AWARD NUMBER: W81XWH-17-1-0327
PR161256

TITLE: Targeting BMPR2 Signaling to Improved Right Ventricular Function in Congenital Heart Disease

PRINCIPAL INVESTIGATOR: Edda Spiekerkoetter, MD

CONTRACTING ORGANIZATION: Stanford University
Stanford, Ca 94305-2004

REPORT DATE: OCTOBER 2020

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
OCTOBER 2020	Final Report	7/1/2017 – 6/30/2020

4. TITLE AND SUBTITLE

Targeting BMPR2 Signaling to improve Right Ventricular Function in Congenital Heart Disease

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0327

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Edda Spiekerkoetter, Mario Boehm, Ross Metzger, Xuefei Tian

E-Mail:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Leland Stanford Junior University
450 Serra Mall
Stanford, Ca 94305-2004

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In our in vitro experiments we found that increasing BMPR2 signaling with FK506 prevented endothelial mesenchymal (EndMT) transition of cardiac endothelial cells in response to TGF-b. Furthermore, increasing BMPR2 signaling decreased collagen production and proliferation of human cardiac fibroblasts. Reduced BMPR2 signaling promoted EndMT and activation of cardiac fibroblasts in vitro, which was blocked by the repurposed drug FK506 (Tacrolimus). In vivo, increasing BMPR2 signaling with FK506 reduced the development of cardiac fibrosis and increased the systolic ejection fraction as well as cardiac strain as assessed by cardiac MRI in a mouse model of increased right ventricular (RV) afterload leading to progressive right heart failure. This model mimicks the RV afterload in congenital heart disease in children. The distribution of the fibrosis characteristic of RV pressure overload was located perivascularly around coronary arteries as well as interstitially in the RV free wall. Lineage tracing experiments documented that the process of endothelial mesenchymal transition only minimally contributed to the observed cardiac fibrosis, but that the fibrosis was rather due to activation and proliferation of resident cardiac fibroblasts. Tacrolimus improved the RV function even in non-BMPR2 deficient animals, suggesting that it might be benefical to improve RV function in situation of RV afterload, irrespectable of their BMPR2 status.

15. SUBJECT TERMS

Endothelial cells, cardiac fibroblasts, cardiac fibrosis, right heart failure, endothelial mesenchymal transition, BMPR2 signaling, repurposing drugs, FK506

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	31	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

- **INTRODUCTION:**

The **goal** of the grant was to ***first*** elucidate the role of the Bone Morphogenetic Protein Receptor 2 (BMPR2) signaling in right ventricle (RV) adaptation to an increased afterload in congenital heart disease, its involvement in cardiac fibrosis and capillary rarefaction. ***Second,*** we aimed to define markers of BMPR2 deficiency in human samples (tissue and blood) to identify those patients who might benefit from BMPR2 upregulating therapies such as the repurposed drug tacrolimus (FK506). Our **specific aims** were: (1) To determine whether reduced BMP signaling facilitates cardiac endothelial-to-mesenchymal transition and activation of cardiac fibroblasts. (2) To determine the role of BMP signaling in the development of RV fibrosis and capillary rarefaction in a murine model of chronic isolated pressure-overloaded RV failure. (3) To develop a non-invasive method of quantifying BMPR2 signaling in plasma and PBMCs as a surrogate biomarker of BMPR2 expression and signaling in human cardiac tissue.

- **KEYWORDS:**

Endothelial cells, cardiac fibroblasts, cardiac fibrosis, right heart failure, endothelial mesenchymal transition, BMPR2 signaling, repurposing drugs, FK506, congenital heart disease, capillary rarefaction, Deep tissue 3D imaging, de-banding.

- **ACCOMPLISHMENTS:**

  - **What were the major goals of the project?**

Major Task 1: Characterize the role of BMP in cardiac endothelial cells and fibroblasts
Major Task 2: To characterize the role of BMPR2 signaling in the right ventricle in heart failure
Major Task 3: To develop a surrogate biomarker in blood to identify patients deficient in BMPR2 who might benefit from BMPR2 activating therapy

Specific Aim 1: To determine whether reduced BMP signaling facilitates cardiac endothelial-to-mesenchymal transition (EndMT) and activation of cardiac fibroblasts.
Major Task 1: Characterize the role of BMP in cardiac endothelial cells and fibroblasts
Aim 1a. *To show that BMP agonists (BMP ligands, FK506) prevent TGF-β1/IL1b induced EndMT in human cardiac endothelial cells (CEC)* ***completed***
Aim 1b. *To show that BMP agonists reduce proliferation and collagen production of cardiac fibroblasts (CFs)* ***completed***
Aim 1c: *To show that reduced BMP signaling facilitates fibrosis by augmenting EndMT in cardiac endothelial cells (ECs) and collagen production in cardiac fibroblasts (CFs), a process reversed by BMP agonists* ***completed***
Milestone(s) Achieved: *Identification of role of BMP agonists and loss of BMRR2 in* *1. EndMT and* *2. fibroblast proliferation and collagen production*
Aim 2: To determine the role of BMP signaling in RV fibrosis and capillary rarefaction in a murine model of isolated pressure-overload RV failure
Major Task 2: To characterize the role of BMPR2 signaling in the right ventricle in heart failure
Aim 2a. *To assess the contribution of fate-mapped endothelial cells to RV fibrosis in a PA banding (PAB) model of pressure-overloaded RV failure in mice* ***completed***
Aim 2b. *To determine whether BMPR2 deficient mice (bmpr2 heterozygous +/- and conditional EC cell specific bmpr2 -/- mice) develop more RV fibrosis and capillary rarefaction than wild type mice*

Aim 2c. *To determine whether increasing BMPR2 signaling with BMP ligands or low-dose FK506 prevent RV fibrosis and capillary rarefaction* ***completed***

Milestone(s) Achieved:

1. We have shown with fate-mapped endothelial cells that they do NOT contribute significantly to the fibrosis development in the RV after banding, but that rather the fibrosis is due to proliferation and collagen production of cardiac fibroblasts.

2. Heterozygous BMPR2 mice (50 % of BMPR2) have worse fibrosis after PAB, but not worse capillary rarefaction.

3. We were able to knock out BMPR2 in endothelial cells to a remaining expression of only 15% after crossing a floxed BMPR2 mouse with an inducible endothelial specific Cre mouse, which allowed us to knock out BMPR2 specifically in endothelial cells. These mice die earlier yet it does not seem because of more fibrosis or more capillary rarefaction.

5. FK506 (Tacrolimus) is able to reduce cardiac fibrosis and improve capillary rarefaction. Furthermore, FK506 improves RV function as assessed by cardiac MRI. The effect of FK506 is independent of a deficiency in BMPR2 levels, as the drug improves fibrosis in WT and BMPR2 heterozygous mice.

Aim 3: To develop a non-invasive method of quantifying BMPR2 signaling in plasma and PBMCs as a surrogate biomarker of BMPR2 expression and signaling in human cardiac tissue

Major Task 3: To develop a surrogate biomarker in blood to identify patients deficient in BMPR2 who might benefit from BMPR2 activating therapy

Aim 3a. *To develop a "BMPR2-signature" from heart tissue and validate candidate biomarkers in PBMCs and plasma in mice after PAB-surgery at various time points. Mice sacrificed at baseline, d7 and d28 to collect heart tissue and PBMCs (n=10 for each time point), during the development of RV failure 50%*

Aim 3b. *To validate the "BMPR2 signature" developed in Aim3a in human heart tissue and PBMCs/Plasma from patients with CHD in comparison to patients with PAH, ischemic/dilated LV cardiomyopathy at different stages of heart failure. Proteomics not evaluated yet.*

Milestone(s) Achieved:

Partial Identification of key molecules of the BMPR2 pathway that are associated with the development of cardiac fibrosis and capillary rarefaction in the heart in PAB mouse model and that correlate with levels in blood. We have begun to correlate BMPR2 and Id1 levels in blood (PBMCs) with RV tissue. Yet, as it is difficult to collect enough blood and isolate PBMCs from mouse blood, we do not have enough samples to show a significant correlation.

1. *Validation of key BMPR2 pathway molecules in blood and RV tissue from patients with CHD; outstanding*
2. *Publications;*
   The PI and co-PI Dr. Reddy have published or resubmitted the following papers during the time of the grand funding:

(1) Boehm et al. Delineating the molecular and histological events that govern right ventricular recovery using a novel mouse model of PA-debanding. *Cardiovasc Res.* 2019 Nov 18. PMID 31738411.

(2) Grinnan D et al. Drug Repositioning in Pulmonary Arterial Hypertension: Challenges and Opportunities. *Pulm Circ.* 2019 Jan-Mar;9(1). PMID:30729869.

(3) Boehm M et al. FK506 improves functional right ventricular adaptation to an increased afterload by reducing cardiac fibrosis. Resubmitted to *Am J Resp Cell Mol Biol*.

(4) Andruska et al. Consequences of BMPR2 deficiency in the pulmonary vasculature and beyond. *Int J Mol Sci* 2018. Aug 24;19(9). PMID 30149506.

(5) Friedberg MK et al. Right ventricular failure in congenital heart disease. *Curr Opin Pediatr*. 2019. Oct;31 5:604-610.

(6) Reddy S. Proceedings from the 2019 Stanford Single Ventricle Scientific Summit: From Discovery to Clinical Applications. *J Am Heart Assoc*. 2020 Apr 4;9(7). PMID: 32188306.

- **What was accomplished under these goals?**

1+2) major activities/objectives:

As described in the SOW above, we have essentially completed Aims 1, 2 and partially Aim 3.
In this grant, we aimed to investigate whether FK506 has additional direct effects on the pressure overloaded RV, with the ultimate goal to improve RV failure in congenital heart disase. We hypothesized that increasing cardiac BMP signaling with FK506 improves RV structure and function in a model of fixed RV afterload independent from its beneficial effects on the pulmonary vasculature. Direct effects on the heart were studied after surgical pulmonary artery banding (PAB) in wildtype and heterozygous *Bmpr2* mutant mice as well as in mice where BMPR2 was deleted in endothelial cells. Function was assessed repetitively via cardiac magnetic resonance (CMR) imaging during continuous FK506 infusion. The contribution of endothelial cells to cardiac fibrosis was assessed by genetic lineage tracing of endothelial cells. Molecular mechanistic studies were performed in human cardiac fibroblasts (hCFs) and cardiac endothelial cells. In mice, low BMP signaling measured directly in the RV exaggerated PAB-induced RV fibrosis and induced capillary rarefaction. FK506 therapy restored cardiac BMP signaling, reduced RV fibrosis in a BMP-dependent manner independent from immunosuppression, preserved RV capillarization through inhibition of endothelial fate conversion and improved RV function over the time-course of disease. Mechanistically as assessed in cell cutlure, FK506 required ALK1 in hCFs as BMPR2 co-receptor to reduce TGFb1-induced proliferation and collagen production.
As a conclusion of this project, we demonstrated that increasing cardiac BMP signaling with FK506 improved RV structure and function independent from its beneficial effects on afterload and independent from baseline BMPR2 levels. Our findings support FK506 as a therapeutic strategy in diseases that have a vulnerable RV such as in congenital heart disease and pulmonary hypertension.

3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative)

In the following I will summarize our findings in a resut summary that we have re-submitted to the American Journal of Molecular and Cell Biology. The respective figures are attached at the end of the report.

Evidence for intrinsic RV abnormalities in PAH patients with low peripheral *BMPR2* expression independent from etiology
Our group has previously suggested that *BMPR2* gene expression in peripheral blood mononuclear cells (PBMCs) is a surrogate for pathway presence and activity in organs such as lung and heart and reported that a 12-months treatment with FK506 increased *BMPR2* expression in PBMCs in three end-stage PAH patients with severe RV dysfunction. Hypothesizing that PAH patients with low BMP signaling in the cardio-pulmonary system would benefit most from pharmacological BMP pathway activation, we first quantified *BMPR2* gene expression in PBMCs from non-PAH patients (n=30) to define an empirical reference threshold of control *BMPR2* levels (Figure 1A). Next, we defined low BMP signaling (≤50% *BMPR2* expression compared to non-PAH controls), and subcategorized a small exploratory PAH patient population of different etiology (n=13 NYHA class II PAH patients; Table E1), including three hereditary PAH cases with *BMPR2* germline mutations, into patients with low and control level BMP signaling (n=6, Figure 1A). We demonstrate in this cohort, that, despite comparable RV afterload assessed as pulmonary vascular resistance index (PVRi, Figure 1B), patients with low BMP signaling present with more severe RV diastolic dysfunction (IVRT/sysTime, Figure 1C) and reduced RV pumping function (global RV strain, Figure 1D). Pearson linear correlation analyses revealed that impaired RV function was related with *BMPR2* transcript expression (r=0.6 and =-0.8, respectively; Figure 1E+F), providing a possible link between the observed intrinsic RV abnormalities and reduced BMP signaling that is independent from PAH etiology.
Low BMP signaling exaggerates RV fibrosis in response to chronic RV pressure overload
Next, we assessed whether low BMP signaling in the RV predisposes to an impaired adaptation to chronic RV pressure overload, as our clinical data and data from patients with hereditary PAH and a BMPR2 mutation would

suggest. Therefore, mice heterozygous for *Bmpr2 (Bmpr2+/-*) and *Bmpr2+/+* littermate controls underwent pulmonary artery banding (PAB) to induce chronic RV pressure overload or Sham control surgery. In these animals, no disease-related mortality was observed and all endpoints were assessed 7 weeks after surgery (Figure 2A). Gene expression analyses for *Bmpr2* and its major downstream signaling target *Id1* confirmed low BMP signaling in the RV of *Bmpr2+/-* mice when compared with controls, suggesting low BMP pathway activity at baseline (Figure 2B). In addition, PAB was sufficient to reduce BMP signaling in the RV of *Bmpr2+/+* animals to levels comparable with *Bmpr2+/-*. This is in line with our observations in PAH patients with low BMP signaling and without *BMPR2* mutation (Figure 1A). On a histological level, chronic RV pressure overload provoked RV cardiomyocyte hypertrophy (cardiomyocyte surface area, Figure 2C), reductions in RV capillarization (capillaries/cardiomyocytes, Figure 2D) and RV fibrosis, characterized by increased collagen amounts (% RV collagen, Figure 2E) and fibroblast accumulation (Vimentin staining, Figure 2F) independent from *Bmpr2* expression after PAB. Intriguingly, animals with low BMP signaling at baseline presented with exaggerated RV fibrosis after PAB (Figure 2E+F). In addition to fibrosis, other mechanisms such as metabolic limitations or changes in calcium cycling that have been previously described can lead to functional RV impairment. We demonstrate a de-regulation of triglyceride synthases (*Dgat1*, *Dgat2*) but not fatty acid transporter *Cd36* mRNA expression without signs of lipid accumulation in RV cardiomyocytes in the pressure overloaded RV of *Bmpr2+/-* mice (Figure E1). We further provide evidence for impaired cardiomyocyte calcium handling as a consequence of low BMP signaling, evidenced by an increased basal intracellular calcium concentration, reduced calcium uptake during contraction and a delayed calcium release during relaxation (Figure E2). As a consequence, *Bmpr2+/-* mice demonstrate reduced exercise capacity in an incremental ramp treadmill exercise protocol (Figure E2), suggesting that several mechanisms related to low BMP signaling contribute to cardio-pulmonary impairment.

FK506 therapy increases BMP signaling, improves RV capillarization and reduces RV fibrosis in an animal model of chronic RV pressure overload

To assess whether FK506 directly affects RV adaptation to chronic RV pressure overload by increasing BMP signaling independent from its documented beneficial effects on the pulmonary vasculature, we utilized a PAB mouse model in which all effects on the RV myocardium are independent from interfering afterload alterations due to surgical stenosis of the main pulmonary artery. In this model, compensatory RV hypertrophy is established within the first week, followed by a progressive decline in cardiac function with impaired exercise capacity and prominent signs of RV failure, such as septal flattening, RV dilatation and activation of the fetal gene program, within 7-8 weeks after disease commencement. Treatment with low-dose FK506 (0.05 mg/kg/d, s.c. via an osmotic mini-pump) was initiated at 1 week after PAB when compensatory hypertrophy was established and animals were sacrificed after additional 7 weeks of treatment (Figure 3A). We did not observe significant disease-related mortality within the experimental groups.

FK506 therapy significantly increased BMPR2 protein levels in the RV after PAB (Figure 3B), together with increased gene expression of its major downstream signaling target *Id1* (Figure 3C), confirming efficacy for low-dose FK506 therapy to increase BMP pathway activity in the RV after PAB. On a structural level, increasing BMP signaling with FK506 had no effect on PAB-induced RV cardiomyocyte hypertrophy (cardiomyocyte surface area, Figure 3D) but preserved the RV capillary vasculature (capillaries/cardiomyocytes, Figure 3E) and significantly reduced RV fibrosis as assessed by total RV collagen content (Figure 3F) and accumulation of fibroblasts (Figure 3G).

Treatment with FK506 directly improves RV function in an animal model of chronic RV pressure overload

Next, we sought to assess the effects of FK506 on RV function using gold-standard non-invasive cardiac magnetic resonance (CMR) imaging in a longitudinal fashion, followed by terminal intra-cardiac catheterization (Figure 4A). Importantly, peak pressure gradient (PPG) measurements across the pulmonary artery band revealed a comparable degree of stenosis between PAB groups before treatment initiation (Figure 4B). Signs of remodeling, including RV free wall hypertrophy and dilatation were prevalent at 1 week after PAB, confirming established remodeling before treatment commencement (Figure 4C). At week 7 after surgery, PAB-challenged placebo-treated animals presented with compression of the LV, septal flattening and severe RV dilatation, which was attenuated by FK506 therapy (Figure 4D). Further, FK506 had no effect on RV hypertrophy, assessed *ex vivo* via Fulton's index (RV/(LV+S), Figure 4E) and RV systolic pressure, measured via closed-chest RV catheterization (RVSP, Figure 4F), both important to maintain RV coupling against the fixed, increased afterload. Importantly, longitudinal CMR imaging uncovered improvements in RV end-diastolic volume, RV stroke volume and RV

ejection fraction over the time course of maintained RV pressure overload when PAB-challenged animals received FK506 therapy (Figure 4G-I). These findings were accompanied by regional deformation improvements in radial, circumferential and longitudinal directions when PAB-operated animals were treated with FK506, assessed by strain imaging, confirming an improved RV performance in these mice (Figure 4J-M). Finally, principal component (PC) analysis was used to reduce 20 metrics of myocardial strain in both ventricles to 3 PCs, which showed that FK506-treated PAB animals cluster from placebo-treated PAB mice along PC1 (Figure 4N+O). Furthermore, vectors superimposed on the PC analysis biplot show a correlation between some strain metrics, and that none are directly aligned with PC1, which suggests that PC1 is strongly influenced by a myriad of strain metrics. This demonstrated that FK506 therapy consistently changed global metrics of inter-ventricular cardiac contractile mechanics, which was associated with improved function.

FK506 administration reduces RV fibrosis through increasing BMP signaling rather than immunosuppression

Given that FK506 at higher doses is clinically well-known to exert prominent immunosuppressive effects, we tested whether the observed beneficial effects of low-dose FK506 therapy on RV structure and function are BMP-dependent or secondary caused by systemic immunosuppression. Of note, chronic BMP pathway inhibition with LDN-193189 in the healthy heart had neither an effect on RV capillarization nor RV fibrosis (Figure E3). We demonstrate that low-dose FK506 therapy (0.05mg/kg/d) improved RV capillarization in a BMP-independent manner as concomitant LDN-193189 administration (2.5mg/kg/d) had no effect on vascularization in the hypertrophied RV (Figure 5A-D). Further, immunosuppression with Cyclosporine (25mg/kg/d), a calcineurin inhibitor that lacks the FKBP12-mediated effect on BMP signaling as opposed to FK506, had no additional beneficial effects on PAB-induced vascular rarefaction (Figure 5A-D), pointing towards a different mechanism of action than immunosuppression for FK506 to preserve the RV vasculature. Furthermore, high-dose FK506 administration (1mg/kg/d) was less effective in reducing RV fibrosis compared to low-dose FK506 in response to PAB and comparable to low-dose FK506 plus LDN-193189 treatment (Figure 5C+E), demonstrating that the anti-fibrotic effects of low-dose FK506 are less a result of immunosuppression but rather of BMP pathway activation and indeed BMP-dependent, as the reduced efficacy with additional LDN-193189 treatment suggests. Along this line, chronic immunosuppression with Cyclosporine rather increased the RV collagen amount when compared to placebo-treated PAB animals, demonstrating that the anti-fibrotic effects of low-dose FK506 are independent from immunosuppression and pointing towards a potential beneficial anti-fibrotic role of T-cell activation in the pressure overloaded RV, which is suppressed with high-dose immunosuppression.

FK506 reduces cardiac fibroblast proliferation and collagen production and preserves endothelial cell function in a BMP-dependent manner

On a cellular level, we provide evidence in primary human cardiac fibroblasts (hCFs) that the anti-fibrotic effects of FK506 are caused by blocking TGF□1-induced proliferation (Figure 6A), reducing expression of extracellular matrix molecules *COL1A1* (Figure 6B) and *COL3A1* (Figure 6C) and decreasing *ACTA2* expression which is required for contractile hCF function (Figure 6D), all in a BMP-dependent manner. Additionally, we show that FK506 potently increases BMP signaling, even under conditions of TGF□ pathway activation, independent from *BMPR2* expression levels in hCFs, as evidenced by increased *ID1* gene expression (Figure 6E+F). Mechanistically, siRNA-mediated gene silencing revealed that ALK1 is the BMPR2 co-receptor required to mediate the anti-fibrotic effects of FK506 (Figure E4).

In human cardiac endothelial cells (hCECs), FK506 improved tube formation capacity after TGF□1+IL-1□ treatment (Figure 6G), similar to our *in vivo* observations, and reduced *COL1A1* expression (Figure 6H). This was accompanied by preservation of endothelial cell-specific *CD31* expression (Figure 6I), suggesting inhibition of endothelial cell-fate conversion. This notion was further supported by morphologic analyses and immunostaining for endothelial (CD31, vWF) and mesenchymal (FN1) markers (Figure E5). In contrast to hCFs, FK506 treatment increased BMP signaling in hCECs by increasing both, *BMPR2* and *ID1* expression (Figure 6J+K), pointing towards cell type-specific regulatory mechanisms that control BMP pathway activity in the heart.

FK506 inhibits cardiac endothelial cell transition towards fibroblast-like cells that minimally contribute to pressure overload-induced RV fibrosis *in vivo*

To address, whether endothelium-derived cells in the RV transition towards a mesenchymal pro-fibrotic fate upon PAB, as our *in vitro* findings and data focusing on the pressure overloaded left ventricle suggest, and thereby contribute to vascular rarefaction and fibrosis, genetic endothelial lineage labeling followed by cell fate mapping using Pdgfb-CreER^{T2}; tdTomato+/- transgenic mice was performed. In these animals, cardiac endothelial cells

and their progeny were genetically marked through tdTomato expression upon Tamoxifen-induced recombination 4 weeks prior to sham surgery or PAB in order to exclude *de novo* cell labeling caused by residual Tamoxifen at the time of surgery. FK506 therapy was initiated directly after disease induction to increase BMP signaling in the RV at the time when resident fibroblasts are activated and fibrosis develops and was continued for additional 3 weeks until RV fibrosis was established (Figure 7A). Cell sorting at the time before surgery revealed tdTomato labeling in >98% of endothelial cells (CD31+CD45-) isolated out of the RV, demonstrating effective genetic endothelial cell labeling via this approach (Figure 7B). This finding was further supported by endothelium-specific co-staining of Isolectin B4 and tdTomato in capillaries and larger blood vessels of the RV free wall, without labeling of neither epi- nor endocardium, excluding epicardial and endocardial contributions to endothelial cell conversion (Figure 7C). Three weeks after PAB, patchy areas of fibroblast accumulation, defined as Vim+IB4- cells, in interstitial and perivascular regions of the RV free wall were apparent without obvious tdTomato co-labeling (Figure 7D; Figure E6), suggesting a non-endothelial cell origin of the Vim+IB4- mesenchymal cells. Quantification of endothelial-to-mesenchymal transition (EndMT) events in perivascular and interstitial fibrotic areas (Figure 7E) further supported this finding as only a minority of cells (<5% of lineage labeled endothelial cells, Figure 7F) transitioned towards a mesenchymal fate or acquired a mesenchymal expression profile, evidenced by Vim+IB4-tdTomato+ labeling. Of note, EndMT events occurred predominantly in cells located at the branching point of capillaries in non-fibrotic interstitial regions (Figure 7E; Figure E7). These rare transitioned cells at the observed timepoint did not contribute to capillary tube formation. Treatment with FK506 further reduced rare EndMT events (Figure 7F) and, importantly, reduced Type 1 Collagen accumulation in the pressure overloaded RV (Figure 7G), again confirming the anti-fibrotic potential of FK506 therapy.

Taken together, we propose that FK506 re-balances TGFb/BMP signaling directly in the pressure overloaded RV by increasing BMP signaling, reduces collagen production and proliferation of cardiac fibroblasts and inhibits endothelial fate conversion, leading to reduced RV fibrosis, preserved RV capillarization, and most important, better RV function.

4) other achievements:

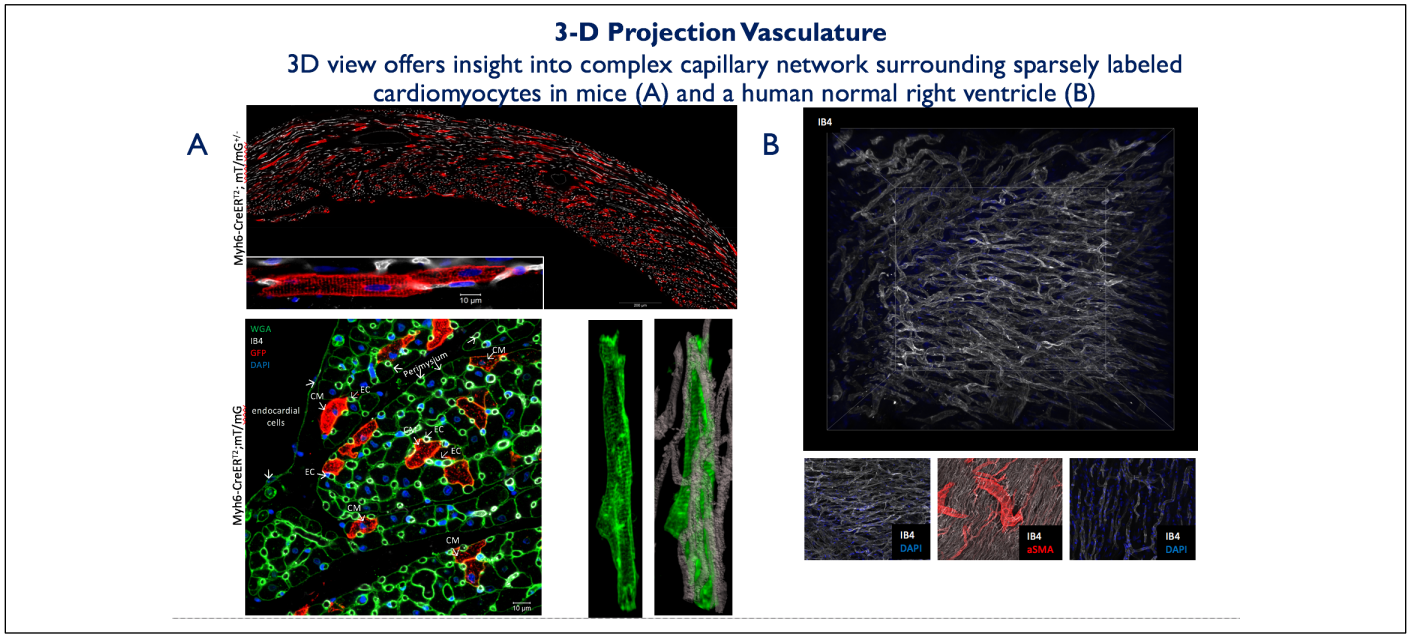
While we identified that reduced BMP signaling does lead to endothelial mesenchymal transition *in vitro,* we discovered through lineage tracing experiments *in vivo* that cardiac fibrosis in pressure-overload RV failure is predominately due to proliferation of resident cardiac fibroblasts and NOT endothelial mesenchymal transition. We did confirm that increasing BMP signaling with FK506 prevented cardiac fibrosis *in vivo* and increased capillary density (using 2-D imaging). Yet, this was true in BMPR2 deficient **and** also wildtype mice, suggesting that BMP-increasing-drugs might be beneficial for RV adaptation, regardless of a BMPR2 deficiency, which made aim 3 not so relevant anymore, as our data suggested that it was not important to identify patients with a BMPR2 deficiency because FK506 would work in all patients. **Stated goals not met**: We therefore did not complete aim 3, as we believe our data show that it des not matter whether pateints have reduced BMPR2 signaling or not. FK506 (Tacrolimus) will improve RV function regardless of whether BMPR2 signaling is reduced or is at normal levels.

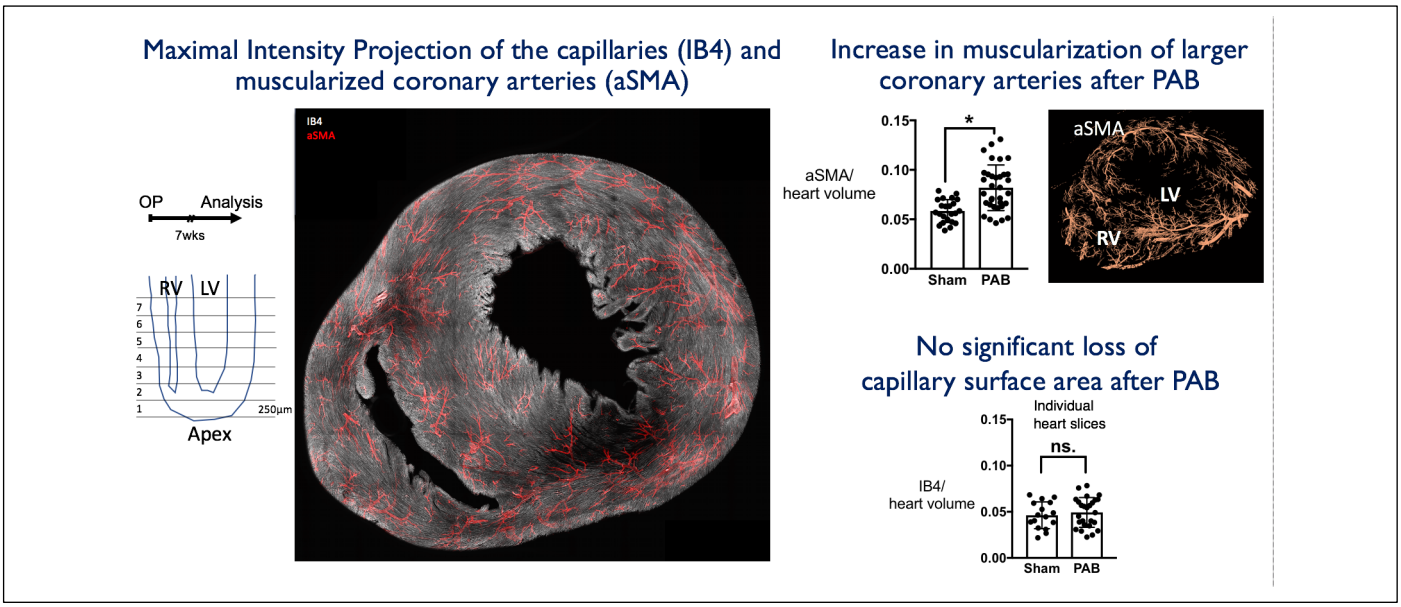
Challenges and Solutions:

We discovered that imaging the right ventricle in 2-D did not give enough granular resolution to really assess the cellular and histological changes responsible for RV failure and to quantify them properly, in particular the vasculature. That's why in the final year of the grant, we applied a **novel 3-D imaging** technique to the heart by imaging 200-700µm thick heart slices to better characterize and quantify the **changes in the capillary network** (**see Figures below**) as well as the spatial and temporal cellular events leading to **cardiac fibrosis** associated with RV adaptation and RV maladaptation/failure. This technique will be useful to determine **which** pathological feature to focus on therapeutically and **when**, in the course of RV adaptation/failure, a **therapeutic intervention** might be **beneficial**.

Given the dual role of fibrosis development, as an adaptive response to prevent cardiomyocyte overstretch vs. increasing stiffness and disrupting cardiomyocyte contraction and relaxation, we further concluded that it is important to pair the histological changes with a thorough characterization of RV function as well as quantitative strain analysis with cardiac MRI imaging. We have therefore formed a **new collaboration with Dr. Kheyfets**

(PhD), Department of Bioengineering at the University of Colorado. In order to reverse and not only prevent development of disease, we faced the problem of not exactly knowing how the RV recovers, leading to the following questions: (1) is cardiac fibrosis and capillary rarefaction reversible? (2) If it is reversible, would it be reversible even at late stages of RV failure? We therefore developed a mouse model of RV recovery by creating a **PA-debanding model** using self-absorbable sutures, which will be available for future studues to learn about **RV-recovery** and the role of therapeutics in the recovery process.





What opportunities for training and professional development has the project provided?

- The project gave the postdoctoral fellow Mario Boehm the opportunity to present his data in form of a poster presentation at the American Heart Association conference. His presentation and also the publication of the "Debanding paper" *(*Boehm et al. Delineating the molecular and histological events that govern right ventricular recovery using a novel mouse model of PA-debanding. *Cardiovasc Res.* 2019 Nov 18. PMID 31738411) were a major reason for him to be recruited back as a group leader at the University of Giessen, Germany.

- **How were the results disseminated to communities of interest?**

"Nothing to Report."

- **What do you plan to do during the next reporting period to accomplish the goals?**

This is the final report. I plan to apply for a DoD extension grant.

- **IMPACT:**
  - **What was the impact on the development of the principal discipline(s) of the project?**

Our finding show that cardiac fibrosis is a promising target in RV failure and that a dysfunctional BMPR2 pathway might promote fibrosis whereas increasing BMPR2 signaling might reduce fibrosis and improve RV function, irrespectible of baseline BMPR2 values. FK506 (tacrolimus) reduces fibrosis and capillary rarefaction and improves RV function, in the setting of persistent RV afterload.
We have patented FK506 for the treatment of PAH. A Phase II clinical trial has already been completed by our group in PAH (www.ClinicalTrials.gov NCT01647945) and a clinical phase IIB efficacy trial in PAH is planned. FK506 is already FDA approved and is well tolerated.
FK506 would be readily available for testing in a clinical trial in patients with congenital heart disease at Stanford Children's Hospital.

  - **What was the impact on other disciplines?**

FK506 as an RV-specific heart failure therapeutic, would present a major advancement not only in the field of CHD but also in other conditions where the RV is uniquely at risk. Examples would be (a) WHO group 1 PAH in its idiopathic or associated form with mixed connective tissue disease such as scleroderma, where RV failure represents the number one cause of mortality and mainly affects women (**b**) Patients with chronic hypoxia and WHO Group 3 pulmonary hypertension (PH) with RV failure, such as pulmonary fibrosis or chronic obstructive lung disease.

  - **What was the impact on technology transfer?**
    - *"Nothing to Report."*
  - **What was the impact on society beyond science and technology?**
    - *"Nothing to Report."*

- **CHANGES/PROBLEMS:** *"Nothing to Report,"*
  - **Changes in approach and reasons for change**
    - *none*
  - **Actual or anticipated problems or delays and actions or plans to resolve them**
    - *none*
  - **Changes that had a significant impact on expenditures**
    - *none*
  - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
    - *Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution*

committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

- **Significant changes in use or care of human subjects**

The Stanford IRB decided that: On the basis of the information provided, the IRB has determined that this project does not meet the definition of human subject research as defined in federal regulations 45 CFR 46.102 or 21 CFR 50.3. No further IRB review is required (see also Appendix for documentation).

- **Significant changes in use or care of vertebrate animals.**
  - none
- **Significant changes in use of biohazards and/or select agents**
  - none

- **PRODUCTS:**
  - **Publications, conference papers, and presentations**
    - **Journal publications.**

1. Boehm M, Tian X, Mao Y, Ichimura K, Dufva MJ, Ali K, Prosseda SD, Shi Y, Kuramoto K, Reddy S, Kheyfets VO, Metzger RJ, **Spiekerkoetter E.** Delineating the molecular and histological events that govern right ventricular recovery using a novel mouse model of PA de-banding. Cardiovasc Res. 2020 Aug 1;116(10):1700-1709. doi: 10.1093/cvr/cvz310. PMID: 31738411

2. Andruska A, Ali K, **Spiekerkoetter E.** Targeting BMPR2 Trafficking with Chaperones - An Important Step Towards Precision Medicine in Pulmonary Arterial Hypertension. Am J Respir Cell Mol Biol. 2020 Aug;63(2):137-138. doi: 10.1165/rcmb.2020-0130ED. PMID: 32339467

3. Grinnan D, Trankle C, Andruska A, Bloom B, **Spiekerkoetter E.** Drug Repositioning in pulmonary arterial hypertension: challenges and opportunities. Pulm Circ. 2019 Jan-Mar;9(1):2045894019832226. doi: 10.1177/2045894019832226. PMID: 30729869

4. Boehm M, Xuefei Tian, Ali MK, Mao Y, Ichimura K, Zhao M, Kuramoto K, Prosseda SD, Fajardo G, Dufva MJ, Qin X, Kheyfets VO, Bernstein D, Metzger RJ, Zamanian RT, Haddad F, and **Spiekerkoetter E**. FK506 improves functional right ventricular adaptation to an increased afterload by reducing cardiac fibrosis. Resubmitted to *Am J Resp Cell Mol Biol*.

5. Andruska A, **Spiekerkoetter E.** Consequences of BMPR2 Deficiency in the Pulmonary Vasculature and Beyond: Contributions to Pulmonary Arterial Hypertension. Int J Mol Sci Aug 24;19(9).p pii: E2499. doi: 10.3390/ijms19092499. Review. PMID: 30149506.

6. Friedberg MK et al. Right ventricular failure in congenital heart disease. *Curr Opin Pediatr*. 2019. Oct;31 5:604-610.

7. **Reddy S**. Proceedings from the 2019 Stanford Single Ventricle Scientific Summit: From Discovery to Clinical Applications. *J Am Heart Assoc*. 2020 Apr 4;9(7). PMID: 32188306.

8. Targeted Proteomics of Right Heart Adaptation to Pulmonary Arterial Hypertension. Myriam Amsallem M*, Sweatt AJ*, Arthur Ataam J, Guihaire J, Lecerf F, Lambert M, Maria Rosa Ghigna MR, Ali MK,

Mao Y, Fadel E, Rabinovitch M, Perez V, **Spiekerkoetter E**, Mercier O, Francois Haddad F*, Zamanian* RT, Eur R J. Accepted for publication Oct 3 2020

- **Books or other non-periodical, one-time publications.** *none*

- **Other publications, conference papers, and presentations.**

American Thoracic Society 2017
Reduced BMPR2 signaling impairs right ventricular heart function and exaggerates cardiac fibrosis upon chronic pressure overload*
M. Boehm, X. Tian, M. Zhao, S. Dannewitz, K. Kuramoto, J. Kuang, S. Reddy, D. Bernstein, E. Ashley and E. Spiekerkoetter
American Thoracic Society International Conference, Washington DC 5/2017
Poster discussion session

American Heart Association conference 2017:
Reduced BMPR2 signaling in the right ventricle impairs functional adaptation to pressure overlaod in pulmonary arterial hypertension.*
M Boehm, Z Tian, F Zhang, S Dannewitz, K Kuramoto, A Ashley, R Zamanian F Haddad, RJ Metzger, E Spiekerkoetter
AHA conference, Anaheim, Nov 11 – 14, 2017
Poster presentation

ATS 2018
Bone Morphogenetic Protein Receptor 2 Expression Is Reduced in Blood Across Pulmonary Arterial Hypertension Subtypes but Does Not Reflect Disease Severity
A94 - BRAVE NEW WORLD: THE CUTTING EDGE OF PH RESEARCHAndrew Sweatt, Richard Wells, Natasha Purington, Haley Hedlin, Deepti Sudheendra, Andrew Hsi, Roham T Zamanian, Edda Spiekerkoetter
American Thoracic Society International Conference, San Diego, CA 5/19-23/2018
Mini Symposium – oral presentation

<u>Invited talks:</u>
- Keystone Meeting, Keystone, CO 1/2018: Heart Failure: Crossing the Translational Divide
Session Chair: PH and RV failure
Increasing BMPR2 Signaling with FK506 (Tacrolimus) improves Right Ventricular Adaptation in Pulmonary Hypertension*

- Wall Center Symposium May 2018
From Precision Biology to Precision Medicine
Title: Targeting the BMPR2 pathway as an example of Precision Medicine

- Pulmonary Grand Rounds, Stanford University, September 2017
Title: What familial PAH can teach us about RV adaptation to pressure overload

- The 5th Annual Drug Discovery and Development Symposium for PH, PVRI and FDA, Bethesda, July 9-10 2018 Invited Faculty –
Panelist. Session: Repurposing Novel Drugs for Pulmonary Hypertension*

- Pulmonary Vascular Research Institute - 13th Annual World Congress
Barcelona Spain, 30 January - 3 February 2019
Invited Speaker
Title: Repurposing drugs for an orphan disease: opportunities and pitfalls*

- **Website(s) or other Internet site(s)**
  none
- **Technologies or techniques**
  none
- **Inventions, patent applications, and/or licenses**
  none
- **Other Products**
  none

- **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
  - **What individuals have worked on the project?**

Name: Edda Spiekerkoetter, MD
Project Role: PI
Nearest person month worked: 12
Contribution to project: Guidance, interpretation of results
Funding Support: DoD, NIH R01, Wall Center Research Fellow

Name: Mario Boehm, PhD
Project Role: Postdoctoral Research Fellow
Nearest person month worked: 12
Contribution to project: PAB surgery and staining.
Funding Support: DoD, Max Kade Foundation.

Name: Xuefei Tian, MD, MS
Project Role: Research Assistant, Lab manager
Nearest Person month worked: 6 months
Contribution to project: Staining, QPCR, Cell culture work, echo
Funding Support: DoD, NIH R01

Name: Mingming Zhao, MD
Project Role: Research Assistant
Nearest Person month worked: 3 months
Contribution to project: PAB surgery, training
Funding Support: DoD

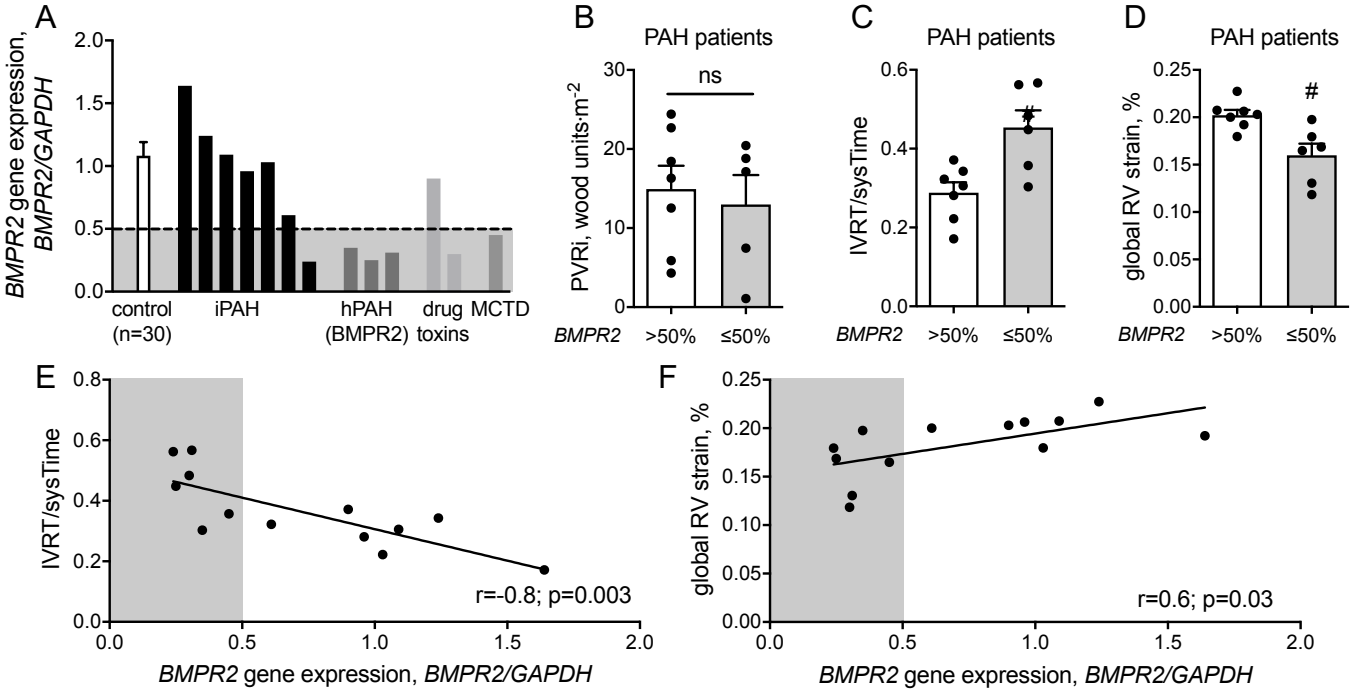
Ross Metzger, PhD
Project Role: co-investigator
Nearest Person month worked: 6
Contribution to project: lineage tracing
Funding Support: DoD, Wall Center for pulmonary vascular Disease Stanford

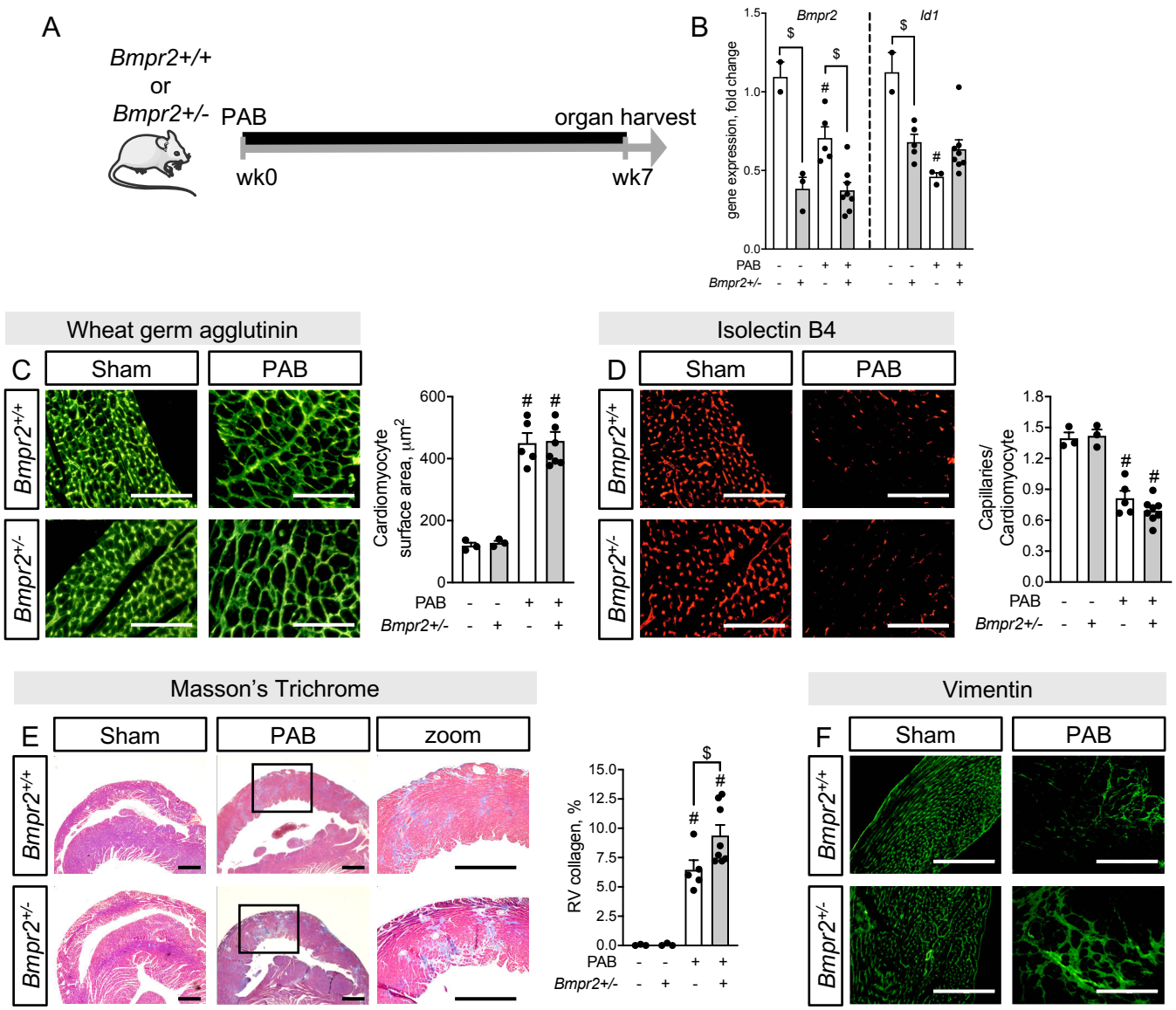
Sushma Reddy, MD
Project Role: co-investigator
Nearest Person month worked: 12
Contribution to project: RV imaging
Funding Support: DoD, Wall Center for pulmonary vascular Disease Stanford

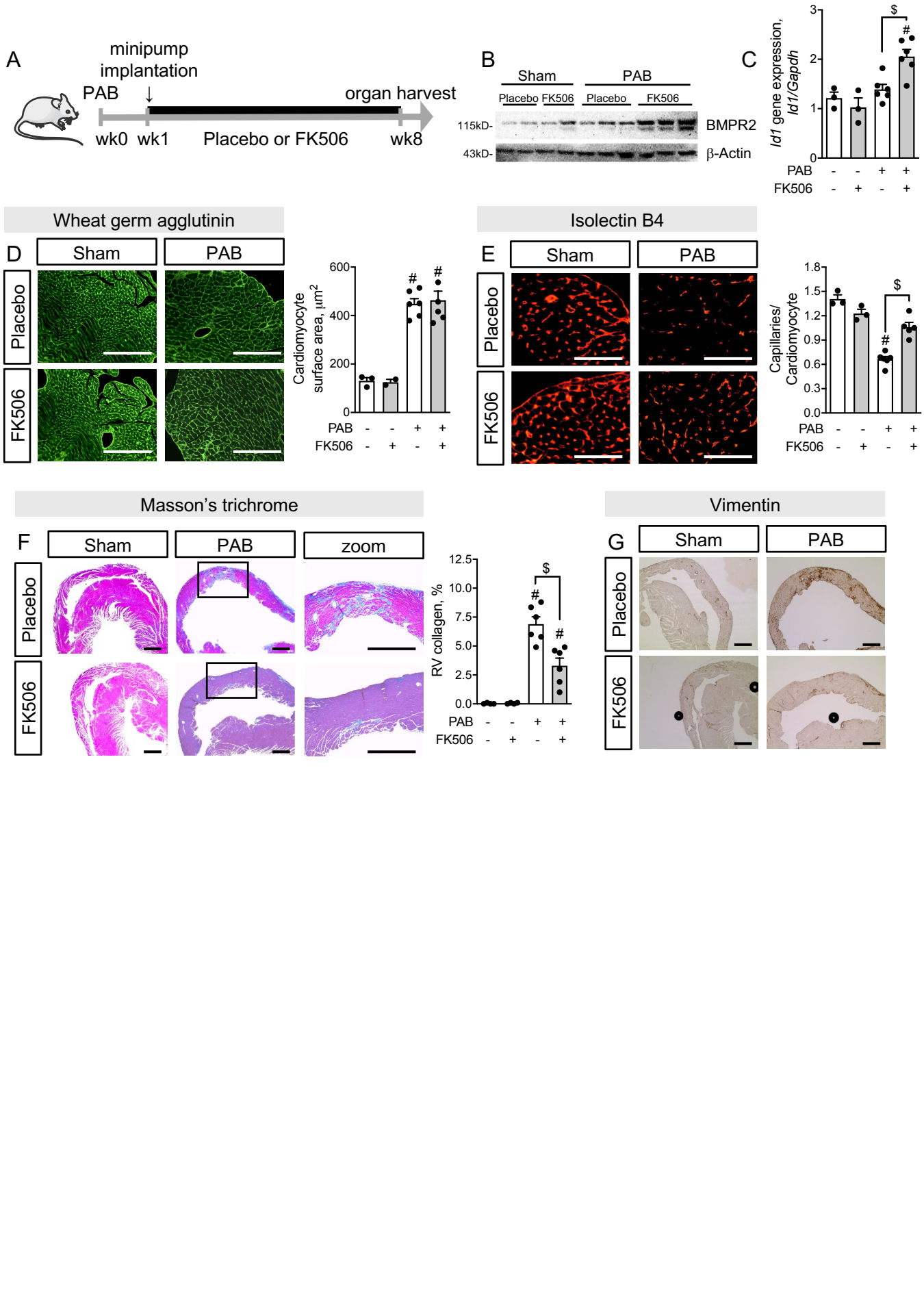
- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
    - *"Nothing to Report."*
- **What other organizations were involved as partners?** *"Nothing to Report."*

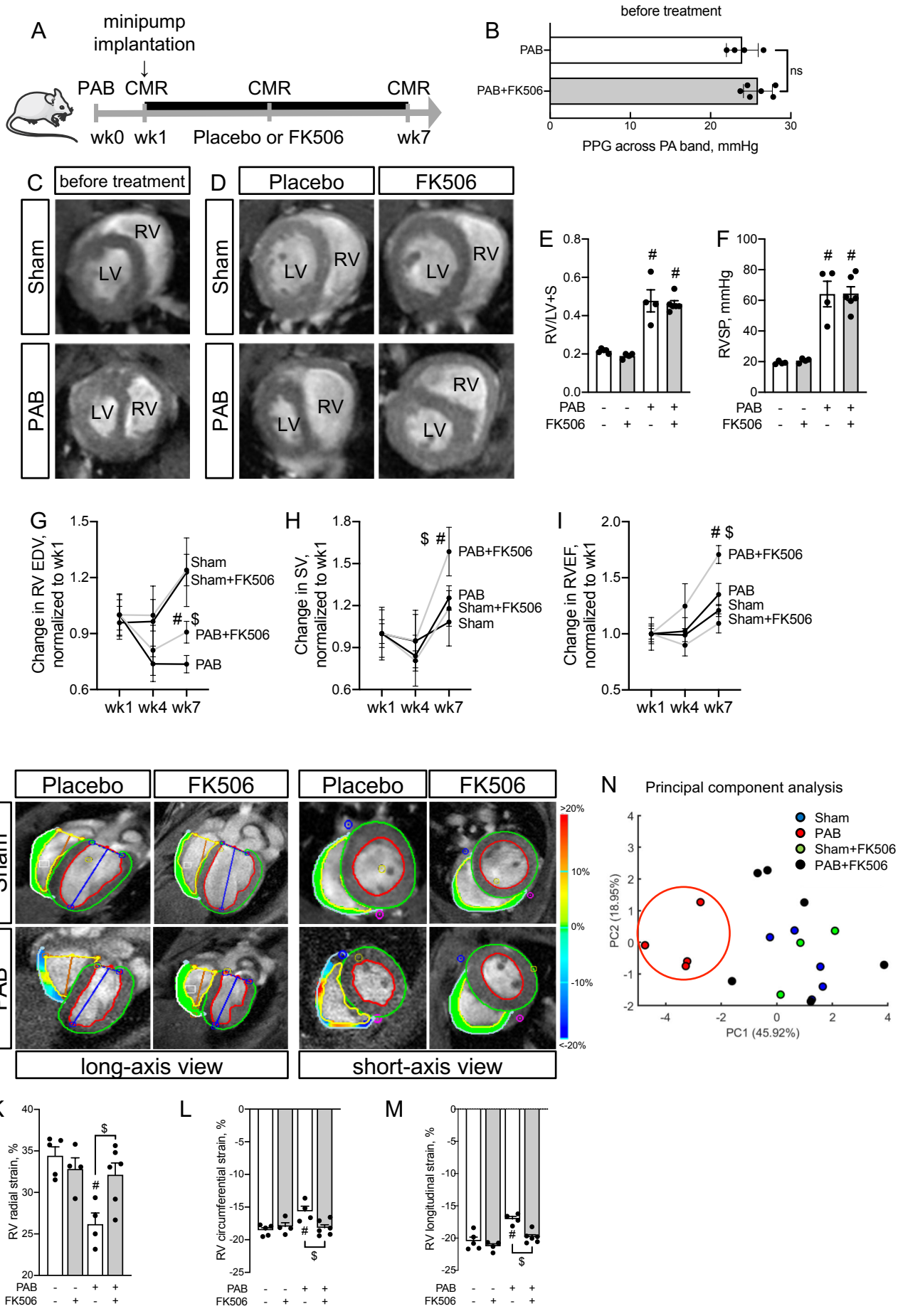
Special Reporting : none

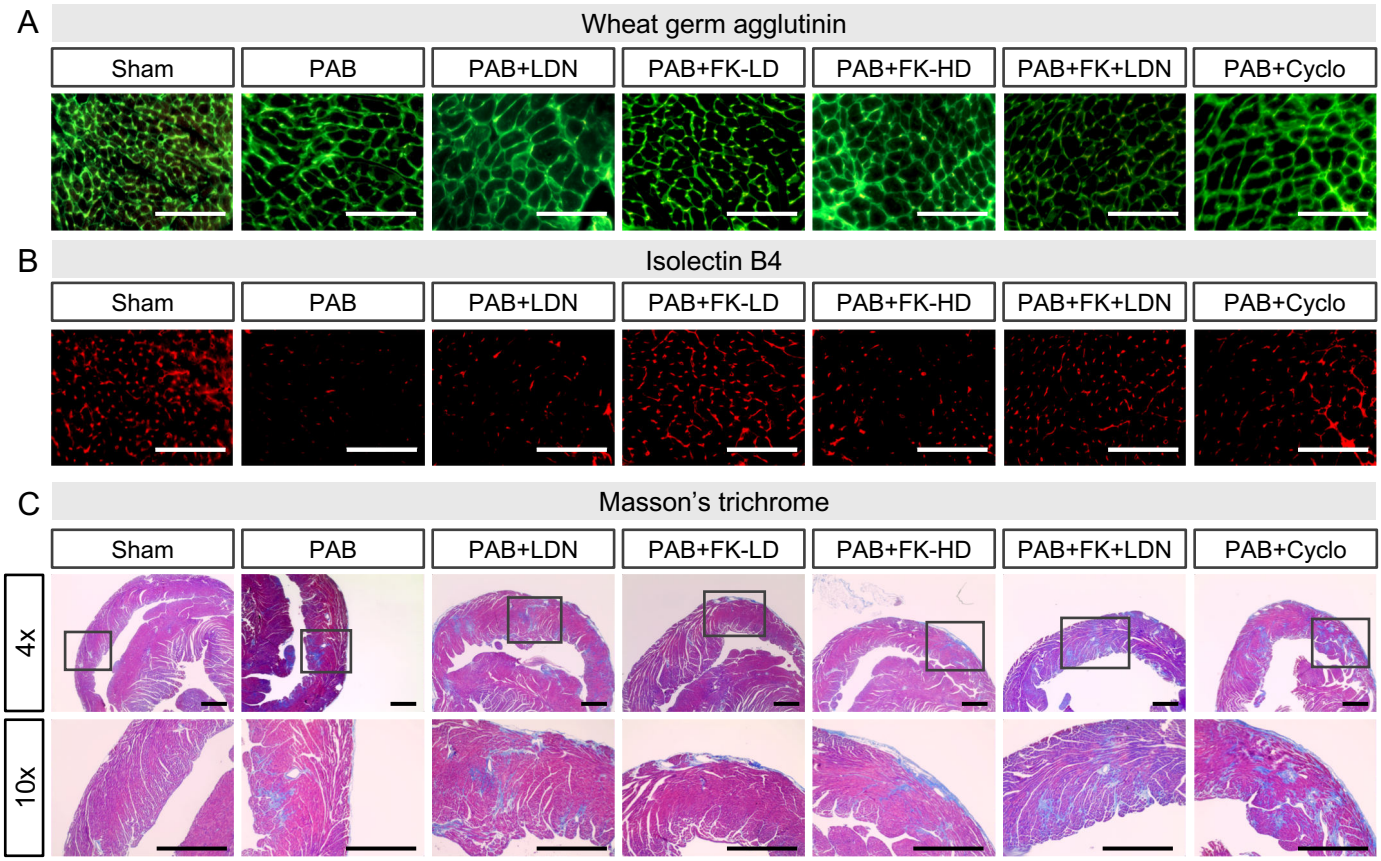
Appendix: Figures

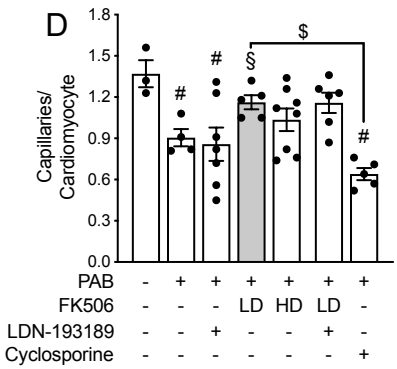

A
BMPR2 gene expression, BMPR2/GAPDH
2.0
1.5
1.0
0.5
0.0
control (n=30)
iPAH
hPAH (BMPR2)
drug toxins
MCTD
B
PAH patients
ns
PVRi, wood units·m-2
30
20
10
0
BMPR2 >50% ≤50%
C
PAH patients
IVRT/sysTime
0.6
0.4
0.2
0.0
BMPR2 >50% ≤50%
D
PAH patients
#
global RV strain, %
0.25
0.20
0.15
0.10
0.05
0.00
BMPR2 >50% ≤50%
E
IVRT/sysTime
0.8
0.6
0.4
0.2
0.0
r=-0.8; p=0.003
0.0 0.5 1.0 1.5 2.0
BMPR2 gene expression, BMPR2/GAPDH
F
global RV strain, %
0.25
0.20
0.15
0.10
0.05
0.00
r=0.6; p=0.03
0.0 0.5 1.0 1.5 2.0
BMPR2 gene expression, BMPR2/GAPDH

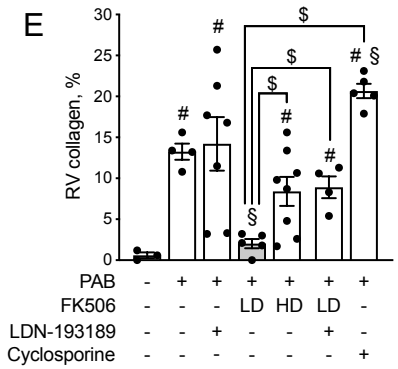

A
Bmpr2+/+
or
Bmpr2+/-
PAB
organ harvest
wk0
wk7
B
Bmpr2
Id1
gene expression, fold change
PAB
Bmpr2+/-
Wheat germ agglutinin
C
Sham
PAB
Bmpr2+/+
Bmpr2+/-
Cardiomyocyte surface area, µm2
Isolectin B4
D
Capillaries/ Cardiomyocyte
Masson's Trichrome
E
zoom
RV collagen, %
Vimentin
F

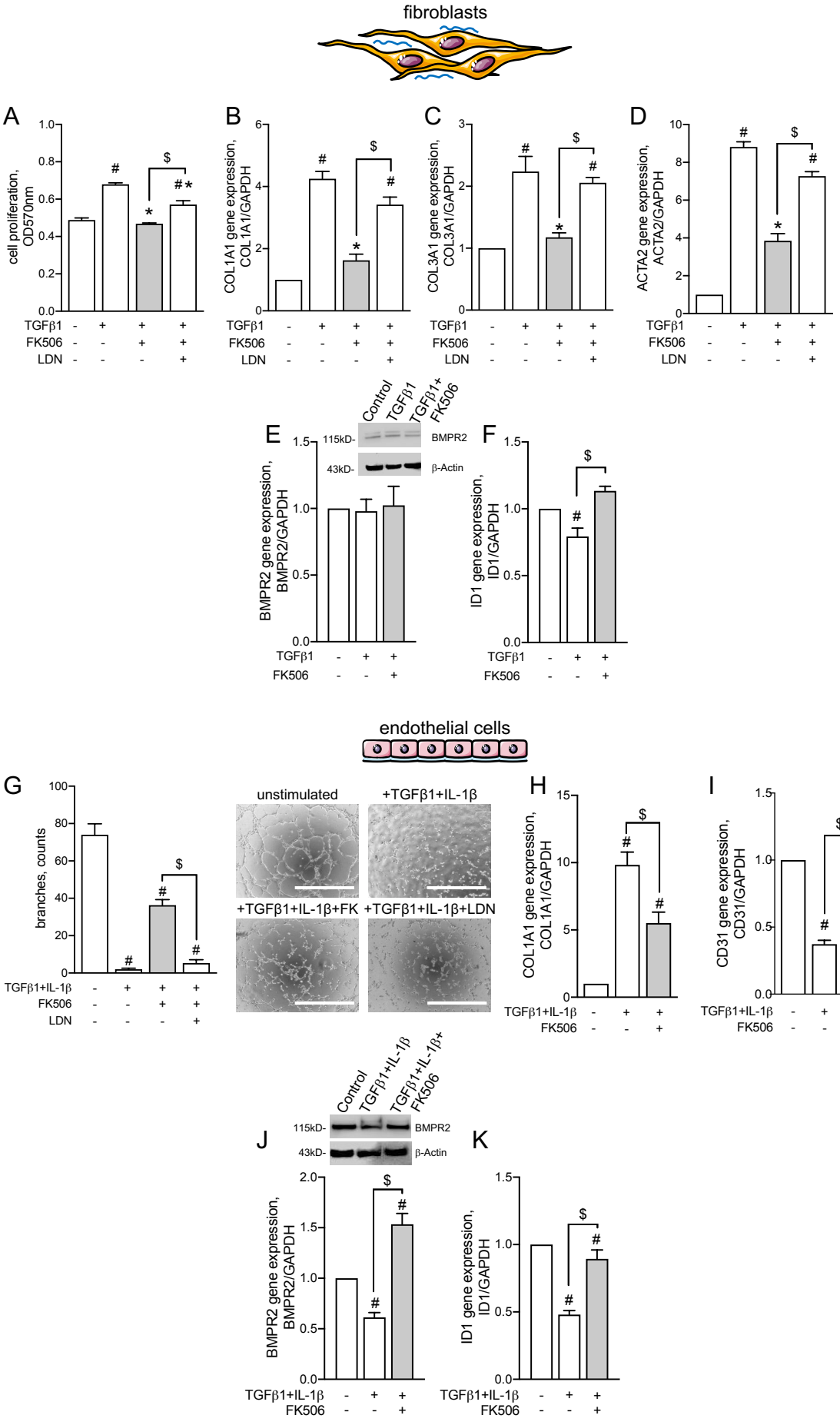

A
minipump
implantation
PAB
organ harvest
wk0 wk1
Placebo or FK506
wk8
B
Sham
PAB
Placebo FK506 Placebo FK506
115kD-
43kD-
BMPR2
β-Actin
C
Id1 gene expression,
Id1/Gapdh
PAB
FK506
Wheat germ agglutinin
D
Sham
PAB
Placebo
FK506
Cardiomyocyte
surface area, μm2
Isolectin B4
E
Capillaries/
Cardiomyocyte
Masson's trichrome
F
zoom
RV collagen, %
Vimentin
G

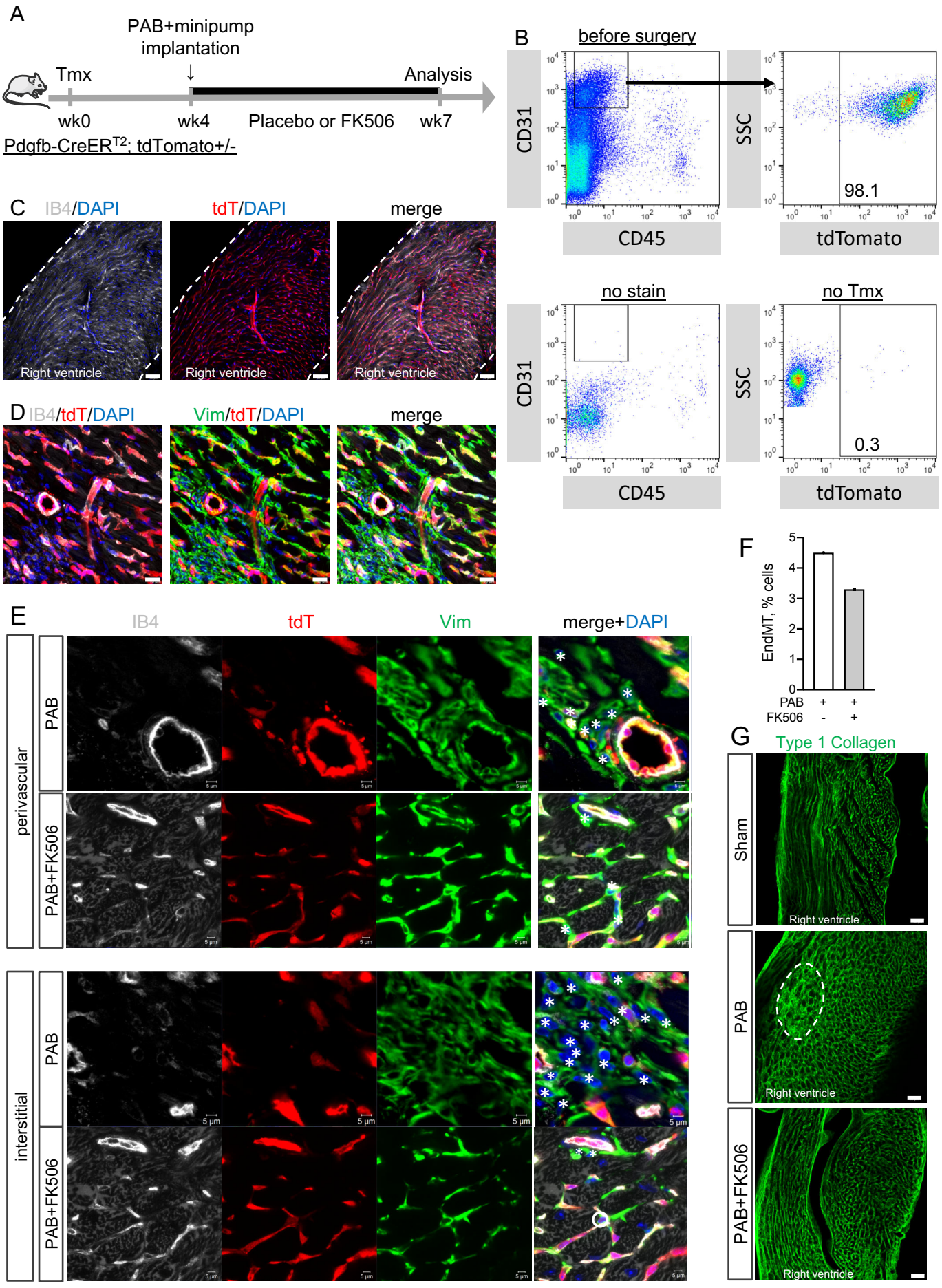

A
minipump implantation
PAB CMR
CMR
CMR
wk0 wk1
Placebo or FK506
wk7
B
before treatment
PAB
PAB+FK506
ns
0 10 20 30
PPG across PA band, mmHg
C
before treatment
Sham
PAB
RV
LV
D
Placebo
FK506
Sham
PAB
RV
LV
E
RV/LV+S
0.0 0.2 0.4 0.6 0.8
#
#
PAB - - + +
FK506 - + - +
F
RVSP, mmHg
0 20 40 60 80 100
# #
PAB - - + +
FK506 - + - +
G
Change in RV EDV, normalized to wk1
1.5 1.2 0.9 0.6
Sham
Sham+FK506
$
PAB+FK506
PAB
wk1 wk4 wk7
H
Change in SV, normalized to wk1
1.8 1.5 1.2 0.9 0.6
$ #
PAB+FK506
PAB
Sham+FK506
Sham
wk1 wk4 wk7
I
Change in RVEF, normalized to wk1
2.0 1.5 1.0 0.5
$
PAB+FK506
PAB
Sham
Sham+FK506
wk1 wk4 wk7
J
Placebo
FK506
Placebo
FK506
Sham
PAB
>20%
10%
0%
-10%
<-20%
long-axis view
short-axis view
N
Principal component analysis
Sham
PAB
Sham+FK506
PAB+FK506
PC2 (18.95%)
PC1 (45.92%)
K
RV radial strain, %
20 25 30 35 40
$
#
PAB - - + +
FK506 - + - +
L
RV circumferential strain, %
0 -5 -10 -15 -20 -25
#
$
PAB - - + +
FK506 - + - +
M
RV longitudinal strain, %
0 -5 -10 -15 -20 -25
#
$
PAB - - + +
FK506 - + - +


A
Wheat germ agglutinin
Sham
PAB
PAB+LDN
PAB+FK-LD
PAB+FK-HD
PAB+FK+LDN
PAB+Cyclo
B
Isolectin B4
Sham
PAB
PAB+LDN
PAB+FK-LD
PAB+FK-HD
PAB+FK+LDN
PAB+Cyclo
C
Masson's trichrome
Sham
PAB
PAB+LDN
PAB+FK-LD
PAB+FK-HD
PAB+FK+LDN
PAB+Cyclo
4x
10x


D
Capillaries/ Cardiomyocyte
1.8
1.5
1.2
0.9
0.6
0.3
0.0
PAB - + + + + + +
FK506 - - - LD HD LD -
LDN-193189 - - + - - + -
Cyclosporine - - - - - - +


E
RV collagen, %
30
25
20
15
10
5
0
PAB - + + + + + +
FK506 - - - LD HD LD -
LDN-193189 - - + - - + -
Cyclosporine - - - - - - +


fibroblasts
A
cell proliferation, OD570nm
B
COL1A1 gene expression, COL1A1/GAPDH
C
COL3A1 gene expression, COL3A1/GAPDH
D
ACTA2 gene expression, ACTA2/GAPDH
TGFβ1
FK506
LDN
Control
TGFβ1
TGFβ1+ FK506
E
115kD-
BMPR2
43kD-
β-Actin
BMPR2 gene expression, BMPR2/GAPDH
F
ID1 gene expression, ID1/GAPDH
endothelial cells
G
branches, counts
unstimulated
+TGFβ1+IL-1β
+TGFβ1+IL-1β+FK
+TGFβ1+IL-1β+LDN
TGFβ1+IL-1β
H
COL1A1 gene expression, COL1A1/GAPDH
I
CD31 gene expression, CD31/GAPDH
Control
TGFβ1+IL-1β
TGFβ1+IL-1β+ FK506
J
BMPR2 gene expression, BMPR2/GAPDH
K
ID1 gene expression, ID1/GAPDH


A
PAB+minipump implantation
Tmx
Analysis
wk0
wk4
Placebo or FK506
wk7
Pdgfb-CreER^{T2}; tdTomato+/-
B
before surgery
CD31
CD45
SSC
tdTomato
98.1
no stain
no Tmx
0.3
C
IB4/DAPI
tdT/DAPI
merge
Right ventricle
D
IB4/tdT/DAPI
Vim/tdT/DAPI
merge
E
IB4
tdT
Vim
merge+DAPI
perivascular
interstitial
PAB
PAB+FK506
F
EndMT, % cells
PAB
FK506
G
Type 1 Collagen
Sham
PAB
PAB+FK506
Right ventricle

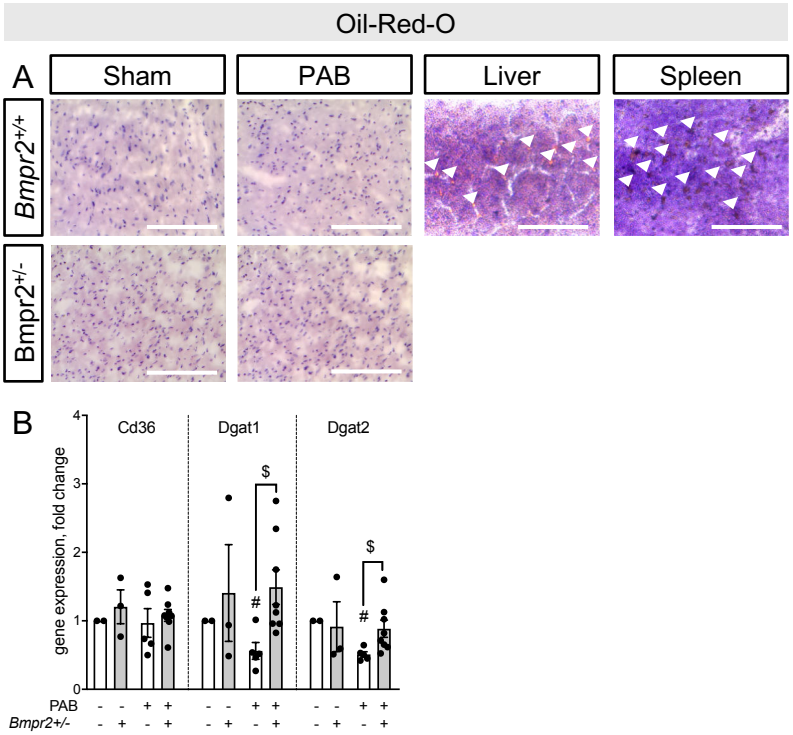


Figure E1: Low BMP signaling alters fatty acid metabolism in the stressed RV

Oil-Red-O staining revealed absent lipid accumulation in RV tissues with low BMP signaling due to reduced BMPR2 expression in *Bmpr2+/-* mice or upon PAB; while lipid accumulation in Liver and Spleen was evident (arrowheads point to adipocytes) (**A**). Gene expression analysis of key enzymes regulating fatty acid metabolism revealed unaltered expression of fatty acid transporter *Cd36*, but reduced triglyceride synthases diacylglycerol O-acyltransferase 1 (*Dgat1*) and 2 (*Dgat2*) expression after PAB, suggesting altered fatty acid metabolism that was restored by low BMP signaling (**B**). n=3 sham-operated animals; n=5-7 PAB mice. Two-way ANOVA followed by Tukey's post-hoc test. #: $p<0.05$ vs. Sham; $: $p<0.05$ vs. *Bmpr2+/+*. Scale bar = 200µm.

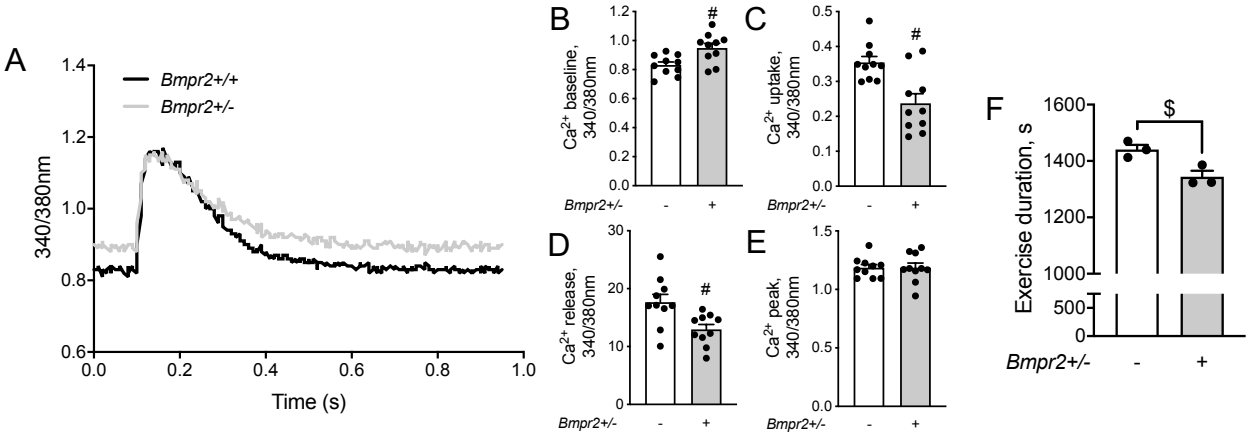


Figure E2: Low BMP signaling alters Calcium handling in cardiomyocytes and slightly reduces exercise capacity in mice

Imaging of calcium cycling in isolated primary cardiomyocytes from *Bmpr2+/-* and littermate control mice revealed an increase in basal intracellular calcium (Ca2+) concentrations in animals with reduced BMP signaling; leading to decreased Ca2+ uptake and release kinetics but similar peak concentrations when compared with controls (**A**-**E**). Exercise testing using a ramped running protocol in these mice demonstrated a reduced exercise capacity of *Bmpr2*-deficient animals as compared with controls (**F**). n=10 cardiomyocytes or n=3 mice per group. Unpaired two-tailed student's t-Test. #: $p<0.05$ vs. *Bmpr2+/+*.

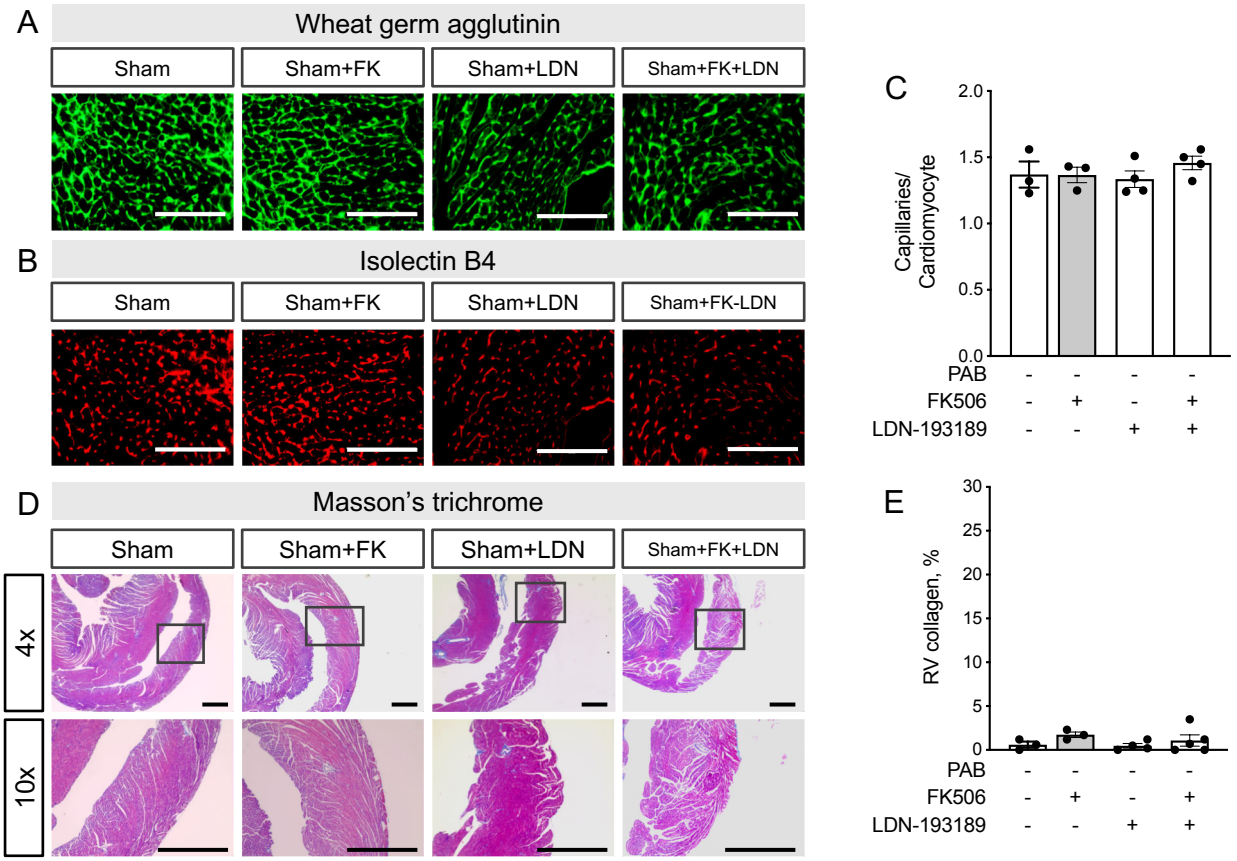


Figure E3: Modulating BMP signaling does not affect RV myocardial remodeling in absence of RV pressure overload

Chronic treatment of Sham-operated animals with either low-dose FK506 (0.05 mg/kg/d), LDN-193189 (2.5 mg/kg/d) or its combination neither affected RV capillarization (**A**-**C**) nor RV fibrotic remodeling (**D**+**E**). n=3-5 animals per group. Two-way ANOVA followed by Tukey's post-hoc test. Scale bar = 100µm.

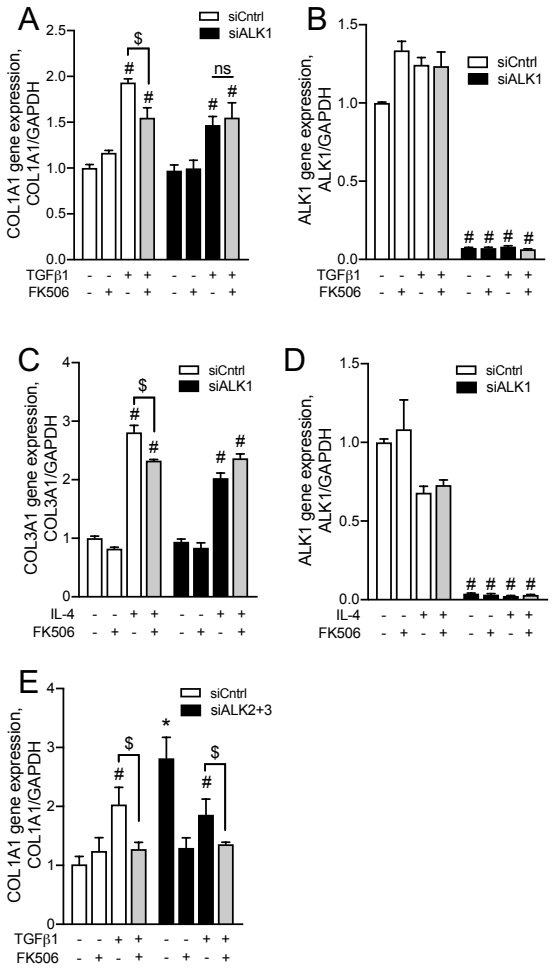


Figure E4: BMP-dependent anti-fibrotic effects of FK506 on cardiac fibroblasts are mediated through ALK1 as BMPR2 co-receptor

TGFβ1- and IL-4-induced collagen production in human cardiac fibroblasts is reduced by co-treatment with FK506 (**A-E**). This effect is lost after siRNA-mediated ALK1 but not ALK2+3 knockdown, suggesting ALK1 as the BMPR2 co-receptor required for the anti-fibrotic effects of FK506. n=3-6 experiments. Two-way ANOVA followed by Tukey's post-hoc test. #: $p<0.05$ vs. unstimulated; $: $p<0.05$ vs. TGFb1; *: $p<0.05$ vs. siCntrl.

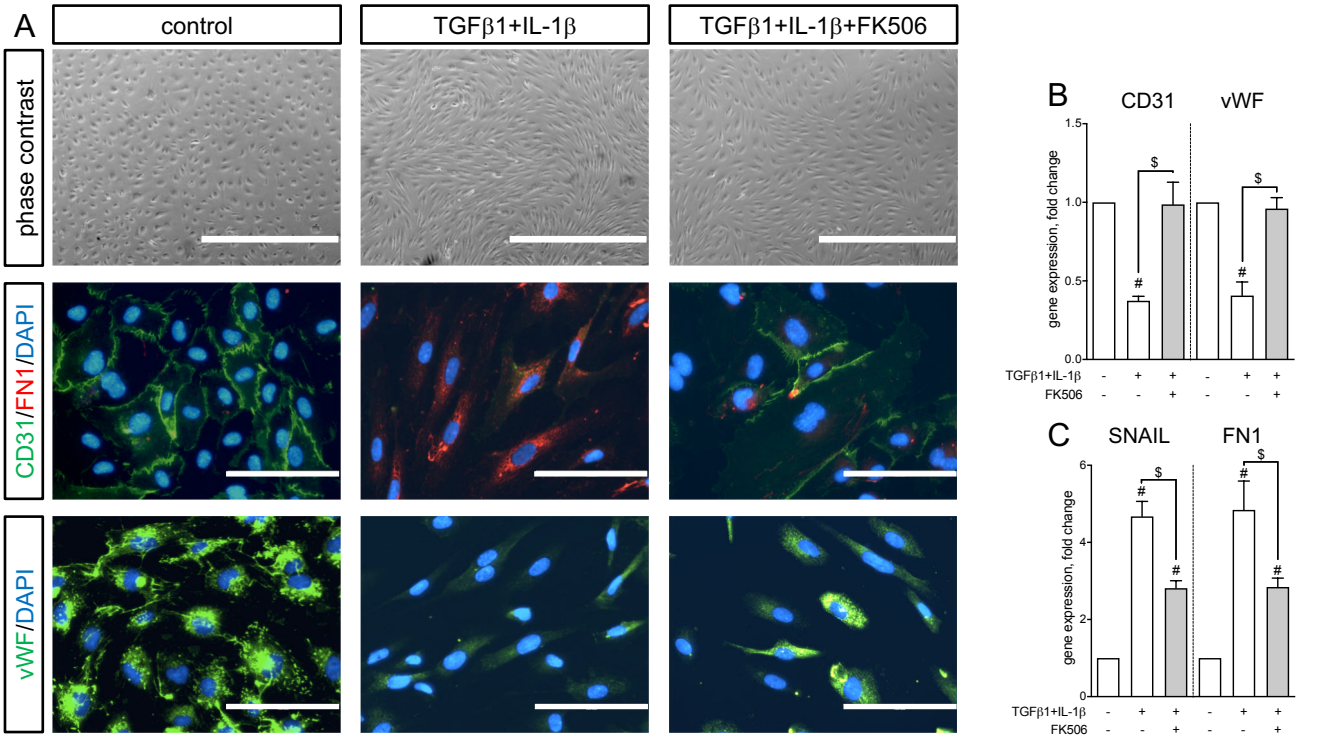


Figure E5: FK506 reduces endothelial-to-mesenchymal transition of cardiac endothelial cells *in vitro*

Cobblestone-like morphology of cultivated cardiac endothelial cells converts towards a spindle-like shape upon TGFβ1-IL-1β treatment; a transition that is rescued by FK506 co-treatment. Morphological changes are accompanied by loss of endothelial cell marker expression (CD31 and von Willebrand factor, vWF) and acquisition of mesenchymal markers (such as FN1), suggesting endothelial-to-mesenchymal transition (EndMT) of cardiac endothelial cells (**A**-**C**); which is reduced by FK506 co-treatment, as evidenced by increased endothelial and decreased mesenchymal marker expression and a reduction in EndMT regulator Snail (**A**-**C**). n=3-6 experiments. Two-way ANOVA followed by Tukey's post-hoc test. #: $p<0.05$ vs. unstimulated; $: $p<0.05$ vs. TGFβ1+IL-1β. Scale bar =1mm (phase contrast) or 100µm.

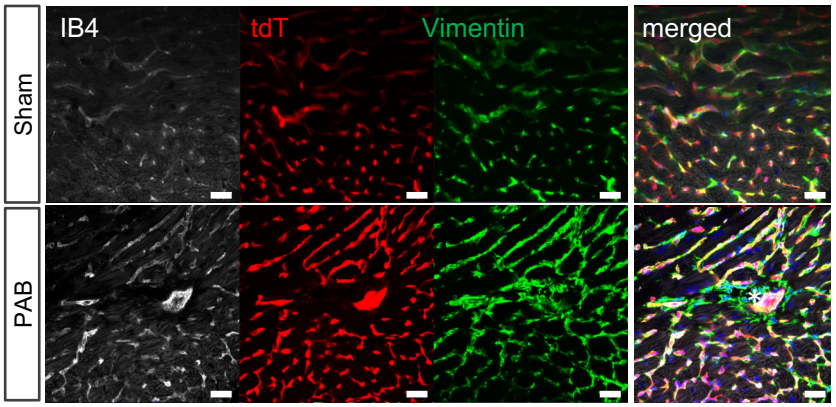


Figure E6: Endothelial lineage labeling reveals minor contribution of endothelial cells to pressure overload-induced RV fibrosis

Endothelial lineage labeling in Pdgfb-CreERT2; tdTomato$^{+/-}$ reporter mice was induced by Tamoxifen administration and 4 weeks later followed by either Sham control surgery (upper panel) or pulmonary artery banding (PAB, lower panel) for induction of RV pressure overload with subsequent development of RV fibrosis. Successful transition of cardiac endothelial cells towards a mesenchymal fate was defined as endothelium marker negative (IB4-), lineage labeling positive (tdT+), mesenchymal marker positive (Vimentin+) staining. The majority of lineage labeled cells co-localize with endothelial staining in close proximity to mesenchymal cells, suggesting that minority of cells convert towards a mesenchymal fate. Scale bar = 20µm.

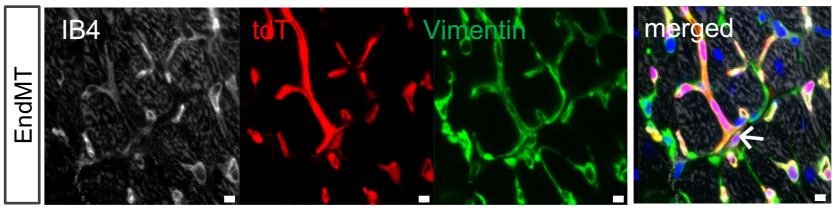


Figure E7: EndMT is a rare event that predominantly occurs at the branching point of capillaries

Endothelial lineage labeling and cell tracing after pulmonary artery banding revealed that endothelial-to-mesenchymal transition (EndMT) is a rare event in the pressure overloaded RV that occurs at the branching point of capillaries in non fibrotic interstitial areas (arrow indicating a cell that underwent EndMT, defined as IB4-, tdT+, Vimentin+). The converted cells do not contribute to capillary tube formation. Scale bar =5μm.

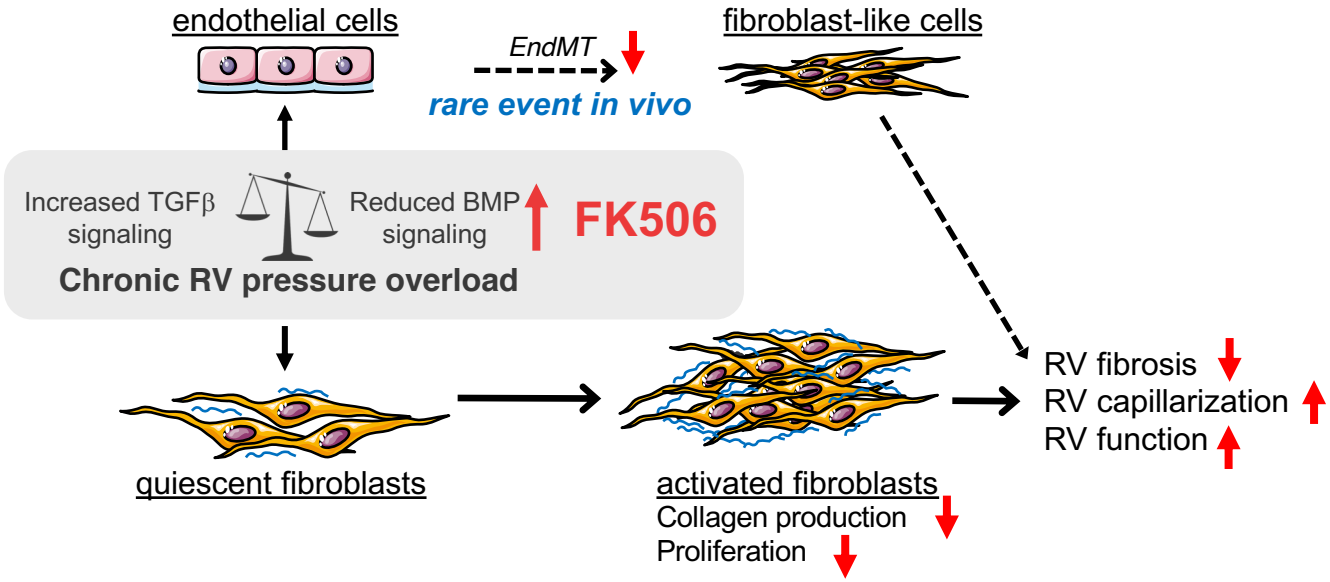

endothelial cells
EndMT
rare event in vivo
fibroblast-like cells
Increased TGFβ signaling
Reduced BMP signaling
FK506
Chronic RV pressure overload
quiescent fibroblasts
activated fibroblasts
Collagen production
Proliferation
RV fibrosis
RV capillarization
RV function

Table E1: Patient characteristics

Peripheral *BMPR2* gene expression, *BMPR2*/*GAPDH*	control *BMPR2* level >50%	low *BMPR2* level ≤50%	
Etiology	6xIPAH 1xdrug&toxin	1xIPAH 1xdrug&toxin 3xHPAH(BMPR2) 1xMCTD	p-value
Sex	♀: 2 ♂: 4	♀: 6 ♂: 1	-
Age, yrs	54.1±12.5	38.3±14.2	0.0516
BMI, kg/m^2	27.2±2.9	29.2±5.0	0.3813
TAPSE, mm	20.7±2.0	19.2±4.2	0.3984
mPAP, mmHg	40.6±14.8	49.0±10.0	0.2973
dPAP, mmHg	24.7±10.5	29.0±3.1	0.4014
PCWP, mmHg	11.2±3.6	10.6±1.9	0.7471
6MWD, m	1746±314	1990±282	0.1717

Abbreviations - IPAH: idiopathic PAH; drug&toxin: drug&toxin-induced PAH; HPAH: hereditary PAH; MCTD: mixed connective tissue disease-associated PAH; BMI: Body Mass Index; TAPSE: Tricuspid Annular Plane Systolic Excursion; mPAP: mean pulmonary artery pressure; dPAP: diastolic pulmonary artery pressure; PCWP: pulmonary capillary wedge pressure; 6MWD: six-minute walking distance.