SUPPLEMENTAL DATA

Distal vessel stiffening is an early and pivotal mechanobiological regulator of vascular remodeling and pulmonary hypertension

Liu et al.; PA Stiffening is a Mechanobiological Regulator of PH

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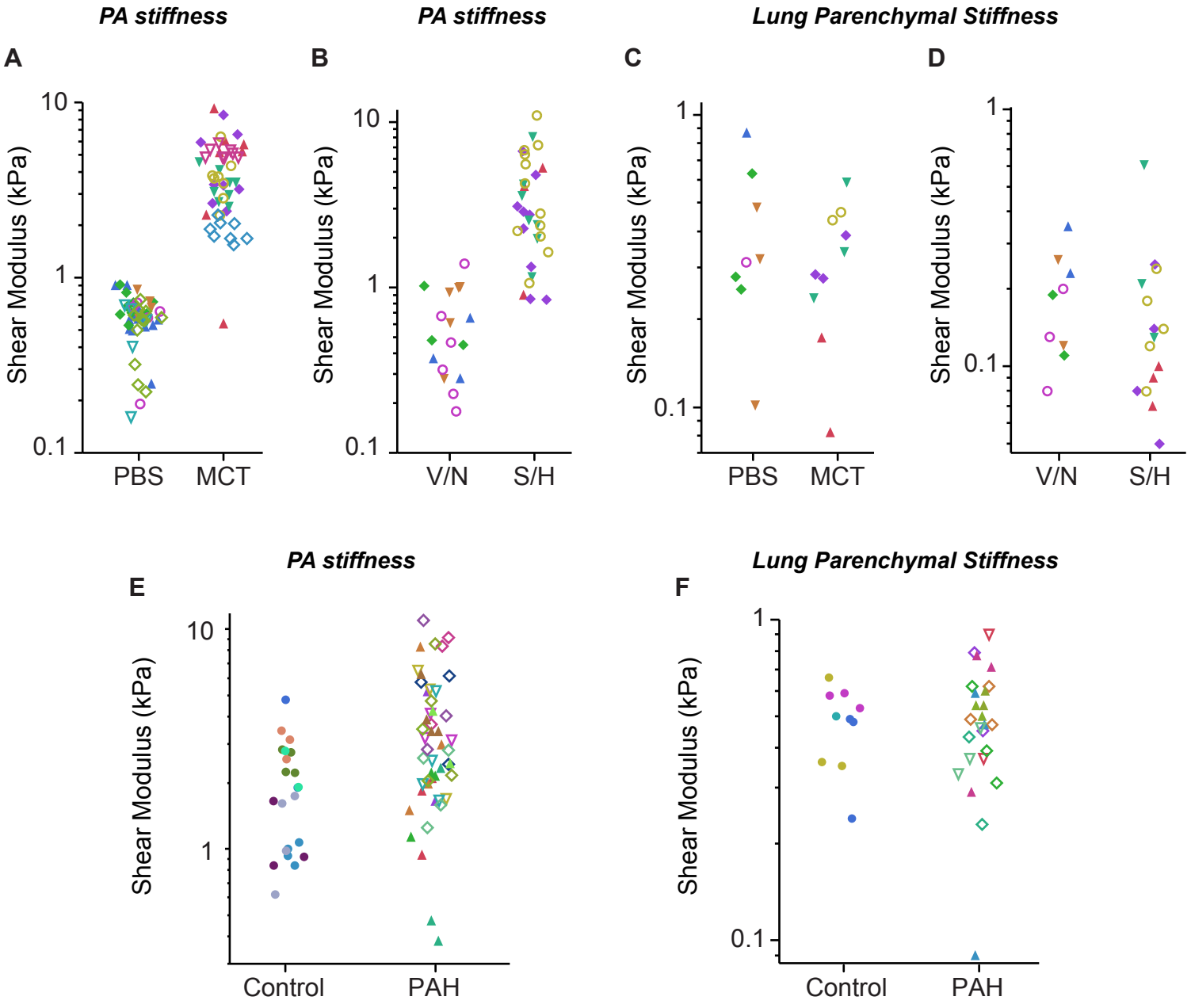
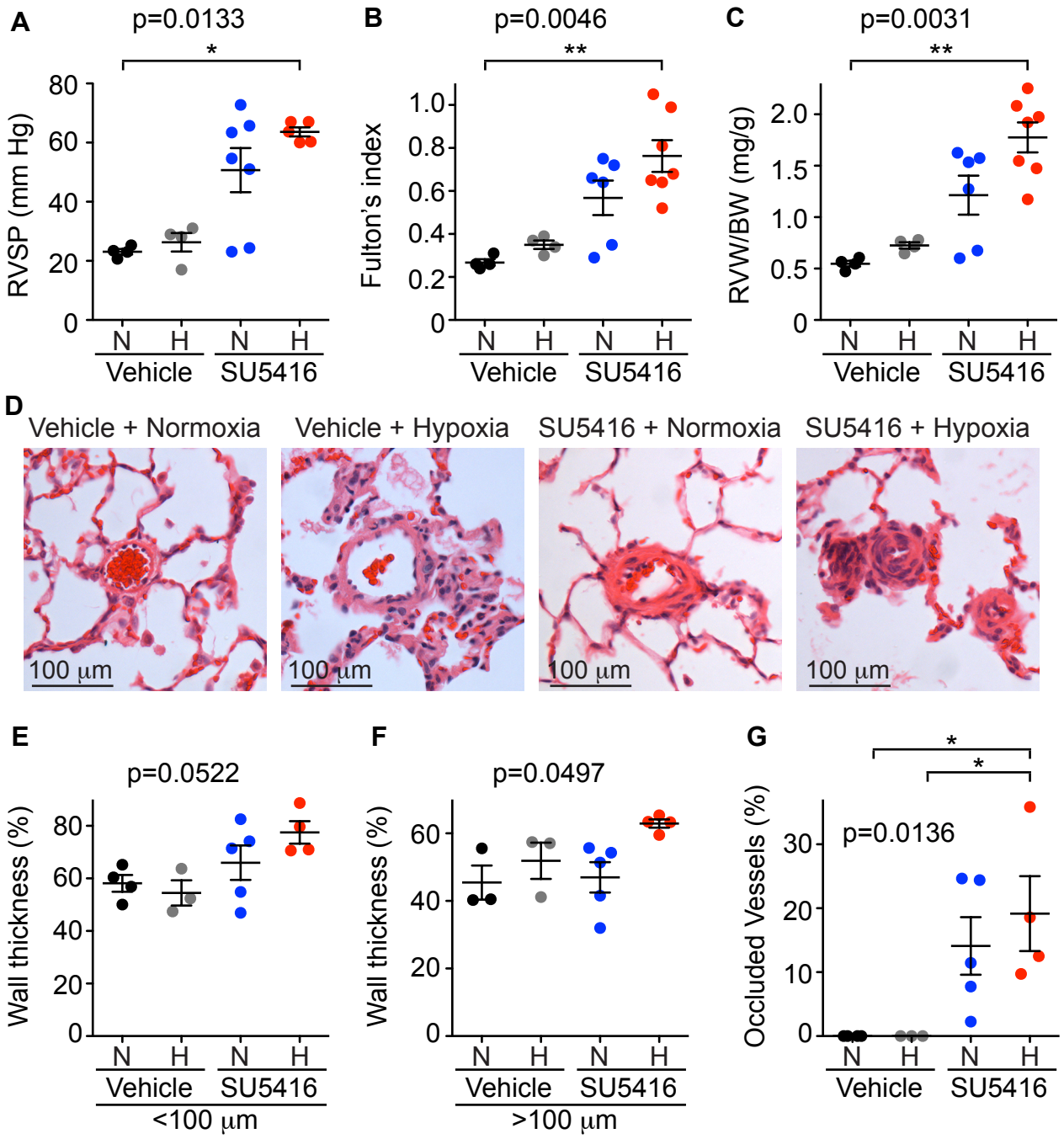
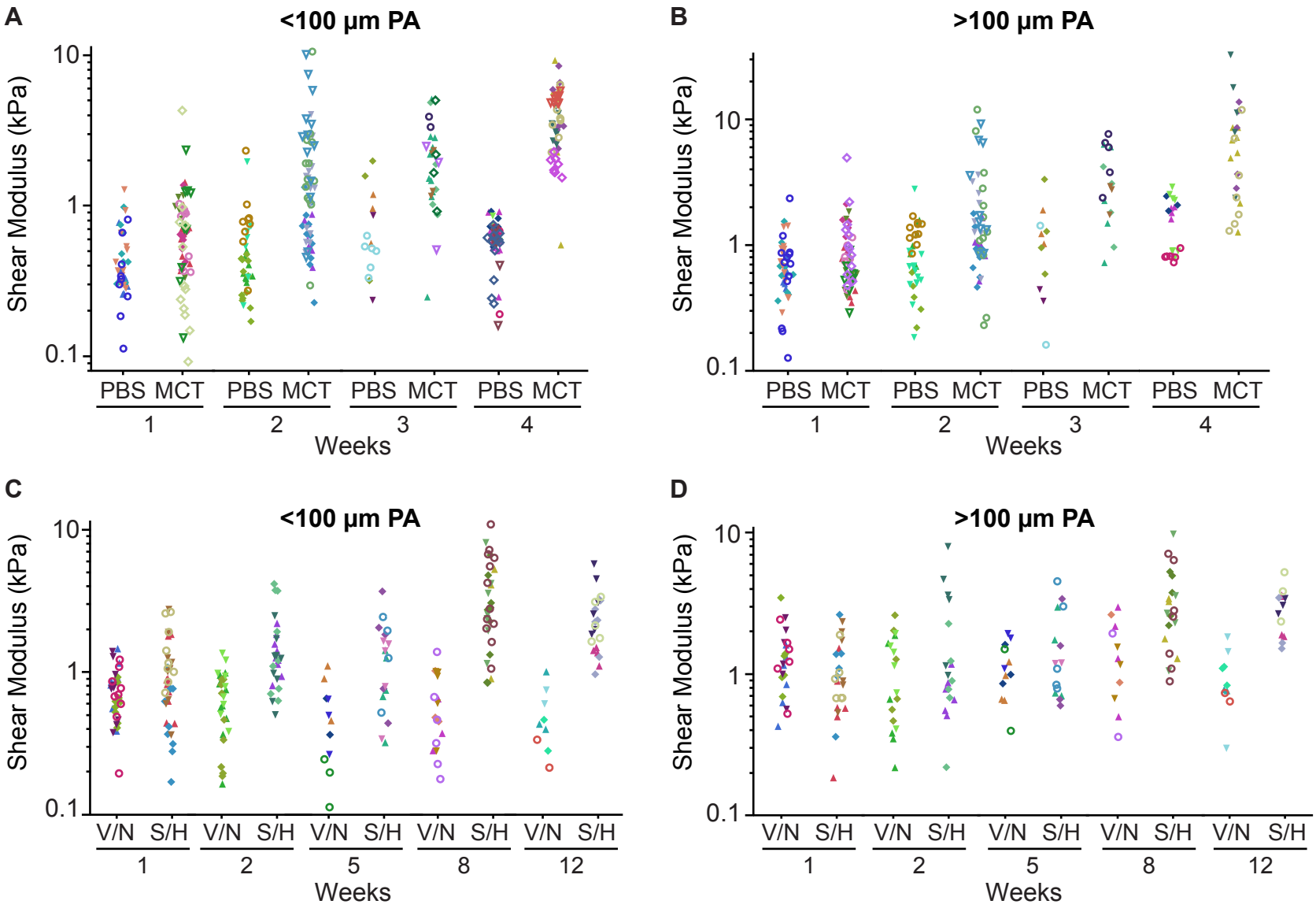


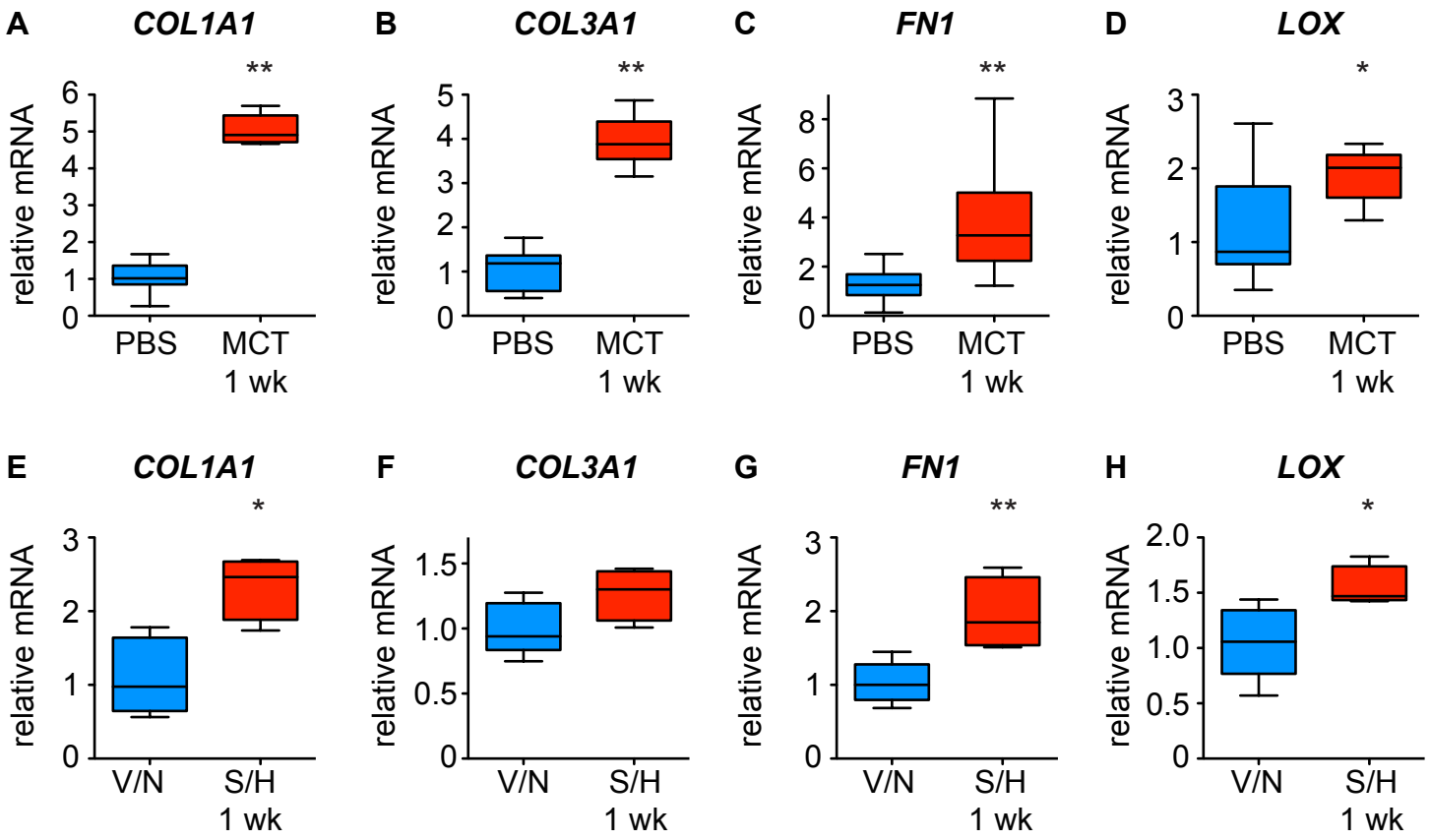
Supplemental Figure 1. Increased PA stiffness in rat pulmonary hypertension models and human PAH. (A, C) Sprague-Dawley rats were treated with monocrotaline (MCT) or PBS (n=6 per group) and lungs harvested after 4 weeks. **(B, D)** Sprague-Dawley rats were treated with SU5416 (S) or vehicle (V), exposed to hypoxia (H) or normoxia (N) for 3 weeks, and then returned to normoxia for an additional 5 weeks (n=4 per group). Pulmonary arterioles (PA) <100 μm **(A-B)** and lung parenchyma **(C-D)** were mechanically characterized via AFM microindentation. Each symbol corresponds to one individual PA measurement and individual rats are identified by unique symbol/color pairs in each panel. **(E-F)** AFM microindentation was used to mechanically characterize PAs **(E)** and lung parenchyma **(F)** in human lung samples from IPAH (n=8; ▲), FPAH (n=3; ▽), APAH (n=6; ◊), and control subjects (n=7; ●). Each symbol corresponds to one individual PA measurement and each subject is represented by a unique symbol/color pair in each panel.



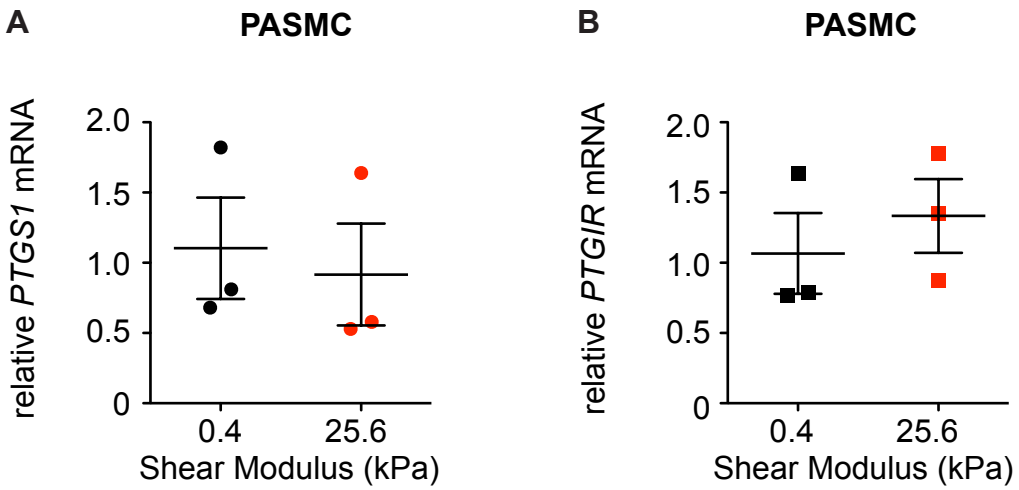
Supplemental Figure 2. Increased RVSP, RVH, and pulmonary vascular remodeling following SU5416 and chronic hypoxia. Male Sprague-Dawley rats were treated with sc SU5416 (20 mg/kg) or vehicle, exposed to hypoxia (n=5 for SU5416; n=4 for vehicle) or normoxia (n=7 for SU5416; n=4 for vehicle) for 3 weeks, and then returned to normoxia for an additional 5 weeks. **(A)** RVSP, **(B)** Fulton's index, and **(C)** RV weight (RVW, mg) normalized for body weight (BW, g). **(D)** Representative 5 μm hematoxylin and eosin (H&E)-stained sections in SU5416 hypoxia-exposed animals and controls. Quantification of wall thickness of **(E)** PAs <100 μm, **(F)** PAs >100 μm, and **(G)** vessel occlusion. Data represent the mean and SEM. Statistical significance was determined by one-way ANOVA followed by Dunn's post test (*p<0.05; **p<0.01; ***p<0.001).



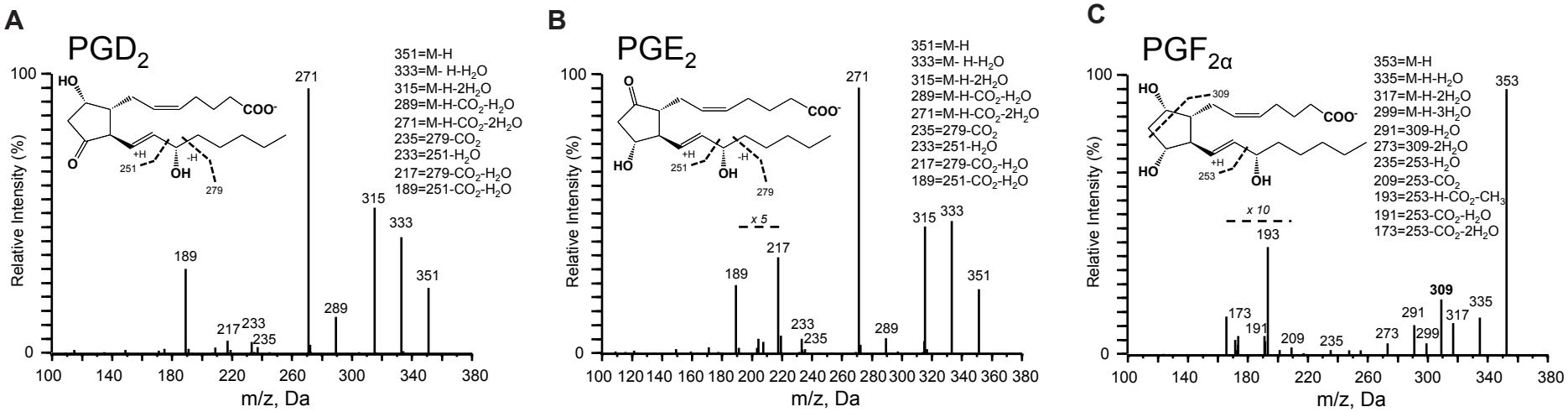
Supplemental Figure 3. Distal PA stiffening occurs early in the MCT and sugen hypoxia models of pulmonary hypertension. Male Sprague-Dawley rats were treated with monocrotaline (MCT) or PBS (n=6-8 per time point) and harvested at serial time points following MCT. AFM microindentation was used to mechanically characterize **(A)** PAs <100 μm and **(B)** PAs >100 μm. Each symbol corresponds to one individual PA measurement and individual rats are identified by unique symbol/color pairs in each panel. Sprague-Dawley rats were treated with SU5416 (S) or vehicle (V), exposed to hypoxia (H) or normoxia (N) (n=4-8 per time point) for 1, 2, or 3 weeks. Animals exposed to 3 weeks of hypoxia were returned to normoxia for an additional 2, 5, or 9 weeks. PAs <100 μm **(C)** and PAs >100 μm **(D)** were mechanically characterized via AFM microindentation. Each symbol corresponds to one individual PA measurement and individual rats are identified by unique symbol/color pairs in each panel.



Supplemental Figure 4. Early matrix deposition in MCT and sugen hypoxia models. (A-D) Male Sprague-Dawley rats were treated with MCT (n=6) or PBS (n=11) and harvested after 1 week. Lungs were harvested and qPCR performed for **(A)** *COL1A1*, **(B)** *COL3A1*, **(C)** *FN1*, and **(D)** *LOX* and normalized to 18S expression. **(E-H)** Male Sprague-Dawley rats were treated with sc SU5416 and exposed to hypoxia (S/H; n=4) or treated with vehicle and kept in normoxia (V/N; n=6) for 1 week. Lungs were harvested and qPCR performed for **(E)** *COL1A1*, **(F)** *COL3A1*, **(G)** *FN1*, and **(H)** *LOX* and normalized to 18S expression. Data represent 25th to 75th percentiles (box), median (line), and 5th and 95th percentiles (whiskers). Statistical significance was determined by the Mann-Whitney U test. *p<0.05; **p<0.01, ***p<0.001.



Supplemental Figure 5. Stiffness does not alter COX-1 or PGI_2 receptor expression in PASMC. Human PASMC were cultured on polyacrylamide substrates with shear moduli of 0.4 and 25.6 kPa. After 48 h, RNA was isolated, reverse-transcribed to cDNA, and qPCR was performed for *PTGS1* **(A)** and *PTGIR* **(B)**. Results were normalized to GAPDH expression. p=NS by the Mann-Whitney U test. Data represent the mean and SEM of three independent experiments.



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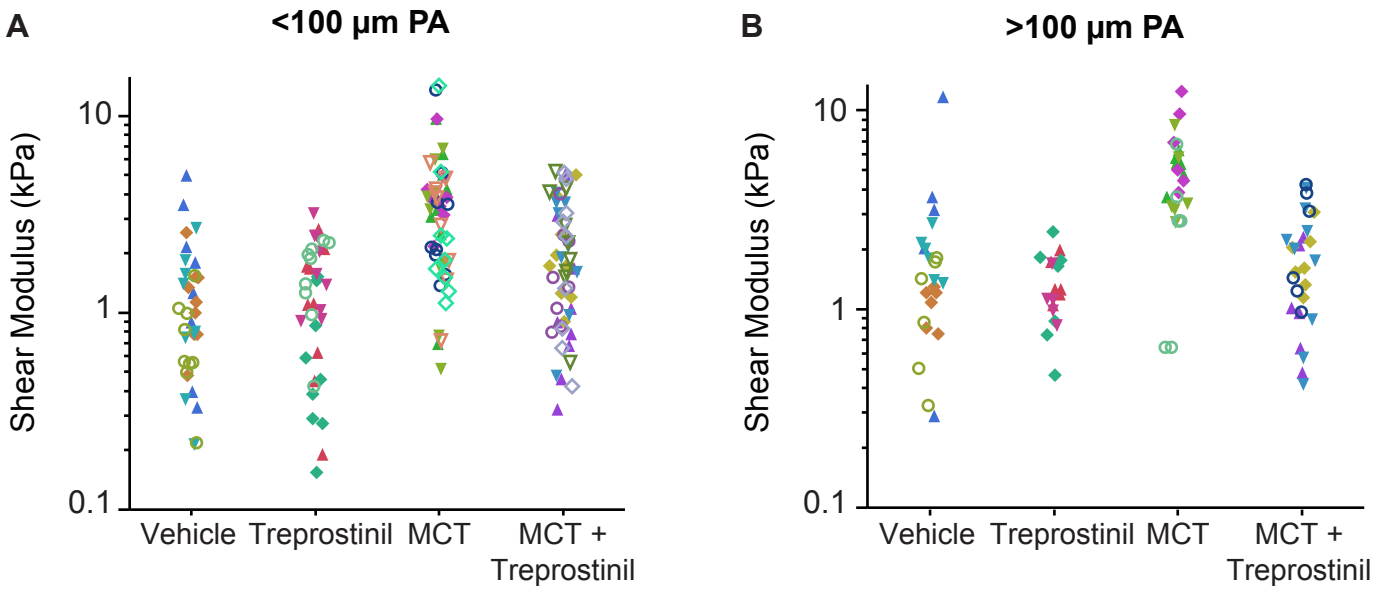
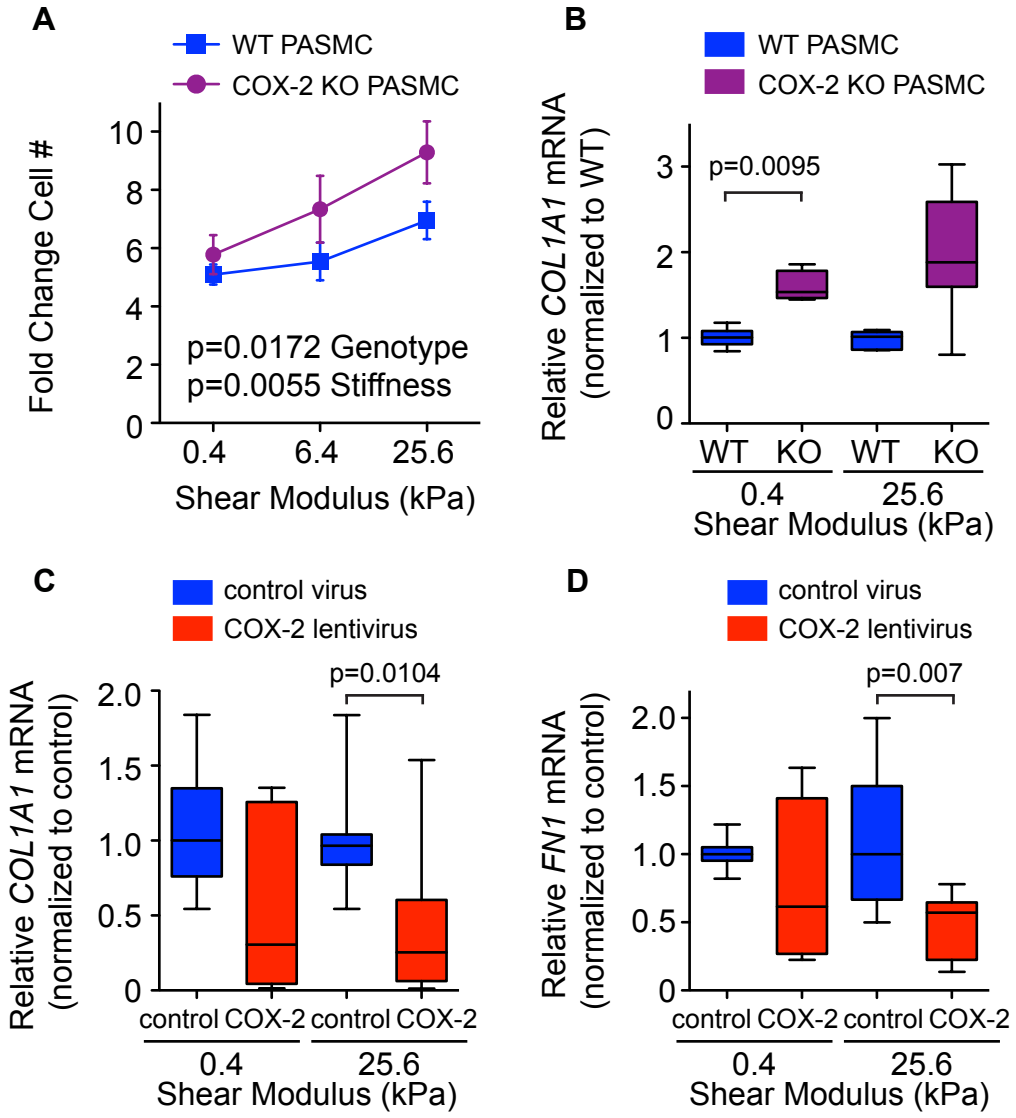
			Prostanoid levels (pg/100 mg lung tissue)									
			MCT					SU5416/hypoxia				
	Q1	**Q3**	**CNT**	**1 week**	**2 weeks**	**3 weeks**	**4 weeks**	**CNT**	**1 week**	**2 weeks**	**8 weeks**	**12 weeks**
PGD_2	**351**	**233**	854	480	1304	967	816	593.3	127.4	433.7	2269.4	503.9
PGE_2	**351**	**189**	1425	110	200	132	1210	1187.6	43.0	295.5	3520.2	597.4
$PGF_{2\alpha}$	**353**	**193**	2588	120	206	139	1073	296.3	17.5	51.8	703.5	126.9

Supplemental Figure 6. Early reduction in prostanoid levels in MCT and SU5416/hypoxia models. Lipid mediators were extracted from lungs of MCT (n=4-5) and SU5416/hypoxia (n=1) exposed rats at serial time points, and prostanoids assessed using LC-MS-MS. Characteristic MS-MS spectra were used for the identification of **(A)** PGD_2, **(B)** PGE_2, and **(C)** $PGF_{2\alpha}$. Da, dalton. **(D)** Prostanoid levels are expressed as pg/100 mg of lung tissue. Q1, M-H (parent ion) and Q3, diagnostic ion in the MS-MS (daughter ion). The detection limit was ~ 0.1 pg. CNT, control.



Supplemental Figure 7. Treprostinil prevents PA stiffening in the MCT model. Sprague-Dawley rats were treated with MCT or vehicle and after 2 wks, had subcutaneous minipumps implanted to deliver intravenous treprostinil (90 ng/kg/min) or saline. PAs <100 µm **(A)** and PAs >100 µm **(B)** were mechanically characterized via AFM microindentation. Each symbol corresponds to one individual PA measurement and individual rats are identified by unique symbol/color pairs in each panel.



Supplemental Figure 8. COX-2 expression alters the stiffness-dependent phenotype in PASMC. (A-B) COX-2 deficient and WT mouse PASMC were plated on discrete stiffness polyacrylamide gels. **(A)** After 48 h, cell density was determined and normalized to 4 h. Statistical significance was determined by two-way ANOVA (p=0.0172 genotype, p=0.0055 stiffness, p=0.5793 interaction). **(B)** RNA was harvested and qPCR was performed for *COL1A1* and normalized to GAPDH expression. **(C-D)** Human PASMC overexpressing COX-2 were plated on discrete stiffness polyacrylamide gels and harvested after 48 h for qPCR for *COL1A1* **(C)** and *FN1* **(D)**. Data represent 25th to 75th percentiles (box), median (line), and 5th and 95th percentiles (whiskers). Statistical significance was determined by the Mann-Whitney U test for pairwise comparisons.

Supplemental Table 1. Demographics and Clinical Characteristics of PAH Patients.

Subject #	Diagnosis	Age	Sex	Race	Ethnicity	Associated Condition	WHO functional class	mPAP (mm Hg)	PVR (Wood Units)†
1	IPAH	29	F	White	Non-Hispanic	–	IV	69	6.29
2	IPAH	30	F	Black	Non-Hispanic	–	III	41	–
3	IPAH	32	F	White	Non-Hispanic	–	IV	49	13.83
4	IPAH	41	F	Hispanic	Hispanic or Latino	–	IV	43	5.47
5	IPAH	41	F	White	Non-Hispanic	–	III	55	9.84‡
6	IPAH	56	F	White	Non-Hispanic	–	IV	57	11.41‡
7	IPAH	18	M	American-Asian	Non-Hispanic	–	III	67	–
8	IPAH	51	M	White	Non-Hispanic	–	IV§	30	5.92
9	FPAH	33	F	White	Non-Hispanic	–	IV	48	11.51
10	FPAH	35	M	White	Non-Hispanic	–	III	–	–
11	FPAH	37	M	White	Non-Hispanic	–	IV	77	14.22‡
12	APAH	35	F	Hispanic	Hispanic or Latino	Drugs and toxins	IV	68	–
13	APAH	40	F	Black	Non-Hispanic	SLE*	III	36	7.43‡
14	APAH	40	F	White	Non-Hispanic	CSTPS**	IV	–	–
15	APAH	64	F	–	Hispanic or Latino	Scleroderma	III	32	5.98‡
16	APAH	71	F	White	Hispanic or Latino	RA***	III	42	2.48‡
17	APAH	35	M	White	Non-Hispanic	CSTPS	III	–	–

* Systemic Lupus Erythematosus
** Congenital Systemic-To-Pulmonary Shunt
*** Rheumatoid Arthritis
§ NYHA Functional Class
† Measured using thermodilution method.
‡ Measured using Fick method.

Supplemental Table 2. Demographics and Clinical Characteristics of Control Donors.

Subject #	Age	Sex	Race	Ethnicity	Type of lethal injury	Reason for no organ transplantation
18	11	M	White	Non-Hispanic	Anoxia	Low PaO2
19	20	M	–	Hispanic or Latino	Head Trauma	Lung trauma
20	24	M	White	Non-Hispanic	Intracranial hemorrhage	Inadequate lung function
21	25	M	White	Non-Hispanic	Intracranial hemorrhage	No recipient
22	26	M	White	Non-Hispanic	Head Trauma	Poor organ quality
23	30	M	White	Non-Hispanic	Head Trauma	Inadequate lung function
24	33	M	White	Non-Hispanic	Anoxia	Infiltrates on chest x-ray

Supplemental Table 3. Stiffness dependent gene expression in PASMC.

Gene	Fold change (0.4 kPa versus 25.6 kPa)
ACE	-6.82
ACE2	-2.85
ALOX5	-1.26
ATP2C1	-29.2
AVP	13.6
AVPR1A	6.3
CHRNA1	5.5
CLIC-5	1.24
CNGB1	7.01
DRD3	3.48
EDN2	4.82
EDNRA	-2.53
EDNRB	-5.17
ITPR1	-2.39
MYLK3	5.06
NOSTRN	-2.95
NPPB	1.56
NPR1	-3.63
NPY1R	-3.89
PRKG2	-2.13
PTGS2	-3.16
SCNN1A	5.03
SPHK2	-1.75
UTS2R	4.69

Supplemental Table 3. Stiffness dependent gene expression in PASMC. PASMC were cultured on polyacrylamide substrates with stiffnesses of 0.4 and 25.6 kPa. After 48 h, RNA was isolated, reverse transcribed, and qPCR performed using the Human Hypertension RT^2 Profiler PCR Array.

Supplemental Table 4. Primer sequences.

Gene	Forward Primer
	Reverse Primer
COL1A1	CACACGTCTCGGTCATGGTA
	AAGAGGAAGGCCAAGTCGAG
FN1	ACCTCGGTGTTGTAAGGTGG
	CCATAAAGGGCAACCAAGAG
PTGS1	TCACACTGGTAGCGGTCAAG
	GTTCTTGCTGTTCCTGCTCC
PTGS2	CCGGGTACAATCGCACTTAT
	GGCGCTCAGCCATACAG
PTGIR	TTGCGGAAAAGGATGAAGAC
	GTGTGCTCCCTGCCTCTC
GAPDH	AATGAAGGGGTCATTGATGG
	AAGGTGAAGGTCGGAGTCAA