SUPPLEMENTAL MATERIALS

HIMF Signaling Mediates the HMGB1-Dependent Endothelial and Smooth Muscle Cell Crosstalk to Drive Pulmonary Hypertension

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Running title: HIMF/HMGB1 mediates EC-SMC crosstalk in PH

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Supplementary Methods

Production of hresistin: hResistin was produced by our laboratory in eukaryotic cell lines (T-REx™ or CHO). Briefly, the pcDNA5/FRT/TOPO TA vector containing C-terminal FLAG-tagged hresistin cDNA was integrated into the genome of the Flp-In™ T-REx™ 293 cell line in a Flp recombinase-dependent manner (Invitrogen, Carlsbad, CA). Production of recombinant hresistin in TREx 293 cells was induced by tetracycline (1 μg/mL) in DMEM supplemented with 5% fetal bovine serum, 100 μg/mL hygromycin B, and 7.5 μg/mL blasticidin. hResistin then was purified from the cell culture medium using anti-FLAG M2 antibody agarose (A2220, Sigma) column chromatography.

Immunohistochemistry: Lung specimens were fixed and sections were stained routinely with hematoxylin and eosin for histologic analysis. Paraffin lung sections were blocked and then incubated with anti-α-SMA (M085129, Dako), anti-Ki67 (ab16667, Abcam), anti-cleaved caspase-3 (9664, Cell Signaling), anti-vWF (A008202, Dako), anti-p62 (ab56416, Abcam), anti-FoxO1 (ab39670, Abcam), anti-BMPR2 (ab130206, Abcam), anti-AMPK (Ab131512, Abcam), anti-p-AMPK (Thr172, 50081, Cell Signaling), anti-RAGE (ab3611, Abcam), or anti-HMGB1 (ab18256, Abcam) antibodies, or a combination of two antibodies for double immunofluorescence staining. Then sections were incubated with the appropriate fluorochrome-coupled secondary antibody (Jackson ImmunoResearch, West Grove, PA). Finally, the sections were mounted in ProLong® Gold anti-fade reagent with DAPI (Invitrogen). Staining was imaged and tissue sections were analyzed by confocal microscopy (Leica SPE DMI8).

Western blot analysis: Mouse lung tissues or cultured cell pellets in RIPA buffer (Sigma) [supplemented with 1 mM PMSF, 1 mM Na_4VO_3, and protease inhibitor mixture (Roche, 116974980011)] were lysed with homogenization beads (0.9-2.0 mm; SSB14B, Bullet Blender) in a Bullet Blender at 4°C, vortexed, and then centrifuged. The concentration of isolated proteins

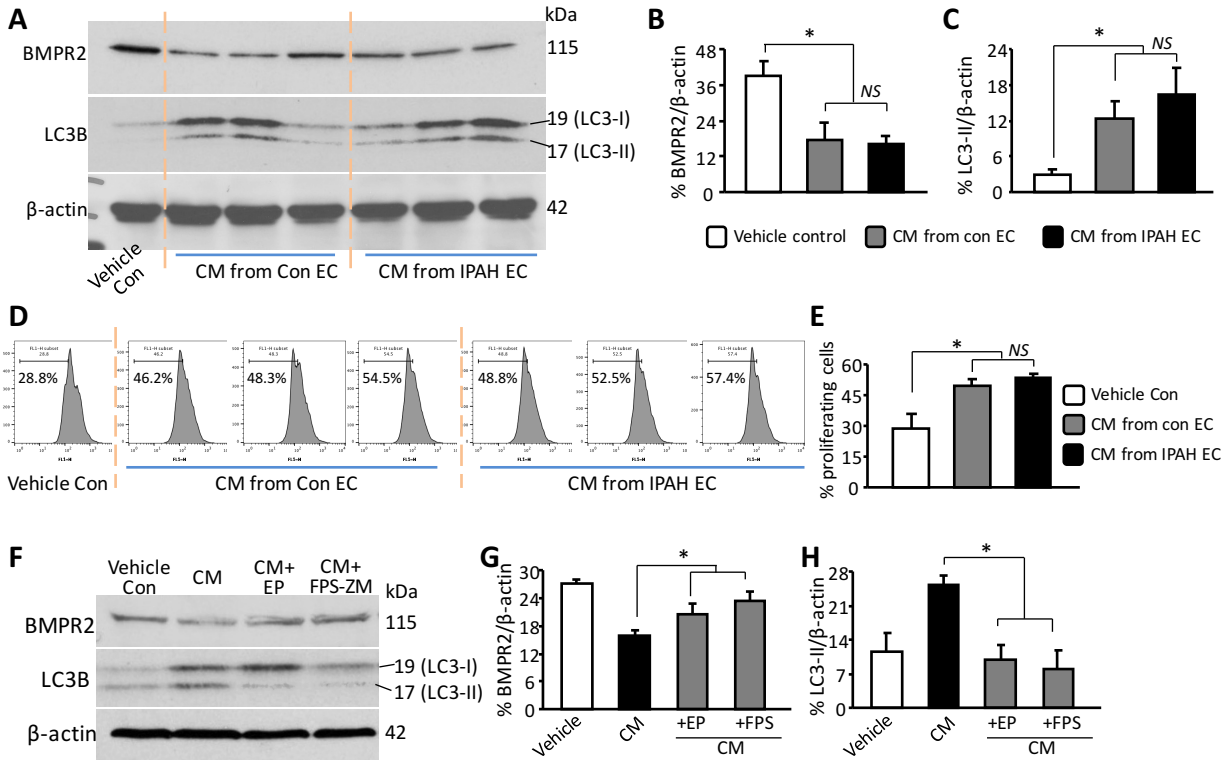
was measured with a BCA kit (Bio-Rad). The supernatants were mixed in sodium dodecyl sulfate (SDS) sample loading buffer (NuPAGE, Invitrogen) at 99°C for 10 minutes and then subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes and blotted with the antibodies to HMGB1, RAGE, LCB-II (ab48394, Abcam), p62, BMPR2, FoxO1 (SAB3500507, Sigma), total Stat3 (9139, Cell Signaling), phospho-Stat3 (Tyr705, 9145, Cell Signaling), Pim-1 (54523, Cell Signaling), or housekeeping protein β-actin (A1978, Sigma) overnight at 4°C, and then probed with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) for 2 hours. Protein bands were visualized by chemiluminescence (ECL; Amersham Pharmacia Biotech, Arlington Heights, IL).

Flow cytometry-based assay: To track proliferation, we treated hPVSMCs labeled with the fluorescein-based dye 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE, C34554, Thermo Fisher) as described in the section "Human Cell Culture and In Vitro Treatment" of the main text. After an additional 48 hours, we analyzed cells by fluorescence-activated cell sorting to determine CFSE fluorescence of the daughter cells according to instructions in the CellTrace™ CFSE Cell Proliferation Kit (Thermo Fisher). For apoptosis assays, these treated hPVSMCs were processed with the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (APOAF, sigma) according to the manufacturer's instructions. Only cells that were Annexin V-FITC-positive and propidium iodide-negative were counted as apoptotic cells.

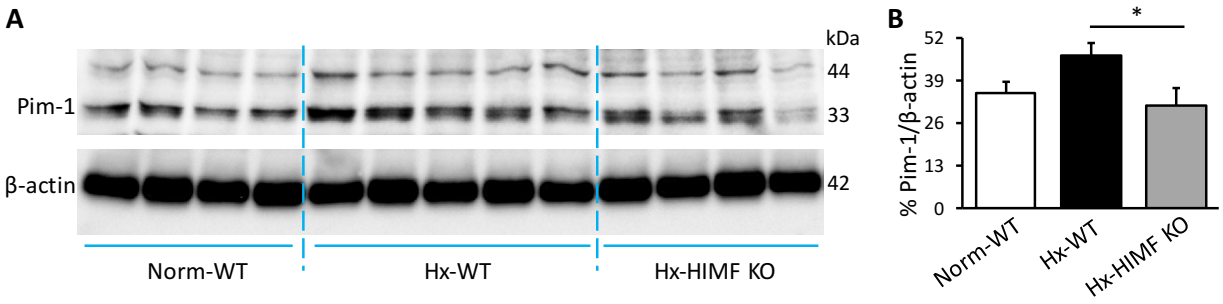
ELISA: We measured HMGB1 levels in medium from hresistin-treated human cells and in the cell-free bronchoalveolar lavage fluid (BALF) from mice by a commercial ELISA kit (LS-F4038, LifeSpan) according to the manufacturer's instructions.

BALF collection: After wild-type and humanized mice were euthanized, their lungs were lavaged and BALF was collected in three lung flushes by delivering 700 μL of sterile saline through a catheter inserted into the trachea. Samples were centrifuged (10 min, 1,000*g*, 4°C) and the aliquots of cell-free BALF (supernatant) were prepared and stored at −80°C for further assay.

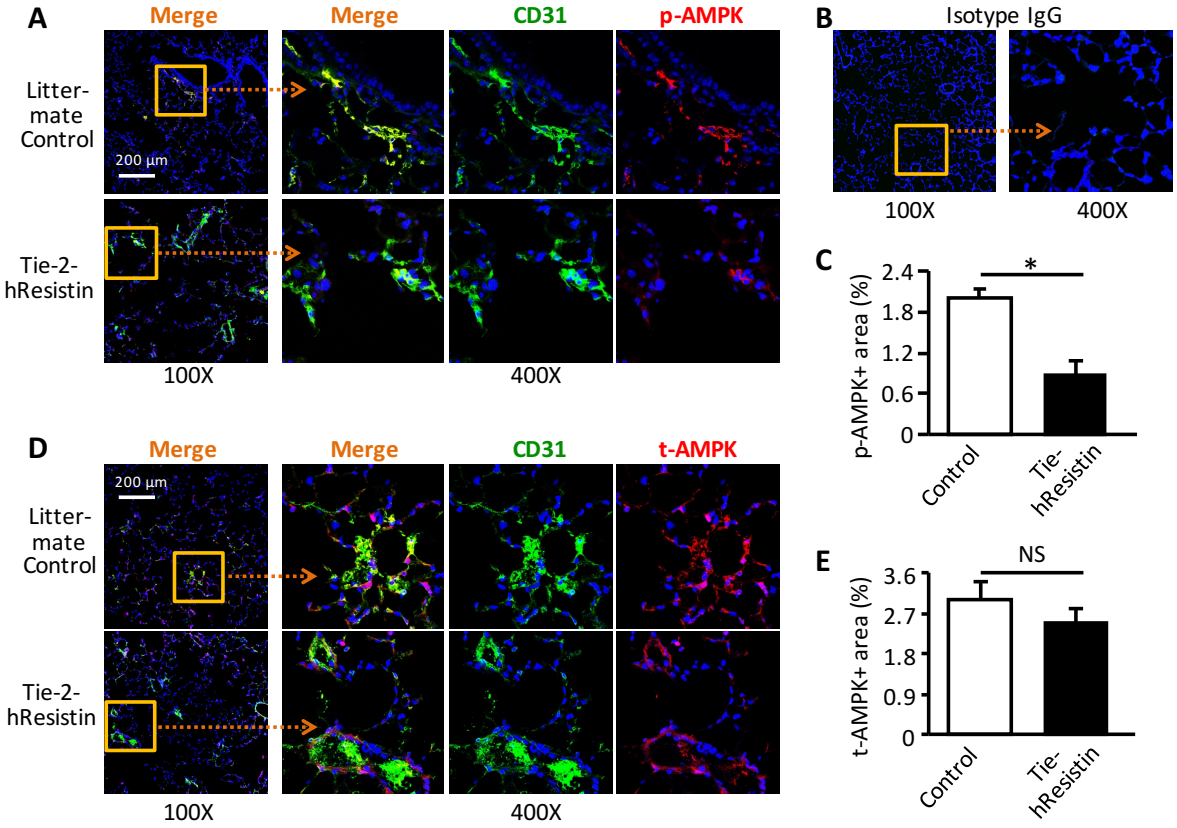
Supplementary Results



Supplemental Figure I. HIMF/DAMP-regulated proliferation-related elements in pulmonary vascular cells derived from patients with IPAH. **A,** Western blot analysis of PVSMCs. Human pulmonary artery ECs from individuals without (donor, Con, n = 3) and with IPAH (n = 3) were treated with 0.1 μg/mL human (h) resistin protein. The resulting conditioned medium (CM) was collected and applied to human PVSMCs from individuals without PAH. These CM-treated PVSMCs were then collected and analyzed for expression of BMPR2 and LC3B. **B** and **C,** Quantitative analysis of western blotting data in A for the expression of BMPR2 (B) and LC3-II (C). Data represent means ± SEM (n = 3). **p*<0.05. NS, not statistically significant. **D,** Flow cytometry analysis of PVSMCs. The CM from hresistin-treated IPAH or non-PAH (donor) pulmonary artery ECs was collected as described in A. The CFSE-labeled PVSMCs were treated with CM, and proliferation was analyzed by flow cytometry. **E,** Quantitative analysis of data in D. Data represent means ± SEM (n = 3). **p*<0.05. **F,** Human pulmonary artery SMCs from individuals with IPAH (n = 3) were treated with CM from the 0.1 μg/mL hresistin-stimulated non-PAH PMVECs. These CM-treated PVSMCs were then collected and analyzed by western blotting for expression of BMPR2 and LC3B. The results shown are representative of n = 3 IPAH PVSMCs. Experiments were repeated three times. **G** and **H,** Quantitative analysis of western blotting data in F for the expression of BMPR2 (G) and LC3-II (H). Data represent means ± SEM (n = 3, pooled data of three repeat experiments). **p*<0.05.



Supplemental Figure II. Pim1 expression in hypoxic mouse lungs. A, Western blot analysis of PIM1 expression in lung lysates collected from wild-type (WT) mice and HIMF knockout (KO) mice after 4 days of hypoxia (Hx). WT mice exposed to normal room air served as the negative control (Norm-WT). **B,** Quantitative analysis of data in A. Data represent means ± SEM (n = 4-5 per group). *p<0.05.



Supplemental Figure III. AMPK signals in the lungs of mice that overexpress human resistin. A, Lung sections from the Tie-2-hresistin humanized mice or littermate controls were immunofluorescently stained with anti-CD31 (green) and anti-phospho AMPK (p-AMPK, red) antibodies. Signals are digitally merged in left panels. Boxed areas are enlarged and displayed along with separate channels in the right panels. Magnification: 100X (left panels) and 400X (enlarged). Representative images are from 4-5 individual samples per group. **B,** Lung sections were double stained with isotype control antibodies to replace the CD31 (mouse IgG) and p-AMPK (rabbit IgG) primary antibodies. **C,** Quantitative analysis of data in A. Percentage of area positive for p-AMPK was determined with Adobe Photoshop software. Data represent means ± SEM (n = 4-5 per group). *$p<0.05$. **D,** Lung sections of the transgenic mice were immunostained with anti-CD31 (green) and anti-total AMPK (t-AMPK, red) antibodies. **E,** Quantitative analysis of data in D. Data represent means ± SEM (n = 6 per group). NS, not statistically significant.

Major Resources Tables

Animals (in vivo studies)

Species	**Vendor or Source**	**Background Strain**	**Sex**
Wild-type mouse	Charles River Laboratories	C57BL/6J	Male
Wild-type rat	Charles River Laboratories	Sprague-Dawley	Male
RELMα$^{-/-}$ mouse	Dr. Sem Phan, University of Michigan	C57BL/6J	Male
Tie-2-hRETN transgenic mouse	Dr. Roger Johns, Johns Hopkins University	C57BL/6J	Male

Animal breeding

Parent	**Species**	**Vendor or Source**	**Background Strain**
Male	RELMα$^{-/-}$ mice	Dr. Sem Phan, University of Michigan	C57BL/6J
Female	RELMα$^{-/-}$ mice	Dr. Sem Phan, University of Michigan	C57BL/6J
Male	TRE-hResistin or tTA-Tie2 mice	Dr. Roger Johns, Johns Hopkins University	C57BL/6J
Female	TRE-hResistin or tTA-Tie2 mice	Dr. Roger Johns, Johns Hopkins University	C57BL/6J

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration
HMGB1	Abcam	ab18256	WB and IF: 1 μg/mL
RAGE	Abcam	Ab3611	WB: 1 μg/mL IF: 10 μg/mL
vWF	Dako	A008202	IF/IHC: 5 μg/mL
α-SMA	Dako	M085129	IF/IHC: 5 μg/mL
FLAG	Sigma	A2220	IF: 5 μg/mL
Cleaved caspase-3	Cell Signaling	9664	IF: 1 μg/mL
Ki67	Abcam	ab16667	IF: 5 μg/mL
SQSTM1/p62	Abcam	ab56416	WB: 1 μg/mL IF: 5 μg/mL
FoxO1	Abcam	ab39670	IF: 5 μg/mL
FoxO1	Sigma	SAB3500507	WB: 2 μg/mL
BMPR2	Abcam	ab130206	WB: 1 μg/mL IF: 5 μg/mL
LC3B	Abcam	ab48394	WB: 1 μg/mL
Annexin V-FITC	Sigma	APOAF	FACS: 0.5 μg/mL
Mouse IgG	Abcam	Ab37355	IF: 5 μg/mL
Rabbit IgG	Abcam	Ab37415	IF: 5 μg/mL
Pim-1	Cell Signaling	54523	WB: 1 μg/mL
Pim-1	ThermoFisher	710504	IF: 10 μg/mL
AMPK	Abcam	ab131512	IF: 10 μg/mL
p-AMPK (Thr172)	Cell Signaling	50081	IF: 5 μg/mL
CD31	Abcam	ab9498	IF: 0.5 μg/mL
β-actin	Sigma	A1978	WB: 0.5 μg/mL

FACS, fluorescence-activated cell sorting; IF, immunofluorescence; IHC, immunohistochemistry; WB, western blot.

Cultured Cells

Name	Vendor or Source	Sex
Human pulmonary microvascular ECs	Lonza (CC-2527)	Unknown
Human pulmonary artery SMCs	Lonza (CC-2581)	Unknown
Pulmonary artery ECs isolated from patients with IPAH	PHBI	Male and Female
Pulmonary artery SMCs isolated from patients with IPAH	PHBI	Male and Female
Pulmonary artery ECs isolated from control (donor) patients	PHBI	Male and Female
Pulmonary artery SMCs isolated from control (donor) patients	PHBI	Male and Female

IPAH, idiopathic pulmonary hypertension; ECs, endothelial cells; SMCs, smooth muscle cells; PHBI, Pulmonary Hypertension Breakthrough Initiative.