Materials and Methods

All studies were approved by the Institutional Animal Care and Use Committee at the University of Iowa (PHS Animal Welfare Assurance #A3021-01).

Experimental strategy. *Egfr*$^{Wa2/+}$ (*Control*) and littermate *Egfr*$^{Wa2/Wa2}$ (*Wave*) mice bred on a C57/BL6 background were maintained on normal chow diet (Harlan Teklad 7004 Rodent Diet). Echocardiograms were performed on surviving mice at 1.5, 6, and 12 months of age. Cardiac magnetic resonance imaging (MRI) was performed on a subset of mice from each group at 6 months of age. Cardiac catheterization was performed on subsets of mice from each group at 6 or 12 months of age. Following completion of imaging and catheterization studies, mice were euthanized (pentobarbital, 150 mg/kg intraperitoneal injection), at age 1.5, 6, or 12 months, and tissue was collected for subsequent histological analysis.

Pharmacologic intervention. In a previous study, we found that administration of the PPAR-γ agonist pioglitazone attenuated aortic valve calcification and protected aortic valve function in hypercholesterolemic mice.[1] We hypothesized that pioglitazone would attenuate aortic valve calcification and protect valve function in normocholesterolemic Wave mice. Therefore, 16 mice (7 Control and 9 Wave mice) were treated with pioglitazone (20 mg/kg per day in chow) from 1 to 6 months of age, then euthanized.

Echocardiography. Midazolam (0.15 mg, subcutaneous) was used to produce light conscious sedation. Parasternal long- and short-axis views were obtained using high-frequency echocardiography (Vevo 2100®, VisualSonics, Toronto, Canada) to assess aortic valve function and aortic root dimensions. LV mass, volumes, and systolic function were assessed using the bi-plane area-length method, previously validated in our laboratory.[2] M-mode images were then acquired in order to measure systolic aortic valve orifice dimension as previously described.[3] Anatomical M-mode imaging was accomplished by redirection of the cursor superimposed on a short-axis 2D image of the aortic valve, as shown in Supplemental Figure IV. Color Doppler was used in a parasternal long-axis plane to determine the presence of aortic regurgitation. Echocardiographic severity of AR was graded by measuring length of the maximum diastolic regurgitant jet, and dividing by the end-diastolic long-axis length of the LV. Trace or mild AR was recorded when jet length was < 25% of LV length, moderate: 25 – 50%, severe: > 50%.

Cardiac MRI. Mice were deeply sedated with midazolam (8 mg/kg, subcutaneous) and morphine (4 mg/kg, subcutaneous), a regimen that produces only modest depression of heart rate and left ventricular contractility, and MRI was performed using an Innova® 4.7T instrument (Varian, Palo Alto, CA) as described previously.[4] The presence and qualitative severity of aortic regurgitation were assessed by observing dephasing of the blood signal in the left ventricular outflow tract during early diastole. Regurgitant fraction was then calculated as the difference between left and right ventricular (RV) stroke volumes divided by LV stroke volume.

Invasive hemodynamic measurements. Immediately prior to euthanasia, the peak aortic valve gradient was measured. Mice were anesthetized using ketamine/acepromazine (87.5/12.5 mg/kg, intraperitoneal). A 1.4F microtransducer-tipped catheter (Millar, Houston, TX) was inserted into the right common carotid artery and advanced retrograde into the LV. Pressure was then continuously recorded as the catheter tip was pulled back into the ascending aorta. Aortic valve peak systolic gradient was calculated as the difference between peak LV pressure and peak aortic pressure. Aortic pulse pressure was calculated as the difference between systolic and diastolic aortic pressures.

Valve histology. Tissue was frozen in optimal cutting temperature compound (OCT, 10.24% polyvinyl alcohol 4.26% polyethylene glycol 85.5% non-reactive ingredients). Sections, 10-microns thick, were obtained from proximal, mid, and distal aortic valve from each mouse. Slides were stained with Alizarin Red, Masson's Trichrome or Picrosirius Red, or Oil Red-O to quantitate the amount of calcium, collagen and lipid, respectively, as described previously.[1] Movat's pentachrome stain was used to quantitate proteoglycan content in the aortic valve.

Immunofluorescence was used to quantitate levels of α-SMA, TGF-β1, p-Smad2, osterix, osteocalcin, intact versican, cleaved versican, biglycan, p-HH3, and activated caspase-3. The method for discriminating between true immunofluorescence in the valve vs. autofluorescence in the valve annulus is shown in Supplemental Materials and Methods Figure IB below. Confirmatory quantitation of apoptosis in the aortic valve was performed using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, In Situ Cell Death detection Kit®, Roche). The percentage of valve exhibiting positive staining was reported as "% positive".

Gene expression in aortic valve and proximal aorta. Aortic roots from individual mice (6 Control mice and 6 Wave mice, at 12 ± 1 months of age) were frozen in OCT, after which the aortic valve was dissected free from supporting structures, under microscopic guidance, and transferred to Trizol reagent. RNA was prepared using RNeasy mini kit (Qiagen). Complementary DNA was generated using MMLV reverse transcriptase with random hexamers. ADAMTS1, ADAMTS5, biglycan, and versican mRNA levels were each measured in a well along with a β-actin reference standard, using qRT-PCR FAM and VIC fluors, respectively (TaqMan®, Integrated DNA Technologies, Coralville, IA). RNA from the ascending aorta was isolated at 6 months of age with Trizol reagent, followed by separation with RNeasy minicolumn(Quiagen). Smad3 and Smad7 mRNA levels were measured as described above. Sources for primers are shown in Supplemental Materials and Methods Table 1 below.

Myocardial gene expression. RNA was isolated with Trizol reagent from the left ventricle of 7-11 mice, and cDNA was generated using Superscript-III reverse transcriptase and random hexamers. Transcript levels were measured with SYBR Green and Bio-Rad CFX96 Real-Time system. Expression of genes of interest was normalized to 18S rRNA. The primer sequences utilized to interrogate myocardial gene expression are shown in Supplemental Materials and Methods Table 2 below.

T-tubule imaging in myocardium. The structure and organization of cardiomyocyte t-tubules were assessed using methods previously described in rats.[5] Briefly, whole hearts were removed, then perfused retrograde at room temperature for 30 minutes with Ca^{2+}-free Tyrode's solution via Langendorff apparatus. The membrane-binding lipophilic marker, MM 4-64 (AAT BioQuest, CA) was added to perfusate for 30 minutes. T-tubules were imaged in intact hearts using a confocal microscope (LSM 510, Zeiss, Germany) at 63x magnification. Left ventricular T-tubule organization was reported as TT_{power}, in each of 10 confocal micrographs from each mouse, using software designed for this purpose.[5] Lower values for TT_{power} indicate disruption of T-tubule organization.

Blood chemistries. Total plasma cholesterol and inorganic phosphorus were measured with a spectrophotometer using the reagent sets (C7510 and P7516, respectively, Pointe Scientific, Inc., Canton, MI). Plasma calcium was determined spectrophotomically using C503 reagent set (Teco Diagnostics, Anaheim, CA).

Statistical analysis. All continuous variables are reported as mean ± standard error. Group data for continuous variables were compared between Wave *vs*. age-matched Control mice, using Student's t-test. Presence or absence of a discrete condition (severe aortic stenosis or moderate/severe AR) was compared between groups using z-testing.[6] Data reporting the effect of pioglitazone treatment upon valve calcification, where $N \leq 4$, were first subject to Mann-Whitney Rank Sum testing in order to confirm that data conformed sufficiently to a normal distribution.[6] Statistical significance was set at $p < 0.05$, except where a group of data were utilized in two discrete comparison, in which case Bonferroni correction was utilized to set statistical significance at $p < 0.025$.

References

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2. Hill, J.A., Mohsen, K, Kutschke, W., Davisson,R., Zimmerman, K., Wang, Z., Kerber, R.E., and Weiss, R.M.: Cardiac hypertrophy is not a required compensatory response to short-term pressure overload. *Circulation*. 2000;101:2854-2862.
3. Weiss RM, Lund DD, Chu Y, Brooks RM, Zimmerman KA, Accaoui RE, Davis MK, Hajj GP, Zimmerman MB, Heistad DD. Osteoprotegerin inhibits aortic valve calcification and preserves valve function in hypercholesterolemic mice. *PloS One*. 2013;8:e65201.
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5. Wei S, Guo A, Chen B, Kutschke W, Xie YP, Zimmerman K, Weiss RM, Anderson ME, Cheng H, Song LS. T-tubule remodeling during transition from hypertrophy to heart failure. *Circ Res*. 2010;107:520-31.
6. Glantz SA. Primer of Biostatistics. 2012; McGraw-Hill, New York.

Methods and Materials Table 1. Primers and probes for assessment of gene expression in the aortic valve and proximal aorta.

Gene	TaqMan Primers/probe	Source
ADAMTS1	Mm.PT.58.29991678/FAM	Integrated DNA Technologies*
ADAMTS5	Mm.PT.58.28649059/FAM	Integrated DNA Technologies
Biglycan	Mm.PT.58.6381330/FAM	Integrated DNA Technologies
Versican	Mm.PT.58.13796540/FAM	Integrated DNA Technologies
Smad3	Mm.PT.56a.10139890/FAM	Integrated DNA Technologies
Smad7	Mm.PT.56a.6640883/FAM	Integrated DNA Technologies
Beta-actin	4352341E/VIC	Applied Biosystems**
GAPDH	4352339E/VIC	Applied Biosystems

*Coralville, IA; **Carlsbad, CA

Materials and Methods Table 2. Primer sequences for myocardial gene expression.

(18S-fwd) CTTTCGCTCTGGTCCGTCTT; (18S-rev) CTTTCGCTCTGGTCCGTCTT,
(α-MyHC-fwd) CCTGTCCAGCAGAAAGAGC,
(α-MyHC-rev)CAGGCAAAGTCAAGCATTCATATTTATTGTG,
(β-MyHC-fwd) CAGGACACCAGCGCCCA,
(bMyHC-rev) CCCTTGGAGCTGGGTAGCAC,
(Anp-fwd) AGGAGAAGATGCCGGTAGAAGA,
(Anp-rev) GCTTCCTCAGTCTGCTCACTCA,
(Bnp-fwd) AAGGTGCTGTCCCAGATG,
(Bnp-rev) TTGGTCCTTCAAGAGCTGTC,
(Sk actin-fwd) CGACATCAGGAAGGACCTGTATGCC,
(Sk actin-rev) AGCCTCGTCGTACTCCTGCTTGG,
(Mcip1.4-fwd) AGCTCCCTGATTGCTTGTGT,
(Mcip1.4-rev) TGGAAGGTGGTGTCCTTGT,
(Col1a1-fwd) AATGGCACGGCTGTGTGCGA,
(Col1a1-rev) AACGGGTCCCCTTGGGCCTT,
(Col3a1-fwd) TGGCACAGCAGTCCAACGTA,
(Col3a1-rev) TGACATGGTTCTGGCTTCCA,
(Serca-fwd) TGTAAGTGGCCAGATTGCTC,
(Serca-rev) CCTAAACAACTGAAGTTAGG.