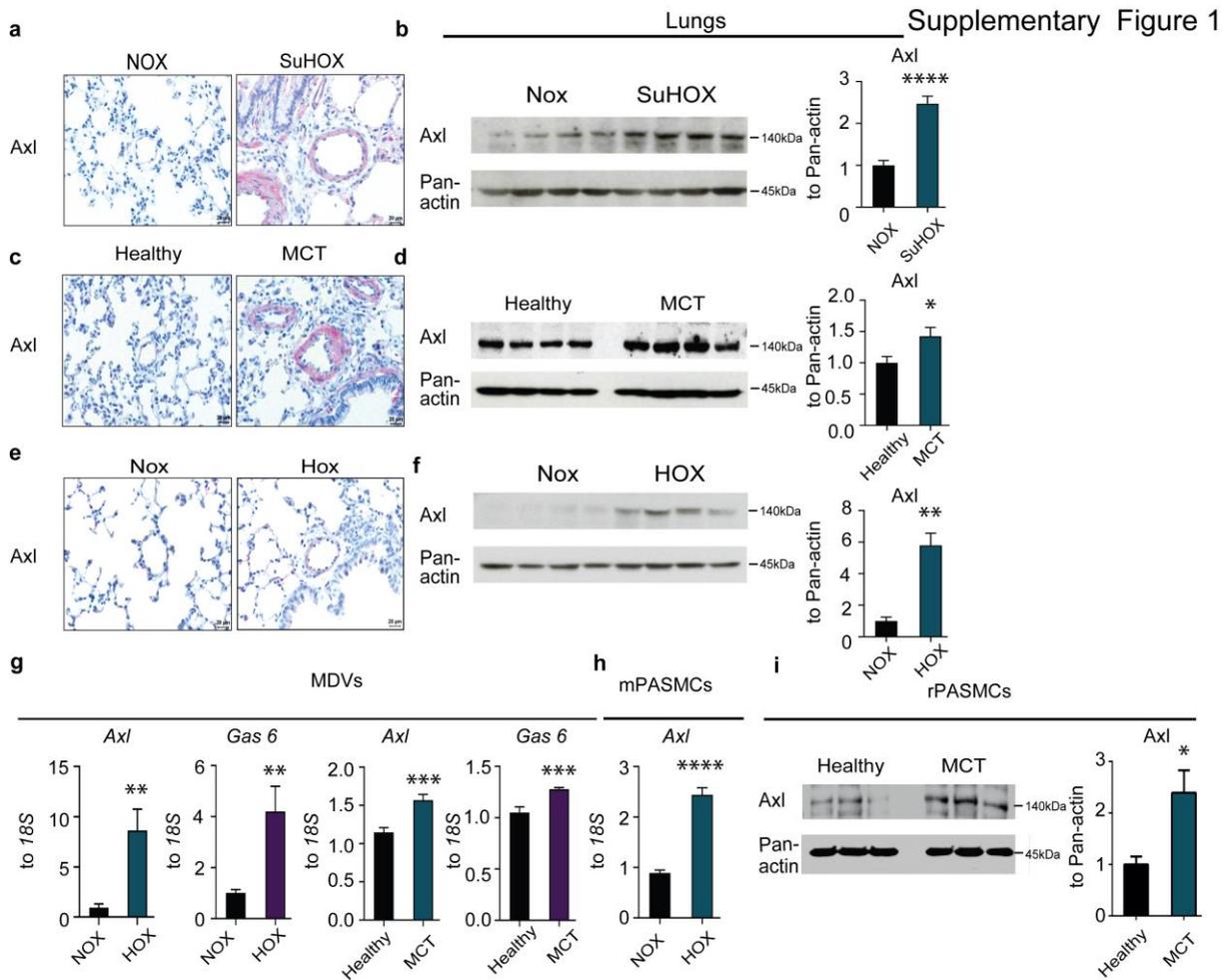


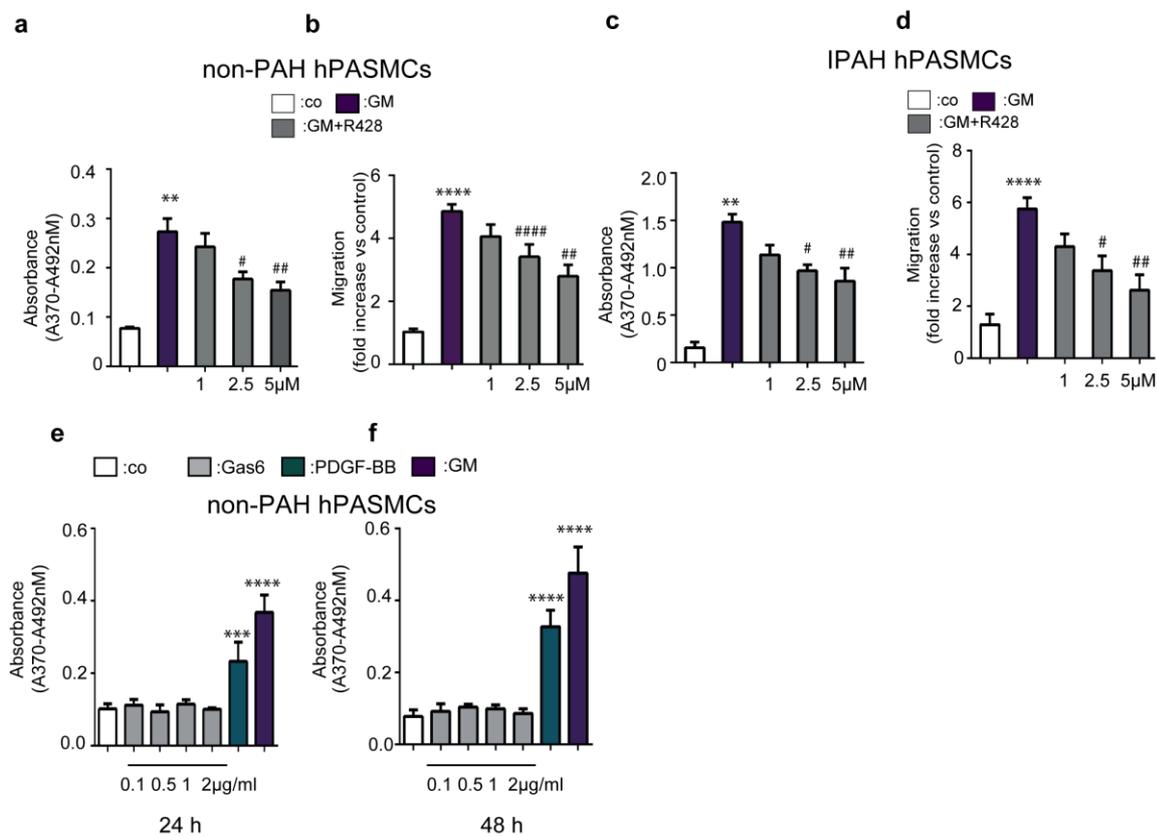
Supplementary Information



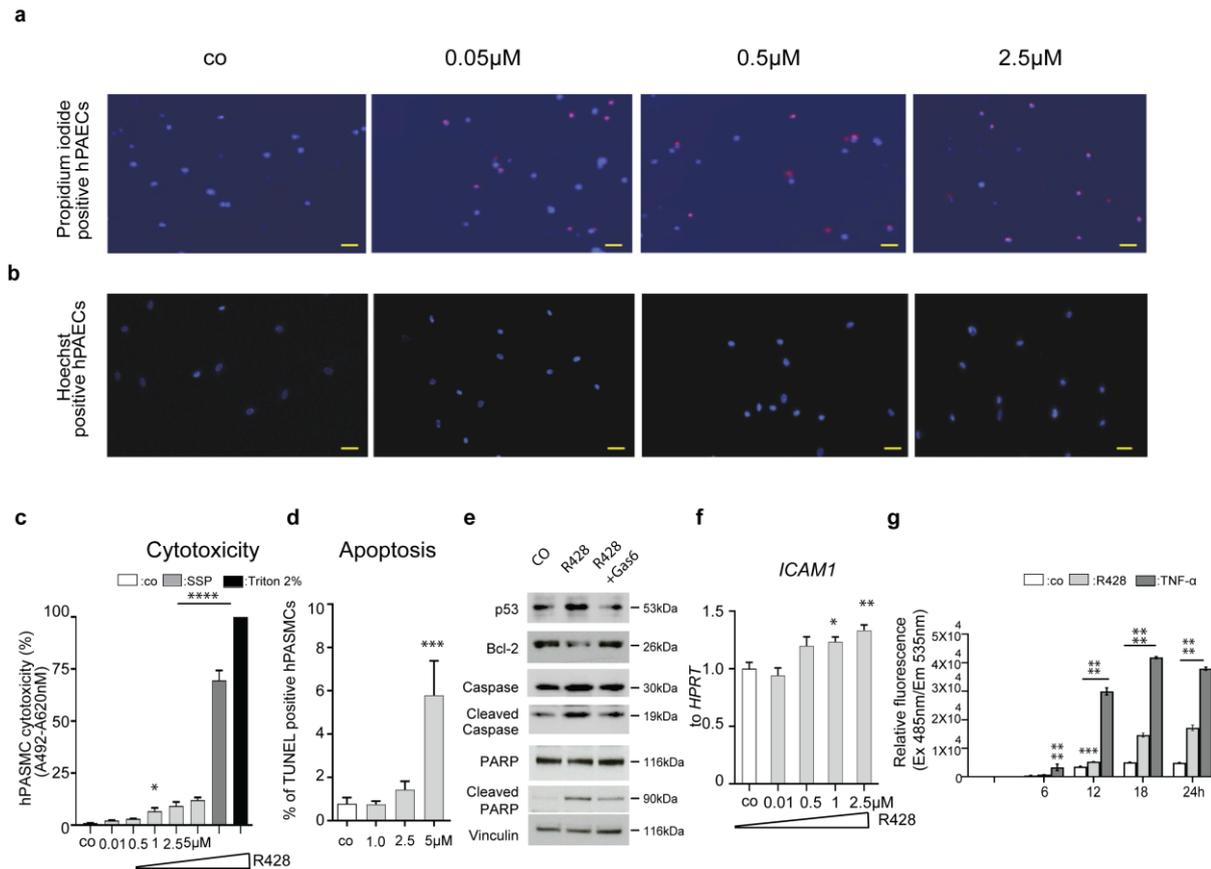
Supplementary Figure 1. Analyses of Axl expression in experimental pulmonary hypertension (PH). **a, c, e** Representative images of immunohistological staining of Axl expression in lung sections derived from **a** the Sugden 5416/hypoxia (SuHOX) experimental rat PAH model (normoxic rats [NOX], $n = 3$; SuHOX, $n = 3$), **c** the monocrotaline (MCT) experimental PAH rat model (healthy, $n = 3$; MCT, $n = 3$), and the chronic-hypoxia experimental PH mouse model (healthy, $n = 3$; MCT, $n = 3$). **b, d, f** Western blot analysis and subsequent densitometric quantification of Axl expression in lung homogenates from **b** the SuHOX (NOX, $n = 8$; SuHOX, $n = 9$), **d** the MCT (healthy, $n = 8$; MCT, $n = 8$), and the chronic-hypoxia (NOX, $n = 4$; HOX, $n = 4$) experimental PH models. Pan-actin served as a loading control. Statistical analysis was performed using Student's t-test; **** $P < 0.0001$ for SuHOX versus NOX, * $P <$

0.05 for MCT versus healthy, and $**P < 0.01$ for HOX versus NOX **g** Real-time quantitative PCR analyses of *Axl* mRNA expression from laser-assisted microdissected vessels (MDVs) in HOX ($n = 6$) versus NOX ($n = 6$) mouse lungs; and healthy control ($n = 5$) and MCT-injected rat lungs ($n = 5$). Expression levels were normalized to the *18S* house-keeping gene. **h** Relative mRNA expression of *Axl* from mouse pulmonary arterial smooth muscle cells (PASMCs), normalized to *18S*, as a reference gene. Data from three independent experiments are presented as the n -fold change ($2^{-\Delta\Delta Ct}$) compared with NOX control cells. **i** Western blot analysis and subsequent densitometric quantification of *Axl* expression in PASMCs isolated from the MCT rats (healthy, $n = 6$; MCT, $n = 6$). Pan-actin served as a loading control. Statistical analysis was performed using Student's t-test; $***P < 0.001$, $*P < 0.05$ for MCT versus healthy controls and $**P < 0.01$, $****P < 0.0001$ for HOX versus NOX. All the data represent mean \pm SEM.

Supplementary Figure 2



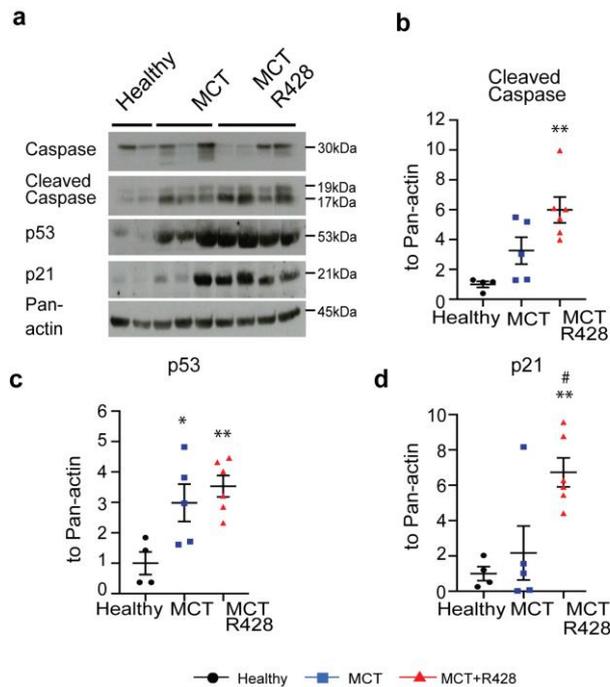
Supplementary Figure 2. Evaluation of the impact of R428 and growth arrest-specific protein 6 (Gas6) on proliferation and migration of human pulmonary arterial smooth muscle cells. Cultured human pulmonary arterial smooth muscle cells (hPASMCs) from **a, b** healthy individuals and **c, d** patients with idiopathic pulmonary arterial hypertension (IPAH) were exposed to growth medium (GM, consisting of 5% fetal bovine serum [FBS], human basic fibroblast growth factor [FGF-2], human epidermal growth factor [EGF], and insulin) for 24 h with or without R428 at indicated concentrations. Control cells were exposed to dimethyl sulfoxide (DMSO; co). **a, c** Proliferation potential was assessed by 5-bromo-2'-deoxyuridine (BrdU) colorimetric enzyme-linked immunosorbent assay (ELISA). Four hours after BrdU addition, assessment of DNA synthesis was completed [$A_{370nm} - A_{492nm}$]. Data from four independent experiments performed in triplicate are presented as mean \pm SEM. **b, d** Migration efficiency was determined by transwell migration assays. Human PASMCs were exposed to GM for 16 h with or without increasing concentrations of R428, as indicated. Data from three independent experiments performed in triplicate are presented as mean \pm SEM of the *n*-fold change normalized to DMSO-treated control cells. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Newman–Keuls post-hoc test for multiple comparisons. $**P < 0.01$ and $****P < 0.0001$ versus DMSO-treated control cells; $\#P < 0.05$, $##P < 0.01$ and $####P < 0.0001$ versus GM-treated cells. **e, f** Cultured hPASMCs from healthy individuals were stimulated for 24 h and 48 h with indicated concentrations of Gas6, PDGF-BB (30 ng/ml), and GM with or without R428 at indicated concentrations. Control cells were left in serum-free conditions (co). Proliferation potential was assessed by BrdU colorimetric ELISA. Four hours after BrdU addition, assessment of DNA synthesis was completed [$A_{370nm} - A_{492nm}$]. Data from three independent experiments performed in triplicate are presented as mean \pm SEM. Statistical analysis was performed using one-way ANOVA with Newman–Keuls post-hoc test for multiple comparisons. $***P < 0.001$ and $****P < 0.0001$. All the data represent the mean \pm SEM.



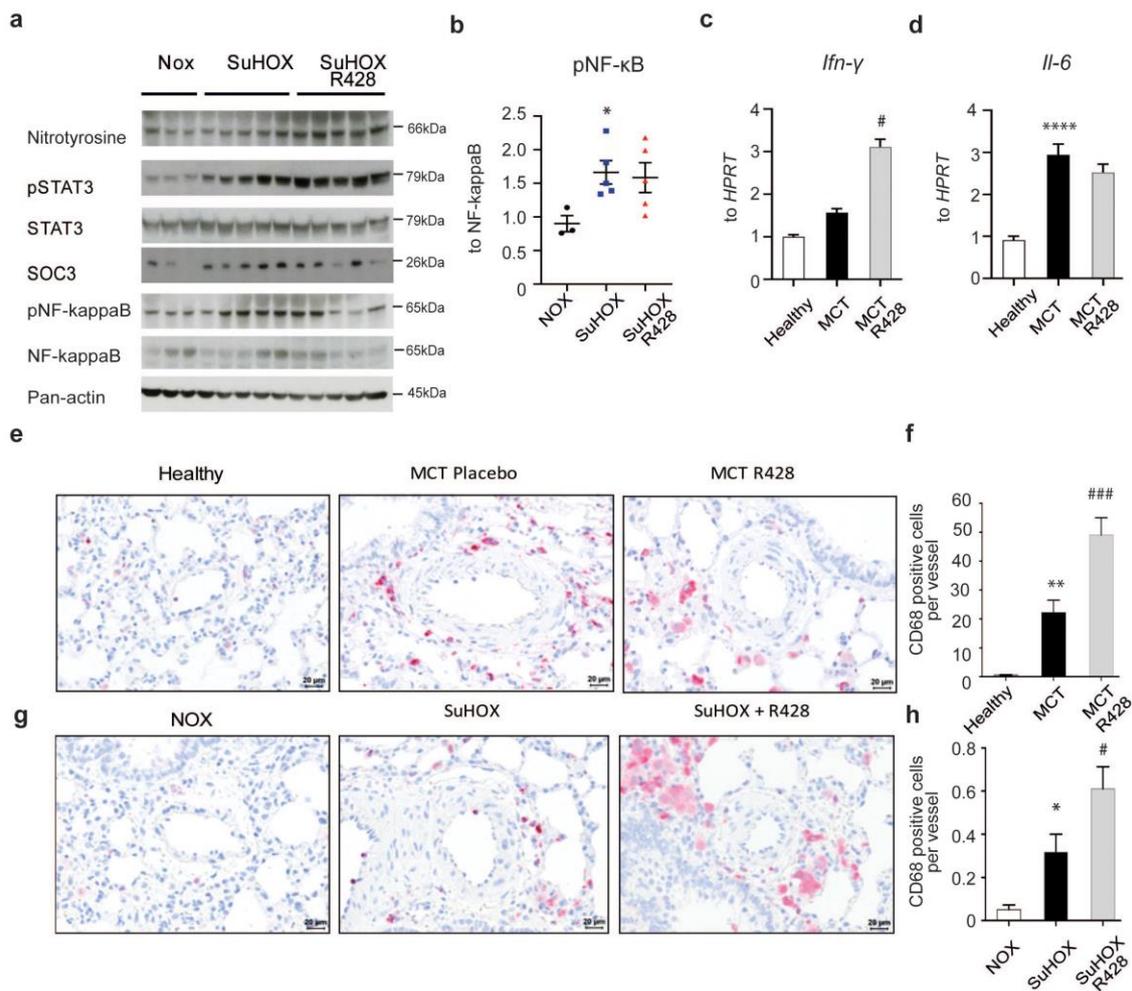
Supplementary Figure 3. Evaluation of the impact of R428 and growth arrest-specific protein 6 (Gas6) on cytotoxicity, apoptosis and permeability of human pulmonary vascular cells. Representative images of immunofluorescent staining with (a) propidium iodide (red) and (b) Hoechst (blue) of human pulmonary arterial smooth muscle cells (hPAECs) exposed to increasing concentrations of R428. Control cells were exposed to dimethyl sulfoxide (DMSO; co). DNA in (a) was visualized using 4',6'-diamidino-2-phenylindole (DAPI; blue). Scale bar: 50 μ m. **c** Cytotoxicity in human pulmonary arterial smooth muscle cells (hPASMCMs) was determined by detecting LDH release into the cell culture media [$A_{492nm} - A_{620nm}$]. Cells treated with Staurosporine (SSP) were used as positive control. Data are reported as percentage release of LDH compared to DMSO-treated control (co) cells. Data from three independent experiments performed in triplicates are presented. Statistical analysis was performed using one-way ANOVA with Newman-Keuls post-hoc test for multiple comparisons; * $P < 0.05$, **** $P < 0.0001$. **d** Quantitative analyses of TUNEL-positive hPASMCMs

exposed to increasing concentrations of R428. Data from three independent experiments performed in triplicate are presented as mean \pm SEM of the *n*-fold change normalized to DMSO-treated control (co) cells. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Newman–Keuls post-hoc test for multiple comparisons. ****P* < 0.001 versus DMSO-treated control cells. **e** Representative images of western blots of apoptotic markers p53, B-cell lymphoma 2 (Bcl-2), Caspase, Cleaved caspase, and poly (ADP-ribose) polymerase (PARP) expression in cells exposed to R428 (5 μ M) for 24 h and subsequently stimulated with growth arrest-specific 6 (Gas6; 200 ng/ml). Vinculin served as a loading control. **f** Relative mRNA expression of intercellular adhesion molecule 1 (*ICAM1*) expression in cultured healthy hPASCs treated for 24 h with indicated concentrations of R428. Data are presented as mean \pm SEM of the *n*-fold change ($2^{-\Delta\Delta C_t}$) normalized to hypoxanthine guanine phosphoribosyl transferase (*HPRT*) as a reference gene, compared with DMSO-treated control cells. Data from three independent experiments are presented. **P* < 0.05 and ***P* < 0.01 versus DMSO treated control cells. Statistical analysis was performed using one-way ANOVA with Newman–Keuls post-hoc test for multiple comparisons. **g** Quantitative analyses of vascular permeability determined by FITC-dextran permeability assay. Data from two independent experiments performed in fourplicate are presented. ****P* < 0.001 and *****P* < 0.0001 versus DMSO-treated control cells. For statistical analysis two-way analysis of variance with Tukey`s post-hoc test for multiple comparisons was performed. All the data represent the mean \pm SEM.

Supplementary Figure 4



Supplementary Figure 4. Effect of R428 on apoptotic response in experimental Monocrotaline rat model of pulmonary hypertension (PH). **a** Representative western blots of markers of apoptosis in total lung homogenates of rats exposed to monocrotaline (MCT) injection and controls (healthy, $n = 4$; MCT, $n = 5$; MCT + R428, $n = 6$). **b** Subsequent densitometric quantification of Cleaved Caspase, **c** p53 and **d** p21 expression in total lung homogenates of rats exposed to MCT injection and controls (healthy, $n = 4$; MCT, $n = 5$; MCT+R428, $n = 6$). Data are presented as mean \pm SEM and statistical analysis was performed using one-way analysis of variance with Tukey's post-hoc test for multiple comparisons. * $P < 0.05$ for MCT versus healthy controls; ** $P < 0.01$ for MCT + R428 versus healthy controls; # $P < 0.05$ for MCT + R428 versus MCT. Pan-actin served as a loading control. All the data represent the mean \pm SEM.

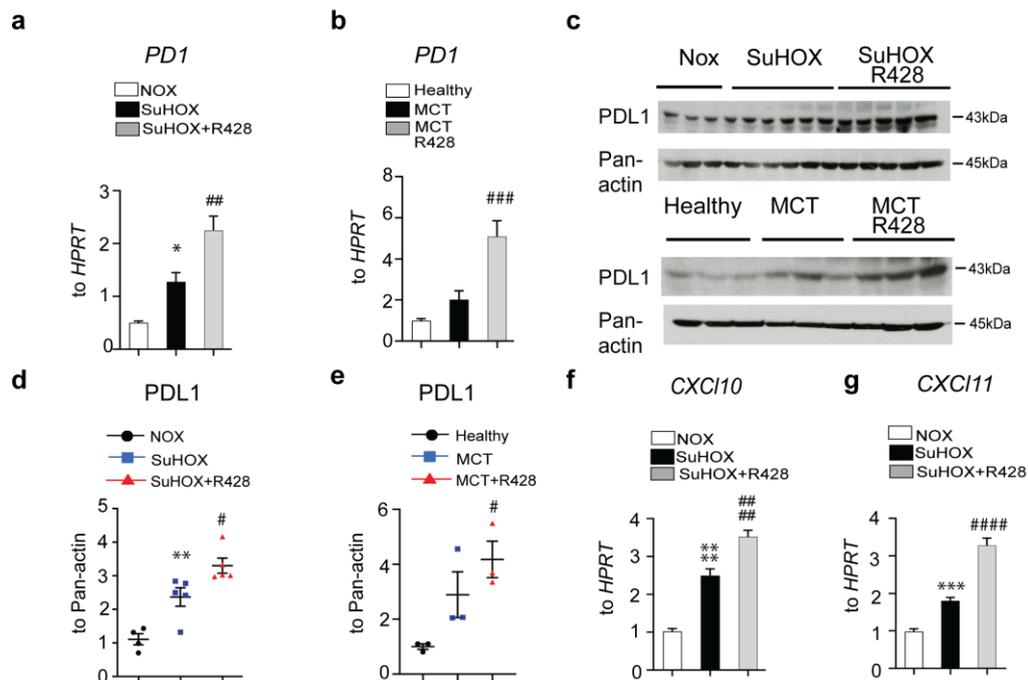


Supplementary Figure 5. Effect of R428 on pro-inflammatory response in experimental rat models of pulmonary hypertension (PH).

a Representative western blots of Nitrotyrosine, phosphorylated signal transducer and activator of transcription 3 (phospho-STAT3), suppressor of cytokine signaling 3 (SOCS3), and phospho- and total p65 of nuclear factor kappa-light-chain-enhancer of activated B-cells pathway, and **b** subsequent densitometric quantification of phospho-65 in total lung homogenates of all three experimental groups of the Sugen 5416/hypoxia (SuHOX) experimental rat model of PH (healthy, $n = 3$, SuHOX, $n = 5$, SuHOX + R428, $n = 5$). The levels of phospho-STAT3 were controlled by total STAT3 protein. Pan-actin served as a loading control. **c**, **d** Real-time quantitative PCR analyses of **c** interferon γ (*Ifn- γ*) and **d**

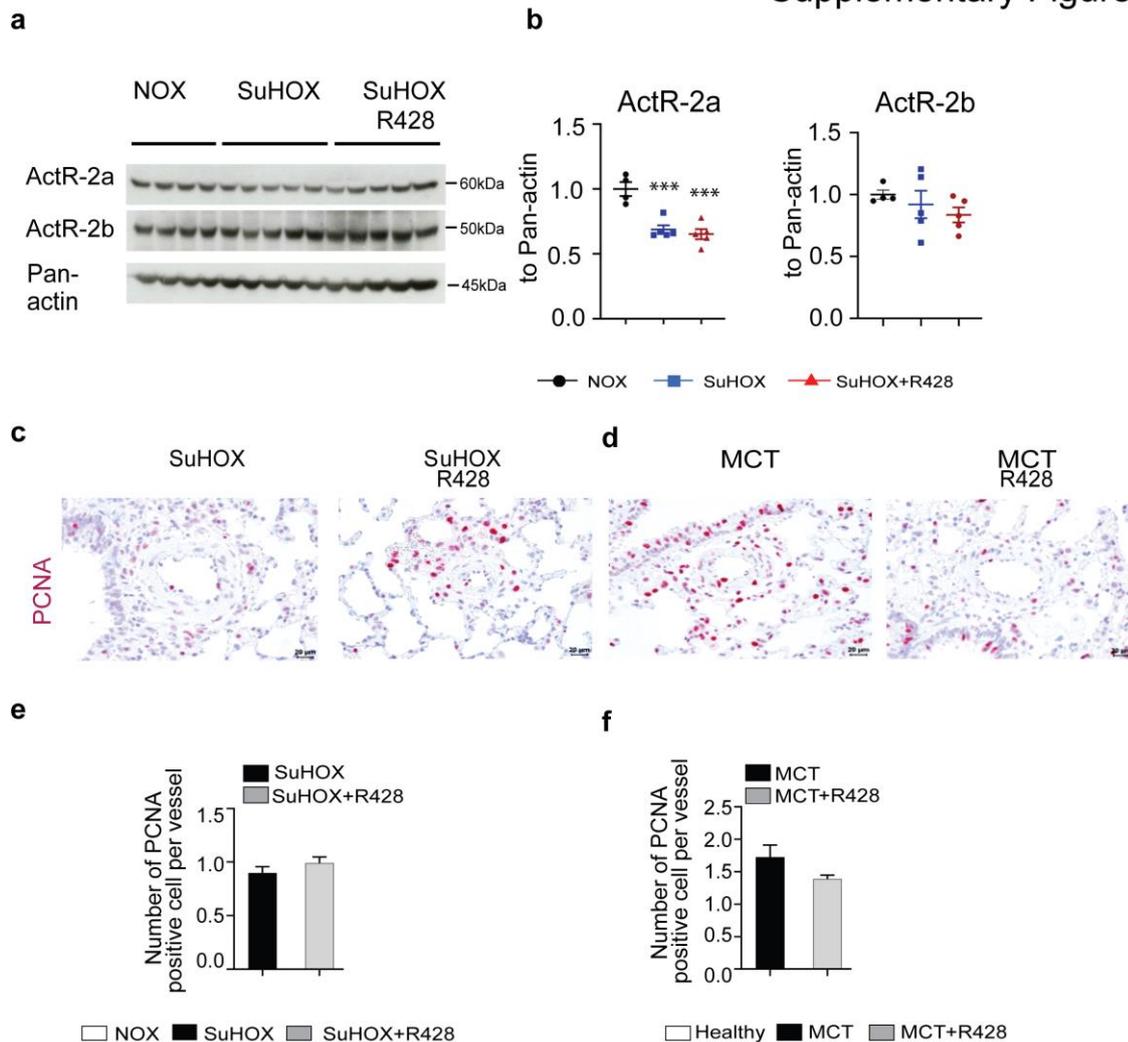
interleukin 6 (*Il-6*) expression in mRNA from lungs of rats exposed to monocrotaline (MCT) and controls. Data are presented as mean \pm SEM of the *n*-fold change ($2^{-\Delta\Delta Ct}$) normalized to hypoxanthine guanine phosphoribosyl transferase (*HPRT*) as a reference gene, compared with healthy controls (healthy, *n* = 5; MCT, *n* = 5; MCT + R428, *n* = 6). **e, g** Representative images and **f, h** quantitative analyses of immunohistological staining of CD68 expression in lung sections derived from the MCT and the SuHOX experimental rat models of PH upon R428 treatment (**[e,f]** healthy, *n* = 6; MCT, *n* = 5; MCT+R428, *n* = 6; and **[g,h]** NOX, *n* = 6; SuHOX, *n* = 6; SuHOX+R428, *n* = 6). Statistical analysis was performed using one-way analysis of variance with Tukey's post-hoc test for multiple comparisons. **P* < 0.05, ***P* < 0.01, and *****P* < 0.0001 for MCT and SuHOX versus healthy and normoxic (NOX) controls, respectively; #*P* < 0.05 and ###*P* < 0.001 versus MCT + Placebo (MCT) and SuHOX controls, respectively. All the data represent the mean \pm SEM.

Supplementary Figure 6



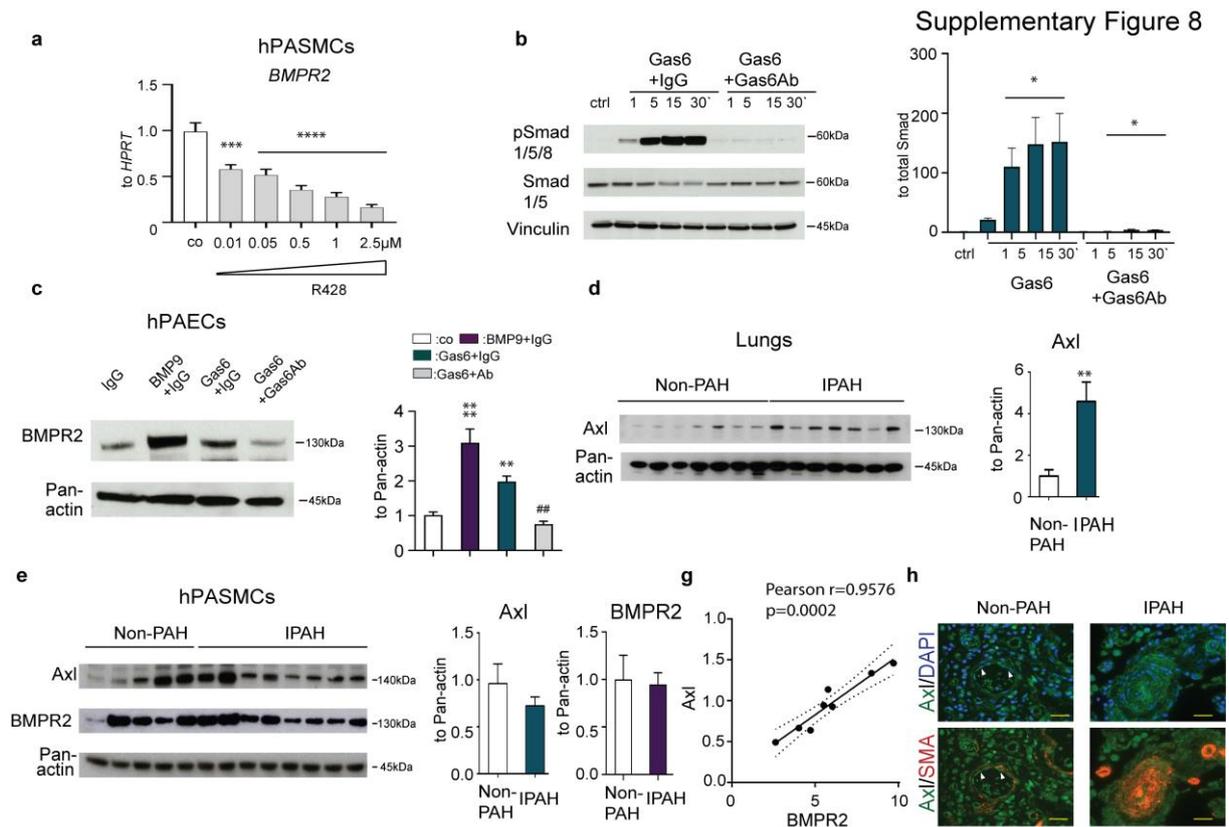
Supplementary Figure 6. Effect of R428 on programmed cell death ligand 1 (PDL1)/programmed cell death 1 (PD1) axis in experimental rat models of pulmonary hypertension (PH). Real-time quantitative PCR analyses of mRNA expression of *Pd1* in total lung homogenates of **a** Sugen 5416/hypoxia (SuHOX) and **b** monocrotaline (MCT) experimental rat models of PH. Data are presented as mean \pm SEM of the *n*-fold change ($2^{-\Delta\Delta Ct}$) normalized to hypoxanthine guanine phosphoribosyl transferase (*HPRT*) as a reference gene, compared with normoxic (NOX, *n* = 5; SuHOX, *n* = 6; SuHOX + R428, *n* = 7) or healthy control rats (healthy, *n* = 6; MCT, *n* = 5; MCT+R428, *n* = 6). **c** Western blots and **d**, **e** subsequent densitometric quantification of PDL1 in total lung homogenates of **d** SuHOX and **e** MCT experimental rat models upon R428 administration. Representative samples from all three experimental groups are shown (NOX, *n* = 4; SuHOX, *n* = 5; SuHOX + R428, *n* = 5 and healthy, *n* = 3; MCT, *n* = 3; MCT+R428, *n* = 3). Pan-actin served as a loading control. **f**, **g** Real-time quantitative PCR analyses of mRNA expression of C-X-C motif chemokine ligands *Cxcl10* and *Cxcl11* from total lung homogenates of SuHOX experimental rat model of pulmonary hypertension. Data are presented as mean \pm SEM of the *n*-fold change ($2^{-\Delta\Delta Ct}$) normalized to *HPRT* as a reference gene, compared with normoxic controls (NOX, *n* = 6; SuHOX, *n* = 6; SuHOX + R428, *n* = 7). Statistical analysis was performed using one-way analysis of variance with Tukey's post-hoc test for multiple comparisons. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001 for SuHOX versus NOX controls; #*P* < 0.05, ###*P* < 0.001 for MCT + R428 versus MCT controls; #*P* < 0.05, ##*P* < 0.01, and ####*P* < 0.001 for SuHOX +R428 versus SuHOX controls. All the data represent the mean \pm SEM.

Supplementary Figure 7



Supplementary Figure 7. Analyses of activin A receptor type 2A (ActR-2a) and activin A receptor type 2B (ActR-2b) expression in human pulmonary arterial smooth muscle cells (hPASMCs). **a** Western blots and **b** subsequent densitometric quantification of ActR-2a and ActR-2b in total lung homogenates of Sugen 5416/hypoxia (SuHOX) experimental rat model upon R428 administration. Representative samples from all three experimental groups are shown (NOX, $n=4$; SuHOX + placebo [SuHOX], $n=5$; SuHOX + R428, $n=5$). Pan-actin served as a loading control. Statistical analysis was performed using one-way analysis of variance with Tukey's post-hoc test for multiple comparisons. $***P < 0.001$ for SuHOX (or SuHOX+R428) versus NOX **c,d** Representative images of immunohistological staining of proliferating cell nuclear antigen (PCNA) in lung sections derived from **h** the SuHOX (SuHOX, $n=6$; SuHOX+R428, $n=6$) and **i** the MCT (MCT,

$n = 5$; MCT+R428, $n = 6$) experimental rat PH models. Scale bars: 20 μ m. **e, f** Quantitative analysis of immunofluorescent staining of pulmonary vascular proliferative cells using PCNA antibody. Statistical analysis was performed using Student's t-test. All the data represent the mean \pm SEM.



Supplementary Figure 8. Analyses of *BMPR2* and *Axl* expression in human pulmonary arterial smooth muscle cells (hPASMCs). **a** Real-time quantitative PCR analyses of mRNA expression of bone morphogenetic protein receptor 2 (*BMPR2*) in healthy cultured hPASMCs treated for 24 h with indicated concentrations of R428. Data are presented as mean \pm SEM of the n -fold change ($2^{-\Delta\Delta C_t}$) normalized to hypoxanthine guanine phosphoribosyl transferase (*HPRT*) as a reference gene, compared with DMSO-treated control cells. Data from three independent experiments are presented. Statistical analysis was performed using one-way ANOVA with Newman–Keuls post-hoc test for multiple comparisons. *** $P < 0.001$ and **** $P < 0.0001$ versus DMSO treated control (co) cells. **b** Western blot analysis and subsequent

densitometric quantification of Smad1/5/8 activation in hPAECs stimulated with Gas6 and pre-treated with Gas6Ab and IgG control. Data from three independent experiments are presented.

c Western blot analysis and subsequent densitometric quantification of BMPR2 expression in hPAECs stimulated for 48h with BMP9 and Gas6 followed by pre-treatment with Gas6Ab and IgG control. Data from three independent experiments are presented. **b, c** Statistical analysis was performed using one-way ANOVA with Newman–Keuls post-hoc test for multiple comparisons. * $P < 0.05$ and **** $P < 0.0001$ versus control (co) IgG treated cells; ## $P < 0.01$ versus Gas6+Gas6Ab treated cells. **d** Western blot analysis and subsequent densitometric quantification of Axl receptor expression from total lung homogenates isolated from patients with idiopathic pulmonary arterial hypertension (IPAH; $n = 8$) and non-PAH control individuals ($n = 7$). Statistical analysis was performed using Student's t-test; ** $P < 0.01$. **e** Western blot analysis and subsequent densitometric quantification of Axl and BMPR2 receptor expression in hPASMCs of patients with idiopathic pulmonary arterial hypertension (IPAH; $n = 8$) and non-PAH control individuals ($n = 5$). Pan-actin served as a loading control. **g** Correlation analysis of Axl and BMPR2 expression in hPASMCs. Pearson correlation coefficient (r value) and the significance (P value) of correlation analysis is indicated on the graph. **h** Representative examples of immunofluorescence staining of Axl (green) expression in tissue sections of non-PAH ($n = 4$) and IPAH ($n = 4$) human lung specimens. Sections were double stained with α -smooth muscle actin (α -SMA) (red) antibody. Nuclei were visualized using 4',6'-diamidino-2-phenylindole (DAPI; blue). Scale bars: 50 μ m. All the data represent the mean \pm SEM.

Gene	Host	Company	Cat. No.	Dilution in 5% BSA/TBS/T
Actr2A	Ms	Santa Cruz Biotechnology, Dallas, US	sc-515826	1:500
Actr2B	Ms	Santa Cruz Biotechnology, Dallas, US	sc-134245	1:500
AXL (C89E7)	Rb	Cell Signaling Technology, Inc., Danvers, US	#8661S	1:1000
BCL-2 (C-2)	Ms	Santa Cruz Biotechnology, Dallas, US	sc-7382	1:500
BCL-2 (N-19)	Rb	Santa Cruz Biotechnology, Dallas, US	sc-492	1:500
BMPR2	Rb	Abnova Ltd, Cambridge, UK	abx007889	1:1000
BMPR2 (E-1)	Ms	Santa Cruz Biotechnology, Dallas, US	sc-393304	1:1000
BMPR2	Ms	BD Biosciences, San Jose, CA, US	612292	1:500
Caspase-3	Rb	Cell Signaling Technology, Inc., Danvers, US	#9662S	1:1000
Cleaved Caspase-3 (Asp175)	Rb	Cell Signaling Technology, Inc., Danvers, US	#9661S	1:500
Cyclin D1 (H- 295)	Rb	Santa Cruz Biotechnology, Dallas, US	sc-753	1:500
Gas6	Gt	R&D Systems, Minneapolis, US	AF885	1:500
ID1 (B-8)	Ms	Santa Cruz Biotechnology, Dallas, US	sc-133104	1:500

NFκB p65 (F-6)	Ms	Santa Cruz Biotechnology, Dallas, US	sc-8008	1:500
Nitrotyrosine (1A6)	Rb	Merck Millipore, Burlington, MA, US	06-284	1:500
p21 (F-5)	Ms	Santa Cruz Biotechnology, Dallas, US	sc-6246	1:500
p27 (F-8)	Rb	Santa Cruz Biotechnology, Dalls, US	sc-1641	1:500
p53 (1C12)	Ms	Cell Signaling Technology, Inc., Danvers, US	#2524S	1:500
Pan-Actin	Rb	Cell Signaling Technology, Inc., Danvers, US	#4968S	1:2000
PD-L1 (CD274)	Rb	Abxexa Ltd, Cambridge, UK	abx001385	1:1000
pAXL (Y779)	Ms	R&D Systems, Minneapolis, US	MAB6965	1:500
pSmad 1/5/9	Rb	Cell Signaling Technology, Inc., Danvers, US	#13820S	1:500
pSmad 2	Rb	Cell Signaling Technology, Inc., Danvers, US	#3108S	1:500
pSmad 3	Rb	Cell Signaling Technology, Inc., Danvers, US	#9520S	1:500
pSTAT3 (S727)	Rb	Cell Signaling Technology, Inc., Danvers, US	#9134S	1:500
pNFκB p65 (S536)	Ms	Santa Cruz Biotechnology, Dallas, US	sc-136548	1:500
Smad 1/5/8 (N- 18)	Rb	Santa Cruz Biotechnology, Dallas, US	sc-6031-R	1:1000

Smad2/3	Rb	Cell Signaling Technology, Inc., Danvers, US	#3102	1:1000
SOCS3	Rb	Cell Signaling Technology, Inc., Danvers, US	#2923	1:500
STAT3 (79D7)	Rb	Cell Signaling Technology, Inc., Danvers, US	#4904S	1:1000

Supplementary Table 1: List with primary antibodies used for Western blot analyses.

Gene	Species	Forward	Reverse
18S	<i>Homo sapiens</i>	CTCAACACGGGAAACCTCAC	CGCTCCACCAAATAAGAACG
AXL	<i>Homo sapiens</i>	GCAGGCTGAAGAAAGTCCCT	CCTGTTTCATCCTCACCCAGG
AXL	<i>Rattus norvegicus</i>	TCCACCATCTGGTGAGTGA	CCTCCCCATATCTCGTCTCCT
BMP R2	<i>Homo sapiens</i>	CCACTGAATCGCTGGACTGT	ATGGTTGTAGCAGTGCCTCC
BMP R2	<i>Rattus norvegicus</i>	CACTACGGCTGCTTCCCAGAA	AGACCAGCACCCCTTGTTTCAC
B2M	<i>Homo sapiens</i>	CTCGCGCTACTCTCTTTCT	CATTCTCTGCTGGATGACGTG
Cxcl10	<i>Rattus norvegicus</i>	TGCAAGTCTATCCTGTCCGC	TGACCTTCTTTGGCTCACCG

Cxcl11	<i>Rattus norvegicus</i>	CCGAGTAACGGCTGTGACAA	TATGAGGCGAGCTTGCTTGG
E-Selectin	<i>Homo sapiens</i>	TGGTGAGGTGTGCTCATTCC	AGAGAAATGGCAGGTGGAGC
GAPDH	<i>Homo sapiens</i>	CAGAAGACTGTGGATGGCCC	AGTGTAGCCCAGGATGCCCT
GAS6	<i>Rattus norvegicus</i>	CCCCCGTGATTAGACTACGC	GATCCAGGTGCTATCCGAGC
HPRT	<i>Homo sapiens</i>	TGAGGATTTGGAAAGGGTGTT	ATGTAATCCAGCAGGTCAGCA
HPRT	<i>Rattus norvegicus</i>	AGGCCAGACTTTGTTGGATT	GCTTTTCCACTTTTCGCTGAT
ICAM1	<i>Homo sapiens</i>	GGTAGCAGCCGCAGTCATAA	TCCCTTTTTGGGCCTGTTGT
ID1	<i>Rattus norvegicus</i>	CAGCTGGAGCTGAACTCTGA	GCTCCTTGAGGCGTGAGTAG
ID1	<i>Homo sapiens</i>	CGGAATCCGAAGTTGGAACC	CAGCGACACAAGATGCGAT
ID2	<i>Rattus norvegicus</i>	CCAGAGACCTGGACAGAACC	CGACATAAGCTCAGAAGGGAAT
ID2	<i>Homo sapiens</i>	TGAAAGCCTTCAGTCCCCTG	TGAGCTTGGAGTAGCAGTCG
IFNγ	<i>Rattus norvegicus</i>	GTGTCATCGAATCGCACCTG	ACCGACTCCTTTTCCGCTTC
IL-6	<i>Rattus norvegicus</i>	AGCGATGATGCACTGTCAGA	GGAACTCCAGAAGACCAGAGC
PD-1	<i>Rattus</i>	CCCAACGGACATGACTTCCA	ATGGCCCCACAGAGGTAGAT

	<i>norvegicus</i>		
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Supplementary Table 2: List with primer sequences used for quantitative real-time PCR analyses.

Product	Company	Cat. No.
PathScan Phospho-Axl (panTyr) Sandwich ELISA	Cell Signaling Technology, MA, USA	7042C
In Situ Cell Death Detection Kit TMR red	Roche, Basel, Switzerland	12156792910
Rat Anti-cardiolipin antibody IgG (ACA-IgG) ELISA	MyBioSource, San Diego, CA, USA	MBS265605
Rat Neopterin ELISA	MyBioSource, San Diego, CA, USA	MBS2515927
Rat Phosphatidylserine ELISA	MyBioSource, San Diego, CA, USA	MBS024773
Recombinant Human/Murine/Rat BMP-2	PeproTech, London, UK	120-40
Recombinant Human BMP-4	PeproTech, London, UK	120-05
Recombinant Human BMP-6	PeproTech, London, UK	120-06
Recombinant Human BMP-7	PeproTech, London, UK	120-03
Recombinant Human BMP-9	Biolegend, San Diego, CA, USA	553102
Recombinant Human BMP-10	R&D Systems, Minneapolis, USA	885-GSB
Recombinant Human Gas6	PeproTech, London, UK	100-14
Recombinant Human PDGF-BB	PeproTech, London, UK	120-02

Supplementary Table 3: List with recombinant proteins and ELISA kits.