Cell culture systems

Porcine and human valve cell assays.

Porcine aortic valve (AoV) interstitial (pAVIC) and endothelial cells (pAVEC) were isolated from AoV cusps of juvenile staged pigs as described,[1] and plated at $4x10^4$ cells/cm^2 in growth media containing DMEM, 10% L-glutamine, 10% Fetal Bovine Serum (FBS) and1% penicillin/streptomycin (pen/strep). Human mitral valve interstitial cells (hVICs) were a kind gift from Dr. Alain Colige (University of Liege).[2]

Isolation of murine cardiac endothelial cells.

Murine cardiac endothelial cells (mCECs) were isolated from post natal day 6-8 *Tgfβ1*$^{fl/fl}$ mice using a magnetic bead isolation technique (Invitrogen).[3] Sheep anti-rat IgG DynaBeads (Invitrogen) were coated with either CD31 or ICAM-2 (BD Biosciences) according to the manufacturer's instructions (Invitrogen). Whole hearts were dissected and cells were dissociated by incubation in 1mg/ml collagenase/dispase solution (Roche) for 45 minutes at 37°C. The resulting cell suspension was incubated with CD31-conjugated DynaBeads (BD biosciences) and bound cells were isolated using the magnetic bead separation system (Invitrogen). The cell pellet consisting of antibody-conjugated cells was resuspended in endothelial cell media (EBM-2 containing 0.5% FBS, 1% pen/strep, and 0.75% Fibroblast Growth Factor) and plated onto a gelatin-coated T-75 flask. Confluent endothelial cells were further purified using ICAM-2-conjugated Dynabeads (BD Biosciences) and maintained on gelatin-coated flasks for subsequent experiments.

AoV explant assays.

AoV explants were isolated from post natal *Tgfβ1*$^{fl/fl}$ mice and cultured on membrane filters as previously described.[4]

Transwell and calcification assays.

For calcification assays, $4x10^4$ pAVICs were seeded on glass coverslips in 24-well cell culture plates and allowed to reach confluency. Cultures were then incubated for 1-7 days, changing media every 48 hours to allow for calcification (n=4). For transwell-culture systems endothelial cells (HUVECs, pAVECs, or mCECs) were seeded into transwell inserts (Millipore) at a density of $2x10^4$ and cultured overnight to allow for cell attachment. Transwell inserts were then placed into wells containing pAVICs or hVICs, and VIC media was replaced with mCEC media (see above) and cultured for 5-7 days with a media change every 48 hours with or without TGFβ1 (10ng/ml) or SB431542 (100nM) (n=4). Following culture, cells were fixed in 4% paraformaldehyde (PFA)/PBS and subject to immunohistochemistry or quantitative PCR (qPCR) (see below).

Treatment of primary valve cells

For treatments, pAVICs were grown to confluency and fresh media was supplemented with the following. TGFβ1 (10ng/ml), Y27632 (10μmol/L), or BSA control (0.0002% final concentration) for 48 hours (n=4). For cultures treated with both TGFβ1 and Y27632, cells were pre-treated with Y27632 30 minutes prior to the addition of Tgfβ1. 5ng/ml Leptomycin B or ethanol vehicle (0.1% final concentration) for 6 hours, then replaced with VIC media for an additional 7 days (n=3). 100nM SB431542 was added to mCEC media and supplemented every other day for 7 days (n=3). Following treatments, cells were subject to RNA or protein isolation, or fixed for immunostaining (see below). AoV explants from *Tgfβ1*$^{fl/fl}$ post natal pups were infected immediately with $1×10^{10}$ PFU AdV-Cre or control AdV-GFP in serum free medium for 6 hours. Following infection, explants were incubated in VIC media (see above) for an additional 48 hours. Following infection

and incubation, treated explants were removed from the membrane filter and processed for histological, Western blot or RNA analysis (see below).

Generation of mice

Tgfβ1$^{fl/fl}$ female mice (Jackson Laboratories, stock #010721, C57BL/6J) were bred with *Nfatc1ENCre*[5] males (backcrossed to C57BL/6J) to generate heterozygous offspring (*Tgfβ1*$^{fl/+}$*;Nfatc1ENCre*). Heterozygous males were then bred with *Tgfβ1*$^{fl/fl}$ females to create homozygotes (*Tgfβ1*$^{fl/fl}$*;Nfatc1ENCre*$^{+}$) and *Cre* negative (*Tgfβ1*$^{fl/fl}$*;Nfatc1ENCre*$^{-}$) littermate controls. *Tgfβ1*$^{fl/fl}$*;Nfatc1ENCre*$^{+}$ and control mice were then harvested at post natal (PND3-5), 3 and 6 months of age and analyzed (see below). To determine recombination, *Nfatc1ENCre* mice were crossed with *Rosa26R-Tomato* reporter mice (Jackson Labs, stock #007676, C57BL/6J) and sacrificed at post natal day 1. Genotyping was performed as previously described for adult genomic DNA.[5, 6] *Reversa* hypercholesterolemic and normocholesterolemic control mice were obtained from Dr. Donald Heistad.[7] Normocholesterolemic animals were generated following deletion of *microsomal triglyceride transfer protein (Mttp)* using the interferon-inducible Mx1-Cre transgene and receiving 4 injections of polyinosinic-polycytidylic acid (225 μg IP) at 2-day intervals and maintained on a chow diet for 6 or 12 months. While hypercholesterolemic animals maintained *Mttp* function and were fed Western diet for 6 months and then given 4 injections of polyinosinic-polycytidylic acid (225 μg IP), switched to a chow diet, and followed up for an additional 6 months. Hearts from these mice were harvested at 3, 11, and 22 months of age for immunohistological analysis (see below).

Human AoV specimens

Human diseased aortic valve specimens (n=3) were obtained from patients undergoing valve replacement surgery. Patients with a history of infective endocarditis, rheumatic heart disease, or a genetic syndrome were excluded. Control AoVs (n=3) were obtained from age-matched individuals at the time of autopsy who died of non-cardiac causes. AoV tissues were fixed in 10% formalin, dehydrated through a graded ethanol series, washed in xylenes, and embedded in paraffin wax. Studies were approved by the Institutional Review Boards at Cincinnati Children's Hospital Medical Center and the University of Cincinnati (RBH).

Histology

Animal models: Hearts were dissected from *Tgfβ1*$^{fl/fl}$*;Nfatc1ENcre*$^{+}$ mice and controls (*Tgfβ1*$^{fl/fl}$*;Nfatc1ENcre*$^{-}$) at PND3-5, 3 and 6 months of age, and hypercholesterolemic and normocholesterolemic *Reversa* mice at 3, 11 and 22 months. Whole hearts were fixed in 4% PFA/PBS overnight and processed for paraffin embedding and cut at 6μm-thick as previously described.[8] Tissue sections containing AoVs were then subject to Pentachrome, Trichrome or von Kossa staining as described and counterstained for 20 minutes in 1% Alcian Blue.[4, 8] Valve thickness was determined using Image Pro Plus software by tracing the perimeter of the AoV cusps identified by Alcian Blue staining, and measuring the internal area (pixels) for n=4 mice per group (n=4 per genotype) for *Tgfβ1*$^{fl/fl}$*;Nfatc1ENcre*$^{+}$ mice compared to *Tgfβ1*$^{fl/fl}$*;Nfatc1ENcre*$^{-}$ controls. For Alizarin Red staining, tissue sections were stained with freshly prepared 2% Alizarin Red S (pH 4.1-4,3, Sigma) for 10 minutes. Quantification of von Kossa and Alizarin Red reactivity were performed using Image Pro Plus software following auto-contrast of colored images in Photoshop. Quantitation of von Kossa and Alizarin Red reactivity was calculated as a percentage of positive staining (black) over the total valve area as identified by Alcian Blue and statistical significance was determined by Student's t-test in

Tgfβ1$^{fl/fl}$;Nfatc1ENcre^{+} mice compared to *Tgfβ1$^{fl/fl}$;Nfatc1ENcre^{-}* controls.

In vitro assays: Following treatment, post natal AoV explants were mounted onto glass slides, fixed in 4% PFA and subject to von Kossa staining and Alcian Blue counterstaining as described above. Quantitation of von Kossa reactivity was calculated as a percentage of positive staining (black) over the total valve area and statistical significance was determined by Student's t-test in AdV-Cre treated explants compared to AdV-GFP controls. For cultured pAVICs, Alizarin Red reactivity was calculated as an average of positive (red) area (in pixels) in 10 fields per sample. Statistical significance in treated cells was determined using the Student's t-test as compared to respective vehicle treated controls, or pAVICs cultured alone for transwell assays.

Immunohistochemistry
Animal models: For antibody staining, fixed tissue sections of AoV from human subjects, *Tgfβ1$^{fl/fl}$;Nfatc1ENcre* mice, *Reversa* hypercholesterolemic, or respective control mice were subjected to antigen retrieval by boiling for 10 minutes in unmasking solution (Vector Laboratories) prior to overnight incubation at 4°C with primary antibodies against Sox9 (Millipore, 1:500), Cleaved Caspase 3 (Cell Signaling 1:200) Tgfβ1 (Abcam 1:200), Col2a1 (Abcam, 1:100), Runx2 (Santa Cruz, 1:100) or pSmad2 (Cell Signaling, 1:100). For human tissue, colormetric detection of Sox9 was performed using diaminobenzidine according to the manufacturer's instructions (ABC staining system, Santa Cruz Biotechnology) and slides were counterstained with hematoxylin for 5 minutes. For tissue sections and cultured cells, immunofluorescent staining against Sox9 and Tgfβ1 was performed using a donkey-anti-rabbit-488 Alexa-Fluor secondary antibody (Invitrogen, 1:400) and visualized using an Olympus BX51 microscope.

In vitro assays: For cultured cells, differential Interference Contrast (DIC, Nomarski) images were taken after 1 and 7 days of culture using an Olympus BX51 microscope equipped with a DIC prism and analyzer using polarized light. In addition, cells were incubated overnight with anti-Sox9 diluted in block (as above) or no primary antibody (block alone) at 4°C, followed by a 1 hour incubation with donkey-anti-rabbit-488 Alexa-Fluor secondary antibody (Invitrogen, 1:400). Reactivity was visualized and captured using an Olympus BX51 microscope and Cellsense software set at the same exposure as primary antibody treated samples. Quantitation of Sox9 nuclear localization in vitro was performed blinded and calculated as the number of cells exhibiting nuclear Sox9 staining over the total number of cells as detected by DAPI nuclear stain, in a total of 10 fields per sample (n=3). Staining was considered nuclear if the intensity of nuclear staining (co-localized with nuclear DAPI) was greater than the intensity of cytoplasmic staining. Statistical significance was determined against respective controls using the Student's t-test.

Western Blotting and ELISA
For nuclear and cytoplasmic protein extracts, cells were collected in NE-PER extraction reagent (Pierce) supplemented with complete EDTA free protease inhibitor cocktail, and lysed according to the manufacturer's protocol. Total protein extracts were obtained by lysing cells in RIPA buffer supplemented with complete EDTA free protease inhibitor cocktail. Protein extracts were collected and 5-10μg of total, nuclear, or cytoplasmic protein was run on a 12% SDS PAGE gel and transferred to nitrocellulose membranes using an iBlot dry blot system (Invitrogen). Membranes were blocked in 2% BSA for 1 hour and probed with antibodies against Sox9 (1:2000, Millipore), pSmad2 (1:1000, Cell Signaling), tSmad2 (1:1000, Cell Signaling) Actin (1:5000, Cell Signaling) TBP (1:1000,

Abcam) Cleaved Caspase-3 (1:1000, Cell Signaling) or Histone H3 (1:1000, Cell Signaling) followed by incubation with anti-rabbit-horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000, Cell Signaling). Membranes were then washed three times in 1× TBST for 10 minutes. Western blots were developed using Super Signal West Femto Substrate (Pierce) and BioMax MR film (Eastman Kodak). Densitometry was performed using ImageJ software and calculated as a ratio of Sox9 band density over the loading control in n=3 samples. Statistical significance in treated samples was determined using the Student's t-test comparing loading control normalized band density values.

Secreted Tgfβ1 levels were determined using the Quantikine ELISA kit (R&D Systems). pAVICS or pAVECS were serum starved for 6 hours prior to collection of cell culture supernatant. Samples were activated and assayed according to the manufacturers protocols. Optical densities of samples were determined using a microplate reader set to 450nm, and Tgfβ1 concentrations were calculated using a standard curve (n=5). Statistical significance was determined using the Students t-test comparing absolute secreted levels in pAVECS versus pAVICs.

Polymerase Chain Reaction

Changes in gene expression were determined by quantitative real-time PCR using a Step One Plus Real Time PCR system (Applied Biosystems) according to the manufacturer's instructions using TaqMan assays (Applied Biosystems) for *Tgfβ1, Sox9, Col2a1* and *Runx2;* normalized to *18s.* RNA was extracted from pAVICs, pAVECs, and HUVECs using standard Trizol protocols (Invitrogen) and cDNA and PCR reactions were performed as previously described.[9] Significant differences in gene expression were reported as a fold change compared to control (n=3-6).

Transfections and Luciferase Assays

The pGL3-Col2a1 constructs were obtained from Dr. Michael Underhill.[10] The 4x48bp-Col2a1 construct contains the minimal promoter of murine *Col2a1* in addition to the Sox9-responive first intron, while the $^{-}$89-$^{+}$6bp-Col2a1 lacks the first intron. For luciferase assays, pAVICs were plated at 4x10^{4}/cell (24-well plate) 16-20 hours prior to transfection with Lipofectamine and Plus Reagent (Invitrogen) according the manufacturer's instruction. 500ng of 4x48bp-Col2a1 or $^{-}$89-$^{+}$6bp-Col2a1 were transfected into each well along with 50ng pGL4 (*Renilla* luciferase, Promega). For Tgfβ1-treated assays, TGFβ1 (10ng/ml) or BSA was added to the pAVIC media following transfection. Alternatively, pAVICs were co-transfected with 500ng WT-Sox9 or pmutant-Sox9 obtained from Dr. Martin Cheung.[11] All transfections were performed in 0.5mL OptiMem for 4 hours before the addition of 0.5mL normal growth media. Cell lysates were collected 48 hours following transfection according to the manufacturer's instructions for dual luciferase assays (Promega). Data for TGFβ1-treated assays are represented as a fold change in luciferase activity of TGFβ1 treatment compared to BSA-treated controls (n=6). For the p-mutant co-transfections, data are represented as an average percent of luciferase activity of the pGL3-*4x48Col2a1* co-transfected with WT-Sox9 (set at 100%) and normalized to pGL4 *Renilla* signal (n=3). Statistical analysis was performed using the Student's t-test.

Echocardiography

Transthoracic echocardiography was performed on *Tgfβ1$^{fl/fl}$;Nfatc1ENCre^{+}* and *Tgfβ1$^{fl/fl}$;Nfatc1ENCre^{-}* mice (n=6) at 3 and 6 months of age using a VisualSonics 2100 system (Toronto, Canada). Mice were anesthetized with 1% isofluorane inhalation and

placed on a heated platform. Two-dimensional imaging was recorded with a 40-hertz transducer to capture long- and short-axis projections with guided M- Mode, B-Mode and PW Doppler recorded. The average reading for each parameter measured was recorded from at least 10 frames from each animal and the standard deviation calculated. Statistical significance was determined using Student's t-test ($P<0.05$) comparing absolute peak velocities of *Tgfβ1$^{fl/fl}$;Nfatc1ENCre^{+}* against *Tgfβ1$^{fl/fl}$;Nfatc1ENCre^{-}* littermate controls.

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