO-TEK plate reader. Background was determined in parallel using a sham extracted pair of empty microfuge tubes starting with 200 uL of 10% formic acid. A standard curve performed with each assay from a 1 mg/ml stock calcium solution prepared in deionized water from heat-dried calcium carbonate.

Urinary calcium and creatinine measurements – SM-caPTH1R;LDLR+/- and LDLR+/- male sibling cohorts were place on HFD for 3 months, then individually house overnight with ad libitum access to water in metabolic cages to collect feces-free urine during a 24 hour fast (n = 7 SM-caPTH1R+/-;LDLR+/- mice vs. 4 LDLR+/- sibling cohort controls). The next day, urinary calcium and creatinine concentrations were determined using the BioAssay Systems Quantichrom kit DICA-500 (Hayward, CA) and the Cayman Chemical Company creatinine assay kit 500701 (Ann Arbor, MI), respectively. All samples were measured in duplicate, and

data presented as the mean +/- S.E. for the urinary calcium concentration (mg/dL) normalized to urinary creatinine concentration (mg /dL).

Quantitative assessment of aortic distensiblity by ex vivo aortic video plethymography -- Aortic distensibility was measured implementing a modification of the method of Mecham et al.[23] using a plethysmography system purchased from Living Systems Instrumentation (LSI) (Burlington, VT). Briefly SM-caPTH1R;LDLR+/- (n = 4) and LDLR+/- (n =5) sibling controls were maintained on HFD for 3 months beginning at 5- 8 weeks of age. Mice were euthanized by exanguination under ketamine-xylazine anesthesia using protocols approved by the Washington University Animal Studies Committee. After careful dissection in supine position to reveal the ventral aspect of the thoracic aorta in situ, the native length (15 – 20 mm) of the thoracic aorta from the left subclavian artery to the diaphragm was recorded with a digital Vernier caliper (ILAC-MRA) and landmarked under dissection scope magnification (Olympus SZ-PT). The thoracic aorta was then transected at the landmarks, excised by gentle lifting at the caudal end with careful dissection to separate from the adjacent vertebrae, and rinsed in HEPES-buffered saline solution (HBSS) supplemented with 0.02% sodium azide. In a small puddle of HBSS that maintains hydration and lumen patency, the aorta held firmly gently in place on a glass plate via the cephalad end with a cotton swap. The adherent adventitial was then removed by a repetitive, gentle "rolling and stroking" action with an un-treated cotton swab (cephalad to caudad strokes). Any residual fat was removed by extremely cautious dissection with a scalpel under magnification. The cleaned thoracic aortic specimen is then ligated with 4-0 braided suture to two custom-machined, externally etched 0.3 mm metal cannulae (Washington University School of Medicine Machine Shop, St. Louis, MO) mounted onto the Living Systems Instruments (LSI) CH/1/SH chamber (**see Online Figure IX**). The aorta is then extended to native in situ length by adjusting the screw-gear mounts of the CH/1 chamber that orient the cannula longitudinally, using digital Vernier caliper measurement to guide distraction. The vessel is then gently perfused (pressure <50 mmHg) with HBSS to identify "leaks" at thoracic intercostals arteries. Intercostal arteries were ligated with 0.05 mm diameter microsutures using fine forceps (see **Online Figure IX**) -- within the fluid-filled chamber and visualized under the dissecting scope -- to eliminate the possibility of leakage when pressurized. Once all sites of leakage had been identified and sutured, the chamber was moved to the stage of a Nikon inverted microscope (Nikon Eclipse Model TE2000-S) outfitted with a Sony XC-ST30 CCD digital camera, and the image clearly focused by digital projection onto the screen of a Costar video monitor (Carrollton, TX). Intraluminal pressure was applied using a peristaltic pump (PS/200 servo with pump, PM/4 pre-and post- chamber in-line pressure transducers) with native proximal – distal direction fluid flow through the aorta at rates of 0 to 5 ml/min. Changes in outer vessel diameter were measured using the V94 Video Dimension Analyzer. Manual calibration and loading of the scan line with an over-stage micrometer slide was performed prior to each day's measurements (maximum of 2 aortas per day) as per the manufacturer's instructions. The change in thoracic aortic diameter (microns) per mmHg increase in pressure loading over the range of 0 mmHg to 50 mm Hg was measured twice for each aorta, averaged, and recorded. Aortic distensibility data is expressed as the mean +/- S.E. aortic diameter change in microns per mm Hg pressure load.

Quantitative assessment of aortic superoxide levels -- The chemiluminescent substrate lucigenin was utilized to quantify aortic oxidative stress – viz., aortic superoxide – following a modification of previously described methods[24,25]. Briefly, following euthanasia by exsanguination under ketamine-xylazine anesthesia, aortic segments were resected en bloc from diaphragm through the aortic arch, rinse 5 times with intermittent blotting on Kimwipe to

remove adherent blood and coagulum, and aorta placed in 2 mL of Krebs-Ringer bicarbonate buffer on ice until all animal processed. Each aorta is divided into 2 mm segments which are individually incubated in 100 uL of Krebs-Ringer buffer within a 96-well white luminometry microplate (Berthold #23300). Subsequently, 100 uL of 10 uM (2X) lucigenin in Krebs-Ringer bicarbonate buffer added with mixing to each well, and the plate allow to equilibrate in the dark within the luminometer at room temperature for 10 minutes. Subsequently, photons are counted in the Centro XS3 LB 960 Berthold luminometer, integrating light emitted from all aortic segments over a period of 60 seconds. Readings are repeated 3 to 4 times to ensure stability of signal, and the penultimate measurement is used to quantify ROS. Background signal is determined by integrating readings from blank wells containing 200 uL of 5 uM lucigenin in Krebs-Ringer buffer.

Quantitative gelatin zymography of cultured primary cells[26] – Quantitative zymography was used to assess collagenase activity using techniques we've previously detailed[26]. Before each analysis, 0.5 million aortic VSMCs obtained from either SM-caPTH1R;LDLR+/- or LDLR+/- siblings were plated on a 15 cm tissue culture dish, grown until confluent, then seeded at high density 1.0 – 1.5 x 10^5 cells per well (12-well cluster dishes). After recovery from plating, cells were maintained in serum-free DMEM (25 mM glucose) for 24 hours or 48 hours as indicated. Twenty microliter aliquots of serum-free conditioned media were analyzed by zymography (n = 3) 6 using precast 10% Zymogram Gelatin Gels (Invitrogen - Novex) per the manufacturer's instructions (Novex technical bulletin IM-1002, Version B). With constant gentle agitation, gels were renatured for 30 minutes at room temperature, developed overnight at 37 °C, fixed and stained with Colloidal Blue (Novex technical bulletin IM-6025), extensively washed (> 20 hours) to yield uniform background signal, and digital images of stained wet gels captured using a Hewlett-Packard ScanJet 5370C bed scanner. Image analysis was performed by importing scanned JPEG images into Kodak 1D image analysis software, using absolute net pixel intensity of the pro-MMP9 zymogram with uniform rectangular region of interest to quantify metalloproteinase activity[26]. Data are expressed as net pixel intensity of the band, normalized either to mass of extracted tissue protein, or the control treatment condition as indicated. Graphed data are presented as the means ± S.E. of 3 independent replicates.

Statistical analysis – All data points are presented as the mean +/- S.E. of independent replicates (n = 3 to 22, dependent upon the assay). Each in vitro experiment was carried out at least twice. For scoring LacZ histochemical scoring, non-parametric Mann-Whitney U test was implemented. All other statistical analyses performed by one-way ANOVA or Student's t-test analysis as indicated using GraphPad InStat Software (Version 3.06 for Windows). When the ANOVA p was significant ($p < 0.05$), analysis for intergroup differences was then performed using either Student-Newman-Keuls (for all pairwise comparisons) or Dunnett's (for comparison vs. control) post-hoc tests for multiple comparisons with GraphPad Instat.

CITATIONS IN THE SUPPLEMENT

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Online Table I: Serum Biochemistries of LDLR+/- and SM-caPTH1R;LDLR+/- Transgenic Mice

Serum marker	LDLR+/-	SM-caPTH1R;LDLR+/-	p value
Calcium (mmol/L) *	2.08 +/- 0.03	2.11 +/- 0.04	0.567
PTH (pg /mL)	54.0 +/- 10.0	59.9 +/- 12.5	0.71
Osteopontin (nmol / L)	38.0 +/- 0.1	37.0 +/- 0.1	0.74
TRAP (U/L)	48.6 +/- 1.1	46.2 +/- 1.2	0.10
Type I collagen CTX C-terminal peptide (ng/ml)	20.1 +/- 3.2	24.5 +/- 4.8	0.22
8-F-Isoprostane (pg/ml)	41.0 +/- 12.3	46.2 +/- 5.1	0.41

*n = 17 – 23 per group; all other parameters 7 – 13 per group.

SUPPLEMENTARY DATA

Online Figure I: PTH(1-34) down-regulates aortic calcification in LDLR-/- mice fed high fat diabetogenic diets (HFD). LDLR-/- mice were fed HFD for 1 month[27], and subcutaneously dosed once daily for 5 days per week with either vehicle or 400 ng/gm PTH(1-34) precisely as detailed[10]. Following overnight fast, aortic calcium was extracted and quantified as outlined in the online Methods section above (n = 10 / group).

Online Figure II: PTH(1-34) down-regulates serum 8-F-isoprostane, a circulating marker of systemic oxidative stress[28], in LDLR-/- mice fed high fat diabetogenic diets (HFD). LDLR-/- mice were fed HFD for 1 months, and treated once daily 5 days per week with either s.c. vehicle or 400 ng/gm PTH(1-34) precisely as detailed[10]. Following overnight fast, serum was obtained and 8-F-isoprostane levels quantified as an index of systemic oxidative stress[28] using a commercially available EIA from Cayman as outlined in the online Methods section above[26] (n = 10 / group).

Online Figure III: High fat diabetogenic diet (HFD) upregulates aortic expression of Msx2 in male LDLR+/- mice. LDLR+/- mice were fed HFD for 4 months, and total aortic RNA extracted and analyzed for Msx2 gene expression[5,10,11]. Data are presented as the mean +/- S.E.M. relative Msx2 mRNA accumulation observed (n = 5 / group), expressed as % of 18s rRNA signal. As in LDLR-/- mice[5,11,27], HFD upregulates aortic Msx2 expression in LDLR+- mice.

Online Figure IV: High fat diabetogenic diet (HFD) induces diabetes in male LDLR+/- mice. LDLR+/- mice were fed HFD for 4 months, and fast serum glucose and cholesterol levels compared to that observed in LDLR-/- mice fed HFD for 1 month. LDLR+/-, n = 10 / group; LDLR-/-, n = 9 / group. Dashed line, the 14 mmol /L (250 mg/dL) fasting glucose threshold for diabetes in mice[29]. As in LDLR-/- mice[27], HFD induces diabetes in LDLR+/- mice, but with significantly less hypercholesterolemia.

Online Figure V: The SM-caPTH1R does not increase urinary calcium excretion. LDLR+/- and SM-caPTH1R;LDLR+/- mice were placed on HFD for 3 months, then individually house in metabolic cages with ad libitum access to water for assessment of 24 hour fasting urinary calcium and creatinine excretion. The SM-caPTH1R transgene did not increase urinary calcium excretion.

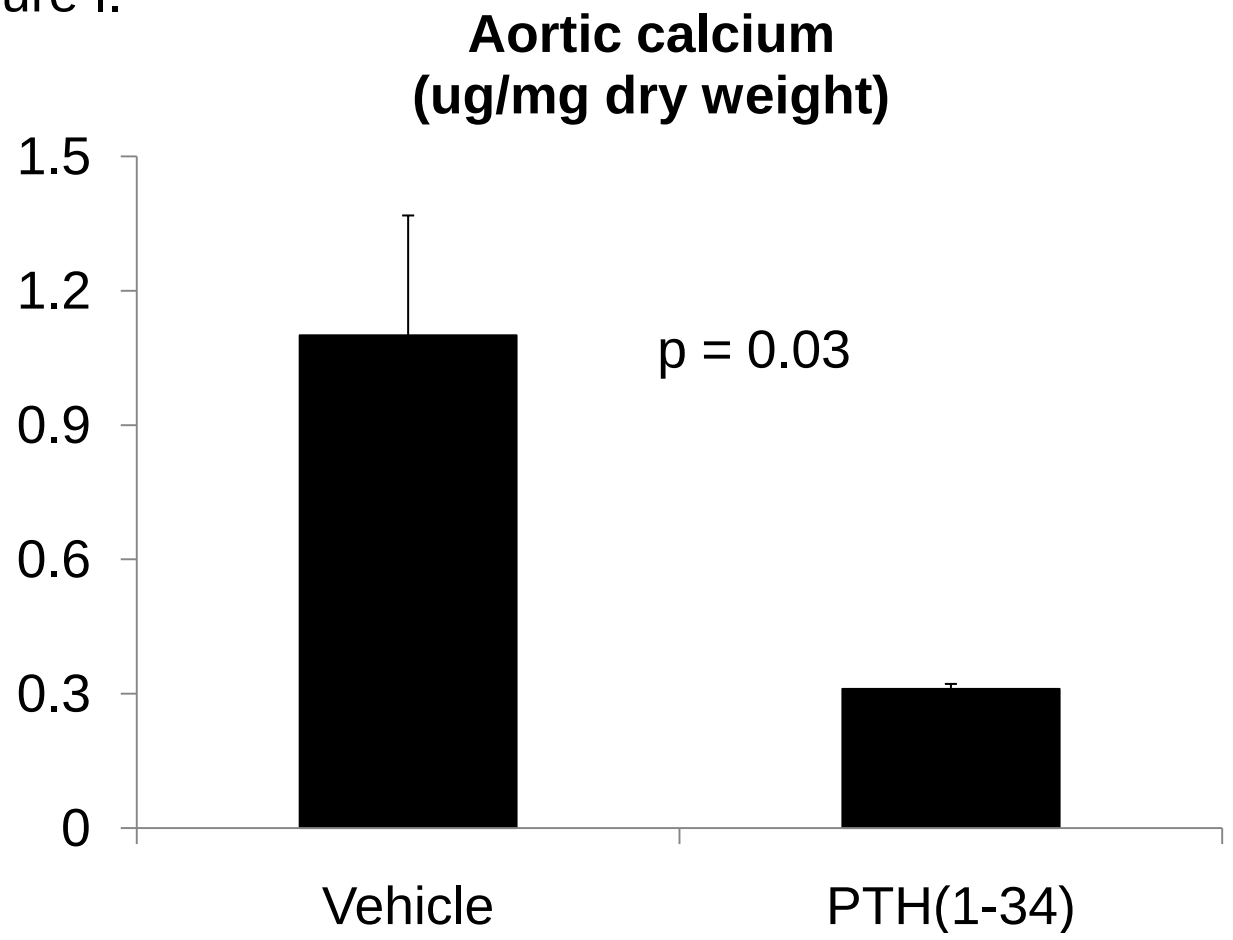
Online Figure VI: The SM-caPTH1R transgene reduces proliferation of primary aortic myofibroblasts. Primary cultures of aortic myofibroblasts were prepared as described in Methods. As a quantitative index of cell proliferation, DNA synthesis was measured using bromodeoxyuridine (BrdU) uptake and incorporation into DNA, implementing a commercially available ELISA. When compared with LDLR+/- sibling control myofibroblasts, myofibroblasts from SM-caPTH1R;LDLR+/- exhibited significantly reduced DNA synthesis.

Online Figure VII: The SM-caPTH1R transgene reduces proliferation of primary aortic myofibroblasts. Primary cultures of aortic myofibroblasts were prepared as described in Methods. As a quantitative index of cell proliferation, DNA synthesis was measured using bromodeoxyuridine (BrdU) uptake and incorporation into DNA, implementing a commercially available ELISA. When compared with LDLR+/- sibling control myofibroblasts, myofibroblasts from SM-caPTH1R;LDLR+/- exhibited significantly reduced DNA synthesis.

Online Figure VIII: The SM-caPTH1R transgene reduces aortic superoxide levels. Aortic superoxide levels were reduced in LDLR-/- mice possessing the SM-caPTH1R transgene (n = 7-11 / group).

Online Figure IX: Ex vivo aortic video plethysmography using customized cannulae on a LSI CH/1/SH vessel chamber.

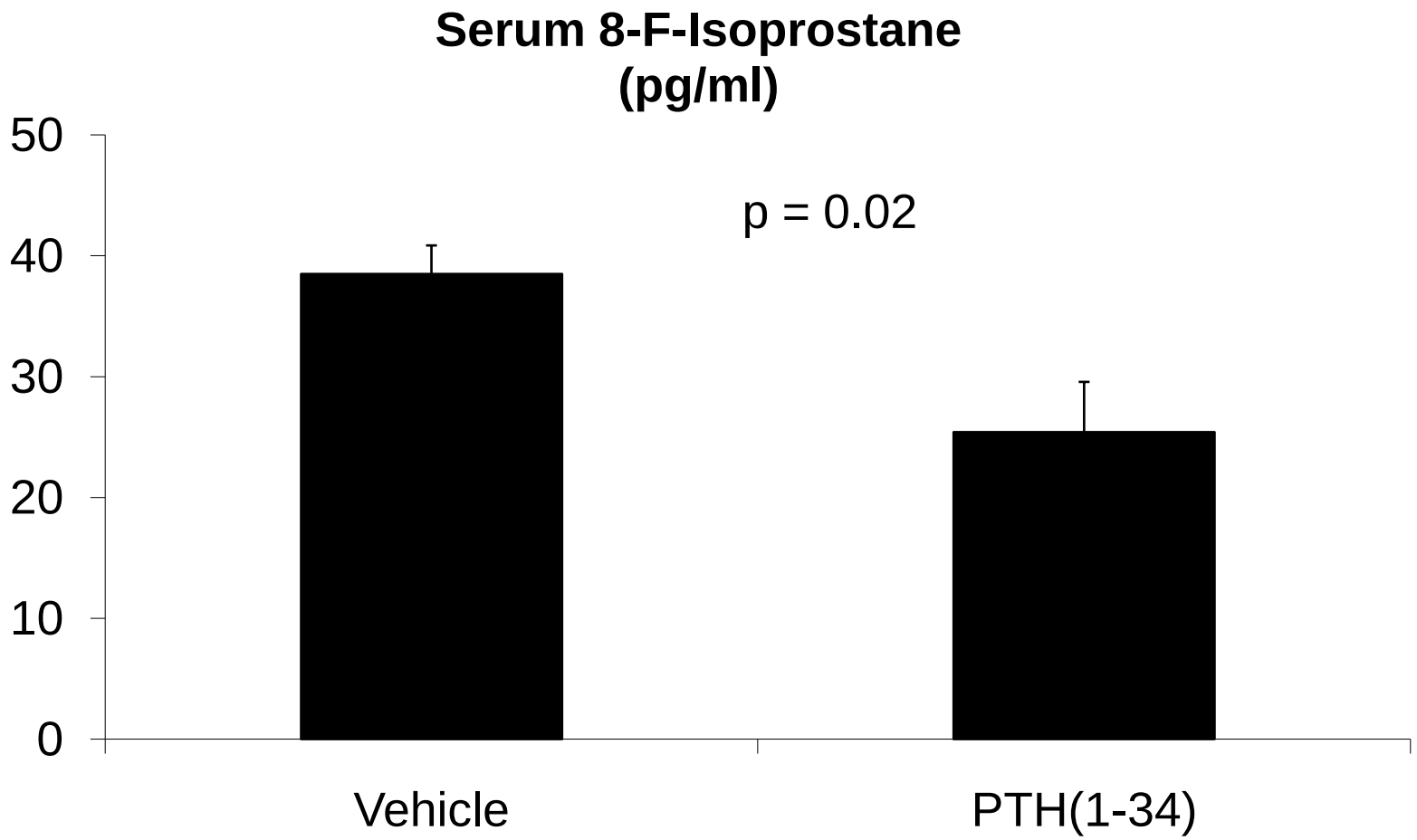
Online Figure I.



Online Figure I: PTH(1-34) down-regulates aortic calcification in LDLR-/- mice fed high fat diabetogenic diets (HFD).

LDLR-/- mice were fed HFD for 1 month[26], and subcutaneously dosed once daily for 5 days per week with either vehicle or 400 ng/gm PTH(1-34) precisely as detailed[10]. Following overnight fast, aortic calcium was extracted and quantified as outlined in the online Methods section above (n = 10 / group).

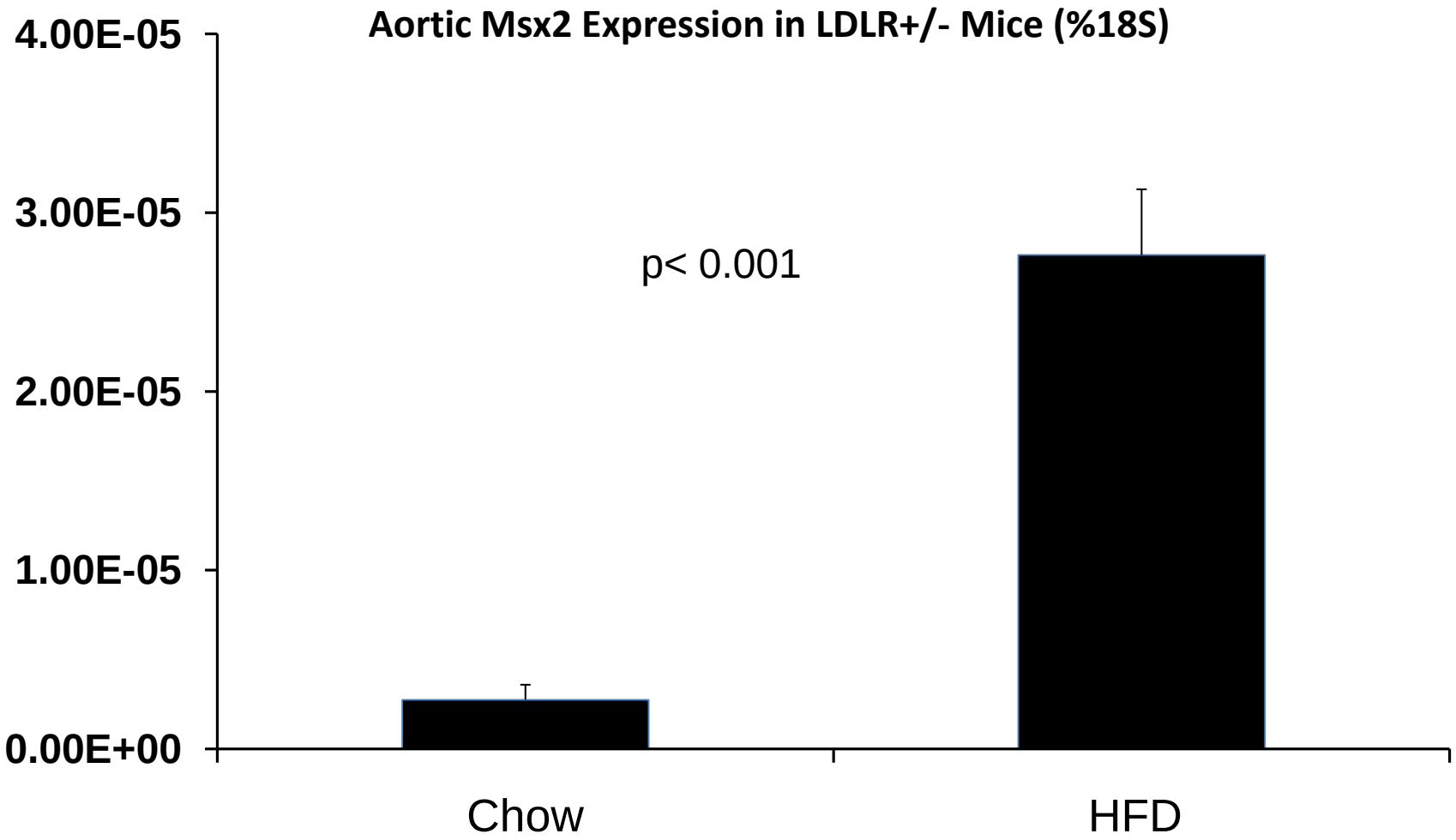
Online Figure II



<u>Online Figure II:</u> PTH(1-34) down-regulates serum 8-F-isoprostane, a circulating marker of systemic oxidative stress[27], in LDLR-/- mice fed high fat diabetogenic diets (HFD).

LDLR-/- mice were fed HFD for 1 months, and treated once daily 5 days per week with either s.c. vehicle or 400 ng/gm PTH(1-34) precisely as detailed[10]. Following overnight fast, serum was obtained and 8-F-isoprostane levels quantified as an index of systemic oxidative stress[27] using a commercially available EIA from Cayman as outlined in the online Methods section above[25] (n = 10 / group).

Online Figure III



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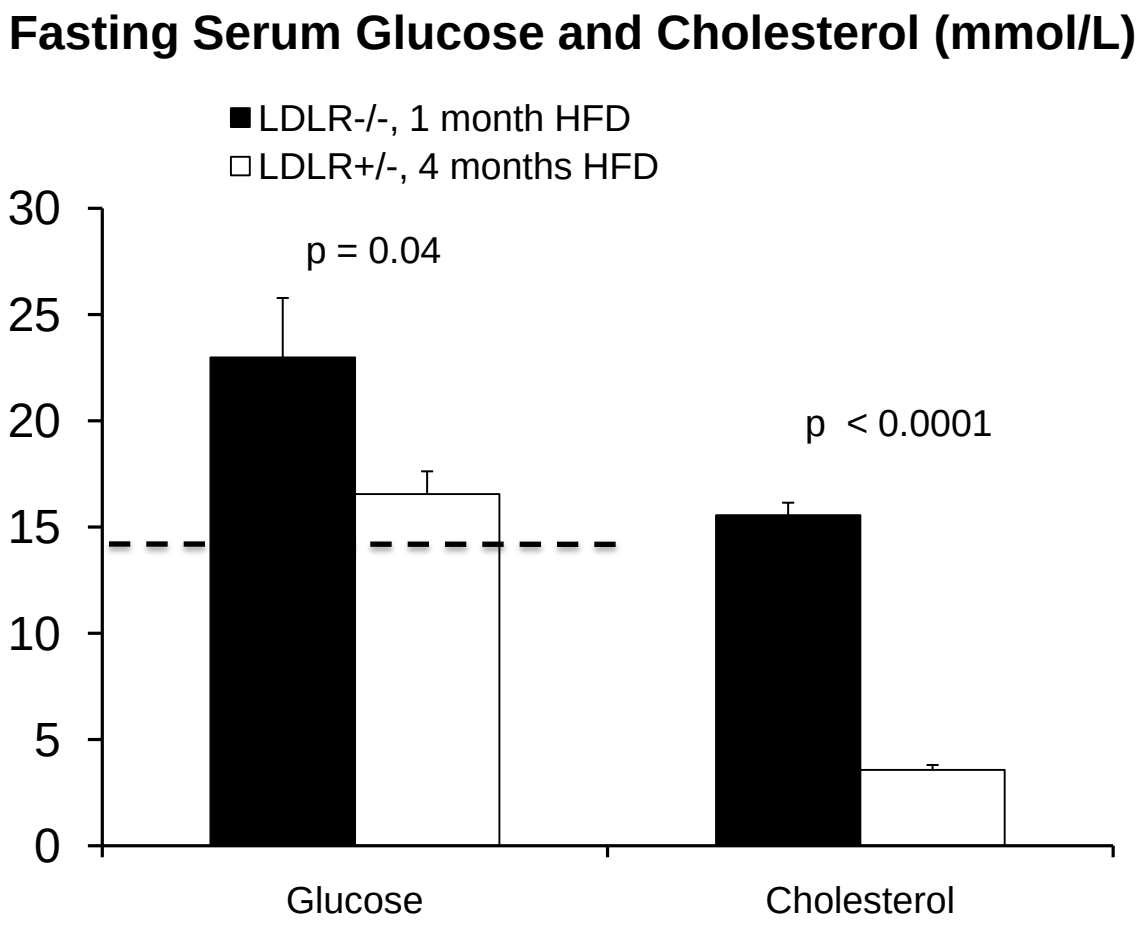
LDLR+/- mice were fed HFD for 4 months, and total aortic RNA extracted and analyzed for Msx2 gene expression[5, 10, 11]. Data are presented as the mean +/- S.E.M. relative Msx2 mRNA accumulation observed (n = 5 / group), expressed as % of 18S rRNA signal. As in LDLR-/- mice[5, 11, 26], HFD upregulates aortic Msx2 expression in LDLR+- mice.

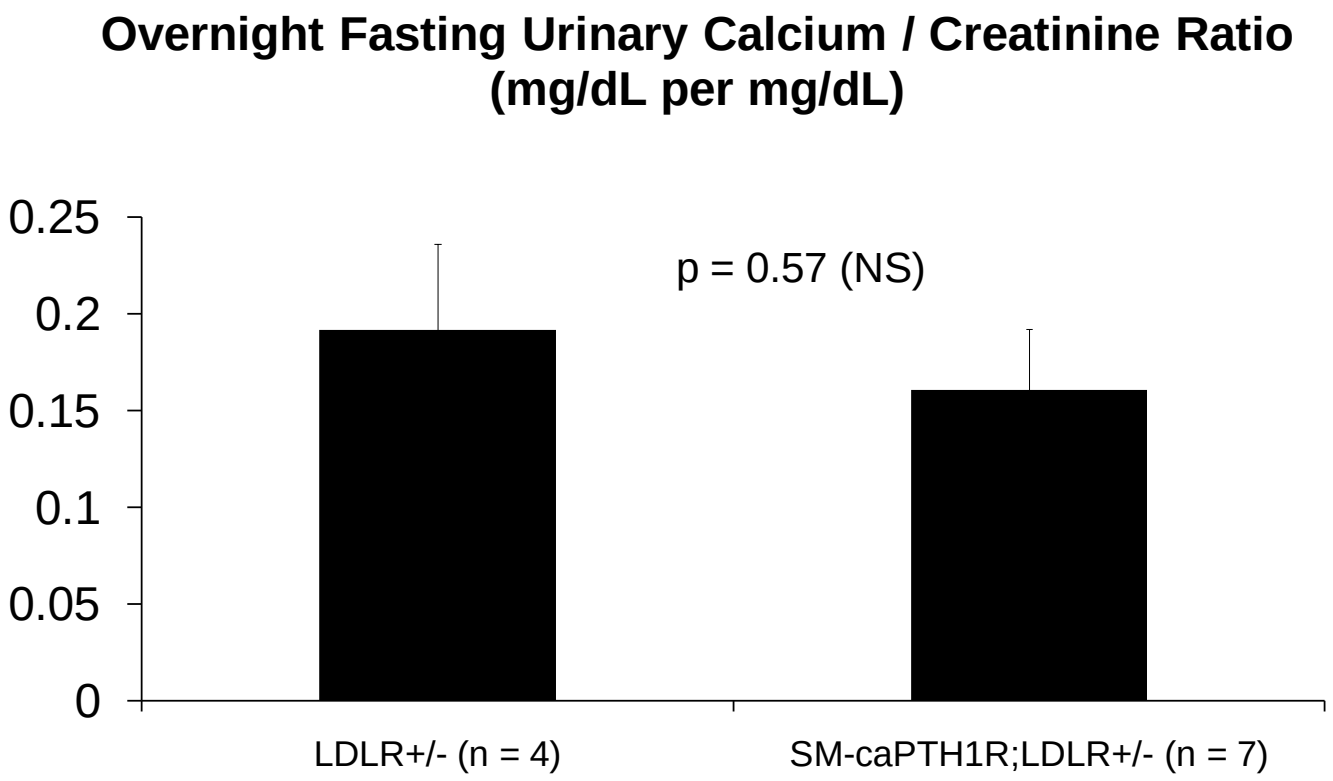
Online Figure IV



Online Figure IV: High fat diabetogenic diet (HFD) induces diabetes in male LDLR+/- mice.

LDLR+/- mice were fed HFD for 4 months, and fast serum glucose and cholesterol levels compared to that observed in LDLR-/- mice fed HFD for 1 month. LDLR+/-, n = 10 / group; LDLR-/-, n = 9 / group. Dashed line, the 14 mmol /L (250 mg/dL) fasting glucose threshold for diabetes in mice[28]. As in LDLR-/- mice[25], HFD induces diabetes in LDLR+/- mice, but with significantly less hypercholesterolemia.

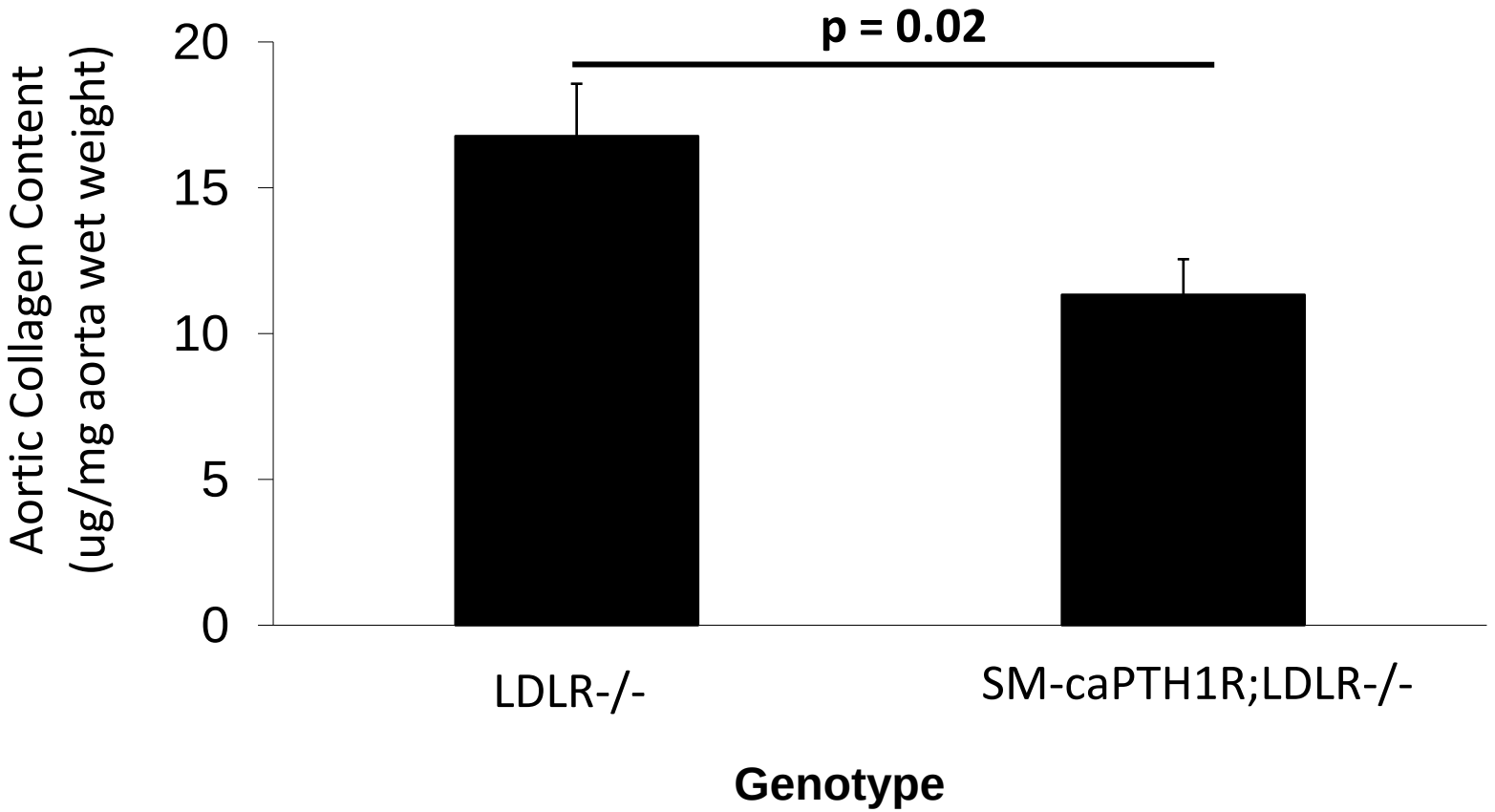
Online Figure V



Online Figure V: The SM-caPTH1R does not increase urinary calcium excretion.

LDLR+/- and SM-caPTH1R;LDLR+/- mice were placed on HFD for 3 months, then individually house in metabolic cages with ad libitum access to water for assessment of overnight fasting urinary calcium and creatinine excretion. The SM-caPTH1R transgene did not increase urinary calcium excretion.

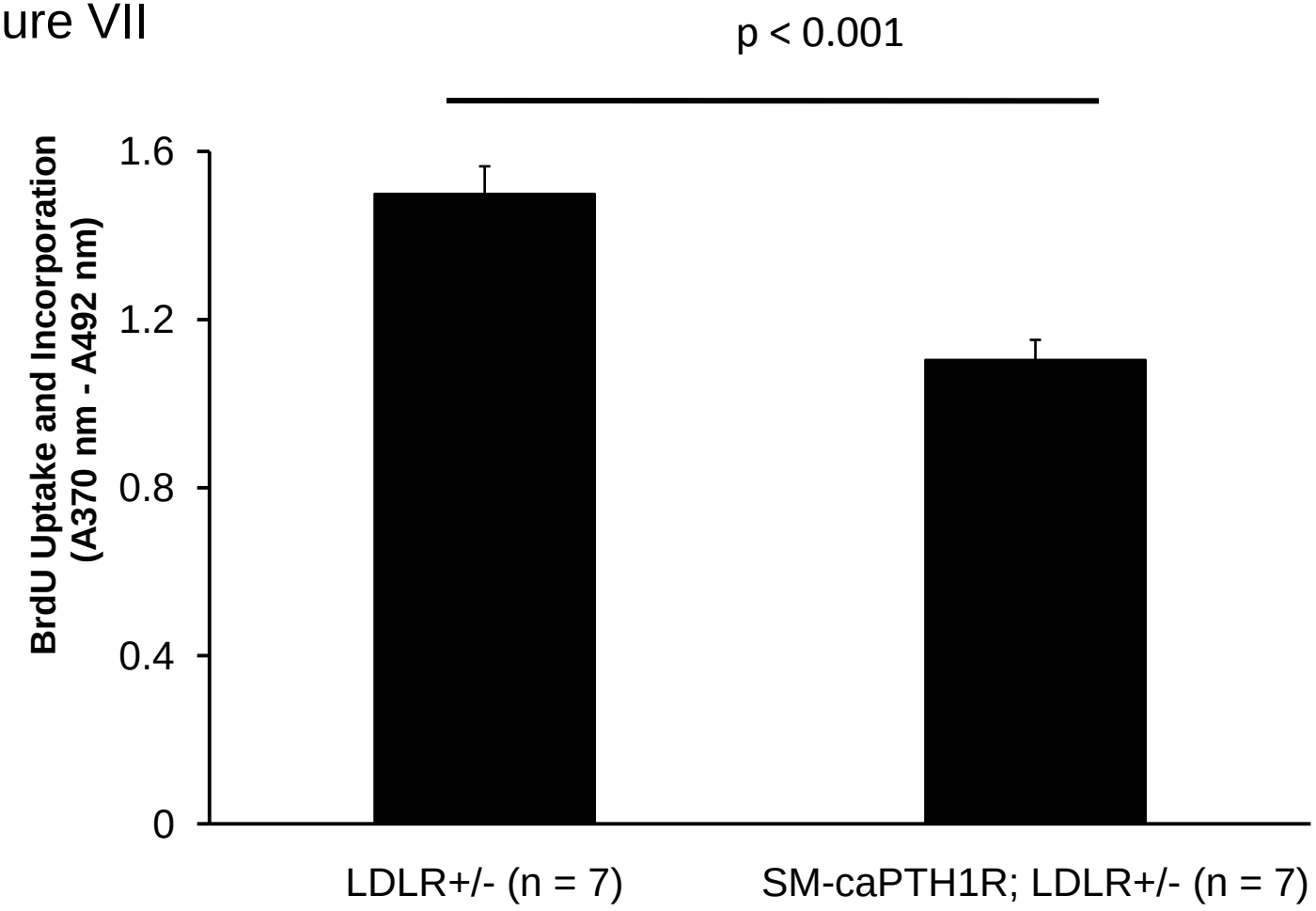
Online Figure VI



Online Figure VI: The SM-caPTH1R transgene reduces aortic collagen content.

Aortic collagen content was also decreased in LDLR-/- mice possessing the SM-caPTH1R transgene (n = 12-14 / group, three months HFD).

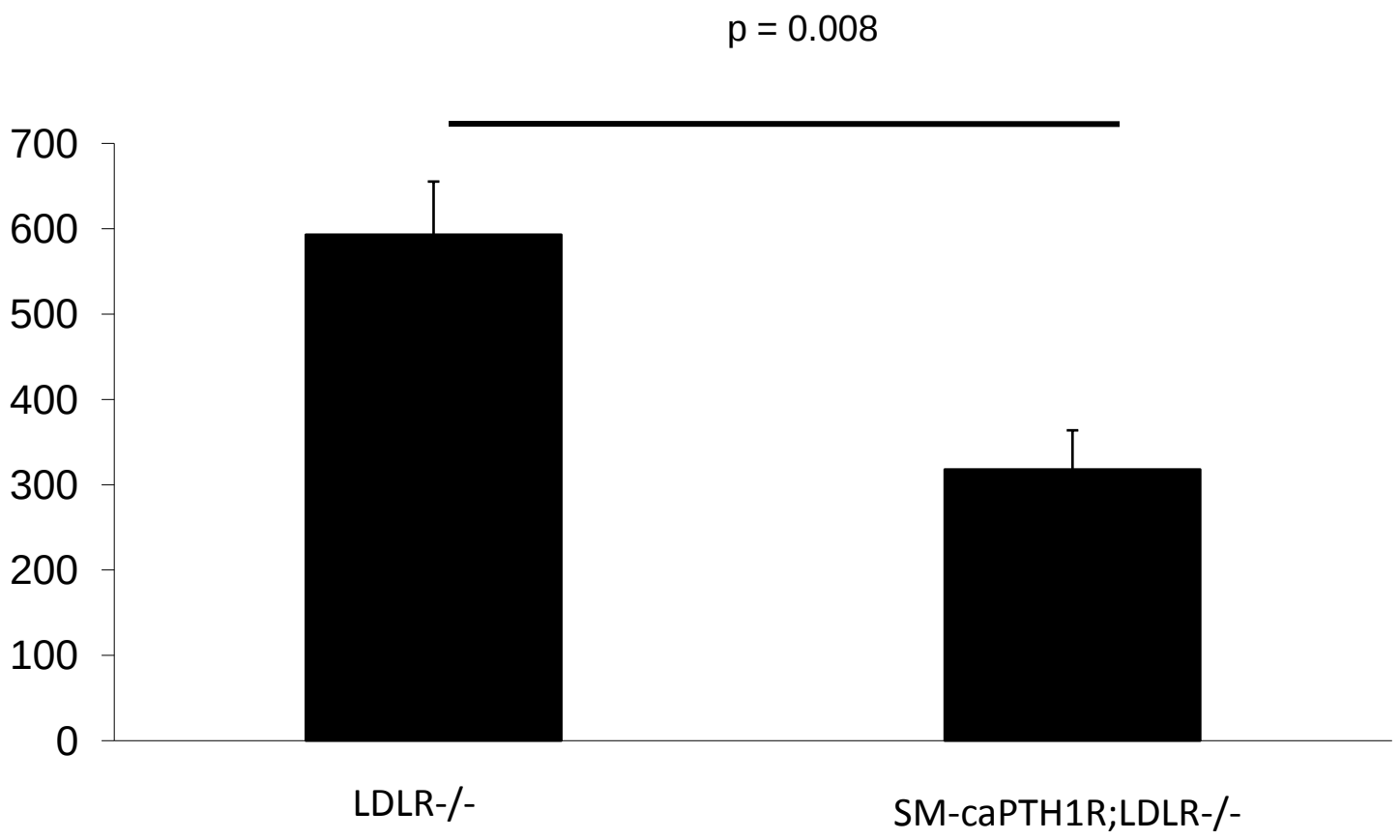
Online Figure VII



Online Figure VII: The SM-caPTH1R transgene reduces proliferation of primary aortic myofibroblasts

Primary cultures of aortic myofibroblasts were prepared as described in Methods. As a quantitative index of cell proliferation, DNA synthesis was measured using bromodeoxyuridine (BrdU) uptake and incorporation into DNA, implementing a commercially available ELISA. When compared with LDLR+/- sibling control myofibroblasts, myofibroblasts from SM-caPTH1R;LDLR+/- exhibited significantly reduced DNA synthesis.

Online Figure VIII

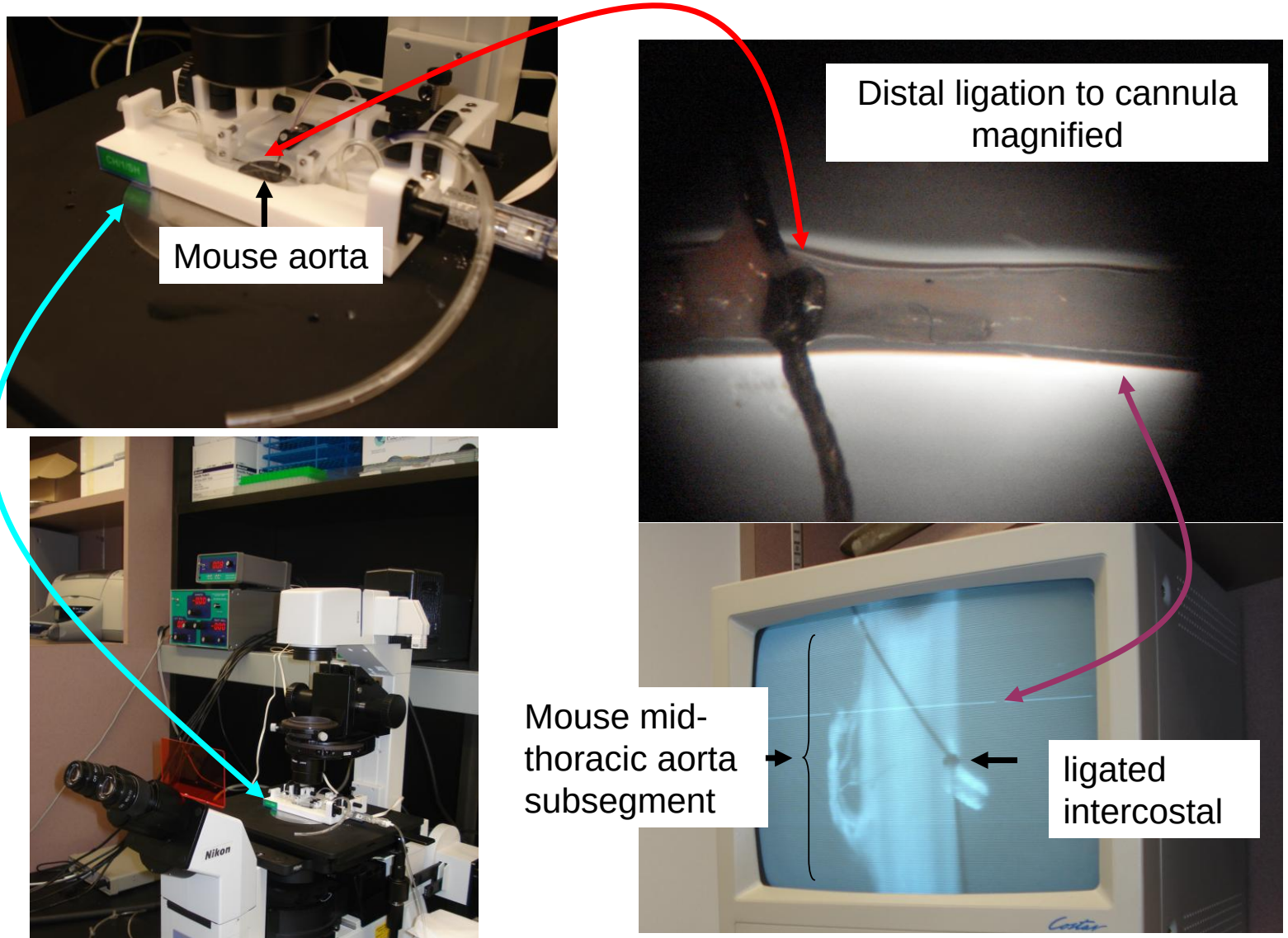


Online Figure VIII: The SM-caPTH1R transgene reduces aortic superoxide levels.

Aortic superoxide levels were reduced in LDLR-/- mice possessing the SM-caPTH1R transgene (n = 7-11 / group).

Online Figure IX

Ex vivo aortic video plethysmography



Video microscopy image of mouse thoracic aorta with ligated intercostal artery