Supplemental Material

SUPPLEMENTARY METHODS

- Animal model and data acquisition

Thirteen Dorsett hybrid sheep were used in this study. In the inferior myocardial infarction (IMI) model, 5 sheep were induced with Propofol (0.5 to 1.5 mg/kg intravenously),and the trachea intubated and ventilated at 15 ml/kg with a mixture of 3% isoflurane and oxygen. Animals were loaded with antiarrhymic drug Amiodarone 200mg PO daily for three days prior to surgery. Intravenous Glycopyrrolate (0.4 mg), Buprenorphine (0.3 mg), and Cefazolin (1 g) were administered 30 minutes before chest wall incision.

Bupivacaine 0.75% was administered locally to block the intercostal nerves prior to chest retractor placement. A left throracotomy was performed between the 4th and 5th intercostal space to access the heart. Following chest retractor placement, the heart was exposed and the pericardium opened. Epicardial 2- and 3-dimensional echocardiography imaging and left ventricular hemodynamic parameters were acquired at baseline and 30-60 minutes post myocardial infarction (MI). Left ventricular hemodynamic data were recorded with a 5F Scisense catheter inserted through the apex. Basic echo views were obtained using a Philips iE33 scanner and a 5-MHz transducer. Images were analyzed offline using QLab 10.5 (Philips, Andover, Mass). LV end-diastolic volume, end-systolic volume, and ejection fraction were measured using 3D full volume data set. MR was quantified by the width of the proximal jet (vena contracta) in the apical long-axis view. The infarct surface area and total LV endocardial surface area were measured at end-diastole using customized 3D analysis software (Omni4D,). The second and third obtuse marginal branches of the left circumflex coronary artery were ligated at their origin to create myocardial infarction (MI) that results mitral regurgitation. Lidocaine 0.8mg/kg intravenous bolus(es) were administered prior to coronary artery ligation and after coronary artery ligation. Sham animals were treated identically except that the coronary arteries were not ligated and thus no myocardial infarction created. Three-dimensional (3D) echocardiographic analysis included LV end-systolic and end-diastolic volumes integrated from multiple rotated views derived from the full 3D dataset using Omni4D software (M.D.H., Boston, MA); infarct size as endocardial surface area (ESA) measured at end diastole based on visualized wall motion hinge points; and total LV remodeling reflected by the increase in total LV ESA from immediately to 6 months post-MI[1-4].

Following 6 months after MI creation, prior to euthanasia, the animals were anesthetized as described above and echocardiography and hemodynamic data repeated to assess severity of mitral regurgitation and hemodynamics. Upon completion of data collection, animals were euthanized via intravenous injection of 100 mg/kg IV pentobarbital (while on 3% isoflurane). Under sterile conditions, the hearts were excised and the mitral valve leaflets immediately submerged in a solution of 5% heat inactivated FBS, 4% Penicillin/Streptomycin/Amphotericin B, 1% L-Glutamine and 0.2% gentamycin sulfate in EBM-2 medium (Lonza Inc., GA, USA, #CC-3156), and kept on ice at 4°C until processed. Animals were monitored by qualified AAALAC-certified veterinary staff. These studies conform to National Institutions of Heath guidelines for animal care and received Institutional Animal Care Committee approval.

-Immunohistochemistry

A portion of each excised mitral valves was frozen in OCT compound (Sakura Finetek, Tokyo, Japan) and sectioned in 7 μm slices. Sections were then incubated with 0.3 % hydrogen

peroxide to inhibit endogenous peroxidase activity, and then incubated with primary mouse anti-sheep CD45 (1:10 dilution; AbD Serotec, NC, USA, cat# MCA2220GA), anti-sheep CD14 (1:10 dilution; AbD Serotec, NC USA, cat# MCA920GA) or anti-human α-SMA (1:150 dilution; DAKO, CA, USA, cat#, M0851) antibodies. After washing with PBS, secondary antibody (biotinylated anti-mouse IgG, 1:100 dilution; Vector Laboratories, CA, USA) was applied, followed by avidin-peroxidase complex (Vectastain ABC kit; Vector Laboratories, CA, USA). The reaction was visualized with 3-amino-9-ethyl carbazole substrate (AEC; Sigma-Aldrich, MO, USA). Sections were counterstained with Mayer's hematoxylin solution (Sigma-Aldrich, MO, USA) and mounted. Isotype-matched IgGs applied to the tissue sections served as control. Images were captured with a digital camera (Eclipse 50i, Nikon Instruments, Melville, New York) with a cooled CCD camera (DS-Fi1c, Nikon Instruments, Melville, New York) using imaging software NIS-Elements (version 3.1). Fluorescence microscopy visualized simultaneously fluorescein-labeled endothelial cells (von Willebrand Factor, DAKO, CA, USA, cat# A0082) detected by fluorescein--conjugated streptavidin (Alexa Fluor 488 dye, Life Technologies, CA, USA) and CD45 (Alexa Fluor 594 dye, Life Technologies, CA, USA). Nuclei were counterstained with 4'-6'-Diamidino-2-phenylindole (DAPI, Vector Laboratories, CA, USA). Images were captured and processed with the epifluorescence microscope (Eclipse Ti-E, Nikon Instruments, NY, USA).

Fibrosis: Sections from each sham and IMI-6 MV) were stained with Masson Trichrome; five high powered fields (HPF) were analyzed from each section to determine % Positive Area for collagen. In parallel adjacent sections were stained for CD45+ cells. Sections were analyzed at 400X magnification (Eclipse 50i, Nikon Instruments, Melville, NY, USA) with a cooled CCD camera (DS-Fi1c, Nikon Instruments, Melville, NY, USA) using imaging software NIS-Elements AR (Advanced Research) 3.1 (Nikon Instruments, Melville, NY, USA).

- Flow cytometry

Ovine MVs were minced and digested for 30 minutes with Liberase (Roche Diagnostics, IN, USA), a blend of highly purified collagenases I and II, in a 37C water bath. Tissue pieces were rinsed with wash buffer (5% FBS, 1% Penicillin/Streptomycin/Amphotericin B, 1X Hank's Basic Salt Solution, 126 µmol/L $CaCl_2$ and 80 µmol/L $MgSO_4$ in H2O) 4 times, to extract the cells, and the isolated cells were fixed using Flow Cytometry Fixation Buffer (R&D Systems, MN, USA) and labeled in Flow Cytometry Permeabilization/Wash Buffer I (R&D Systems, MN, USA #FC005) for 45 minutes (100,000 cells/100 µl buffer), following the manufacturer's protocol. Murine anti-sheep CD45-FITC and -APC (1:50 dilution; AbD Serotec, NC, USA, cat# MCA2220F, #MCA896GA, LYNX Rapid APC Antibody Conjugation Kit, #LNK031APC), murine anti-human VE-Cadherin-PE and -FITC (1:100; R&D Systems, MN, USA FAB9381Pand AbD Serotec, NC, USA, # AHP628F, respectively), and murine anti-human α-smooth muscle actin-APC and -PE (1:100; R&D Systems, MN, USA, # IC1420P and # IC1420A) were used to detect CD45, VE-Cadherin, and αSMA respectively. All antibodies were shown to cross-react with their ovine homologs. Flow cytometry samples were analyzed on a BD FACS Calibur system. Data were analyzed using FlowJo version 10 software (Tree Star, Inc. Ashland, OR).

- RNA isolation and quantitative real-time PCR (qPCR)

Total cellular RNA was extracted from mitral VEC clones (D1, E5 and E10-2) and CAEC (n=2) with an RNeasy Micro extraction kit (Qiagen, Valencia, CA). Reverse transcriptase reactions were performed using an iScript cDNA Synthesis Kit (Bio-Rad, CA, USA #170-8890). qPCR was performed using Kapa Sybr Fast ABI Prism 2x qPCR Master Mix (KAPA BioSystems, MA, USA # KK4604). Amplification was carried out in an ABI 7500 (Applied Biosystems, Foster City, CA). A standard curve for each gene was generated to determine amplification efficiency. RPS9 was used as housekeeping gene expression reference. Fold increases in gene

expression were calculated according to 2 delta C_T method [5, 6], with each amplification reaction performed in triplicate.

- Migration assay

Mitral VEC clones (D1, E5, E10-2) or CAEC were treated ± TGFβ for 4 days to induce EndMT. The cells were treated for 30 minutes ± the CD45-selective PTPase inhibitor (1μM)[7] prior to trypsinization. 20,000 cells (± PTPase inhibitor 1μM) in 0.1% BSA/EBM-2 (Lonza) were placed in the upper chamber of 6.5mm Transwells containing fibronectin-coated (0.2 ug/cm2) polycarbonate membranes with 8.0μm pores. The lower chambers contained 0.1% BSA/EBM-2 media alone or EBM with serum and basic FGF as a chemoattractants. Cells were allowed to migrate for 6 hours at 37°C. Cells that migrated through the pores were fixed with methanol and stained with Eosin-Y, Azure A and Methylene Blue for visualization and quantification using Three Step Stain Set (VWR, PA, USA #48218-567). In parallel, an aliquot of cells used for the migration assay were also analyzed for CD45 by flow cytometry to verify response to TGFβ1.

Online TABLE I

Sheep ID	EDV(mL)	ESV(mL)	SV(mL)	EF(%)	VC(mm)
6 months IMI					
2082	**94**	**51**	**43**	**45**	**4.6**
2077	**119**	**90**	**29**	**25**	**7.4**
2057	**97**	**53**	**43**	**45**	**4.1**
2102	**88**	**46**	**42**	**48**	**3.3**
2071	**81**	**42**	**38**	**48**	**4.1**
Sham					
2095	**54**	**16**	**38**	**70**	**1.4**
2137	**53**	**21**	**32**	**60**	**0**
4048	**49**	**15**	**34**	**69**	**0.0**
4064	**54**	**17**	**37**	**69**	**1.0**
4033	**47**	**14**	**33**	**69**	**1.1**
Normal					
2115	**69**	**28**	**41**	**59**	**0.17**
3011	**57**	**21**	**36**	**64**	**0.00**
5149●	**105**	**51**	**54**	**51**	**0.00**

- Sheep 5149 was larger (67kg) than other sheep in the study (~45kg), which could account for the larger LV

Online Table I - Echocardiographic measurements ischemic, sham and normal sheep. Volumes were calculated using three-dimensional data (full volume acquisition) on an Echomachine Philips IE33, using Philips Qlab software. EDV = End-diastolic volume; ESV = End-systolic volume; SV = Stroke volume; EF = Ejection fraction; VC = Vena contracta of the MR jet (proximal dimension reflecting orifice size). Sheep 2095 and 2137 were analyzed at 6 months and 4048, 4064, 4033 at 2 months.

Online TABLE II

	Normal *n = 4*	6 months IMI *n = 5*	Statistical Significance
VEC VE-Cadherin^{+}/CD45^{-}/αSMA^{-}	38.7 ± 9.7	4.6 ± 6.6	*P = 0.0004*
VEC^{CD45+} VE-Cadherin^{+}/CD45^{+}/αSMA^{-}	1.5 ± 1.9	9.0 ± 5.1	*P = 0.028*
VEC-EndMT VE-Cadherin^{+}/CD45^{-}/αSMA^{+}	1.5 ± 1.5	3.8 ± 3.6	*P = 0.272*
VEC^{CD45+}-EndMT VE-Cadherin^{+}/CD45^{+}/αSMA^{+}	0.4 ± 0.3	17.4 ± 4.6	*P = 0.001*
Quiescent VIC VE-Cadherin^{-}/CD45^{-}/αSMA^{-}	57.5 ± 9.8	47.1 ± 12.8	*P = 0.222*
Activated VIC VE-Cadherin^{-}/CD45^{-}/αSMA^{+}	0.1 ± 0.2	5.3 ± 5.1	*P = 0.082*
Hematopoietic cells VE-Cadherin^{-}/CD45^{+}/αSMA^{-}	0.5 ± 0.8	8.8 ± 6.0	*P = 0.036*
Fibrocytes VE-Cadherin^{-}/CD45^{+}/αSMA^{+}	0.0 ± 0.0	5.8 ± 2.9	*P = 0.011*

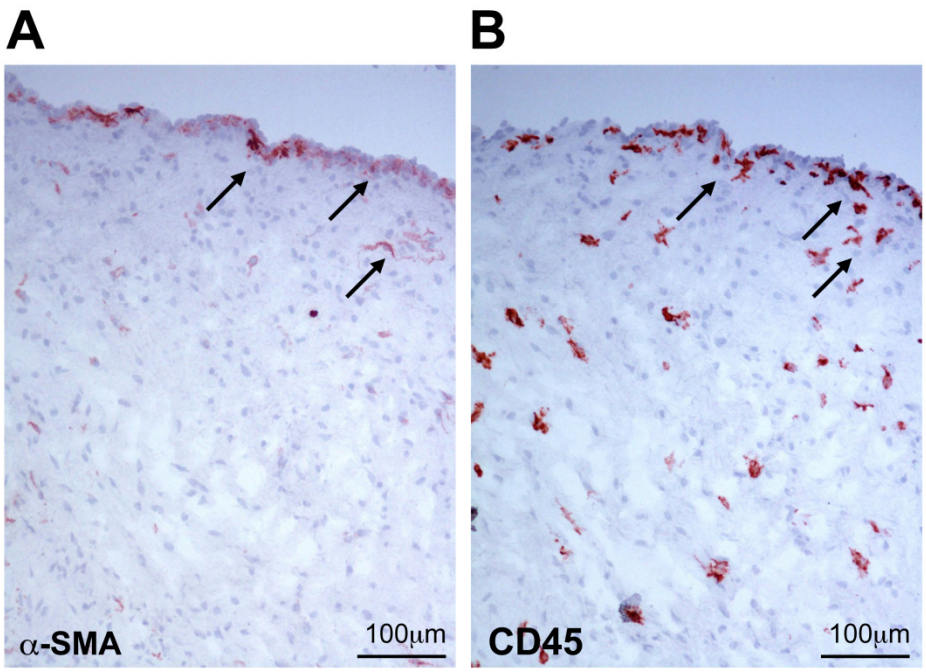
Online Table II. Endothelial, interstitial and hematopoietic cell populations in MVs from IMI infarcted sheep versus normal sheep as percentage of total cells within each cell population. Statistical significance measured using student t-test analysis.

Online TABLE III

Student t-tests performed on fold increases (mean ± SD) in Figure 4C.

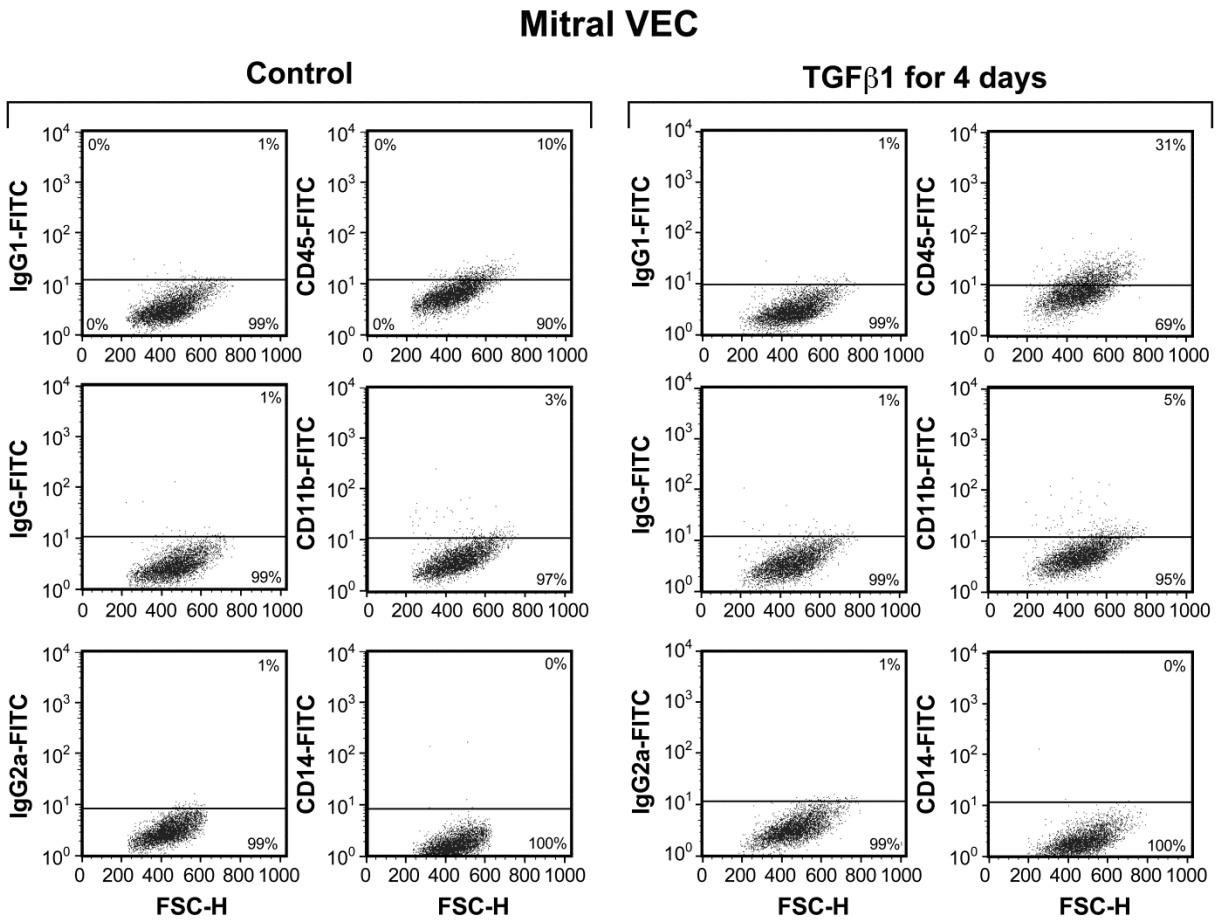
Control vs TGFβ1 (p values)									
VE-cadherin	**αSMA**	**Slug**	**MMP2**	**NFATc1**	**Collagen1**	**Collagen3**	**TGFβ1**	**TGFβ2**	**TGFβ3**
0.0005	0.0131	0.0009	0.0133	0.0095	0.0009	0.0079	0.0019	0.00001	0.0082
TGFβ1 vs TGFβ1 + PTPase (p values)									
VE-cadherin	**αSMA**	**Slug**	**MMP2**	**NFATc1**	**Collagen1**	**Collagen3**	**TGFβ1**	**TGFβ2**	**TGFβ3**
0.1666	0.0143	0.0060	0.0196	0.0022	0.0115	0.0179	0.0344	0.00005	0.0051

Supplementary Online Figures



Online Figure I

Online Figure I - A) αSMA and B) CD45 in the endothelial and subendothelial layers of IMI mitral valves Adjacent sections of valve leaflets were analyzed by immunohistochemistry. Arrows mark sites of potential co-localization. Scale bar, 100 μm.



Online Figure II

Online Figure II – Mitral VEC clone E10 treated without (control) or with TGFβ1 for 4 days to induce EndMT. Cells were analyzed by flow cytometry using anti-CD45-FITC (top row), anti-CD11b-FITC (middle row) or anti-CD14-FITC (bottom row). IgG isotype-matched controls-conjugated to FITC were used to establish background. As expected, mitral VEC clone E10 expressed CD45 at a low level without TGFβ1 and at a higher level (31%) after TGFβ1 treatment, but neither CD11b nor CD14 were detected. The anti-CD11b and anti-CD14 were validated using ovine peripheral blood leukocytes as a positive control (not shown).

References

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