SUPPLEMENTAL MATERIAL

Deficiency of natriuretic peptide receptor 2 promotes bicuspid aortic valves, aortic valve disease, left ventricular dysfunction, and ascending aortic dilatations in mice

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Detailed Methods

Unless otherwise noted, all materials were purchased from Sigma-Aldrich (Oakville, Ontario, Canada).

Mice

All animal procedures were approved by the Animal Care Committees of the University of Toronto and The Centre for Phenogenomics, in accordance with guidelines of the Canadian Council on Animal Care. Heterozygous Npr2+/− (JAX STOCK Npr2tm1Gar/J; #007658) and homozygous Ldlr−/− (JAX B6.129S7-Ldlrtm1Her/J; #002207) mutant mice were obtained from The Jackson Laboratory (JAX; Bar Harbor, ME) and housed in a pathogen-free facility. Npr2+/− mice were obtained from JAX on a mixed 129/B6 background1 at N2 to C57Bl/6 and were subsequently backcrossed with C57Bl/6J mice (JAX #000664) to N3 prior to experimental use. Npr2+/− mice (N3 to C57Bl/6) were then bred to Ldlr−/− mice to generate Npr2+/−;Ldlr−/− mice (Npr2tm1Gar;Ldlrtm1Her; N4 to C57Bl/6 at experimental use). Due to well-defined mortality and infertility in homozygous Npr2 mutants,1 Npr2 mice were maintained via heterozygous sibling breeding, while Npr2;Ldlr mice were maintained by breeding Npr2+/−;Ldlr−/− siblings together. Mice were genotyped for Npr2 by the standard polymerase chain reaction (PCR) genotyping protocol for stock number 007658 (version 1.0, The Jackson Laboratory). In brief, DNA was isolated from neonatal tail clips by alkaline lysis with 25 mM NaOH/0.2 mM EDTA at 98°C for 1 hour followed by neutralization with 40 mM Tris HCl. PCR was performed with JAX primers 9950 (common forward): 5’-CGGCTATCAGGCTCAGTTTT-3’, 9951 (wild-type reverse): 5’-CAGCATTCTGGAGGCTAAGG-3’, and oIMR7415 (mutant reverse): 5’-GCCAGAGGCCACTTGTGTAG-3’. Bands were produced by the wild type allele (Npr2+) at 490 bp and by the mutant allele (Npr2tm1Gar) at 234 bp. Mice were genotyped for Ldlr by PCR using DNA from tail clips as above, with primers P15 (wild-type forward): 5’-AAGACGTGCTCCCAGGATGACTTC-3’, P16 (common reverse): 5’-GTGCTCCTCATCTGACTTGTCCCTTG-3’, and P17 (mutant forward): 5’-CGCATTGCTGAGTAGGTGTCATTC-3’. Bands were produced by the wild type allele (Ldlr+) at 377 bp and by the mutant allele (Ldlrtm1Her) at 274 bp.

Quantitative Real Time PCR

Neonatal mouse hearts or lungs (postnatal day 4-5) were dissected and stored in RNAlater (Qiagen, Valencia, CA) at 4°C overnight. RNA was extracted using the RNeasy Fibrous Tissue Mini Kit, quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Mississauga, Canada), with reverse transcription using the Superscript III kit (Thermo Fisher Scientific). Transcript expression of wild type (Npr2+) mRNA (targeting within exons 3-7 of accession number: NM_173788.3, forward
primer: 5’ - TGGCCCCATCCCTGATGAAC - 3’ and reverse primer: 5’ - CATCTTCCCTGGTACCCCCT - 3’ in Npr2+/+, Npr2+-/-, and Npr2-/- mice was quantified by real-time polymerase chain reaction using SYBR green for detection (SsoAdvanced, Bio-Rad Laboratories, Mississauga, Canada) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh; accession number: NM_001289726, forward primer: 5’ - GACAACCTTGCCATGTCGGAAGG - 3’ and reverse primer: 5’ - ACCAGTGATGCGAGGTATG - 3’) as the housekeeping gene on a CFX384 real-time PCR detection system (Bio-Rad Laboratories). Gene expression was quantified by the delta-delta Ct method (2-ΔΔCt).

**Primary Cell Isolation & Culture**

Primary porcine aortic VICs were isolated from normal aortic valves from 8-month-old pigs immediately after death (Fearmans Pork, Burlington, Canada) by collagenase digestion as described previously. VICs were seeded on uncoated tissue-culture-treated polystyrene (TCPS) at 10,000 cells/cm² and media was changed every two days unless noted otherwise. Primary mouse lung fibroblasts were isolated from neonatal (postnatal day 4-5) male and female mice by collagenase digestion. In brief, mouse pups were euthanized by decapitation and lungs were removed, rinsed, pooled by genotype, and stored in ice-cold phosphate buffered saline (PBS) containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 μg/ml Amphotericin B (Gibco, Burlington, Canada). Lungs were rinsed in sterile PBS, minced with scissors, and digested by 300 units/ml collagenase IV (Gibco) and 0.2% trypsin-EDTA reconstituted in PBS for 80 minutes at 37°C, with vortexing for 20 seconds every 20 minutes and a 50% volume addition of collagenase/trypsin solution after 40 minutes of incubation. After 80 minutes, an equal volume of complete medium (Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) (Gibco), 100 units/ml penicillin, 100 μg/ml streptomycin) was added, lungs were vortexed for 1 minute, and the cell suspension was collected by removing undigested tissue pieces with 70 μm and 40 μm cell strainers. Cells were pelleted, re-suspended in complete medium, seeded on uncoated tissue-culture-treated polystyrene (TCPS) at 10,000 cells/cm², and media was changed every two days unless noted otherwise. In all experiments, VICs and lung fibroblasts were cultured in complete medium at 37° C and 5% CO₂ unless noted otherwise.

**Flow Cytometry**

Freshly isolated porcine VICs were grown to 80% confluency, serum starved in 0.1% FBS for 24 hours, then cultured with with/without CNP (CNP-22, 100 nM in water) (Bachem, Torrance, CA) and/or the specific NPR2 inhibitor P19 (0.5 μM, in water) (Phoenix, Burlingame, CA) or the specific NPR3 inhibitor AP-811 (100 nM, in water) (California Peptide, Salt Lake City, UT) for 4 days. αSMA expression was quantified by flow cytometry. In brief, VICs were detached with HyQTase (SV30030, GE Healthcare Life Sciences, Pittsburgh, PA), fixed in 1% paraformaldehyde (PFA) for 15 minutes at room temperature, permeabilized in ice-cold methanol, and immuno-labelled with FITC-conjugated mouse monoclonal anti-αSMA primary antibody (F3777, Sigma-Aldrich) or FITC-conjugated mouse monoclonal IgG2a antibody (F6522, Sigma-Aldrich) as control at 1:250 in cell staining buffer (1X PBS with 1% BSA, 0.1% saponin and 0.01% sodium azide). Cells were analyzed on a FACSCanto (BD, Mississauga, Canada) with an argon-ion 488 nm laser beam.

**cGMP Enzyme Immunoassay**

Intracellular cGMP levels were assayed using a cGMP Direct Biotrak competitive enzyme immunoassay (RPN226, GE Healthcare Life Sciences). Primary porcine VICs were pre-treated with 0.5 mM IBMX for 15 minutes prior to CNP treatment (0-1000 nM for 10 minutes). VICs were then lysed, acetylated, and incubated in microplate wells with rabbit anti-cGMP antibody and standard amounts of cGMP conjugated to horseradish peroxidase (as per manufacturer’s recommended protocol #3). Donkey anti-rabbit antibodies coating the wells held the antibody–antigen complex in place while color development proceeded with the addition of 3,30,50,50-tetramethylbenzidine/H₂O₂. Reactions were stopped by addition of 1 M sulphuric acid and absorbance was recorded at 450 nm.
cGK1 Activity Assay
Primary porcine VICs were assayed for cGK1 activity using the CycLex Cyclic GMP dependent protein kinase (cGK) Assay Kit (CY-1161, CycLex, Nagano, Japan). In some experiments, VICs were treated with CNP (100 nM) for 0-30 minutes. In other experiments, VICs were treated with CNP (100 nM), and/or the specific cGK1 inhibitor Rp-8-pCPT-cGMPS (C240, “Rp-cGMP”, 30 μM, Sigma-Aldrich) or the specific cGK1 activator 8-pCPT-cGMPS (C5438, “cGMP”, 100 μM, Sigma-Aldrich) for 10 minutes. VICs (5x10⁶) were then harvested, lysed in 50 μl of the cell lysis buffer, and protein concentrations were equalized for each condition. For each well, 10 μl of cell lysate was combined with 90 μl of kinase reaction buffer containing cGMP and incubated for 1 hour at 30°C. After washing wells, 100 μl of horseradish peroxidase-conjugated anti-phospho-G-kinase substrate antibody was added to each well and incubated for 1 hour at room temperature. 100 μl of tetramethylbenzidine was added to each well and incubated for 15 minutes at room temperature; absorbance was then measured at 450 nm.

Alcian Blue Guanidine-HCl Solubilization
Freshly isolated porcine VICs were cultured for 5 days with or without CNP (1 μM) and/or TGF-β1 (5 ng/ml), and synthesized proteoglycan in the culture media was quantified using a modified Alcian blue guanidine-HCl solubilisation assay. In brief, 1% Alcian blue stock solution was prepared using 3.822 g of guanidine-HCl, 100 μl of H₂SO₄, 1 g of Alcian blue 8GX, and 100 ml of distilled H₂O. Guanidine-HCl-propanol was prepared with 27.64 g of guanidine-HCl, 6.67 ml of 1-propanol, 13.33 ml distilled H₂O, and 50 μl of Triton X-100. 50 μl of culture media were combined with 50 μl of 8 M guanidine-HCl and incubated for 15 minutes at room temperature. 50 μl of SAT reagent (9% v/v glacial acetic acid + 0.75% v/v Triton X-100) was added and incubated for 15 minutes at room temperature, then 750 μl of Alcian blue working solution (33.3% v/v SAT reagent, 60% v/v Alcian blue stock solution) and incubated for a further 15 minutes at room temperature, then centrifuged at 12,000g for 30 minutes. The supernatant was then removed, pellets were resuspended in 50 μl of DMSO, and centrifuged at 12,000g for 30 minutes. The supernatant was again removed, 500 μl of guanidine-HCl-propanol were added, and incubated on a shaker overnight at room temperature. Absorbance was then measured at 620 nm.

Immunofluorescence
Freshly isolated neonatal mouse lung fibroblasts were cultured in complete DMEM supplemented with TGF-β1 (5 ng/ml) for 72 hours with/without CNP (100 nM). Cells were then fixed in 10% neutral buffered formalin, permeabilized with 0.1% Triton X-100 for 10 minutes, blocked with 3% BSA for 30 minutes at 37°C, incubated with Cy3-conjugated anti-αSMA primary antibody (C6198, 10 μg/ml, Sigma-Aldrich) in 3% BSA for 30 minutes at 37°C, and counter-stained in Hoescht. Five 40x field-of-views were imaged per well using an Olympus IX71 microscope (Olympus, Richmond Hill, Canada), QImaging Retiga 2000R, and QCapture v. 2.9.13 (Quantitative Imaging Corporation, Surrey, Canada), and cells with/without αSMA-positive stress fibers were counted.

Traction Force Microscopy
Traction force microscopy methods including gel fabrication/functionalization, imaging, and analysis are as follows: In brief, freshly isolated Npr2⁺/⁺ and Npr2⁺/- mouse lung fibroblasts were seeded directly on 11 kPa collagen-coated polyacrylamide (PA) gels (modified from Chen et. al.) and treated with/without CNP (1 μM). Gels were fabricated in glass-bottom Petri dishes with a microwell diameter of 20 mm, overall diameter of 35 mm, and #0 cover glass bottom (D35-20-0-N, In Vitro Scientific, Sunnyvale, CA). The cover glass was treated with 0.1 M sodium hydroxide for 10 minutes, and then with (100% 3-aminopropyl)trimethoxysilane (3-APTMS; 281778, Sigma-Aldrich), which was removed after 3 minutes. The glass was thoroughly rinsed with deionized water to remove all trace of 3-APTMS. The cover glass was treated with 1% solution of glutaraldehyde (340855, Sigma-Aldrich) and left to incubate for 30 minutes at room temperature, after which the solution was removed and glass thoroughly rinsed with deionized water again. PA substrates with 11 kPa elastic modulus as determined by compression testing
were obtained by mixing 8% acrylamide (161-0140, Bio-Rad), 0.2% bis-acrylamide (161-0142, Bio-Rad), and 10 mM HEPES buffer (H0887, Sigma-Aldrich). Polymerization was initiated by adding 1/200 (v/v) 10% ammonium persulfate (215589, Sigma-Aldrich) and 1/2000 (v/v) n,n,n',n'-tetramethylethlenediamene (161-0800, Bio-Rad) and mixed by pipetting up and down. Fluorescent beads (0.18-μm diameter; excitation maximum of 441 nm and emission maximum of 485 nm; 9003-53-8, Polysciences Inc., Warrington, PA) were added to the gel solution (1/150 v/v) immediately after addition of the polymerizing agents and mixed thoroughly to ensure uniform distribution of the beads. 4 μL of the gel solution was pipetted on the treated cover glass in the microwell of the Petri dish. Polymerized gels were activated for protein conjugation by placing them in 0.5 M N-sulfosucciniidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (sulfo-SANPAH, CovaChem, Loves Park, IL) solution in 0.25% dimethyl sulfoxide (D2650, Sigma-Aldrich), 50 mM HEPES and deionized water, and exposed to UV light for 10 minutes. Photoactivation was repeated with fresh sulfo-SANPAH solution. The gels were thoroughly rinsed with 50 mM HEPES, and then sterilized under UV for 30 minutes. The gels were incubated with 100 μg/mL of rat tail type I collagen (354236, BD) in PBS at 4 °C overnight. The collagen-functionalized gels were rinsed three times with PBS prior to cell seeding.

24 hours after seeding, fibroblasts were rinsed twice with PBS to remove red blood cells. Prior to imaging after 24-72 hours, fibroblasts were stained with 5 μM CellTracker Red (Thermo Fisher Scientific) for 30 minutes at 37°C, then rinsed with PBS and cultured in complete DMEM. Fibroblasts were imaged on a Nikon Ti-Eclipse inverted confocal microscope using a heated stage at 37°C and a 60x oil immersion objective. A Z-stack of the cell and embedded fluorescent beads was captured, and cells were then removed from the substrate to return the gel to its initial, undeformed state using 10 μL of 10% SDS. After 90 seconds, another Z-stack was captured. Images from before and after the SDS cell lysis were extracted. Three custom ImageJ plugins were used to calculate the displacement and traction fields generated by the fibroblasts. First, the images were aligned in the xy-plane using the Template Matching and Slice Alignment plugin, using the normalized correlation coefficient algorithm with the subpixel registration option checked. Second, the displacement of the beads was tracked using the Particle Image Velocimetry (PIV) plugin. Lastly, the traction field was calculated using the Traction Force Microscopy plugin, which uses the Fourier transform traction cytometry (FTTC) method with zero-order Tikhonov regularization, as described previously.

Notch Activation
Notch ligand was immobilized to tissue culture plates as previously described. In brief, 48-well plates were incubated at 37°C for 30 minutes with 100 μl/well of 20 μg/ml of goat anti-human IgG antibody in PBS/- (AP113, EMD Millipore, Billerica, MA), rinsed with PBS/-, incubated with DMEM at 37°C for 30 minutes, and rinsed with PBS. Wells were then incubated overnight at 4°C with either 10 μg/ml of recombinant human Jagged1 Fc chimera protein (“hr-Jag1-Fc”,1277-JG, R&D Systems, Minneapolis, MN) or recombinant human IgG1 Fc (“hr-IgG1-Fc”, 110-HG, R&D Systems) in PBS/-. Immediately before seeding at subconfluence on coated plates, hr-Jag1-Fc or hr-IgG1-Fc solutions were removed, and primary porcine VICs or primary mouse lung fibroblasts were then seeded and treated with with/without CNP (CNP-22, 100 nM in water) and/or the specific NPR2 inhibitor P19 (0.5 μM, in water) or the specific NPR3 inhibitor AP-811 (100 nM, in water). After 2 hours of treatment, cells were fixed and stained for NICD (1° = rabbit polyclonal ab8925, Abcam, 1:800, overnight at 4°C; 2° = goat anti-rabbit Alexa Fluor 568, A11011, 1:200, Thermo Fisher Scientific), imaged, and quantified (cells with/without positive nuclear NICD) as above. RNA was also extracted from cultured neonatal mouse lung fibroblasts 24 hours after seeding on Jag1-coated or IgG-coated well plates using the RNeasy Mini Kit (Qiagen), and transcript expression of Hey1 (forward primer: 5' - CCGACGAGACCAGAATCAATAAC - 3' and reverse primer: 5' - TCAGGTGATCCACAGTCATCTCTG - 3'), Hes1 (forward primer: 5' - GCTACCAGCCAGTGTC - 3' and reverse primer: 5' - AAGTGCAATCCAAAATCAGTGT - 3'), Snail1 (forward primer: 5' - CTTGTGTCCTGCAGGACCCTG - 3' and reverse primer: 5' - CTTCACATCCAGTGGGT - 3'), Notch1 (forward primer: 5' - GCCAGCAGCAGATGTATGAA - 3')...
3’ and reverse primer: 5’ - GCCTGACACCTCTGGACAAC - 3’), Dll4 (forward primer: 5’ - ACCTTTGGCAATGTCTCCAC - 3’ and reverse primer: 5’ - GTCTGCCTCCCCGGTGTGTG - 3’), Jagl (forward primer: 5’ - TGGTATTTTGTTACGGCCAGT - 3’ and reverse primer: 5’ - GTACTGCGACAAGTGACATCC - 3’), Nppe (forward primer: 5’ - ATCGGTCTCCCTGAGATTG - 3’ and reverse primer: 5’ - GTCGTCTCCCGGTGTGTG - 3’), Jag1 (forward primer: 5’ - TGGTATTTGTCAGGCCCAGT - 3’ and reverse primer: 5’ - GTACTGCGACAAGTGACATCC - 3’), Npr2 (forward primer: 5’ - ATCGGTCTCCCTGAGATTG - 3’ and reverse primer: 5’ - GTCGTCTCCCGGTGTGTG - 3’), or Npr3 (forward primer: 5’ - AGTAGAAGGCCATTAGCAAGC - 3’ and reverse primer: 5’ - GGCCCTTTGAAACTGAGACT - 3’) was quantified by delta-delta Ct with the housekeeping gene Gapdh as above.

**Mouse Studies**

All mice used in diet studies were male. At 21 days after birth, all mice were weaned and fed a normal diet (ND; Harlan Teklad Global 2918, 6.2% fat by weight, 0% cholesterol by weight, 18% kcal/g from fat; Harlan, Indianapolis, Ind). At 1.5 months of age, Npr2+/- mice and Npr2+/- littermate controls were randomized into groups fed the ND or Harlan Teklad TD.88137, a Western diet (WD) commonly used in dietary mouse models of CAVD10-13 that consists of 21.2% fat and 0.2% cholesterol by weight, with 42% kcal/g from fat, for a duration of 4 or 8 months (n = 6-15 per age/diet/genotype sub-group). From 1.5 months of age, Npr2+/-;Ldlr-/- mice (n = 18) and Npr2+/-;Ldlr-/- littermate controls (n = 13) were exclusively fed the WD (TD.88137) diet for a duration of 10 months. One week prior to their respective 4, 8, or 10 month endpoints, mice underwent echocardiography, and blood pressure was measured immediately prior to sacrifice at 4, 8, or 10 months of age.

**Echocardiography**

Transthoracic cardiac/valvular imaging was performed using a Vevo 2100 high frequency ultrasound system (Fujifilm VisualSonics, Toronto, Canada), with an 18-38 MHz MS400 linear array transducer (with a central frequency of 30 MHz) as described previously. Briefly, mice were anesthetized by induction for 5 minutes with 5% isoflurane and maintained at 1.5% isoflurane with an oxygen flow rate of 2.0 L/min on a thermal platform set to 42°C that maintained mouse body temperature at 37°C. Ventral chest fur was removed with depilatory cream, and Aquasonic 100 ultrasound transmission gel (Parker Laboratories, Fairfield, NJ) was applied to the chest. Imaging windows were as specified previously: the right parasternal longitudinal section RL1 was utilized for pulsed-wave and color Doppler measures of transvalvular and left ventricular outflow tract (LVOT) blood velocities; the left and right parasternal longitudinal sections LL2 and RL5 were used for B-mode measures of LVOT and aortic annulus diameters; the left parasternal longitudinal section LL4 was used for M-mode measures of LVOT dimensions; and the right parasternal longitudinal section RL4 was used for B-mode measures of ascending aorta/aortic arch diameters. Calculation of cardiac function parameters from M-mode measures of left ventricular dimensions were performed as described previously. In brief, LV mass was calculated using the uncorrected cube assumption/Penn algorithm as LV mass = 1.055*[(LVAWD + LVEDD + LVPWD)³ – (LVEDD)³], where LVAWD = left ventricular end-diastolic anterior wall thickness, LVEDD = left ventricular end-diastolic diameter, and LVPWD = left ventricular end-diastolic posterior wall thickness. Fractional shortening was calculated as FS = 100*[(LVEDD – LVESD)/LVEDD], where LVESD = LV end-systolic diameter. Ejection fraction was calculated by the Teichholz equation, as EF = 100*[(LVEDV – LVESV)/LVEDV], where LVESV = (7*LVESD³)/(2.4 + LVESD) and LVEDV = (7*LVEDD³)/(2.4 + LVEDD). To account for variability in cardiac function between experimental groups, Doppler measures of transvalvular blood velocity were expressed as the Dimensionless Index (DI), where peak velocity of the aortic jet is normalized to peak velocity of the LVOT immediately proximal to the aortic valve. Doppler measures of blood velocity were all made with the intercept angle ≤45° between the targeted vessel and ultrasound beam, and then angle-corrected. In all cases, each echocardiographic parameter was taken as the average measurement over 3 cardiac cycles.
Blood Pressure
Blood pressure measurements were performed as described previously\(^\text{20}\) with the following modifications to minimize the impact of anaesthetic effects. Briefly, mice were anesthetized by induction for 3 minutes with 3% isoflurane, placed on a thermal pad set to 37°C, and maintained at 2% isoflurane during the catheterization procedure. Ventral chest fur was removed with depilatory cream, and the right common carotid artery was catheterized using a 1.4 French blood pressure probe (Millar Instruments Inc., Houston, TX) that was passed into the ascending aorta. Data were monitored and collected using a PowerLab 4/ST data acquisition system (AD Instruments, Canada) and LabChart\(^{\text{TM}}\) acquisition software. Blood pressure was allowed to stabilize for 3 minutes at 1.5% isoflurane, and measurements were subsequently collected for 1 minute at 1% isoflurane. Data are reported as the mean values for the duration of the collection period. Values for systolic (SBP) and diastolic (DBP) blood pressure for each mouse were calculated as the average of this 1 minute of data, while mean arterial pressure (MAP) was calculated using the formula MAP = DBP + (1/3)*pulse pressure.

Blood Biochemistry
Prior to sacrifice and dissection, mice were fasted overnight (~14 hours) with free access to water, weighed, then anesthetized by induction for 5 minutes with 5% isoflurane and maintained at 2% isoflurane on a thermal pad set to 42°C. The brachial artery was severed and blood was collected in K\(_2\)EDTA Microtainer tubes (BD). Tubes were inverted 8-10x and stored on ice, then promptly centrifuged at 4°C and 4000g for 20 minutes to obtain plasma supernatant, which was then aliquotted and stored at -80°C. Plasma samples were then thawed on ice and immediately analyzed with an AU480 Chemistry System (Beckman Coulter, Brea, CA) using the Total Cholesterol (OSR6116), HDL-Cholesterol (OSR6195), and Triglycerides (OSR60118) system reagents; LDL-cholesterol levels were calculated using the Friedewald formula\(^\text{21}\): LDL-Cholesterol = Total Cholesterol – HDL-Cholesterol – (Triglycerides/5).

Tissue Collection
Mouse hearts were dissected and slowly flushed with 5 ml PBS+/- and then 5 ml of 10% neutral buffered formalin (NBF) with a 25G needle through the apex of the left ventricle. All tissues were then immersion-fixed in 10% NBF for 24 hours, weighed, and stored in 70% ethanol.

After fixation, tibias were separated from femur and foot, cleaned of all muscle and connective tissue, and imaged with an Olympus SZ61 stereomicroscope (Olympus), Canon Powershot A620 camera (Canon, Mississauga, Canada), and PSRemote v. 1.9.1 (Breeze Systems Ltd, Surrey, UK). Tibial lengths were measured from the malleolus to the condyles of the tibial tuberosity. To check for presence of a bicuspid aortic valve, after fixation the aortic arch was removed from all mouse hearts at the level of the sinotubular junction in order to expose the coronary sinuses and leaflets of the aortic valve. Hearts were mounted in Aquasonic 100 ultrasound transmission gel (Parker Laboratories), immersed in 70% ethanol, and examined with an Olympus SZ61 stereomicroscope (Olympus). 25G needles were used to probe the exposed aortic valve to identify leaflets and cusp attachments. Images were acquired with a Canon Powershot A620 camera (Canon), and PSRemote v. 1.9.1 (Breeze Systems Ltd).

Bone Mineral Density
After tissue collection from 8-month mice as above, internal organs and visceral fat were excised from carcasses, and carcasses were then positioned, pinned in place, and frozen at -20°C. Carcasses were thawed immediately prior to measurement of bone mineral density by dual-energy x-ray absorptiometry (DEXA) on a PIXI-mus Small Animal Densitometer (LUNAR, Madison, WI) as per the manufacturer’s standard protocol. Regions of interest (ROIs) were drawn around the femur, tibia, tibial growth plate, humerus, and L3-L6 vertebrae, and bone mineral density was calculated for each ROI.

Histology and Immunohistochemistry
After immersion-fixation, mouse hearts were embedded intact in paraffin and 4-chamber view (coronal/longitudinal) serial sections through the LC and RC leaflets (or in BAVs, through the LC and RC/NC leaflets) were cut at 5 μm. Ascending aortic segments were cut between the sinotubular junction and immediately distal to the brachiocephalic artery branch, paraffin-embedded, and 5 μm serial cross-sections were taken immediately proximal to the brachiocephalic artery branch, at level AA3 (see Figure 7). Movat’s pentachrome staining (RT26385, Electron Microscopy Sciences, Hatfield, PA) as described previously was used to assess valvular morphology, dimensions, and proteoglycan/collagen/elastin content. Collagen content of valves, myocardium, and ascending aortas was also assessed by staining with picrosirius red, 0.1% in saturated picric acid for 90 minutes with 0.2% phosphomolybdic acid pre-treatment for 10 minutes. Tissue calcification was visualized using 2% aqueous alizarin red,4 (stained at pH 4.1-4.3 for 3 minutes), by von Kossa (stained in silver nitrate under fluorescent light for 1 hour, treated with sodium thiosulphate for 3 minutes, and counterstained in neutral red for 1 minute), and by OsteoSense 680EX (PerkinElmer, Waltham, MA; stained 1:200 overnight at 4°C). Imaging of OsteoSense 680 was performed with a Nikon A1+ microscope (Nikon Instruments, Melville, NY). Ascending aortic elastin was stained by Verhoeff-Van Gieson (VVG); in brief sections were stained in Verhoeff’s haematoxylin solution (20 ml of 5% haematoxylin in absolute alcohol, 8 ml of 10% aqueous ferric chloride, 8 ml Verhoeff’s iodine) for 10 minutes, differentiated in 2% ferric chloride, and counterstained in Van Gieson solution (45 ml of saturated aqueous picric acid, 5 ml of 1% acid fuchsin in distilled water) for 1-2 minutes. One section (left and right coronary leaflets) was stained per mouse. Immunohistochemical staining was performed largely as previously described. In brief, sections underwent antigen retrieval (tris-EDTA buffer at pH 9.0 for 30 minutes at 98°C, or sodium citrate buffer at pH 6.0 for 20 minutes at 95°C), endogenous peroxidases were blocked with 3% H2O2/methanol, were blocked in normal serum and stained with primary antibodies for αSMA (ab32575, 0.3 μg/ml, tris-EDTA, Abcam, Cambridge, MA), and pVASP (ab194747, 15.7 μg/ml, tris-EDTA, Abcam), for 1 hour at room temperature, followed by a biotin-labeled secondary antibody, an avidin-biotin-peroxidase conjugate (ABC Elite), a NovaRED or DAB HRP substrate, and a hematoxylin counter-stain (Hematoxylin QS), all from Vector Laboratories as per manufacturer’s instructions (Burlington, Canada). Other sections underwent antigen retrieval with EDTA buffer (pH 8.0 for 20 minutes at 95°C), were blocked in normal serum and stained with a primary antibody for RUNX2 (12556S, 1:25 and 1:100, Cell Signaling Technology, Danvers, MA), for 1 hour at room temperature, followed by a fluorescent secondary antibody (goat anti-rabbit Alexa Fluor 568, 1:100, A11011, Thermo Fisher Scientific) and counter-staining with Hoescht. Negative controls included no primary and IgG controls (AB-105-C/AB-108-C, R&D Systems). One section was stained by IHC per mouse: stained sections were imaged using an Olympus BX41 microscope, Olympus SC30 camera, and cellSens Standard v. 1.4. Images were corrected for background illumination using light field and dark field images in ImageJ (NIH, Bethesda, MD). IHC positive stain area was calculated automatically using color separation in ImageJ as described previously, with an application of the Threshold_Colour plugin v. 1.9. For RUNX2, imaging was performed with an Olympus IX71 microscope, QImaging Retiga 2000R, and QCapture v. 2.9.13 (Quantitative Imaging Corporation).

Statistical Analysis
Quantitative data are presented as mean ± standard error. Integrated traction force was assessed by a one-tailed unpaired Welch’s t-test. Two-tailed Fisher’s Exact tests were used for incidence rates of bicuspid aortic valve. When a Student’s t-test determined an absence of a diet effect (ND vs. WD) within a single genotype and timepoint, ND and WD mice were pooled for subsequent analysis. Student’s t-test (two-tailed, unpaired) was used for comparisons between two groups; one-way and two-way ANOVA with Student-Neuman-Keuls’ post-hoc tests were used as appropriate to evaluate statistically significant differences in multiple group comparisons. When data failed the Kolmogorov-Smirnov test for normality, it was transformed to ranks prior to ANOVA analysis. Two-tailed, one-sample t-tests (Prism 5, GraphPad, San Diego, CA) were used to compare the indexed transvalvular velocity of the single 8-month Npr2+/-;BAV mouse to those of the 8-month Npr2+/-; TAV, and 4-month Npr2+/-;BAV.
groups. A one-way ANOVA with Student-Neuman-Keuls post-hoc testing (SigmaPlot 10, Systat Software, San Jose, CA) was used to analyze the 10-month Npr2+/-;Ldlr-/-;BAV mice (n = 2) vs. the other 10-month groups (Npr2+/+;Ldlr-/- and Npr2+/-;Ldlr-/-;TAV). All three groups passed tests for equal variance (Levene) and normality (Kolmogorov-Smirnov) (SigmaPlot 10).

References


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Supplemental Figure 1. Mice heterozygous for Npr2 are dwarfed but do not have impaired bone calcification. A, Quantification of tibial lengths revealed that Npr2+/− mice were dwarfed by ~9% vs. age-matched littermate controls, and that this dwarfing was maintained throughout adulthood. B, Dual-energy x-ray absorptiometry (DEXA) of bone mineral density (BMD) determined that despite the dwarfing present in A, there was no impact on bone calcification in 8 month Npr2+/− mice when compared with Npr2+/+ age-matched littermate controls (“Body” = whole skeleton, “Hum.” = humerus, “L3-L6” = L3-L6 vertebrae). Mean ± SEM; *** p < 0.001; n = 13-27 mice per group. At 4/8 months there was no effect of diet in age/genotype-matched mice; normal and Western diet-fed mice were pooled for analysis of Npr2 effect at each timepoint. Mice in the 10 month groups were exclusively fed the Western diet.
Supplemental Figure II. Dietary and genetic induction drive hyperlipidemia and obesity to an equal degree in Npr2+/+ and Npr2+-/ mice. A,B, Fasting plasma total cholesterol and LDL cholesterol were mildly elevated by the TD.88137 western diet (WD) in Npr2+/+ and Npr2+-/ mice vs. normal diet (ND) controls, with no impact of Npr2 genotype. Npr2+/+;Ldlr-/- and Npr2+-/;Ldlr-/- mice were severely hyperlipidemic by 10 months, and again there was no impact of Npr2 genotype on blood lipid levels. C, The WD induced increases in body weight in Npr2+/+ and Npr2+-/ mice at both 4 and 8 months of treatment, and there was no impact of Npr2 genotype on weight gain at 4, 8, or 10 months. Mean ± SEM; ** p < 0.01, *** p < 0.001; n = 6-18 mice per group.
Supplemental Figure III. Aged Npr2+/− mice develop mild hypotension. A-C, As was the case with the onset of LV dysfunction, 8 month Npr2+/− mice had statistically-significant reductions in systolic, diastolic, and mean arterial (MAP) blood pressure vs. age-matched Npr2+/+ littermate controls as quantified by catheterization of the ascending aorta via the carotid artery, while 4 month Npr2+/− mice did not. Mean ± SEM; * p < 0.05; n = 10-12 mice per group. At 4/8 months there was no effect of diet in age/genotype-matched mice; normal and Western diet-fed mice were pooled for analysis of Npr2 effect at each timepoint.
**Supplemental Figure IV. Immunohistochemistry of 4-month mouse aortic valves.** All images are coronal/longitudinal cross-sections of the aortic valve; AA = ascending aorta, LVOT = left ventricular outflow tract. At 4 months there was no effect of diet in age/genotype-matched mice; normal and Western diet-fed mice were pooled for analysis of \( Npr2 \) effect at each timepoint. Representative images of Movat’s pentachrome, picrosirius red (PSR), and alizarin red staining show that loss of \( Npr2 \) expression drove accumulation of proteoglycan-rich lesions, fibrosis, and small microcalcific regions (black arrows) in \( Npr2^{+/−} \) mice, primarily in those with BAVs. All scale bars = 100 μm.
Supplemental Figure V. Deficiency of NPR2 and hypercholesterolemia accelerates myofibrogenesis and osteogenesis. A,B, Representative immunohistochemistry of αSMA (scale bar = 100 μm) and RUNX2 (scale bar = 25 μm) shows that loss of Npr2 promotes myofibrogenic and osteogenic differentiation respectively in Ldlr-/- mice fed a Western diet for 10 months.
Supplemental Figure VI. Deficiency of NPR2 accelerates atherogenesis. A, B, Representative H&E and picrosirius red (PSR) staining show that loss of Npr2 in hypercholesterolemic Ldlr−/− mice promotes development of atherosclerotic plaque in the ascending aorta after 10 months on the Western diet. C, Quantification of plaque area in the ascending aorta demonstrates an increase in atherosclerotic plaque size in Npr2+/−;Ldlr−/− mice fed a Western diet for 10 months. All scale bars = 500 μm. Mean ± SEM; * p < 0.05; n = 6-9 mice per group.
Supplemental Figure VII. CNP/NPR2 and Notch signaling. A,B, Left: Quantification of immunofluorescence staining for cleavage and nuclear localization of the Notch intracellular domain (NICD, produced by Notch receptor activation) determined that CNP activated Notch signaling, but did not do so via NPR2 when primary porcine VICs (A) or primary Npr2+/+ and Npr2+-/- neonatal mouse lung fibroblasts (B) were cultured with/without CNP (100 nM) and the NPR2 inhibitor P19 (0.5 μM); n = 4. Jag1 = wells coated with the human Notch ligand Jagged1 (positive control), IgG = plates coated with human IgG (negative control). Right: Representative NICD immunofluorescence images of (un)treated primary porcine VICs or neonatal mouse lung fibroblasts; scale bar = 20 μm. C, Left: Treatment of primary Npr2+/+ mouse lung fibroblasts with CNP (100 nM) and the NPR3 inhibitor AP-811 (100 nM) abrogated CNP-induced activation of NICD cleavage and nuclear localization; UD = undetected, n = 4. Right: Representative NICD immunofluorescence images of (un)treated neonatal mouse lung fibroblasts; scale bar = 20 μm. D, qPCR found that activation of Notch signaling and NICD cleavage (via seeding of Npr2+/+ fibroblasts on Jag1-coated wells) induced upregulated expression of the Notch target genes Hey1/Hes1/Snai1, Notch1 itself, and the Notch ligand Jag1 vs. IgG-coated controls; expression of the angiogenic Notch ligand Dll4 was unchanged. Nppc (the CNP gene) was upregulated by activation of Notch signaling, while Npr2 was downregulated and Npr3 was unchanged; n = 2. Mean ± SEM; * p < 0.05, ** p < 0.01, *** p < 0.001.
Supplemental Figure VIII. Full transvalvular velocity profiles of mice. Representative pulsed Doppler velocity spectra of full transvalvular blood flow profiles from all genotypes demonstrate stenotic flow acceleration in Npr2+/−;TAV and Npr2+/−;BAV mice. At 4/8 months there was no effect of diet in age/genotype-matched mice; normal and Western diet-fed mice were pooled for analysis of Npr2 effect at each timepoint. Mice in the 10 month groups were exclusively fed the Western diet. Note changing velocity scales between images.
Supplemental Figure IX. Microcalcifications in mouse aortic valves. All images are coronal/longitudinal cross-sections of the aortic valve; AA = ascending aorta, LVOT = left ventricular outflow tract. At 8 months there was no effect of diet in age/genotype-matched mice; normal and Western diet-fed mice were pooled for analysis of Npr2 effect at each timepoint. Mice in the 10 month groups were exclusively fed the Western diet. A, B Representative images of von Kossa (phosphate) and OsteoSense 680EX (hydroxyapatite) staining on sections as serial as possible to those stained with alizarin red in Figure 5C demonstrate specific and consistent detection of leaflet microcalcifications in Npr2+/- mice with TAVs and BAVs. All scale bars = 100 µm.