## ONLINE SUPPLEMENT

# Bone morphogenetic protein 9 is a mechanistic biomarker of portopulmonary hypertension

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## MATERIALS AND METHODS

De-identified clinical data, animal model and in vitro data, analytic methods, and non-commercial study materials are available for the purpose of reproducing the results by contacting the corresponding author.

### Reagents

Recombinant human sEng-Fc (6578-EN), murine sEng-Fc (1320-EN), human ALK1-Fc (370-AL) and murine ALK1-Fc (770-MA) were obtained from R&D Systems expressed as IgG Fc fusion proteins. Recombinant murine ALK3-Fc and sEng-Fc were kindly provided by Acceleron Pharma, Inc., for *in vivo* mouse studies. Human recombinant mature BMP9 and mature BMP10 homodimers were obtained from R&D Systems. Recombinant BMP9 complexed with its prodomain, e.g., BMP9 pro-complex, was produced as previously described.(1) Human pulmonary microvascular endothelial cells (PMVEC, Lonza, CC-2527) were maintained in EBM-2 media (Lonza CC-3156) containing EGM-2 MV SingleQuot (Lonza CC-4147). Bovine aortic endothelial cells (BAEC, Lonza, BW-6001) were cultured in EBM-2 containing 10% fetal bovine serum (FBS, Invitrogen).

### High content, in-cell western, immunoblot, and immunoprecipitation analyses

For a high content SMAD1/5/8 nuclear translocation assays, $1x10^4$ PMVEC were plated into collagen-coated black 96-well cell carrier ultra plates (Perkin Elmer, #6055700). The following day, the cells were treated in EBM-2 media containing 0.5% FBS. The treatments included varying concentrations of recombinant BMP9 pro-complex, pooled AB serum (Sigma H4522), and varying concentrations of human ALK-1 Fc and anti-BMP9 (R&D MAB3209) in the presence of either 50 pg/mL BMP9 or 8% AB serum (diluted in EBM-2 media+0.5% FBS). ALK1-Fc and anti-BMP9 were incubated with either BMP9 or AB serum for 1 hr at room temperature before being added to cells. The cells were treated

for 30 min at 37C and then fixed in 4% formaldehyde (ThermoFisher, #28908) for 15 min. The cells were blocked and permeabilized in PBS containing 0.3% Triton X-100 and 5% goat serum (Sigma-Aldrich G9023) for 1 hour. The cells were stained overnight in anti-phospho-SMAD1/5/8 (Cell Signaling Technology, 13820S) diluted 1:500 in PBS containing 0.3% Triton X-100 and 1% BSA (Sigma A7979). The next day, the cells were washed 3x in PBS and stained with Alexa Fluor 488 goat anti-rabbit IgG (ThermoFisher A11008) diluted 1:1000, Hoechst 33342 (ThermoFisher, H3570) diluted 1:1000, and HCS Cell Mask Deep Red Stain (ThermoFisher, H32721) diluted 1:10,000 in PBS containing 0.3% Triton X-100 and 1% BSA for 1 hour. Cells were imaged on an Operetta High Content Imager (Perkin Elmer) with a 20x 0.45 NA objective at room temperature in PBS. Nuclear intensity was measured in Harmony software by calculating the intensity of phospho-SMAD1/5/8 staining in the nucleus as defined by Hoechst 33342 staining. Whole cells were defined by HCS Cell Mask Deep Red Stain.

A high-throughput in-cell western assay was used to measure phosphorylated SMAD1/5/8 in cultured cells as previously described.(2) Briefly, primary cultured bovine aortic endothelial cells (Lonza) or human pulmonary microvascular endothelial cells (Lonza) were seeded into 96-well plates, grown to confluence in recommended media, and treated under low (1%) serum conditions with varying concentrations of BMP9 for 30 min at 37C in the presence or absence of BMP9 ligand trap ALK1-Fc. Fixed and permeabilized cells are assayed for anti-phospho-SMAD1/5/8 immunoreactivity (Cell Signaling Technologies, Clone 41D10) and developed by enhanced chemiluminescence (APU4, Surmodics).

Immunoblots for assaying SMAD1/SMAD3 activation were performed using a monoclonal antibody recognizing both phosphorylated SMAD1 and SMAD3 (EP823Y/ab52903, Epitomics/Abcam). Immunoblots for BMP9 were performed using recombinant mature BMP9 or BMP9 immunoprecipitated from human AB serum pool (Sigma) using mouse monoclonal anti-human mature BMP9 (R&D Systems MAB3209) and magnetic anti-mouse IgG beads (Invitrogen), and visualized using biotinylated goat anti-human BMP9 (BAF3209, R&D Systems) and HRP-Streptavidin (R&D Systems).

**Biomarker measurements**

Ethylenediaminetetraacetic acid (EDTA)-treated plasma samples were collected from peripheral venous blood and stored at -80°C until analysis. sEng levels were measured by sandwich enzyme-linked immunoabsorbent assay (ELISA, Quantikine, R&D Systems) according to manufacturer's protocol. A capture ELISA was used to measure BMP9 levels in human plasma as follows: Mouse monoclonal anti-BMP9 (MAB3209) was diluted in phosphate buffered saline (PBS, 5 µg/mL) and

immobilized overnight at 4℃ on Costar EIA/RIA Easy-Wash 96 well plates, and blocked with 1% bovine serum albumin (BSA Fraction V, BP1600-100, Fisher Scientific) in phosphate buffered saline (PBS) for 1 hr at 25℃. Wells were incubated with recombinant BMP9 protein or plasma at 1:4 dilution, found to yield optimal detection range and sensitivity, in the presence of heat inactivated goat serum (0.1%) to eliminate non-specific binding of detection antibody. Captured BMP9 was detected using biotinylated goat anti-human BMP9 (BAF3209, 72 µg/mL, R&D Systems) diluted in 1% BSA and 0.1% heat inactivated goat serum in PBS. Streptavidin-HRP (DY998, R&D Systems) was reacted with wells in 1% BSA PBS and bound BMP9 quantified using recombinant human BMP9 as a standard (3209-BP, R&D Systems). HRP activity was detected using chromogenic substrate 3, 3', 5, 5'-tetramethylbenzidine (Sigma, Abs 650 nM) at 10 minutes. Analytes were measured at minimum in duplicate within the sensitive and unsaturated range of this assay. To confirm the specificity of the measurements, BMP9 ligand traps including human and murine ALK1-Fc were added at varying concentrations to plasma samples. Under these conditions, all detected BMP9 activity could be inhibited with the addition of ALK1-Fc, whereas incubation of some plasma samples without goat serum resulted in artefactually high measurements of BMP9 that were felt to be non-specific on the basis of lack of inhibition with ALK1-Fc. BMP9 levels in mouse plasma were measured following the same procedure and reagents, using recombinant mouse BMP9 standard. All measurements were performed on samples within 7 years of being drawn, with samples having been stored between 0.5 – 7 years. All assays were calibrated with newly reconstituted commercial recombinant human mature BMP9 protein, as well as two standardized human samples (aliquoted at time of collection to avoid freeze-thaw degradation). Over a 5-year period of repeated measurements, the human samples used to calibrate this assay demonstrated high reproducibility (%CV<10) in relation to recombinant protein standards, with no evidence of degradation after being stored continuously at -80C. All samples measured in this study were subjected to a maximum of 2 freeze-thaws, with which there was no appreciable decrease in measured BMP9 levels.

**Murine models of pulmonary hypertension**

All experiments were done with approval from the Brigham and Women's Hospital Institutional Animal Care and Use Committee. Animals were housed at 24°C in a 12-hour light-dark cycle where food and water were accessible *ad libitum*. Adult male C57BL/6J mice (20-25 g) were purchased from Charles River Laboratory, and were administered weekly subcutaneous injections of SU5416 (20 mg/kg) and exposed to normobaric hypoxia ($FIO_2$=10%) for 3 weeks to induce pulmonary hypertension.

**Murine model of portopulmonary hypertension**

Male C57BL/6J (Charles River Laboratory) mice aged 6-8 weeks were treated with $CCl_4$ in olive oil (1:5, 0.4 ml $CCL_4$ per kg body weight, i.p. three times per week) or olive oil only (n=10), while receiving phenobarbital (0.35 g/L) via drinking water and weekly injections of Phytonadione (0.4 mg per kg body weight, s.c.) to prevent excessive bleeding. After 16 weeks of treatment with $CCl_4$, mice underwent cardiac ultrasound assessments (Vevo 2100, VisualSonics), right heart catheterization via the internal jugular vein (1.2 Fr pressure catheter, Transonic) under anesthesia (0.5-2% inhaled isoflurane), assessments of RV hypertrophy (RV/LV+S), histomorphometric analysis of pulmonary vascular remodeling as previous,(3) and measurements of plasma biomarkers. Measurements and analysis of physiological parameters and vascular remodeling were performed in blinded fashion.
To model more prolonged and severe disease, in some experiments mice were treated with $CCl_4$ for 20 weeks.

**Murine model of hypoxia combined with ALK1-Fc**

Mice received either USP saline, murine ALK1-Fc (5 mg/kg i.p. weekly over 3 weeks), or control proteins (ALK3-Fc or sEng-Fc, 5 mg/kg i.p. weekly over 3 weeks) under normoxia or normobaric hypoxia ($FIO_2$=0.10, BioSpherix hypoxia chamber) for a period of 3 weeks. Measurements and analysis of physiological parameters and vascular remodeling were performed in blinded fashion.

**Rat models of pulmonary hypertension**

All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). All studies were approved by the Brigham and Women's Hospital Institutional Animal Care and Use Committee on Animals. Animals were housed at 24°C in a 12-hour light-dark cycle where food and water were accessible *ad libitum*. Adult male Sprague-Dawley rats (150-170 g) were purchased from Charles River Laboratory. PH was induced in one of two ways: a single subcutaneous injection of monocrotaline (MCT, 40 mg/kg, Oakwood) followed by 3 weeks of normoxia, or a single subcutaneous injection of VEGF receptor antagonist (SUGEN5416, 20 mg/kg), followed by 3 weeks of chronic, normobaric hypoxia in a regulated chamber via infusion of $N_2$ gas (FIO2=10%, BioSpherix) followed by 3 additional weeks of normoxia.

**Animal echocardiography and right heart catheterization**

Echocardiography was performed and analyzed using a Vevo 2100 small animal ultrasound with MS550D transducer (VisualSonics) applied to the anterior chest wall with mice in supine position. Following anesthesia induction with 2.5% isoflurane and maintenance with 1.5% isoflurane, mice were intubated intratracheally and mechanically ventilated using a rodent ventilator (TV=6 mL/kg,

f=150/min. Pulse-wave Doppler was used to measure flow across the pulmonary valve, and pulmonary artery acceleration time (PAT), in short axis view at the level of the aortic valve. Minimally invasive right heart catheterization was performed via right jugular vein cannulation using a 1.2F Scisense Pressure catheter (Transonic Scisense, Canada) to measure right ventricular systolic pressure (RVSP). At completion of the right heart catheterization, mice were euthanized, and plasma was collected by cardiac puncture, lungs lobes were isolated *en bloc* or perfused with fixative (1.0% paraformaldehyde in phosphate buffered saline), and lung, liver and spleen tissues collected. Measurement and analysis of echocardiographic and hemodynamic parameters were done in a blinded manner.

**Study population**

All enrollees were 18 years or older, and provided written consent approved by the Institutional Review Boards at Partners Health Care, Inc. (Boston, USA) or Tufts Medical Center (Boston, USA). Derivation cohort subjects were enrolled at the California PH Forum 2007 meeting, the Pulmonary Hypertension Association Scientific Sessions 2008, 2010, and 2012 meetings, and the Pulmonary Hypertension clinical service at Massachusetts General Hospital (MGH), including 90 patients with a confirmed Group I PAH diagnosis, 20 patients with WHO Group 2 PH, 21 patients with WHO Group 3 PH, and 49 healthy controls. The validation cohort included 63 PAH patients and 11 healthy controls without known cardiopulmonary disease, all of whom were enrolled at the Tufts Medical Center Pulmonary Hypertension Clinic. Patients with Group 2 and 3 PH were enrolled at MGH at time of diagnostic catheterization. Forty-two patients with liver disease without known PAH were enrolled from the MGH Liver Center. In all cohorts, patients with PAH, PoPH or PH included a mixture of incident patients at time of initial diagnostic catheterization, and prevalent patients. Plasma samples were collected between January 2005 and August 2014. In all enrolled patients, including patients enrolled from sites other than MGH or Tufts, comprehensive data including initial diagnostic and contemporaneous right heart catheterization, clinical laboratory, exercise capacity, pulmonary function testing and radiologic data were requested from pulmonary hypertension care providers under release by patients. Clinical diagnoses and disease status were determined by chart review performed independently by two physicians reviewing clinical, diagnostic and treatment data, and adjudicated by a third independent clinician in case of disparity. PAH diagnoses were based upon current WHO criteria, based on a resting mPAP $\geq$ 25 mm Hg, pulmonary vascular resistance (PVR) > 3 Wood units (WU), pulmonary capillary wedge pressures (PCWP) $\leq$ 15 mm Hg, and absence of left-sided cardiac, severe airway disease, or chronic thromboembolic disease. Healthy controls, Group 1 PAH, and liver disease patients were similar in age distribution.

**Clinical and hemodynamic parameters**

New York Heart Association (NYHA) functional class, 6-minute walk distances (6MWD), echocardiograms, laboratory values, and medical therapies were ascertained contemporaneously within one year of the sample collection. Echocardiographic estimates of right ventricular systolic pressure were calculated using 4 x (peak tricuspid regurgitation velocity)$^2$ + estimated right atrial pressure. When available, hemodynamic data obtained by right heart catheterization was performed simultaneously with plasma sample collection, or within 1 year of sample collection, with a mean time between catheterization and plasma collection of 1.5 months. Cardiac output was determined by either Fick's method with measurement of $O_2$ consumption and/or thermodilution. Pulmonary vascular resistance was calculated as (mPAP-PCWP)/cardiac output. Survival data was obtained retrospectively through chart review and social security death index database search. The degree of liver disease in patients with PoPH hypertension and patients with liver disease without PAH was assessed in at least one of the following ways: When available, liver biopsy samples were assessed for the degree of fibrosis by a certified pathologist and scored according to the Ishak fibrosis scoring system where 0 signifies no fibrosis, 1-2 represent portal fibrosis, 3-4 represent bridging fibrosis, 5 signifies incomplete fibrosis, and 6 signifies cirrhosis; alternatively, liver synthetic dysfunction was assessed by calculating the Model for End-Stage Liver Disease (MELD) score using the UNOS modified scoring system where MELD score = 10*(0.957*ln(serum creatinine)+0.378*ln(serum bilirubin)+1.12*ln(INR)+0.643).

**Statistical analyses**

Statistical analyses were performed using GraphPad Prism 7.0 (San Diego, California, USA) and STATA 13.0 (StataCorp, College Station, Texas, USA). The normality of all variables was tested using the Shapiro-Wilk test. Normally distributed variables are presented as mean ± standard error of the mean (SEM), whereas non-normally distributed variables are presented as median ± interquartile range (IQR). Normally distributed variables were compared using either the Student's T-test for two groups, or one-way ANOVA with post-hoc testing for multiple comparisons as indicated, with a multiplicity adjusted $p<0.05$ considered statistically significant. Non-normally distributed variables were compared using the Mann-Whitney test for two groups, or the Kruskal-Wallis test with post-hoc testing for multiple comparisons as indicated, with a multiplicity adjusted $p<0.05$ considered statistically significant. Differences in proportions were analyzed by Fisher's exact test or Chi-square test as appropriate. In experiments testing the interaction of two treatments, two-way ANOVA was performed with Sidak's test for multiple comparisons. All statistical tests were two-sided.

Associations between biomarker values and laboratory values were made using Spearman's rank correlation. To assess the performance of biomarkers in predicting the presence or absence of the disease, receiver operating characteristic (ROC) curves were generated and compared by Delong's test, using a gold standard diagnosis of PoPH based on invasive hemodynamics and radiographic or biopsy-proven cirrhosis. For survival analyses, time to lung transplant or death was determined using the Kaplan-Meier method with Log-Rank testing used to compare survival curves. Additionally, Cox proportional hazards regression models were used to identify univariate predictors of transplant-free survival. The ability of BMP9 to predict transplant-free survival was tested also after adjusting for other known contributors to survival (age, NYHA, 6MWD, RAP). In all Cox proportional hazards regression models, the proportional hazards assumption was met based on Schoenfeld residuals.

**Supplemental References**

E1. Mi LZ, Brown CT, Gao Y, Tian Y, Le VQ, Walz T, Springer TA. Structure of bone morphogenetic protein 9 procomplex. *Proc Natl Acad Sci U S A* 2015; 112: 3710-3715.
E2. Dinter T, Bocobo GD, Yu PB. Pharmacologic strategies for assaying BMP signaling function. *Methods Mol Biol* 2018.
E3. Yung LM, Nikolic I, Paskin-Flerlage SD, Pearsall RS, Kumar R, Yu PB. A Selective Transforming Growth Factor-beta Ligand Trap Attenuates Pulmonary Hypertension. *Am J Respir Crit Care Med* 2016; 194: 1140-1151.
E4. Lanahan AA, Chittenden TW, Mulvihill E, Smith K, Schwartz S, Simons M. Synectin-dependent gene expression in endothelial cells. *Physiological Genomics* 2006; 27: 380-390.

## SUPPLEMENTAL FIGURE LEGENDS

**S1. A high content phospho-SMAD1/5/8 nuclear translocation assay using cultured PMVECs reveals BMP9 to be the principal endothelial signaling molecule in human serum.**

(A-B) Nuclear localization of phosphorylated SMAD1/5/8 was assayed by immunofluorescence using anti-phospho-SMAD1/5/8 in combination with Hoescht 33342, and demonstrated potent activation of endothelial signaling by BMP9 (A, $EC_{50}$~16 pg/mL) and pooled human AB serum (B, $EC_{50}$~1.6%). BMP9-mediated activation of endothelial SMAD1/5/8 was abgrogated by co-treatment with ALK1-Fc or a monoclonal anti-BMP9 Ab (A, MAB3209). Serum-induced activation of endothelial SMAD1/5/8 was similarly inhibited by ALK1-Fc or anti-BMP9 (B), accounting for between 80%-100% of maximal activity, confirming that the principal endothelial BMP signaling activity in circulation is BMP9. (C) An ELISA capture assay detected recombinant mature BMP9 sensitively (~1.6 pg/mL) and without cross-reactivity for BMP10. (D) Detection of recombinant BMP9 (50 pg/mL) by ELISA was dose-dependently neutralized by ALK1-Fc ($IC_{50}$~14 ng/mL). (E) Detection of BMP9 in normal human and

mouse plasma by ELISA was abrogated by the addition of ALK1-Fc (5 µg/mL). (F) Immunoprecipitation of pooled human AB serum with monoclonal anti-BMP9 (MAB3209) and immunoblotting with a polyclonal antibody (BAF3209) under reducing conditions revealed a 12.5 Kd protein seen only with human serum (HS) and MAB3209 but not non-specific control IgG, corresponding with the reduced recombinant human BMP9 monomer.

**S2. Circulating soluble Endoglin (sEng), a BMP9 ligand trap, is elevated in pulmonary arterial hypertension (PAH).** (A) Soluble Endoglin (sEng) competitively inhibits BMP9 signaling in microvascular endothelial cells. Addition of human recombinant sEng expressed as an Fc-fusion protein (hsEng-Fc) inhibited the ability of BMP9 (10 ng/ml) to activate SMAD1/5/8 as detected by a sensitive in-cell western assay, as previously described.(4) Data expressed as mean ± SEM. (B) Circulating sEng was measured in plasma collected from 95 patients with WHO Group 1 PAH and 43 healthy, control individuals. Compared to controls patients with Group 1 PAH have significantly higher levels of circulating sEng (Mann-Whitney test). (C) Circulating sEng was elevated in all etiologies of Group 1 PAH and significantly higher in patients with portopulmonary hypertension (PoPH, n=26) as compared with idiopathic PAH (IPAH, n=35), PAH associated with connective tissue disease (APAH-CTD, n=21), and PAH associated with stimulant exposure (APH-STIM, n=6) (Kruskal-Wallis test with Dunn's multiple comparison). (D) The ratio between BMP9 and sEng levels was also significantly reduced in PoPH compared to controls and other etiologies (Kruskal-Wallis test with Dunn's multiple comparison). (E) ROC analysis of the ability of the BMP9/sEng ratio to distinguish PoPH from other Group 1 PAH was not significantly different than BMP9 alone. Data are expressed as median±IQR, p values as indicated.

**S3. Circulating BMP9 in pulmonary arterial hypertension (PAH) patients is not associated with New York Heart Association (NYHA) Class.** Levels of biomarkers are depicted for control individuals and PAH patients grouped by NYHA Class. Trend analysis was performed with Kruskal-Wallis test for diseased population only. (A) Brain natriuretic protein (BNP) was associated with increasing NYHA Class, whereas no significant trend was observed for (B) sEng or (C) BMP9 levels among Group 1 PAH patients. Data are expressed as median ± interquartile range (IQR); NS signifies $p>0.05$.

**S4. Diminished BMP9 correlates with degree of liver dysfunction.** BMP9 levels exhibit a significant positive correlation with albumin and platelet count (A-B), and a negative correlation with total bilirubin, INR, and MELD score (C-E). There was no significant correlation between BMP9 levels and creatinine (F). Spearman's rank correlation, $\rho$ values as indicated.

**S5. Correlation of BMP9 levels with liver fibrosis and with PoPH in mild liver disease.** (A) BMP9 levels correlated marginally with the histological degree of liver fibrosis, based on liver biopsy samples available from 34 liver disease patients without known PAH, staged for the degree of fibrosis according to the Ishak fibrosis scoring system ($r=-0.31$, $p=0.07$). (B) ROC analysis of BMP9 in PoPH vs. liver disease without PAH among patients with mild liver disease. Diminished circulating BMP9 is a highly specific and sensitive predictor of PoPH among all patients with mild liver disease based on MELD score ≤10; black line = no discrimination.

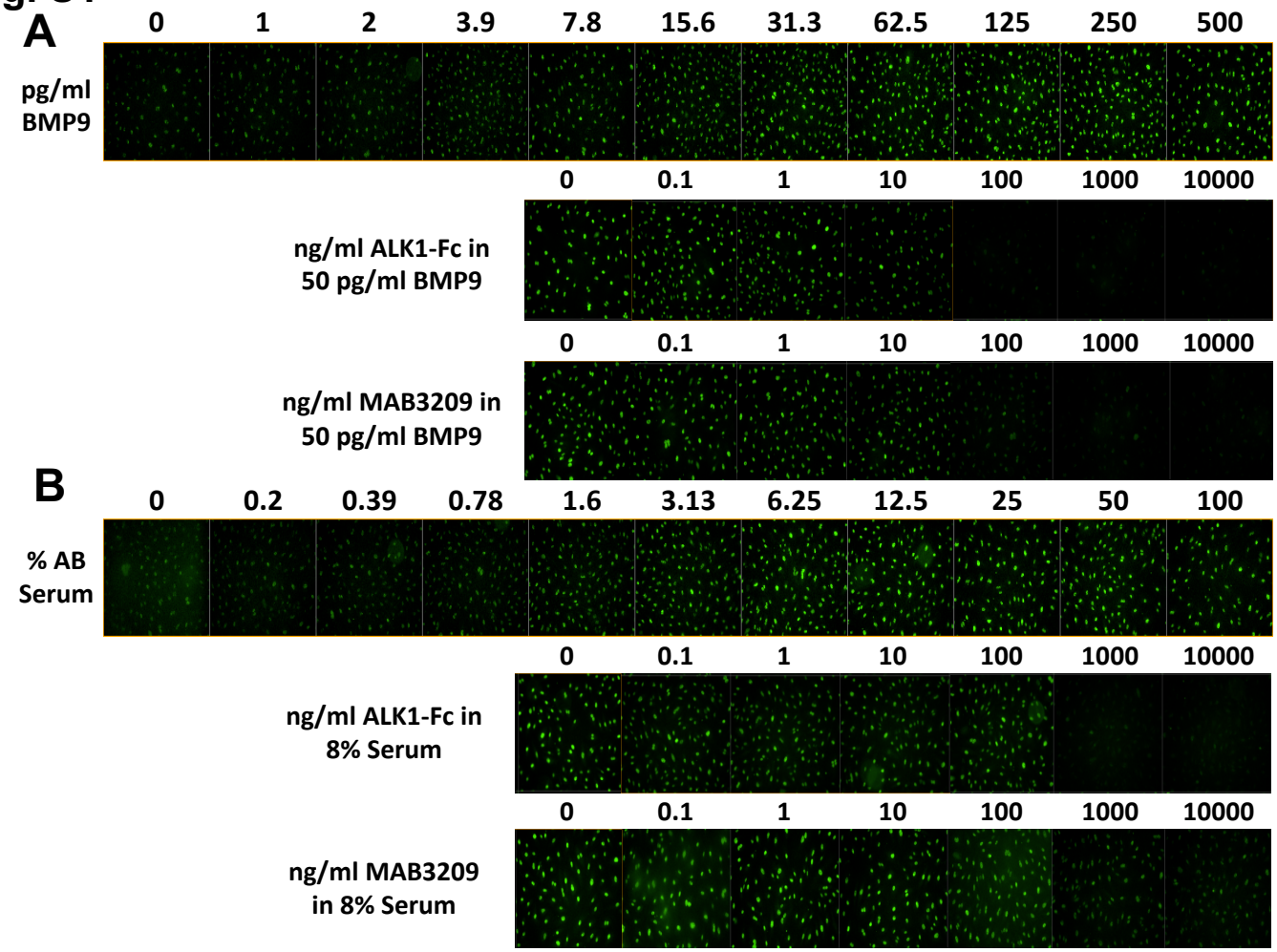
**S6. Mice treated with carbon tetrachloride ($CCl_4$) develop portopulmonary hypertension and reduced levels of circulating BMP9, which correlated negatively with severity of pulmonary hypertension.** Male C57BL/6 mice age 6-8 weeks (n=9) were treated with phenobarbital (0.35 g/L) in the drinking water and intraperitoneal $CCl_4$ in olive oil (1:5, 0.4 ml/kg body weight) or vehicle (n=5) for 16 weeks. Circulating BMP9 correlates negatively with severity of pulmonary hypertension, as evidenced by a negative correlation with right ventricular systolic pressure (A) measured by right heart catheterization and (B) positive correlation with PAT/PET ratio. Spearman's rank correlation, p values as indicated.

**S7. BMP9 ligand trap ALK1- Fc reduces expression of BMP9-responsive genes in the lung tissues of wild-type mice.** ALK1-Fc (5 mg/kg i.p. single injection) was administered to adult C57BL/6 mice, and lung tissues were assayed for mRNA expression of BMP9 responsive genes by quantitative RT-PCR. Both *Id1* and *Id3* were reduced in the lungs of treated versus vehicle-injected control ($p<0.05$, unpaired T-test). Data are expressed as mean ± SEM; *$p<0.05$; **$p<0.01$; n=4 for ALK1-Fc injected mice and controls.

**S8. BMP9 ligand trap ALK1- Fc induces severe pulmonary vascular remodeling in mice treated with hypoxia.** Administration of ALK1-Fc (5 mg/kg i.p. twice in three weeks) to adult C57BL/6 mice exposed to hypoxia ($FIO_2$=0.1) for three weeks increased the frequency of completely muscularized small arterioles (≤50 µm) based on smooth muscle-actin expression to a greater degree than that caused by hypoxia alone, shown by immunofluorescence co-staining for smooth muscle -actin and vWF (bar=50 µm).

**S9. Soluble Endoglin-Fc and ALK3-Fc did not induce pulmonary hypertension in normoxia- or hypoxia-exposed mice.** Administration of sEng-Fc or ALK3-Fc (both 5 mg/kg i.p. twice in three weeks) to adult mice for three weeks did not alter RVSP (A), or right ventricular hypertrophy (RV/(LV+S)) (B) compared to vehicle-treated mice under conditions of normoxia (n=4 per group) or under conditions of hypoxia ($FIO_2$=0.10, n=7-8 mice per group, two-way ANOVA with Sidak's multiple comparison). Data are expressed as mean ± SEM, *p<0.05, ***p<0.001.

Fig. S1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ng/ml MAB3209 in 50 pg/ml BMP9
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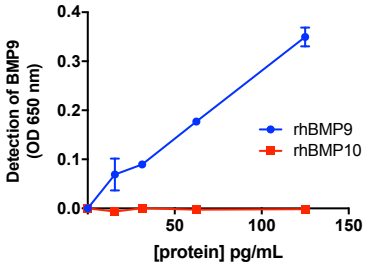
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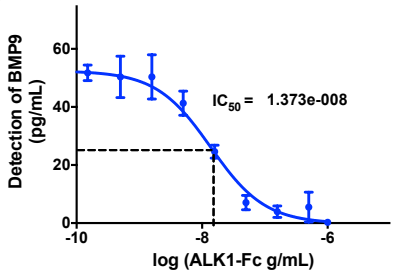
rhBMP10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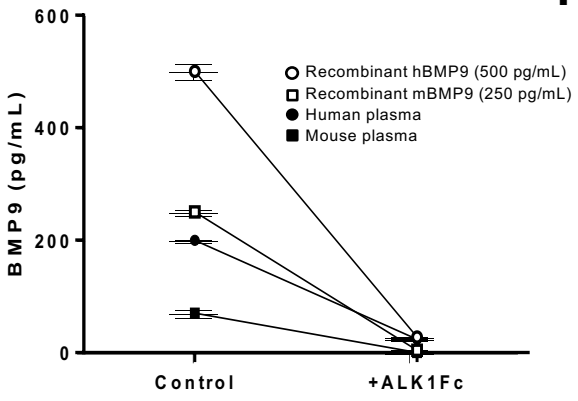
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BMP9 (pg/mL)
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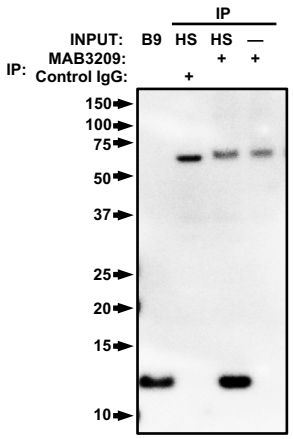
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50
37
25
20
15
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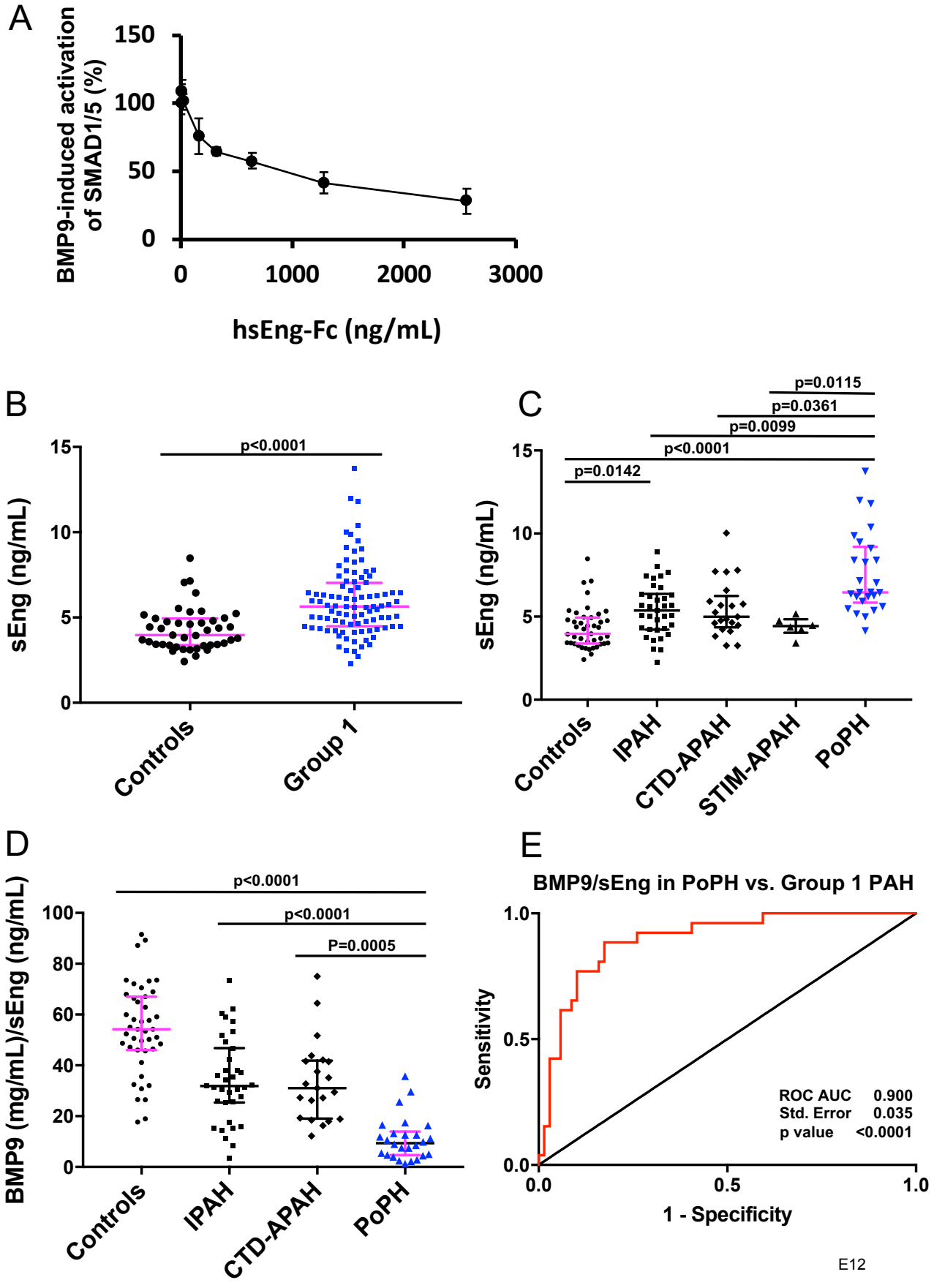
# Fig. S2


A
BMP9-induced activation of SMAD1/5 (%)
150
100
50
0
0
1000
2000
3000
hsEng-Fc (ng/mL)
B
sEng (ng/mL)
p<0.0001
15
10
5
0
Controls
Group 1
C
sEng (ng/mL)
p=0.0115
p=0.0361
p=0.0099
p<0.0001
p=0.0142
15
10
5
0
Controls
IPAH
CTD-APAH
STIM-APAH
PoPH
D
BMP9 (mg/mL)/sEng (ng/mL)
p<0.0001
p<0.0001
P=0.0005
100
80
60
40
20
0
Controls
IPAH
CTD-APAH
PoPH
E
BMP9/sEng in PoPH vs. Group 1 PAH
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0.5
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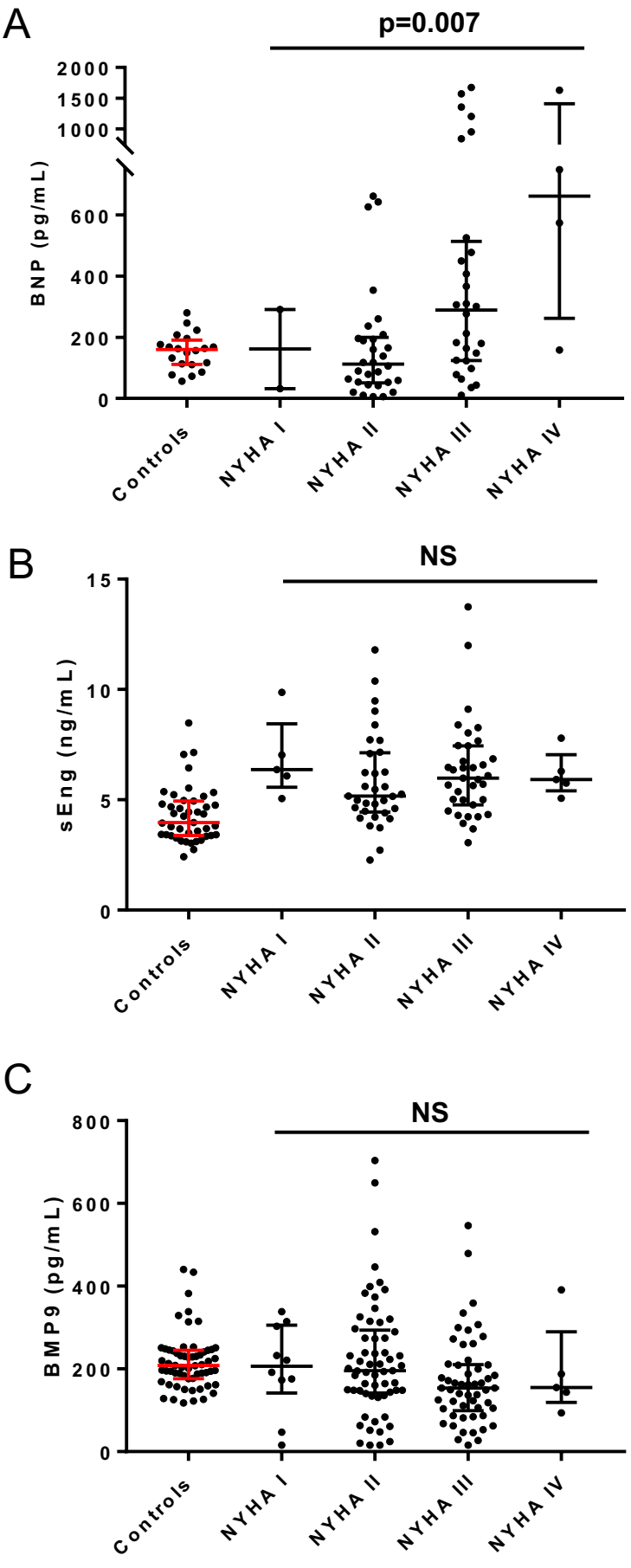
**Fig. S3**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1000
600
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200
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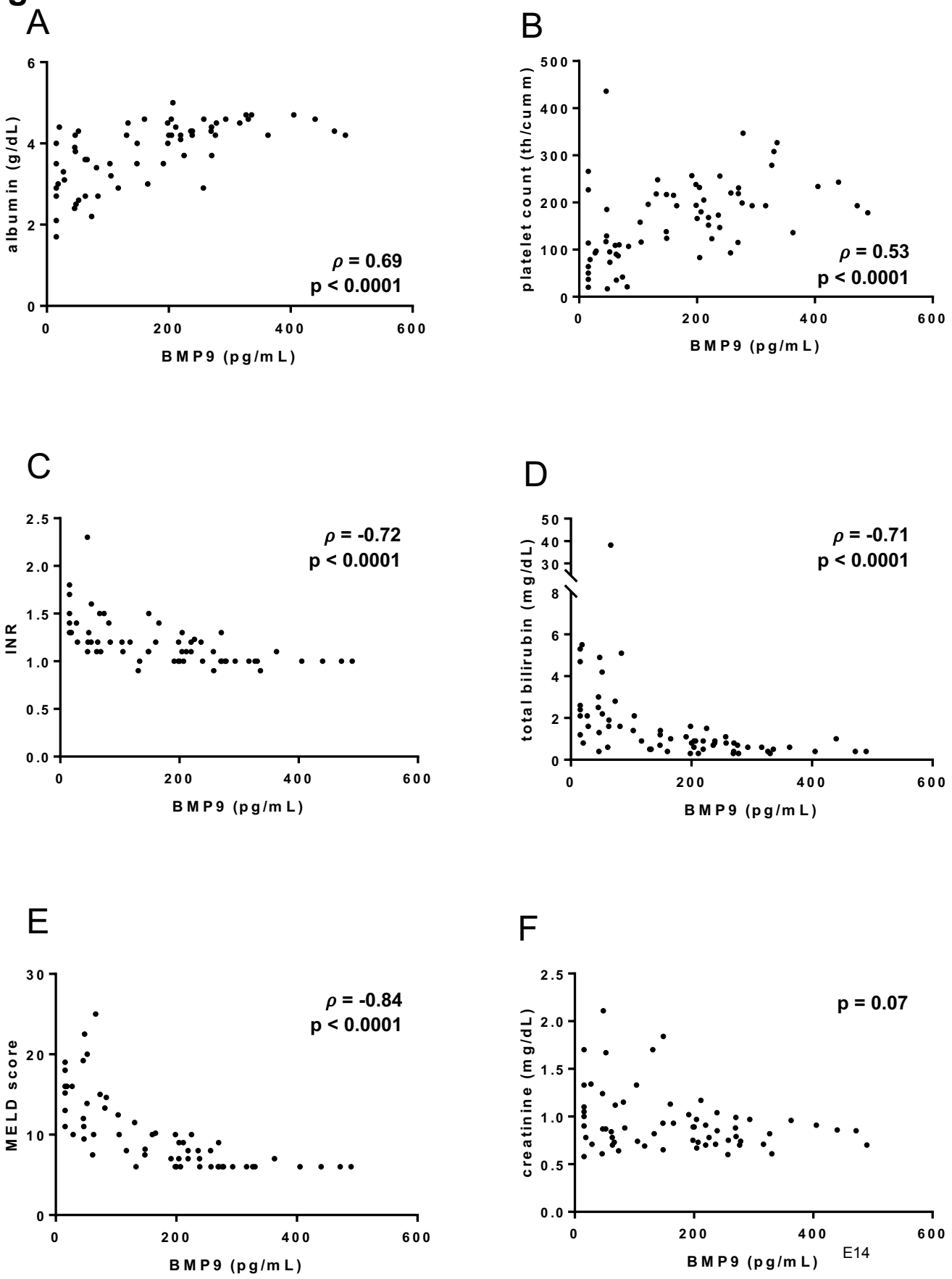
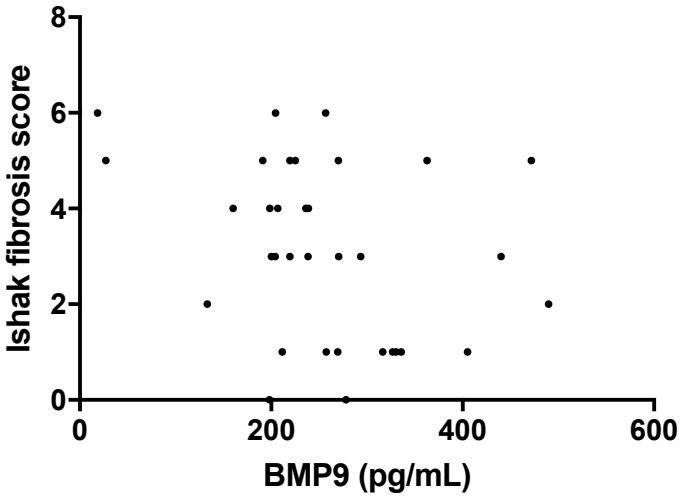
Controls
NYHA I
NYHA II
NYHA III
NYHA IV
B
NS
sEng (ng/mL)
15
10
5
0
Controls
NYHA I
NYHA II
NYHA III
NYHA IV
C
NS
BMP9 (pg/mL)
800
600
400
200
0
Controls
NYHA I
NYHA II
NYHA III
NYHA IV

**Fig. S4**

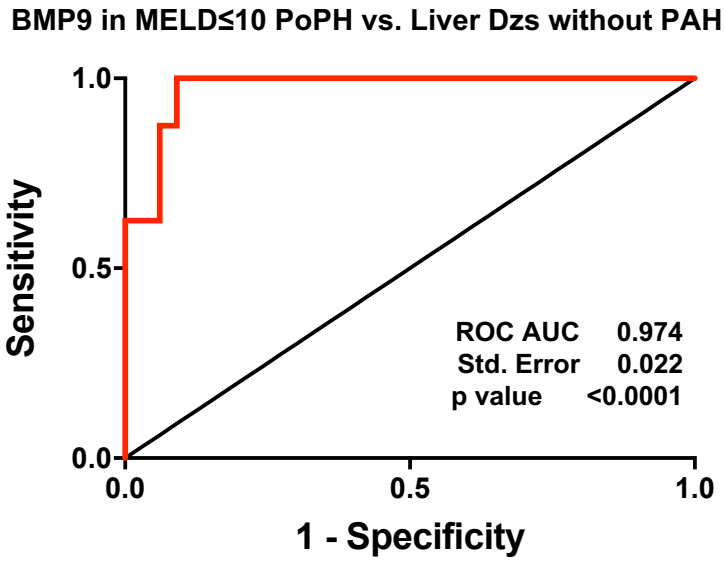

A
albumin (g/dL)
BMP9 (pg/mL)
ρ = 0.69
p < 0.0001
B
platelet count (th/cumm)
BMP9 (pg/mL)
ρ = 0.53
p < 0.0001
C
INR
BMP9 (pg/mL)
ρ = -0.72
p < 0.0001
D
total bilirubin (mg/dL)
BMP9 (pg/mL)
ρ = -0.71
p < 0.0001
E
MELD score
BMP9 (pg/mL)
ρ = -0.84
p < 0.0001
F
creatinine (mg/dL)
BMP9 (pg/mL)
p = 0.07

# Fig. S5

A

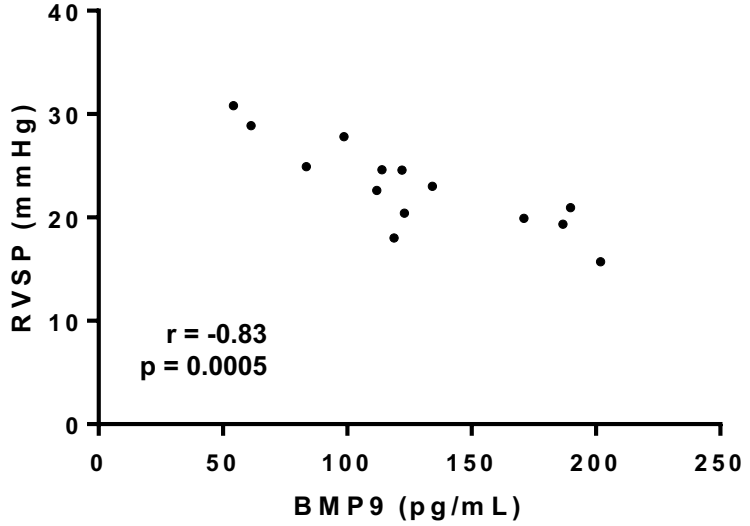

Ishak fibrosis score
8
6
4
2
0
0
200
400
600
BMP9 (pg/mL)

B

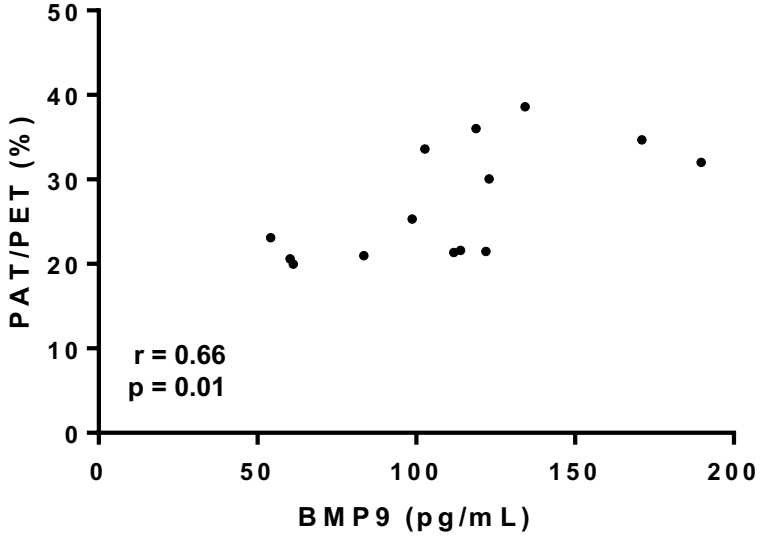

BMP9 in MELD≤10 PoPH vs. Liver Dzs without PAH
Sensitivity
1.0
0.5
0.0
0.0
0.5
1.0
1 - Specificity
ROC AUC 0.974
Std. Error 0.022
p value <0.0001

# Fig. S6

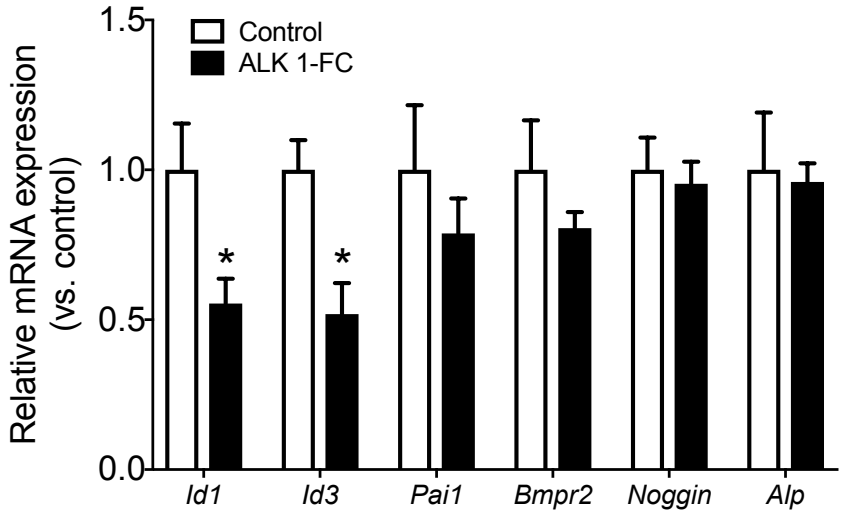
A


RVSP (mmHg)
40
30
20
10
0
r = -0.83
p = 0.0005
0
50
100
150
200
250
BMP9 (pg/mL)

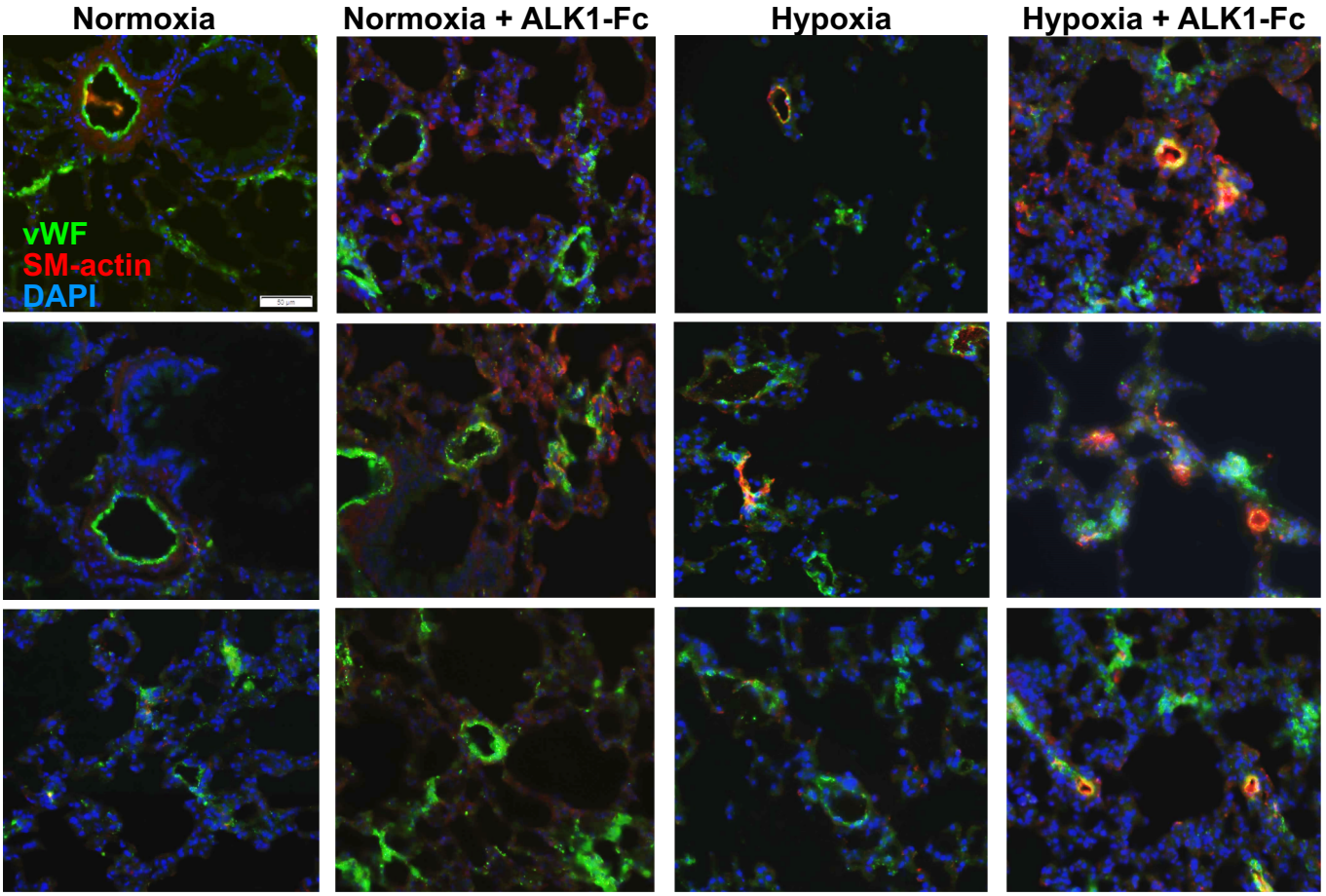
B


PAT/PET (%)
50
40
30
20
10
0
r = 0.66
p = 0.01
0
50
100
150
200
BMP9 (pg/mL)

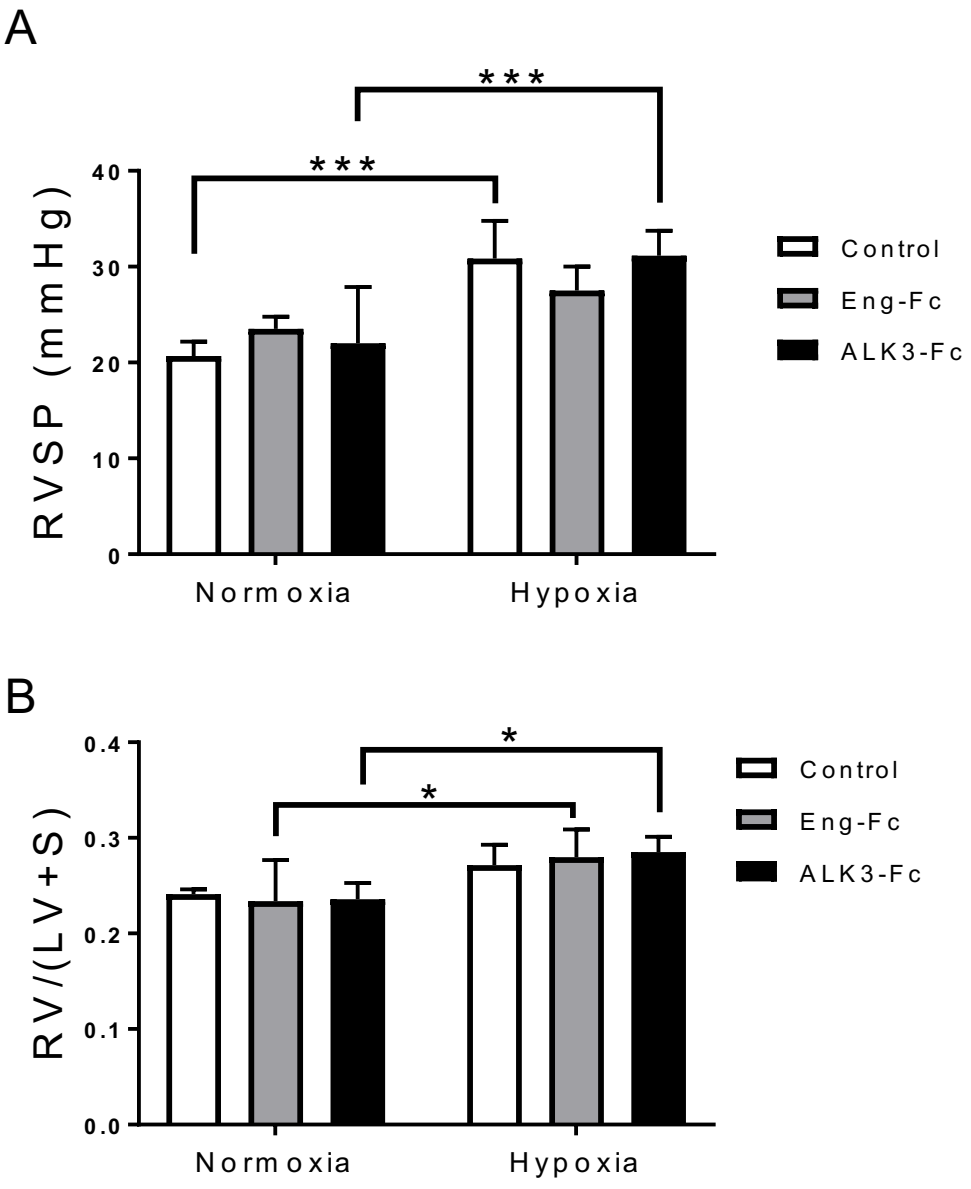
# Fig. S7


Control
ALK 1-FC
Relative mRNA expression
(vs. control)
1.5
1.0
0.5
0.0
*
*
Id1
Id3
Pai1
Bmpr2
Noggin
Alp

# Fig. S8


Normoxia
Normoxia + ALK1-Fc
Hypoxia
Hypoxia + ALK1-Fc
vWF
SM-actin
DAPI

# Fig. S9


A
***
***
RVSP (mmHg)
40
30
20
10
0
Normoxia
Hypoxia
Control
Eng-Fc
ALK3-Fc
B
*
*
RV/(LV+S)
0.4
0.3
0.2
0.1
0.0
Normoxia
Hypoxia
Control
Eng-Fc
ALK3-Fc