RNAseq Reveals a Novel Pathway of Endothelial Dysfunction in Pulmonary Arterial Hypertension

Online Data Supplement

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Expanded Methods

Subjects and cell lines

Commercially available human pulmonary artery endothelial cells (PAECs) (PromoCell GmbH, Heidelberg, Germany) were used for studies not involving IPAH patient vs. donor derived cell lines. PAECs were cultured in EC medium in EC growth medium from ScienCell (Complete medium, with ECGS, 5% fetal bovine serum and penicillin/streptomycin). IPAH patient and unused donor control cells were obtained through the PHBI Network described below. Diagnosis of IPAH was performed as per the latest guidelines (1). All subjects used in this study were screened and found negative for BMPR2 and SMAD9 mutations. Detailed exclusion criteria for unused donor lungs that served as controls are described below.

Pulmonary Hypertension Breakthrough Initiative (PHBI)

In 2006 the cardiovascular medical research and education fund (CMREF) supported the Pulmonary Hypertension Breakthrough Initiative ("PHBI"), a consortium initially comprised of 13 university-affiliated medical institutions devoted to acquiring and archiving blood samples as well as tissue and cells harvested at the time of lung transplantation from PAH patients i.e., familial, IPAH and PAH associated with other conditions (APAH). It is from this resource that we obtained the PAECs used in this project.

Control donor lung exclusion criteria:

Unused donor lungs were not considered for study if the following criteria were met:

1. Lungs from donors ≥ 65 years old (no lower age limit)

2. Lungs from donors with arterial blood gas results showing a PaO2/FiO2 ratio of <300 mmHg (the donor should be mechanically ventilated using a conventional ventilator mode with positive end-expiratory pressure of ≤ 8 mm Hg)
3. Lungs from donors who are seropositive for HIV, HCV or HBV (Hepatitis B surface antigen)
4. Lungs from donors who are high risk for HIV, HCV or HBV infections (e.g., known history of intravenous drug abuse and/or other high-risk behaviors)
5. Lungs from donors with a history of amphetamine/methamphetamine use
6. Lungs noted to have gross aspiration/infiltrates involving both lungs on chest radiographs (unless the infiltrates are due to volume overload and these infiltrates resolved with diuresis)
7. Lungs from donors who have received massive transfusion (≥10 units of packed red blood cells, 10 units of fresh frozen plasma or 10 units of platelets
8. Lungs with unexpected pulmonary problems such as significant emphysema or pulmonary fibrosis noted on radiographic studies or significant pulmonary hypertension (right ventricular dilatation and/or estimated systolic pulmonary arterial pressure ≥45 mmHg) noted on echocardiography
9. Lung from donors who have a smoking history of ≥ 50 pack-years
10. Hospitalisation for >7 days

- *If lungs from cardiac death donors further exclusion criteria apply*

11. Infiltrates on the last chest x-ray (this X-ray must be taken within 24 hrs of donor's death)
12. Fever >38.2°C (100.7°F) within 24 hrs of donor's demise
13. Lungs harvested >60 min after donor's demise

PAEC isolation

Microvascular PAECs were isolated from peripheral lung tissue using magnetic CD31 dynabeads (Invitrogen, Grand Island, NY) following collagenase (0.8mg/ml in Hank's buffered saline solution, HBSS, Invitrogen) digestion of small pieces of tissue at 37^{o}C for 20 minutes and filtration using a 100 μm cell filters to obtain a single cell suspension. Small PAs were dissected down to vessels of <1mm diameter and PAECs were obtained by scraping off the endothelial layer prior to culture. MVPAECs and SPAECs were characterized by cobblestone contact inhibited morphology, by positive immunofluorescence staining for von Willebrand factor (Abcam, Cambridge, MA) and by uptake of dil-conjugated acetylated LDL (Molecular probes). Cells isolated from healthy donors, either from commercial sources or unused donors from the PHBI network, were used for signaling and functional in vitro studies.

RNAseq analysis of PAECs

RNA was extracted from 90-100% confluent PAECs in growth medium using RNeasy Plus Kits (Qiagen, Gaithersburg, MD) as per manufacturer's instructions and strand-specific RNA-Seq libraries were prepared as previously described (2). Briefly, 5μg of total RNA were fragmented using the RNA Fragmentation Reagents (Ambion, Grand Island, NY). The dUTP was used in the second strand synthesis of cDNA. The cDNA molecules were end-repaired and A-tailed. Illumina's Paired-End adaptor oligo was ligated and size selected at an average insert size of 300 bp by gel isolation. The Library was then treated with Uracil-DNA Glycosylase (New England Biolabs) and amplified by Polymerase Chain Reaction; 98°C 30 sec, 15 cycles of (98°C 10 sec, 65°C 30 sec, 72°C 30 sec), 72°C 5 min. The Library was sequenced on 1-2 HiSeq 2000 lanes to obtain an average of ~100 and 150 million uniquely mapped reads for control and IPAH groups,

respectively. The DNAnexus cloud-computing platform (www.dnanexus.com) was used to analyze the data.

Data Preprocessing - The read data was first filtered for sequencing adaptor, primer and other contaminating sequences. Then it was trimmed at 5' end and 3' end to exclude low quality sequences (Q score >30). Data were then uploaded to the DNAnexus platform. Using the DNAnexus tools the reads are aligned to the hg19 reference genome, followed by RNA-transcriptome analysis for transcript assembly and RNA expression analysis resulting in reads per kilobase mapped (RPKM) and read count values for each individual gene.

RNAseq data analysis - Raw expression (RPKM) data was first filtered to exclude any genes that did not meet the pre-specified criteria of >5RPKM in all control or IPAH samples. To allow comparisons across samples the RPKM data distribution for each samples were quantile normalized to match the average distribution of all samples analyzed using preprocessCore package (Benjamin Milo Bolstad preprocess Core: A collection of pre-processing functions. R package version 1.18.0). Fold-differences between control and IPAH patient samples were then calculated and a threshold of 50% average fold-difference in either direction used to select potential genes of interest. Genes that met this threshold in the first RNAseq experiment were then further scrutinized in the second experiment, and those with consistent changes (met threshold in both RNAseq experiments in same direction and $p<0.1$ by t-test in combined data from 2 RNAseq experiments) considered for further investigation by qPCR and functional studies.

Data Deposit - All transcriptome data are being deposited in public databases. Transcriptome data (FASTQ files) is being submitted to the SRA database. Accession numbers will be

available no later than acceptance of the manuscript.

Quantitative polymerase chain reaction

RNA was extracted using spin column based kits (Qiagen and Zymo Research, Irvine, CA) and RT-PCR performed as per manufacturer's guidelines (Applied Biosystems, Grand Island, NY). qPCR was performed using 4ng of cDNA with 1µl 10mM mixed primers, 5µl SYBR green master mix (Qiagen) and 4µl sample. Primer sequences, designed using NCBI's Primer-BLAST function or taken from (3), are shown in Supplementary Table E1. miRNA expression was assessed using TaqMan miRNA assays (Life Technologies).

Immunoblotting

We used 30µg protein samples in RIPA buffer (Pierce, Fisher Scientific) that were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were incubated with antibodies (1:1000 unless indicated) in 5% milk-PBS containing 0.1% Tween-20 – rabbit polyclonal anti-collagen IV (Abcam, Cambridge, MA), rabbit polyclonal anti-ephrinA1 (sc-911), mouse monoclonal anti-beta-actin (both Santa Cruz, CA) overnight at 4^{o}C, incubated with secondary antibodies (anti-mouse IgG 1:5000 or anti-rabbit IgG 1:2500, GE Healthcare) and imaged using enhanced chemiluminescence reagent (BioRad laboratories, Hercules, CA) and a BioRad ChemiDoc XRS system.

siRNA transfections

ON-TARGETplus control and BMPR2 siRNA (Thermo Fisher Scientific, Waltham, MA) or control, EFNA1, COL4-A1 and A2, ALK1, ALK2, ALK3, ALK6 (all Sigma), β–catenin

(Ambion, cat#: 4390824 ID: S437), ID1, PPARγ (both Thermo Fisher Scientific; ID1: L005051-00, PPARγ: L-003436-00) siRNA were transfected into subconfluent passage 3-5 human control PAECs in 6-well plates. Plates were washed with PBS and transfected with total 1ml OPTIMEM (Invitrogen) containing 2µl Lipofectamine RNAiMAX (Invitrogen) and 10-20nM siRNA after 20 minutes incubation of siRNA and RNAiMAX. Five hours later medium was replaced with normal growth media. Effects were assessed 48-72 hours following transfection.

Transfection of SMAD1 and SMAD3 dominant negative constructs

Mutant constructs of SMAD1 and SMAD3 containing three serine to alanine substitutions in their C-terminal sequence and cloned in pCMV5 vectors were supplied by J. Massague (Memorial Sloan-Kettering Institute, New York, NY). Transfection of plasmids was performed using a Nucleofector II (program T-23) with the Basic Endothelial Cell Nucleofection kit (Amaxa, Inc.). Experiments were performed 72 h after nucleofection.

Modified Boyden chamber PAEC migration assay

Coated well inserts (35-3097, BD Biosciences, Franklin Lakes, NJ) were loaded with 250µl cell suspension (20-30,000 cells/well) in OPTIMEM (Invitrogen) and placed in 24 well plate wells containing 750µl of ECM containing either 0.2% or 5% FBS. Migration was allowed for 8 hours, then suspension was removed and the upper membrane scrubbed clean with a wet q-tip. Cells were fixed and stained with IMEB Quick Diff Kit (Fisher Scientific), allowed to dry overnight, mounted and imaged.

Cell adhesion assay

10-20,000 cells per well were added to triplicate 96 well plates coated with bovine gelatin (0.2%, Sigma) or human collagen IV (0.2 mg/ml, Invitrogen) and allowed to adhere for 40 minutes. The remaining cell suspension was then removed, cells washed with PBS then stained with DAPI (Sigma) and imaged. Nuclei were counted by conversion to a 8-bit binary image using the threshold function on the NIH's ImageJ software. The watershed function was used to divide adjacent nuclei and cells counted using the count particle function.

Statistical analysis

Genes analysed by RNAseq and qPCR were compared between control and IPAH cells by expression level using average fold-differences (positive when average IPAH PAEC gene expression levels were higher, and negative when average control PAEC gene expression was higher) and unpaired t-tests. Correlations were assessed by Spearman's rank test. mRNA and protein expression or functional differences between 2 siRNAs was assessed by t-test, and between more than 2 siRNAs/treatment conditions by one-way ANOVA, with Dunnett's or Bonferroni post-hoc testing versus scrambled siRNA. Combinations of treatments and siRNAs were assessed by two-way ANOVA with Bonferroni-corrected post-hoc testing.

Supplemental References

1. McLaughlin VV, Archer SL, Badesch DB, Barst RJ, Farber HW, Lindner JR, Mathier MA, McGoon MD, Park MH, Rosenson RS, Rubin LJ, Tapson VF, Varga J, Harrington RA, Anderson JL, Bates ER, Bridges CR, Eisenberg MJ, Ferrari VA, Grines CL, Hlatky MA, Jacobs AK, Kaul S, Lichtenberg RC, Moliterno DJ, Mukherjee D, Pohost GM, Schofield RS, Shubrooks SJ, Stein JH, Tracy CM, Weitz HH, Wesley DJ. ACCF/AHA 2009 expert consensus document on pulmonary hypertension: A report of the american college of cardiology foundation task force on expert consensus documents and the american heart association: Developed in collaboration with the american college of chest physicians, american thoracic society, inc., and the pulmonary hypertension association. *Circulation* 2009;119:2250-2294.

2. Parkhomchuk D, Borodina T, Amstislavskiy V, Banaru M, Hallen L, Krobitsch S, Lehrach H, Soldatov A. Transcriptome analysis by strand-specific sequencing of complementary DNA. *Nucleic Acids Res* 2009;37:e123.

3. Upton PD, Davies RJ, Trembath RC, Morrell NW. Bone morphogenetic protein (BMP) and activin type II receptors balance BMP9 signals mediated by activin receptor-like kinase-1 in human pulmonary artery endothelial cells. *J Biol Chem* 2009;284:15794-15804.

Table E1: **Primers used for qPCR analysis.**

Gene	Forward Primer	Reverse Primer
Beta-Actin	CATGCCATCCTGCGTCTGGA	CCGTGGCCATCTCTTGCTCG
BMPR2	CTGCGGCTGCTTCGCAGAAT	TGGTGTTGTGTCAGGAGGTGG
NOS3	ACCCTCACCGCTACAACATC	CCCCCGGAGAATGGAGAGAGCTT
DNAJB4	TCGGAGGGTCCAACCCCTTTG	TGTTTGAGGCGGGATGGCCC
HMGA1	GCGCGGCTCCAAGAAGGCAT	TGATGGCTGGGATGCGCGAG
PIR	CGCTACCGCAGCCCCTCCAT	CCTCGCTCCAACCCCTTCCGA
COL4A1	ATCCGGGTCTTCCTGGCCCC	CCGGTGTCACCACGACTGCC
COL4A2	CCAGCCACTGTCACCGAGCG	CAGGCGGTGCGTCTAGCAGG
EFNA1	CACCAGCATGAAGACCGCTGCT	AAGAGGCGTGGGGCAGCACT
GADD45B	TGGAAGAGCTCGTGGCGTGC	ACACCCCCACTGTGAGGCGA
GPRC5B	GGGGCGTCCTCTTTGCGCTC	CGCAGGCTGGCCTTGTGTCA
IQSEC1	CGCTATTTCGTCGAGGGCGAGG	AGGCTCCCACTGACGTGTGCT
ISG20	GGGAGAGTGGCCTGGCTCGT	GCCTGGCCACGGCAAATGGT
PTPRE	AGGAACCCACGGCCTCTGCG	AAGGGCTCCATGGTGGACCGAG
ALK1	ACCATCGTGAATGGCATCGT	TCTGAGCTAGGCCTGAGAGG
ALK3	CCTGTTGTCATAGGTCCGTTTT	ATCCTGTTCCAAATCACGATTG
ALK6	GGGTGGAGTTCAGCCTACTC	AAGTTTGCTTCTCACTTATGGCA
β-Catenin	TCTGATAAAGGCTACTGTTGGATT	TCACGCAAAGGTGCATGATT
ID1	CTGCTCTACGACATGAACGGCT	TGACGTGCTGGAGAATCTCCA
PPARγ	CGCCGTGGCCGCAGATTTGA	AGTTGGTGGGCCAGAATGGCA

Supplemental Figures

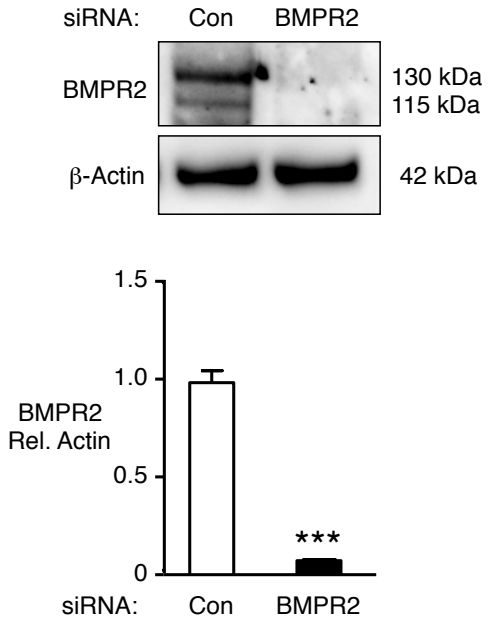


Figure E1: Differentially downregulated genes in 'validation' cohort of PAECs from six IPAH patients and five controls. Data are presented in arbitrary units normalized to 18S relative to the lowest expression value. Box plots are shown. *$p<0.05$ vs. controls by t test.

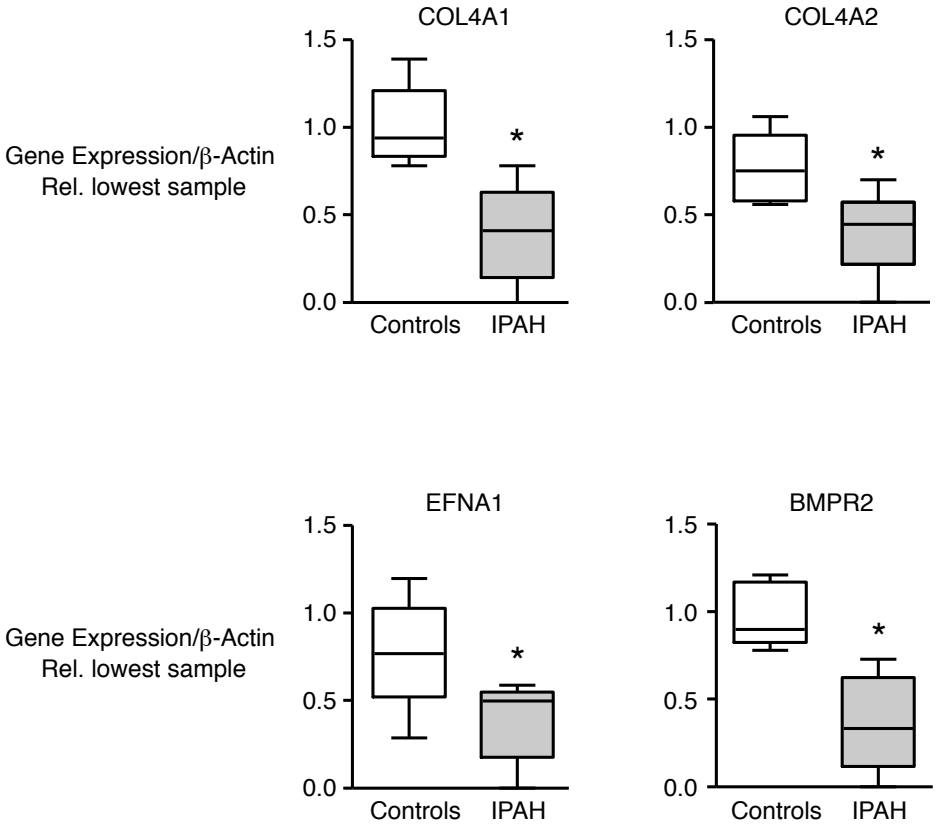


Figure E2: BMPR2 protein following BMPR2 siRNA vs. control (Con) siRNA treated PAECs.

Representative immunoblot following BMPR2 siRNA vs. Con siRNA showing barely detectable BMPR2 48h following siRNA. Dentsitometry reflects data from three experiments. Rel. lowest sample: data are given in arbitrary units, normalised by subtracting the lowest expression value. Bars represent Mean ±SEM. ***$p<0.001$ vs. Con siRNA (Con) by t-test.

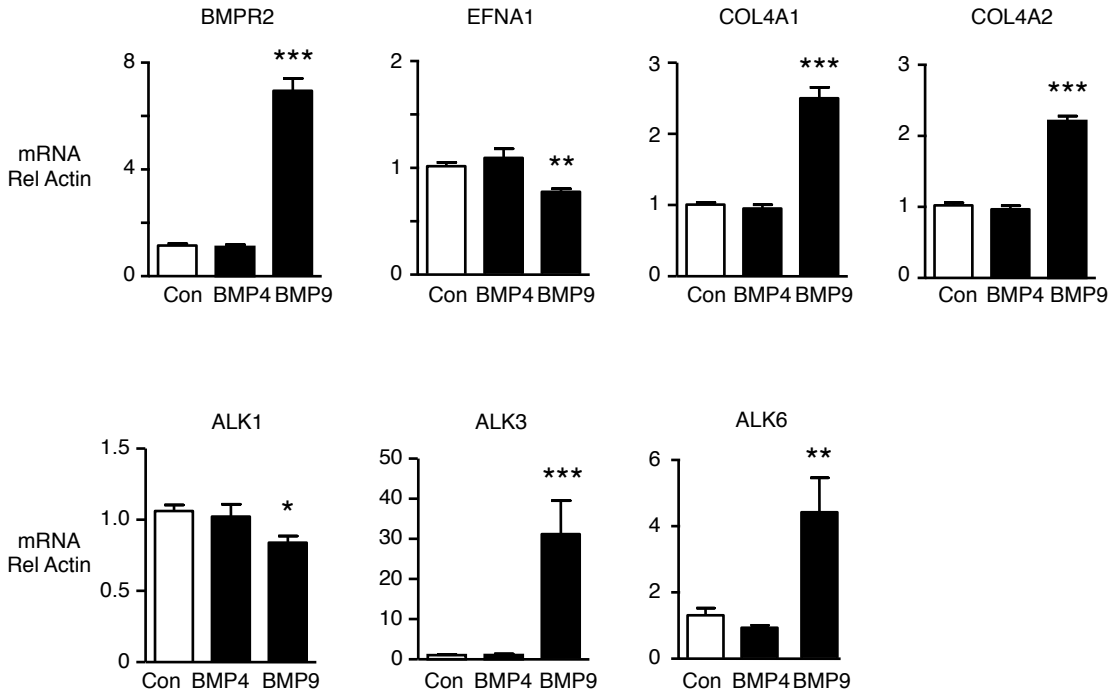


Figure E3: Effects of BMPR2 ligands, BMP4 and BMP9 on RNAseq genes in PAECs.

PAECs with 0.2% FBS-ECM (Con) +/- BMP4 (10ng/ml) or BMP9 (10ng/ml) and 24h later RT-PCR was used to quantify gene expression. ALK1, ALK3 and ALK6 represent type 1 co-receptors for BMPR2. Bars represent Mean±SEM of n=3 separate experiments. *p<0.05, **p<0.01 and ***p<0.001 vs. Con. One-way ANOVA with post-hoc Bonferroni test.

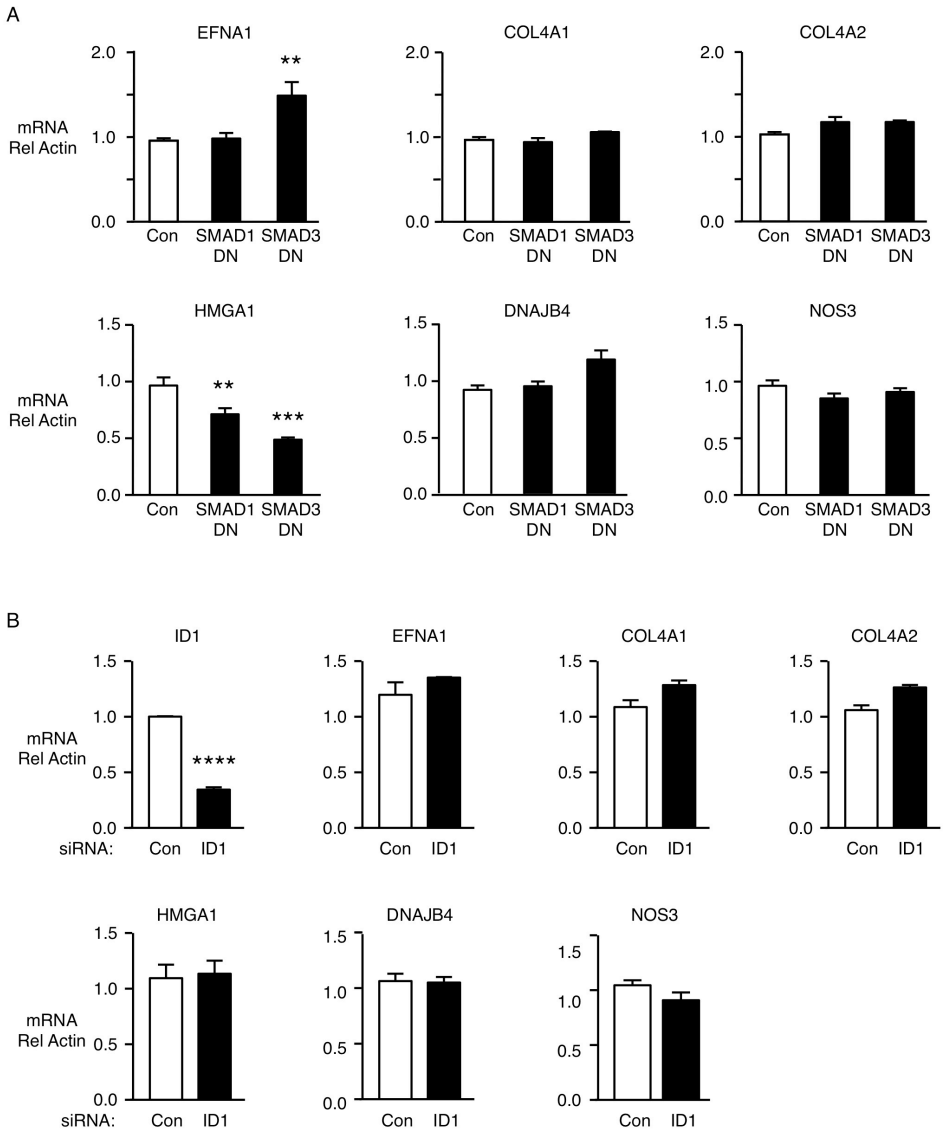


Figure E4: Disruption of SMAD1/3 activation or ID1 downregulation does not alter expression of transcript changes identified in response to loss of BMPR2. **(A)** Mutant constructs of SMAD1 and SMAD3 containing three serine to alanine substitutions in their C-terminal sequence and cloned in pCMV5 vectors were transfected into control PAECs, and gene expression quantified 72h later by qPCR. **(B)** qPCR of transcripts altered by ID1 siRNA vs. scrambled (Con) siRNA. Bar charts show gene expression normalized to beta actin. Bars represent Mean±SEM of n=3 separate experiments. **p<0.01 ***p<0.001 and ****p<0.0001 vs. Con. One-way ANOVA with post-hoc Bonferroni test.

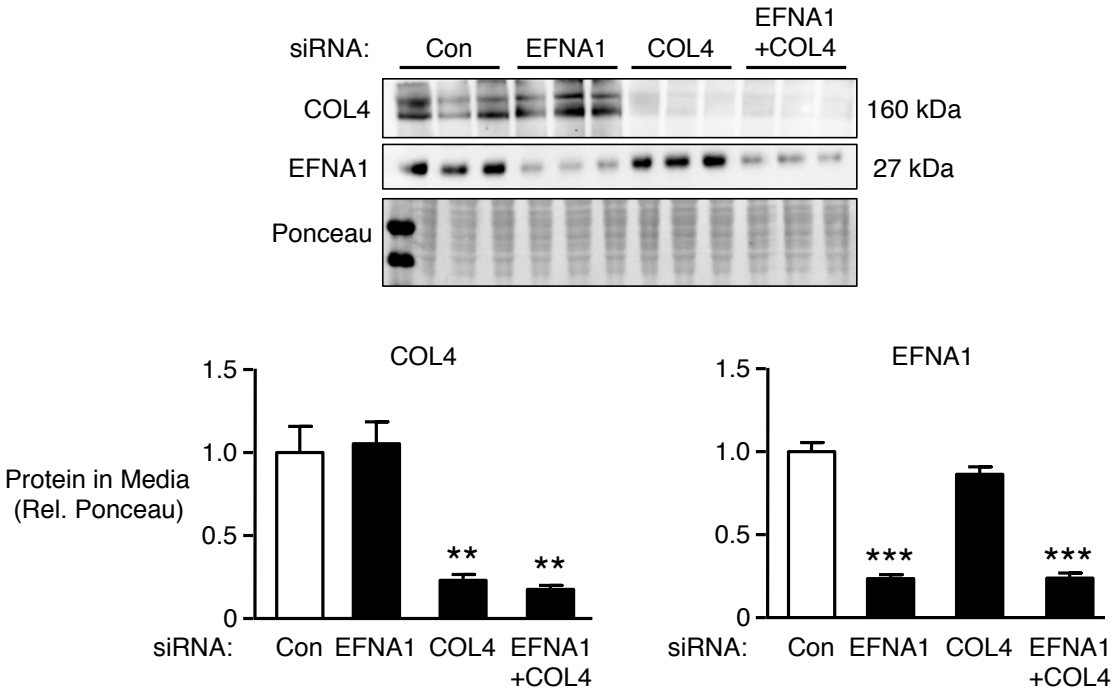


Figure E5: Protein levels following treatment of PAECs with Ephrin A1 (EFNA1) and Collagen IV (COL4) vs. control (Con) siRNA.

Immunoblot analysis of PAEC conditioned media collected for 24 hours after PAECs were transfected with indicated targeted siRNA 72 hours previously. Ponceau is used to control for loading. Bars represent Mean±SEM with 3 separate experiments with **$p<0.01$ and ***$p<0.001$ vs. Con siRNA by one-way ANOVA and Bonferroni post-hoc test.

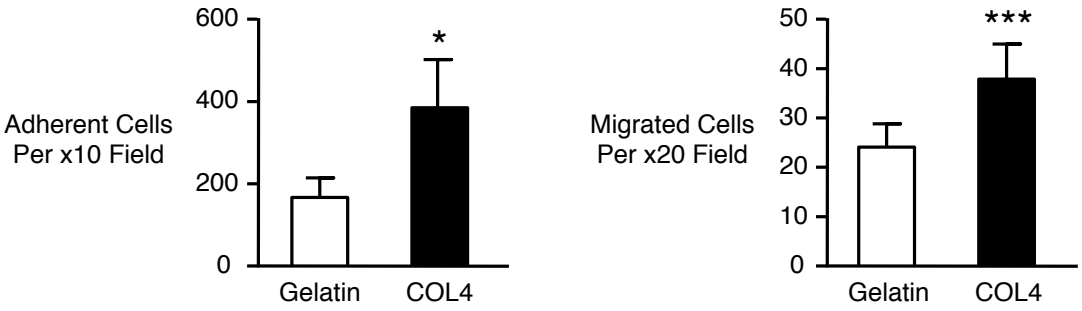


Figure E6: Adhesion and migration of IPAH PAECs in response to gelatin vs. Collagen IV (COL4) coating.

Cells were seeded onto collagen ore gelatin coated wells or inserts and adhesion and migration compared. Bars = Mean ±SEM of n=3. *$p<0.05$, ***$p<0.001$ vs. gelatin by t-test.