SUPPLEMENTAL MATERIAL
Detailed Materials and Methods

Mouse Strains

Wildtype (WT) *C57BL/6* (The Jackson Laboratory) and valve-specific Cad-11 overexpressing *Nfatc1^{Cre};R26-Cad11$^{Tg/Tg}$* (Cad11 OX) mice were bred according to standard protocols and sacrificed at 10 months of age. *Nfatc1^{Cre}* mice were donated by Bingruo Wu (Albert Einstein College of Medicine) and have been previously described in which the Cre reporter gene is expressed in endocardial cells and in the cushion mesenchyme derived from the endocardium but is not expressed in the epicardium or myocardium.[1, 2] *Cad11$^{Tg/Tg}$* mice were created using targeted embryonic stem cell microinjection into *C57BL/6* mice. Full-length Cad-11 cDNA preceded by a floxed stop codon was inserted into the ROSA26 locus via BAC transgenesis. All mice were bred on a *C57BL/6* background for >5 generations. Genotyping was performed by polymerase chain reaction (PCR) using primer pairs for the Cad-11 transgene (F: GTTCCTGGCTGGGTCCCTGA, R: CCAAGCGGCTTCGGCCAGTAA, 200bp) and *Nfatc1^{Cre}* (F: GGCGCGGCAACACCATTTTT, R: TCCGGGCTGCCACGACCAA, 446bp) (**Fig. SI**). All animal work was conducted according to relevant national and international guidelines. Full details of this study were reviewed and approved by the Cornell IACUC (Protocol #2008-0011).

Human and Mouse Aortic Valve Histology and Immunofluorescence

Calcified human aortic valves (CHAV) were obtained from adults undergoing planned, non-elective valve replacement surgery by Dr. Sanjay Samy at Robert Packer Hospital in Sayre, PA. All patients (n=5) were diagnosed with moderate to severe aortic stenosis. Healthy, non-diseased control aortic valves were obtained from patients (n=3) who died from non-valve related illnesses, who had no visible valve pathology, and whose hearts were ineligible for organ donation due to non-valve related defects, provided by Dr. Jonathan Che, Cornell-Weill Medicine and New York Presbyterian Hospital. All samples were procured with informed consent from the patients. All procedures were approved by the Institutional Review Boards at Cornell University, NY Presbyterian Hospital, and Robert Packer Hospital.

CHAV were decalcified in 10% sodium citrate and 22% formic acid in deionized water for 12 hours or until soft and washed in running water for 30 minutes. Decalcified CHAV and non-diseased control valves were paraffin embedded and sectioned at 6 μm. Adult mouse hearts were fixed in 4% paraformaldehyde overnight at 4°C, then dehydrated through an ethanol series and paraffin embedded and sectioned at 8 μm thickness. Following dewaxing and rehydration, sections were stained with Hematoxylin and Eosin (H&E, cellular composition), Von Kossa (phosphate), Alizarin Red (calcium), Alcian Blue (glycosaminoglycans), Picrosirius Red (collagen), Verhoeff-Van Geison (elastin), or Movat's Pentachrome stain. Staining intensity was measured using ImageJ (NIH, Bethesda, MD) using the ImageJ plugin Colour Deconvolution to separate channels, and all values were normalized to total valve area. The aortic vessels were measured for staining intensity to ensure consistent staining.

Sections were additionally processed for immunohistochemistry. For immunodetection, 10mM citrate buffer was used for antigen retrieval, and sections were blocked with 10%

goat serum before primary antibodies (1:400 dilution) were used against Cadherin-11 (rabbit, Invitrogen), Sox9 (rabbit, Abcam), β1 integrin (mouse, BioGenex), alpha-smooth muscle actin (mouse, Sigma), Active RhoA-GTP (mouse, NewEast Biosciences), Runx2 (mouse, Abcam), Ki67 (rabbit, Abcam), and Osteocalcin (mouse, Abcam). Fluorescence-conjugated Alexa Fluor 488 and Alexa Fluor 568 secondary antibodies (Invitrogen, 1:500 dilution) were used according to the primary antibody species. Sections were nuclei counter-stained with DRAQ5 (Abcam, 1:1000 dilution). Signals were detected and images were collected with Zeiss 710 confocal microscopy (Cornell University Life Sciences Core Laboratories Center). Immunoreactivity of proteins stained in tissue sections was measured using ImageJ and quantified by normalizing fluorescent intensity to total valve area and then normalized to controls as demonstrated previously.[3, 4] Sox9 nuclear co-localization was measured using NIH ImageJ software. Green (Sox9) and blue (nuclei) channels were each thresholded and co-localized regions were measured using the ImageJ plugin Colocalization.

Doppler Ultrasound Transthoracic Echocardiography

Mice were bred to 10 months according to standard protocol and anesthetized using 1.5% isoflurane. Chests of adult mice were treated with a chemical hair remover to reduce attenuation. Heart rate and core temperature were continuously monitored throughout the procedure. A long axis 2D cross-sectional and Doppler transthoracic echocardiography was performed using a Visual Sonics Vevo2100 system (Cornell University Life Sciences Core Laboratories Center).

Calcification Analysis and Plasmid Transfection

Porcine aortic valve interstitial cells (PAVICs) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY) and 1% penicillin/streptomycin (Gibco) at 37°C and 5% CO_2 as previously described.[5] PAVICs were used between passages 5-8. For calcification analysis, cells were seeded in 24-well plates at 0.2×10^6 cells/well, incubated for 24 hours, and transfected with either an empty vector (VDF-1012, Amaxa Biosystems) or a Cad-11 Human cDNA ORF Clone (NM_001797, Origene) using X-tremeGENE9 Transfection Reagent (Roche) and left for 24 hours in minimal medium supplemented with DMEM, 5% FBS, and without antibiotics. For 3D calcification analysis, cells were transfected in T-25 flasks, embedded at 200,000 cells per 200μL 3D collagen hydrogel, allowed to polymerize, and remain anchored to the well plate as previously described.[6] The cells were then supplemented with osteogenic growth media (OGM, control medium with 10 mmol/L β-glycerophosphate, 50μg/mL ascorbic acid, and 10 nmol/L dexamethasone) and a ROCK inhibitor (Y27632) (Sigma) at 5μM for up to 10 days with the media changed every 48 hours. RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed into cDNA using the SuperScript III Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instructions. Gene expression and Cad-11 overexpression were confirmed using immunofluorescence and real-time PCR (qPCR) with custom primers (**Table SII**) and SYBR Green PCR master mix. PAVICs were fixed in 4% paraformaldehyde and incubated in 40nmol/L Alizarin Red S (ARS) dye to identify calcific nodules or subjected to Live/Dead Staining (Life Technologies, Carlsbad, CA).

Compaction, Migration, and Cell Morphology Analysis

3D collagen hydrogels were created using 3X Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2mg/mL type I collagen, and 0.1M NaOH to neutralize the solution. Following transfection with either an empty vector or Cad-11 plasmid, PAVICs were left for 24 hours in minimal medium then trypsinized and seeded within the gel at 0.2×10^6 cells/mL. Gels were allowed to polymerize in a 24 well plate at 37°C and 5% CO_2 for 12 hours. They were subsequently released from the sides of the well plate using a pipet tip, allowing them to float freely in regular growth medium and compact for 7 days. PAVIC cells were seeded at 0.2×10^6 cells in a 15.6mm, 24-well plate. Following transfection, PAVICs were left for 24 hours in minimal medium, and a pipet tip was used to make a scratch down the well center. Cells were allowed to migrate, and images were taken every 3 hours for 12 hours. Cell morphology and protein expression was analyzed by seeding transfected PAVICs on a 35mm, coverslip-bottom cell culture dish (MatTek Corporation, Ashland, MA) at low (1×10^4 cells/dish) and high density (5×10^4 cells/dish) seedings. After 48 hours, cells were fixed in 4% PFA, permeabilized in 0.2% Triton-X, blocked with 10% goat serum, and stained for GTP-RhoA, Sox9, or F-actin and nuclei with Alexa Fluor phalloidin 488 (Invitrogen, 1:500 dilution) and DRAQ5, and imaged with a confocal microscope. Fluorescence intensity of GTP-RhoA and Sox9 was normalized to cell area and then to control as previously described.[7] Cells with areas of intense F-actin staining were considered "stress fiber bundle positive," manually counted, and taken as a percentage of cells visible per high powered field.

Quantification of Histological Changes and Statistical Analysis

All data are reported as means with $n\geq3$ independent experiments per condition, and error bars represent standard error of the mean (SEM). Statistical significance was determined using the Student's t-test, one-way ANOVA with Tukey's post hoc paired tests for treatment effects, or Chi-squared analysis for Mendelian ratios. Differences between means were considered significant at $p<0.05$.

References for Detailed Materials and Methods

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