Supplemental Materials

Methods

Model of RV Volume and Pressure Overload

Twelve-week-old male FVB mice were used for all studies. PI was created using two 10.0 silk sutures to entrap the two anterior-most pulmonary valve leaflets, following which a 7.0 silk suture was tied around the main pulmonary artery to create PS (Supplement Figure 1 a). We have previously described the details of each surgical procedure separately.(1, 2) Post procedure Doppler echocardiography demonstrated reversal of flow in the branch pulmonary arteries consistent with hemodynamically significant PI and a pulmonary artery band (PAB) gradient of 20-30 mmHg consistent with mild to moderate PS (Figure 1 b-e). Mice with PI+PS were compared with age-matched sham-operated controls.

Assessment of Cardiac Physiology

Transthoracic echocardiograms (ECHO), cardiac catheterization and exercise testing were performed at 1 and 2 mos (compensated state) and at 3 mos (clinical heart failure) and compared with sham (n=6/sham and PI+PS x 3 time points at 1m, 2m and 3mons). ECHO was performed using a GE Vivid 7 ultrasound platform under isoflurane mask anesthesia to assess RV size and function. Cardiac catheterization was performed using a 1.4-F transducer-tipped micromanometer catheter (Millar Instruments, Houston, TX) inserted via the right jugular vein to determine RV end-diastolic pressure (RVEDP) and dP/dt. Exercise testing was performed using our previously published ramped exercise protocol (Oxymax chamber, Columbus Instruments, Columbus, OH).(2)

RT-PCR

Expression of “hypertrophy/heart failure” genes were assessed in a one-step quantitative qRT-PCR using SYBR green technology (Qiagen) at 1, 2 and 3 mos using β-actin as the housekeeping gene.(2) qRT-PCR was also used to validate selected profibrotic genes obtained from the microarray data. Primer sequences are shown in Supplement Table 1. microRNA (miR) expression of select profibrotic miRs was assessed using a 2 step qRT-PCR (ABI) using U6 as the housekeeping gene.(3) We isolated calcium tolerant adult RV cardiomyocyte and non-cardiomyocyte fractions following retrograde perfusion of the heart and enzymatic digestion to assess miR expression in each cellular compartment. In brief, the heart is resected, cannulated through the aorta and retrograde perfused at 37^{o}C with a calcium free solution (in mM, 120 NaCl, 14.7 KCl, 0.6 KH_2PO_4, 0.6 Na_2HPO_4, 1.2 $MgSO_4$-$7H_2O$, 4.6 $NaHCO_3$, 10 Na-HEPES, 30 taurine, 10 BDM, 5.5 glucose, pH 7.0) for 4 min followed by an enzymatic digestion with collagenase II (Worthington Biochemical Corporation, Lakewood, NJ) 394U/mg, 1.5 mg/ml). The digestion is initially performed in calcium free solution for 2 min and then CaCl2 is added to a 50 mM final concentration for 6 min. The heart is decannulated and the RV and LV are separated. The tissue is cut into small pieces and further digested by gently pipetting with plastic transfer pipettes for 3-5 min. Stop buffer (calcium free solution + $CaCl_2$ 100 mM + 10% bovine calf serum (Hyclone, Logan, UT)) is added and cell suspension is collected in a 15ml tube and centrifuged at 400 rpm for 3 min to isolate cardiomyocytes. Supernatant is centrifuged at 1000 rpm for 5 min to isolate non-cardiomyocytes. Cell morphology is assessed via light microscopy. Cells are also stained with β-actin (secondary antibody Alexa 594) and troponin (secondary

antibody Alexa 488) to confirm myocytes and with the fibroblast marker DDR2 (N-20) (Santa Cruz sc-7555, secondary antibody Alexa 488) and DAPI (ThermoFisher # D1306) for nuclear staining to confirm the presence of fibroblasts via fluorescent microscopy (Supplemental Figure 3A, B). Fold change in expression was compared between PI+PS and sham using the 2 power (−ΔΔCt) method.

Plasma Exosome Expression

Total exosomes were isolated from 100µl of plasma by low-speed centrifugation following manufacturer's guidelines (Invitrogen). The pellet contains exosomes from which exosomal RNA and protein can be isolated. Exosomes were assessed by three methods. (i) Exosome morphology and purity was characterized by transmission electron microscopy (TEM). The samples were prepared using the Exosome-TEM-easy kit (101Bio Cat. # P130).(4) In brief, freshly isolated exosomes were mixed 1:1 with 4% paraformaldehyde for a final concentration of 2% paraformaldehyde and loaded onto Formvar-carbon coated EM grids and washed. EM solution was added to the grids followed by scanning on a JEOL JEM1400 electron microscope at the Cell Sciences Imaging Facility at Stanford University. (ii) Exosomal marker proteins including CD63, HSP 70 and CD9 were detected by immunoblotting using a 12.5% gel (SBI #EXOAB-KIT-1). Rabbit anti-human primary antibodies were used at a dilution of 1:1000 and goat anti-Rabbit IgG HRP conjugated secondary antibody at a dilution of 1:20,000. (iii) For RNA isolation, 100% ethanol is added to the resuspended pellet to bind the RNA. The RNA was washed and eluted for use in downstream reactions. We used an Agilent 2100 Bioanalyzer at the Stanford University PAN facility to further confirm the presence

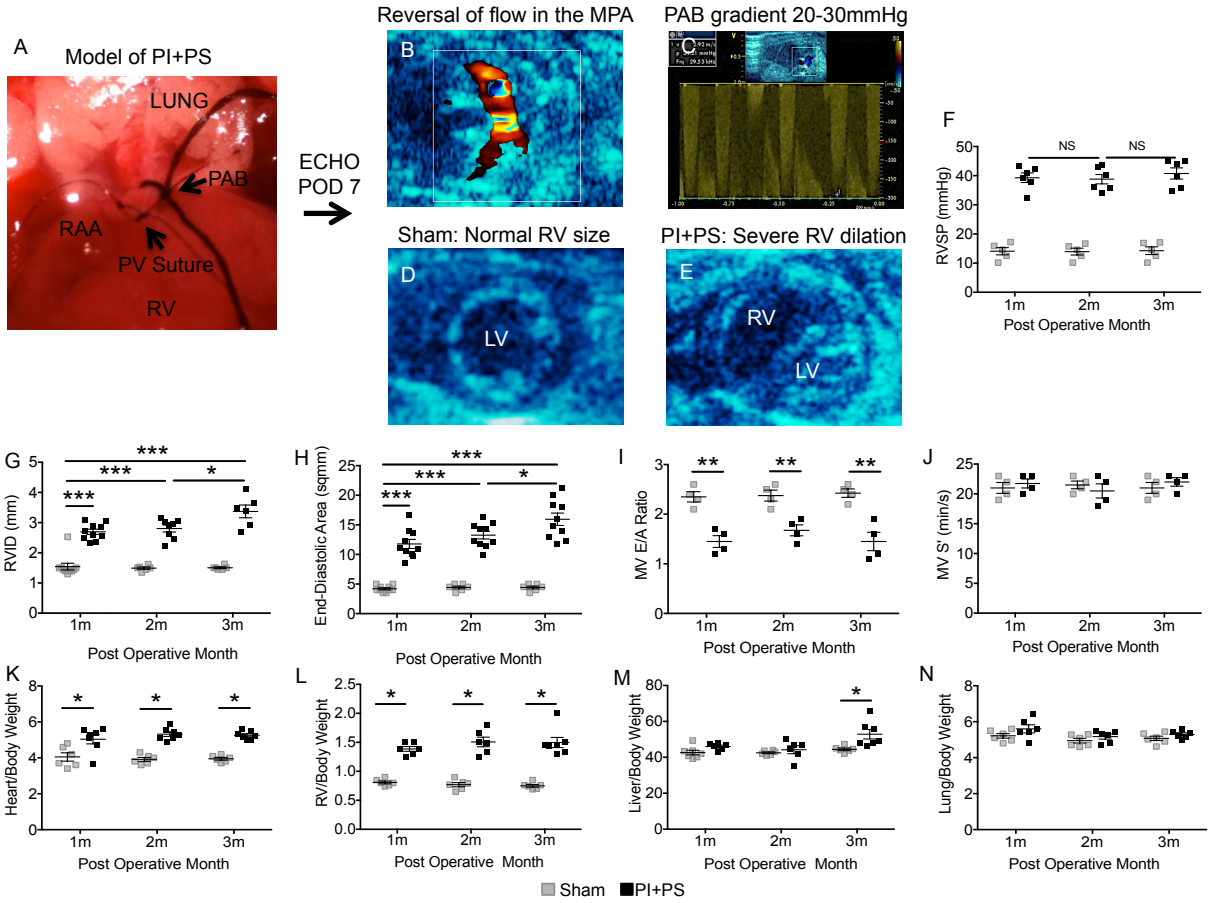
of miRs in the exosomal RNA following which exosomal miR expression was assessed using a 2 step qRT-PCR (ABI). miR expression was also assessed in the non-exosomal fraction of the plasma.

Supplement Table 1. Primers used in qRT-PCR

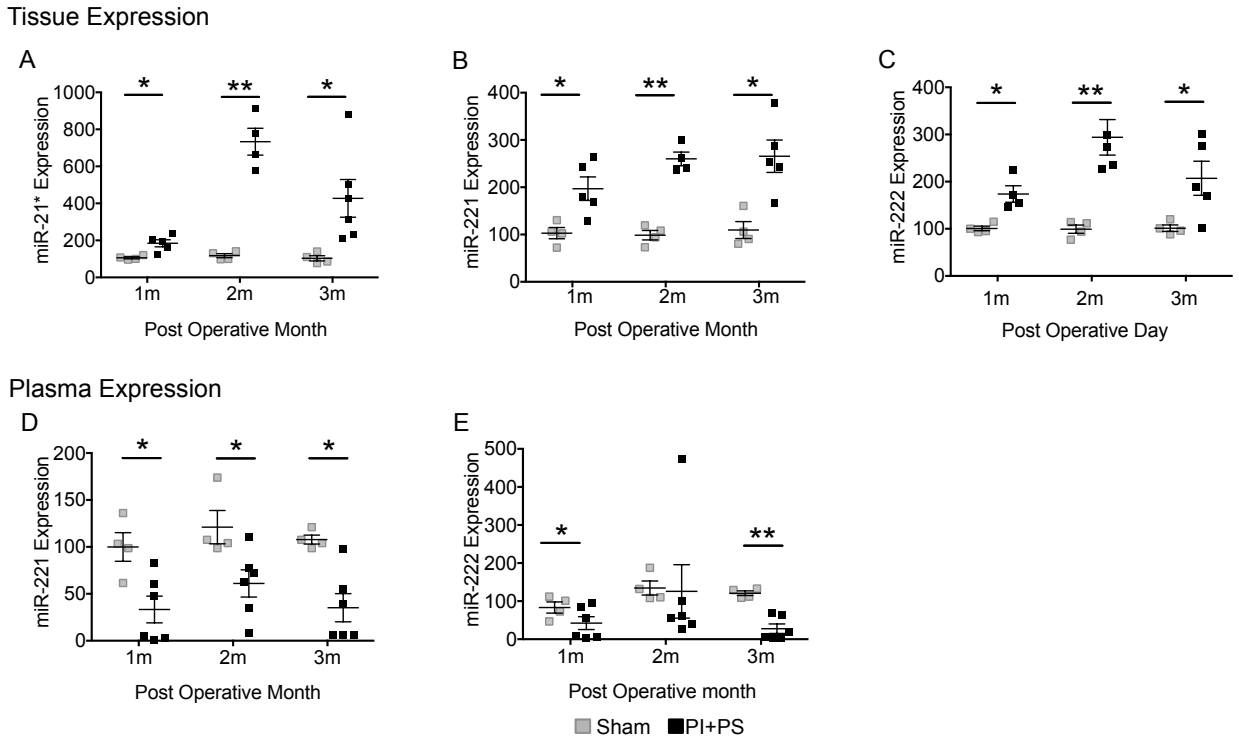
GENE	PRIMER SEQUENCE
SERCA2+	TGGAGAACGCTCACACAAAGA
SERCA2−	ATTCGTTGGAGCCCCATCT
ANP+	TCGTCTTGGCCTTTTGGCT
ANP−	TCCAGGTGGTCTAGCAGGTTCT
TGF-β1+	GACTCTCCACCTGCAAGACC
TGF-β1-	GACTGGCGAGCCTTAGTTTG
TNF-α+	AGCCCCCAGTCTGTATCCTT
TNF-α-	GGTCACTGTCCCAGCATCTT
THBS1+	CAAGTTTGCAACAAGCAGGA
THBS1-	ATGCCATTTCCACTGTAGCC

Supplement Figure Legends

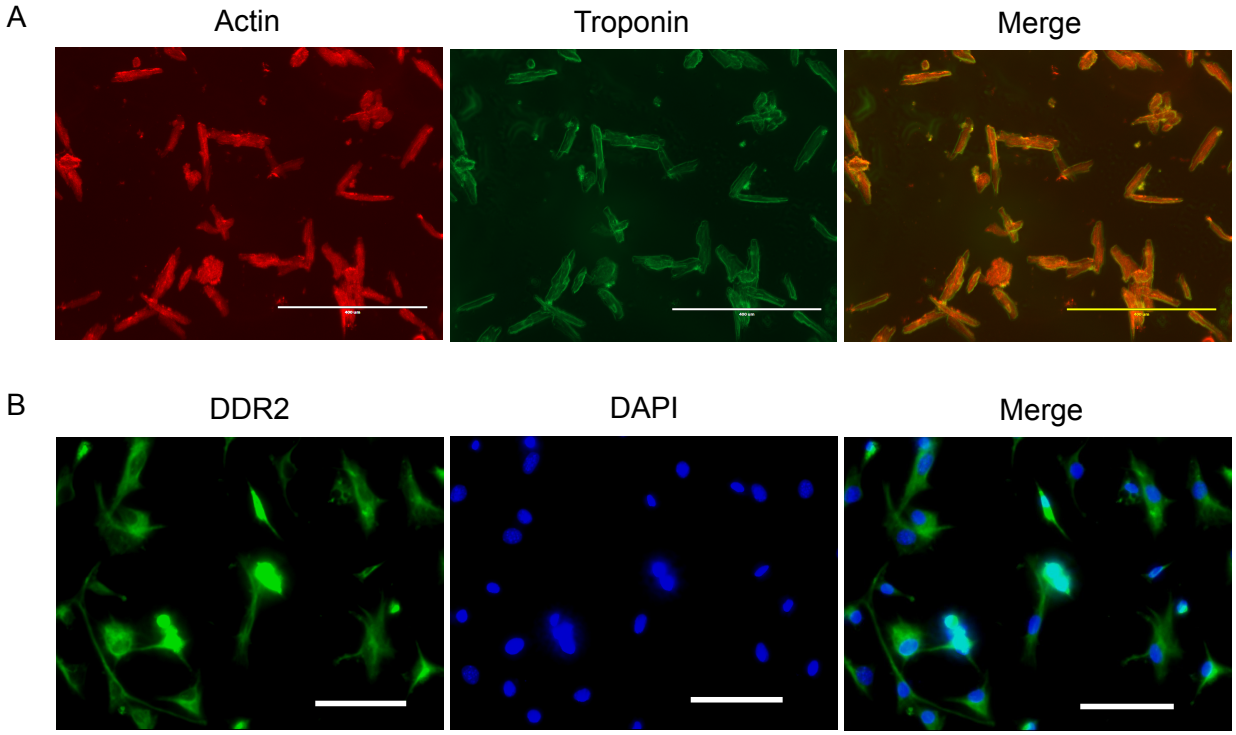
Supplement Figure 1. Murine Model of PI+PS. (A) Via a thoracotomy, the pulmonary valve (PV) leaflets were entrapped with sutures and a pulmonary artery band (PAB) was placed on the main pulmonary artery (MPA), Surgical success was defined by echocardiography (ECHO) on post-operative day 7 (POD) as (B) Reversal of flow in the MPA and (C) PAB gradient of 20-30mmHg, (D) Normal RV size in sham operated controls and (E) RV dilation in PI+PS; (F) RVSP remained stable over time while the RV size progressively increases as shown by (G) RVID and (H) RVED area; Left Ventricular Functional Assessment. (I) Early LV diastolic dysfunction with a decrease in mitral E/A ratio and (J) Preserved systolic function by TDI with a normal LV free wall velocity (S'); Morphometrics. (K) Heart weight and (L) RV mass was increased at all time points while (M) liver mass increased with the onset of clinical heart failure. (N) Lung weight did not increase at any time point. N=4-8/sham/time point, N=4-10/PI+PS/time point for all experiments. RV-right ventricle, PI+PS-pulmonary insufficiency and pulmonary stenosis, RAA – right atrial appendage, LV-left ventricle, RVSP-RV systolic pressure, RVID-RV internal diameter, RVED-RV end-diastole, TDI-tissue Doppler imaging, NS-non significant. Data are presented as Mean±SEM. *p<0.05, **p<0.01, ***p<0.001. ANOVA with multiple testing correction was used.



Supplement Figure 2. Profibrotic miRs are upregulated in the RV. (A-C) The expression of mouse RV profibrotic miR-21* and miR 221/222 cluster were also upregulated during the stages of diastolic (1m and 2m) and systolic dysfunction (3m); (D-E) Mouse plasma miR-221 and miR-222 expression were downregulated at all time points despite tissue level upregulation, miR-21* was not detectable in the plasma. N=4/Sham/time point, N=4-6/PI+PS/time point. RV-right ventricle, PI+PS-pulmonary insufficiency and pulmonary stenosis. Data are presented as mean±SEM. *p<0.05, **p<0.001. ANOVA with multiple testing correction was used.



Supplement Figure 3. Cardiomyocyte and Fibroblast Isolation. Cells were isolated from mice following sham surgery and creation of PI+PS using retrograde perfusion, collagenase digestion and differential centrifugation. A. Cell fraction known to be cardiomyocytes were further stained with cardiac specific Actin and Troponin and confirmed that they were cardiomyocytes. Scale bar represents 400μm; B. Cell fraction known to be non-cardiomyocytes were further stained with the fibroblast marker DDR2 and nuclear staining with DAPI and confirmed that they were fibroblasts. Scale bar represents 50μm.



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