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RESEARCH LETTER

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Suppression of BMP signaling by PHD2 deficiency in Pulmonary Arterial hypertension

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Abstract

BMP signaling deficiency is evident in the lungs of patients with pulmonary arterial hypertension. We demonstrated that PHD2 deficiency suppresses BMP signaling in the lung endothelial cells, suggesting the novel mechanisms of dysregulated BMP signaling in the development of pulmonary arterial hypertension.

KEYWORDS
angiogenesis, BMPR2, hypoxia, pulmonary hypertension

To the Editor,

Pulmonary arterial hypertension (PAH) is characterized by an increase in pulmonary vascular resistance and obliterative pulmonary vascular remodeling that drive right heart hypertrophy, failure, and premature death.[1–3] Due to the poor understanding of the molecular

mechanisms of obliterative vascular remodeling, current therapies result in only modest improvements in morbidity and mortality, with a 5-year survival rate of around 60%.[1,2,4] Bone morphogenetic protein receptor type 2 (*BMPR2*) mutations account for 80% of familial PAH and approximately 20% of sporadic cases, and BMPR2 expression is also reduced in the lung of patients with multiple forms of PAH, including idiopathic PAH (IPAH).[5–8]

Recently, we have reported the first mouse model of pulmonary hypertension (PH) (*Tie2Cre*-mediated disruption of *Egln1*, encoding hypoxia-inducible factor [HIF] prolyl hydroxylase 2 [PHD2], designated $Egln1^{Tie2Cre}$, CKO) with progressive obliterative vascular remodeling, including vascular occlusion and plexiform-like lesion and right heart failure, which recapitulates many features of clinical PAH including IPAH.[9,10] HIF-2α activation secondary to PHD2 deficiency is responsible for the severe phenotype in conditional knockout (CKO) mice, as HIF-2α knockout and inhibition by pharmacologic approach inhibit severe PH in CKO mice.[10–12] However, the effect and mechanisms of how HIF signaling interacts with BMP signaling remain unknown.

The aim of the present study was to determine the effect of HIF on BMP signaling in pulmonary endothelial cells. Both male and female $Egln1^{f/f}$ (wild-type [WT]), $Egln1^{Tie2Cre}$ (CKO), and $Egln1/Hif2a^{Tie2Cre}$ (EH2) mice were generated previously and used for the experiments.[10,13] The animal care and study protocol were approved by the Institutional Animal Care and Use Committee of the Northwestern University and University of Arizona. Right ventricular systolic pressure (RVSP) was measured with a 1.4 F pressure transducer catheter (Millar Instruments) and AcqKnowledge software (Biopac Systems Inc.) as described previously.[11,14] Primary human lung microvascular endothelial cells (HLMVECs) culture and transfection, RNA-sequencing, quantitative reverse-transcription polymerase chain reaction (qRT-PCR), Western blot analysis, and immunostaining analysis were performed as described previously.[10,11] Statistical evaluation was performed on GraphPad Prism 9 (GraphPad Software, Inc.) using one-way analysis of variance for comparison of more than two groups, followed by Tukey posthoc analysis for p value correction for multiple comparisons among groups or an unpaired two-tailed Student's t test for comparisons between two groups. p Values less than 0.05 were considered significant.

First, we carried out RNA-sequencing (RNA-seq) analysis on the lung tissues of WT, CKO, and EH2 mice at the age of 3.5 months as previously described.[10] Expression of several components of BMP signaling, including *Bmpr1b, Bmp3, Bmpr2, Acvrl1, Bmp6, Eng, Acvr2b, and Smad9* was decreased whereas *Grem1*, the negative regulator of BMP signaling was markedly increased in CKO lungs (Figure 1a). The qRT-PCR analysis confirmed the downregulation of *Bmpr2*, *Bmpr1b*, *Acvrl1*, and upregulation of *Grem1* in the CKO lungs (Figure 1b). Western blot analysis also demonstrated a marked decrease of BMPR2 protein and an increase of Gremin-1 (GREM1) protein levels in CKO lungs (Figure 1c). To determine the localization of BMPR2 and GREM1 proteins in the lung, we performed immunostaining against BMPR2 (1:100, Cat#612292; BD Biosciences), GREM1 (1:20, Cat#WH0026585M4; Sigma-Aldrich), and the endothelial cells (ECs) marker CD31 and found a decrease in BMPR2 and an increase in GREM1 in the ECs of CKO lungs (Figure 1d). These data demonstrate the suppression of BMP signaling in the lung ECs of *Egln1*-deficient mice. Genetic deletion of HIF-2α in CKO mice (EH2 mice) completely inhibited the development of PH[10] and the alteration of BMP signaling molecules was also normalized in EH2 mice (Figures 1a–d), suggesting that HIF-2α activation secondary to PHD2 deficiency suppresses BMP signaling in PH in mice.

To further confirm this finding in *Egln1*-deficient PH mice, we knocked down PHD2 in the HLMVECs via siRNA and performed a whole transcriptome analysis. Our data showed that BMP signaling was downregulated in the PHD2-deficient HLMVECs, which is evident through downregulation of *BMP2, BMP3, BMPR1A,* and BMP downstream genes *ID1*, as well as upregulation of BMP negative regulators *NBL and GREM1* (Figure 1e). We then confirmed that GREM1 was upregulated, whereas BMPR1A and ID1 were downregulated in PHD2-deficient HLMVECs via QRT-PCR analysis (Figure 1f). We also found that PHD2 knockdown reduced BMPR2 protein expression and BMP9 (5 ng/ml, Cat#553102; BioLegend) treatment-induced phosphorylation of Smad1/5/9 (indicative of BMPR2 activity, 1:1000, Cat#13820S; Cell Signaling Technology) in IPAH patient-derived pulmonary arterial endothelial cells (PAECs) (obtained from Pulmonary Hypertension Breakthrough Initiative), which is not affected by HIF-2α knockdown (Figure 1g). PHD2 knockdown leads to the stabilization of HIF-1α and HIF-2α,[15] and our vivo data showed that HIF-2α mediates the downregulation of BMP signaling. To determine whether HIF-2α is responsible for the dysregulated BMP signaling, we coknocked down HIF-2α with PHD2 in HLMVECs using siRNA. The qRT-PCR analysis demonstrated that GREM1 upregulation due to PHD2 deficiency was blocked by HIF-2α knockdown (Figure 1h). This data is consistent with the previous finding that hypoxia-induced GREM1 expression is dependent on HIF-2α.[16]

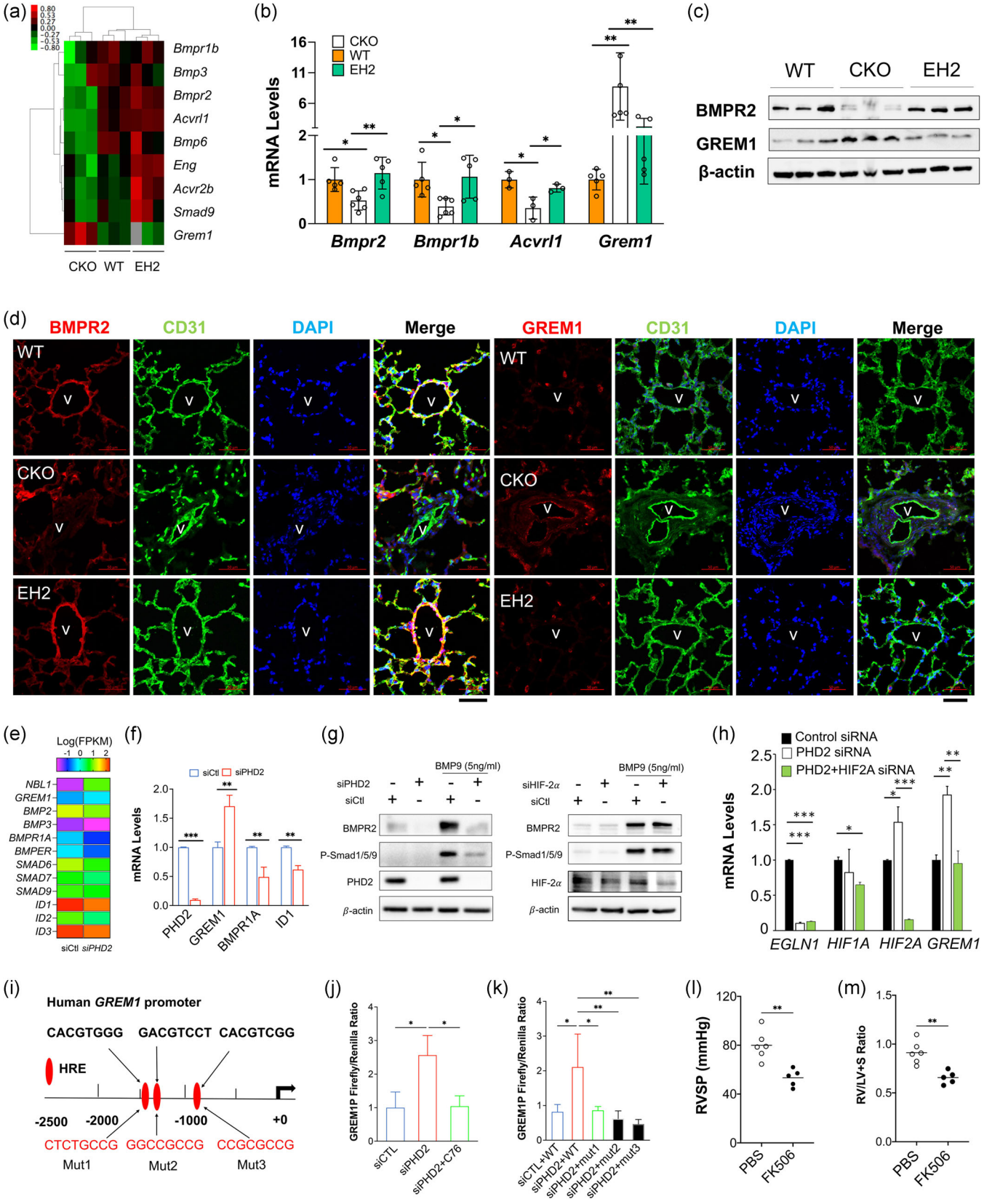

FIGURE 1 (See caption on next page)

It remains unknown if GREM1 is an HIF-2α target gene. To characterize whether GREM1 is a direct transcriptional target of HIF-2α in HLMVECs, we did in silico promoter analysis of the GREM1 promoter and found that there are three putative hypoxia response element (HRE) sites in the human GREM1 proximal promoter (Figure 1i). A 2.5-kb human GREM1 promoter was synthesized by GeneCopoeia and cloned into the upstream of Firefly luciferase reporter gene by replacing SV40 promoter (Cat#E6651; Promega). From assessing the relative luciferase activity, PHD2 knockdown in HLMVECs significantly induced human GREM1 promoter activity, which was abolished by HIF-2α translational inhibitor C76 treatment[11] (Figure 1j), suggesting that GREM1 transcription was controlled by HIF-2α in HMVECs. To further determine which putative HRE sites mediate GREM1 activation, we generated individual HRE site mutation via overlapping PCR and performed a transcriptional assessment. Our data showed that mutations of each HRE all blocked PHD2 deficiency-induced luciferase activities (Figure 1k), suggesting the direct regulation of GREM1 transcription by HIF-2α. However, our data did not support the direct regulation of HIF-2α on BMPR2 expression (Figure 1g). Previous studies showed that miRNA-21, a hypoxia-regulated microRNA, directly targets BMPR2 in PAECs in PAH.[17] It is possible that PHD2 deficiency reduces BMPR2 through upregulation of miRNA-21. Further studies are warranted to address the mechanisms on how HIF regulates BMPR2 and other BMP signaling components.

Impaired BMP signaling has been shown to play an important role in the development of PAH.[18] Given the marked suppression of BMP signaling in CKO lungs, we sought to determine whether pharmacological activation of BMP signaling would attenuate PH in CKO mice. CKO mice at the age of 5 weeks were treated with a potent BMPR2 activator FK506 (Tacrolimus, at a dose of 0.05 mg/kg; Astellas Pharma Inc.)[18,19] or PBS intraperitoneally daily for 9 weeks.[18] FK506 treatment significantly decreased RVSP and RV hypertrophy in CKO mice compared to vehicle (Figure 1l,m), which is in line with the previous observation that activation of BMPR2 reversed severe PAH in monocrotaline and vascular endothelial growth factor receptor blockade and chronic hypoxia-induced PH rats.[18] These data suggest that suppression of BMP signaling contributes to the pathogenesis of severe PH in CKO mice. Recent studies demonstrated that low-dose FK506 treatment was a potential treatment in end-stage PAH patients as is evidenced by marked clinical response, stabilization in cardiac function, and freedom from hospitalization for right heart failure.[19] Taken together, activation of BMP signaling represents a promising approach for the treatment of PAH patients.

FIGURE 1 Endothelial PHD2 deficiency suppressed BMP signaling in lung endothelial cells. (a) RNA-seq analysis demonstrated suppression of BMP signaling by PHD2 deficiency in HIF-2α dependent manner in PH mice. *Egln1*$^{f/f}$ (WT), *Egln1*^{Tie2Cre} (CKO), and *Egln1/Hif2a*^{Tie2Cre} (EH2) mice. (b) mRNA expression of *Bmpr2*, *Bmpr1b, Acrvl1*, and *Grem1* were dysregulated in the lung of CKO mice and normalized in EH2 mice. (c) Downregulated protein expression of BMPR2 and upregulation of GREM1 in the CKO lungs were normalized in EH2 lungs. (d) Decreased BMPR2 and increased GREM1 in the lung ECs of CKO lungs via immunostaining. V, vessel. (e) RNA-sequencing analysis showed that PHD2 deficiency reduced the expression of BMP signaling molecules. HLMVECs were transfected with control siRNA (siCtl) or PHD2 siRNA for 48 h. Three replicates were pooled in an equal amount for RNA-seq analysis. (f) qRT-PCR analysis confirmed the reduction of BMP signaling, including upregulation of *GREM1* and downregulation of *BMPR1A* and *ID1* in PHD2-deficient HLMVECs. $N = 3$. (g) BMPR2 expression and activities were reduced by PHD2 knockdown, but not affected by HIF-2α knockdown in IPAH patient-derived PAECs. IPAH PAECs were transfected with siCtl or PHD2 or HIF-2α siRNA for 48 h, followed by treatment of BMP9 (5 ng/ml) for 16 h. (h) GREM1 is upregulated by HIF-2α secondary to PHD2 deficiency. HLMVECs were transfected with siCtl or PHD2 siRNA or PHD2 plus HIF2A siRNA for 48 h. $N = 3$. (i) A diagram showing three putative hypoxia responsible element (HRE) sites in the promoter region of the human *GREM1* promoter. Red highlighted texts indicate mutated HRE sequences. (j) Luciferase assay demonstrates that human GREM1 promoter activities were induced by PHD2 deficiency in an HIF-2α-dependent manner. HLMVECs were cotransfected with human GREM1 promoter firefly luciferase plasmids, control *Renilla* luciferase plasmids, and siCtl or PHD2 siRNA for 48 h, followed by treatment with dimethyl sulfoxide or HIF-2α translational inhibitor C76 (20 μm) for 16 h. $N = 3$. (k) Mutagenesis studies and luciferase assays demonstrated that HRE sites in the GREM1 promoter mediate GREM1 activation in PHD2-deficient HLMVECs. $N = 3$ to 4. (l) FK506 treatment reduced right ventricular (RV) systolic pressure in CKO mice. (m) RV hypertrophy was inhibited in FK506-treated CKO mice compared to PBS. One-way analysis of variance with Tukey posthoc analysis for multiple group comparisons (b, h, j, k). Student *t* test (f, l, m). CKO, conditional knockout; DAPI, 4′,6-diamidino-2-phenylindole; HIF, hypoxia-inducible factor; HLMVEC, human lung microvascular endothelial cell; IPAH, idiopathic pulmonary arterial hypertension; mRNA, messenger RNA; PAEC, pulmonary arterial endothelial cell; PBS, phosphate-buffered saline; PH, pulmonary hypertension; PHD2, prolyl hydroxylase 2; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; RVSP, right ventricular systolic pressure; siRNA, small interfering RNA; WT, wild-type. $^{*}p < 0.05$; $^{**}p < 0.01$, and $^{***}p < 0.001$

AUTHOR CONTRIBUTIONS

Zhiyu Dai conceived the experiments, interpreted the data, and wrote the manuscript. Bin Liu, Dan Yi, Jingbo Dai, Maggie M. Zhu, designed and performed experiments, and analyzed the data. You-Yang Zhao, S. Paul Oh, and Michael B. Fallon revised the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICS STATEMENT

The animal care and study protocol were approved by the Institutional Animal Care and Use Committee of Northwestern University and the University of Arizona.

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REFERENCE

1. Lau EMT, Giannoulatou E, Celermajer DS, Humbert M. Epidemiology and treatment of pulmonary arterial hypertension. Nat Rev Cardiol. 2017;14:603–14.
2. Thenappan T, Ormiston ML, Ryan JJ, Archer SL. Pulmonary arterial hypertension: pathogenesis and clinical management. BMJ. 2018;360:j5492.
3. 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, Lindner JR, Moliterno DJ, Mukherjee D, Pohost GM, Rosenson RS, Schofield RS, Shubrooks SJ, Stein JH, Tracy CM, Weitz HH, Wesley DJ, ACCF/AHA. A report of the American College of Cardiology Foundation Task Force on expert consensus documents and the American Heart Association. Circulation. 2009;119:2250–94.
4. Humbert M, Guignabert C, Bonnet S, Dorfmüller P, Klinger JR, Nicolls MR, Olschewski AJ, Pullamsetti SS, Schermuly RT, Stenmark KR, Rabinovitch M. Pathology and pathobiology of pulmonary hypertension: state of the art and research perspectives. Eur Respir J. 2019;53:1801887.
5. Frump A, Prewitt A, de Caestecker M. EXPRESS: BMPR2 mutations and endothelial dysfunction in pulmonary arterial hypertension. Pulm Circ. 2018;8:204589401876584.
6. Machado RD, Eickelberg O, Elliott CG, Geraci MW, Hanaoka M, Loyd JE, Newman JH, Phillips JA, Soubrier F, Trembath RC, Chung WK. Genetics and genomics of pulmonary arterial hypertension. JACC. 2009;54:S33–S42.
7. Machado RD, Aldred MA, James V, Harrison RE, Patel B, Schwalbe EC, Gruenig E, Janssen B, Koehler R, Seeger W, Eickelberg O, Olschewski H, Elliott CG, Glissmeyer E, Carlquist J, Kim M, Torbicki A, Fijalkowska A, Szewczyk G, Parma J, Abramowicz MJ, Galie N, Morisaki H, Kyotani S, Nakanishi N, Morisaki T, Humbert M, Simonneau G, Sitbon O, Soubrier F, Coulet F, Morrell NW, Trembath RC. Mutations of the TGF-β type II receptorBMPR2 in pulmonary arterial hypertension. Hum Mutat. 2006;27:121–32.
8. Long L, Ormiston ML, Yang X, Southwood M, Gräf S, Machado RD, Mueller M, Kinzel B, Yung LM, Wilkinson JM, Moore SD, Drake KM, Aldred MA, Yu PB, Upton PD, Morrell NW. Selective enhancement of endothelial BMPR-II with BMP9 reverses pulmonary arterial hypertension. Nat Med. 2015;21:777–85.
9. Dai Z, Zhao Y-YY-Y. Discovery of a murine model of clinical PAH: mission impossible? Trends Cardiovasc Med. 2017;27:229–236.
10. Dai Z, Li M, Wharton J, Zhu MM, Zhao YY. Prolyl-4 Hydroxylase 2 (PHD2) deficiency in endothelial cells and hematopoietic cells induces obliterative vascular remodeling and severe pulmonary arterial hypertension in mice and humans through hypoxia-inducible factor-2α. Circulation. 2016;133:2447–58.
11. Dai Z, Zhu MM, Peng Y, Machireddy N, Evans CE, Machado R, Zhang X, Zhao YY. Therapeutic targeting of vascular remodeling and right heart failure in pulmonary arterial hypertension with a HIF-2a inhibitor. Am J Respir Crit Care Med. 2018;198:1423–34.
12. Pullamsetti SS, Mamazhakypov A, Weissmann N, Seeger W, Savai R. Hypoxia-inducible factor signaling in pulmonary hypertension. J Clin Invest. 2020;130:5638–51.
13. Dai Z, Cheng J, Liu B, Yi D, Feng A, Wang T, An L, Gao C, Wang Y, Zhu MM, Zhang X, Zhao YY. Loss of endothelial hypoxia inducible factor-prolyl hydroxylase 2 induces cardiac hypertrophy and fibrosis. J Am Heart Assoc. 2021;10:2021.03.19 434301.
14. Yi D, Liu B, Wang T, Liao Q, Zhu MM, Zhao YY, Dai Z. Endothelial autocrine signaling through cxcl12/cxcr4/foxm1 axis contributes to severe pulmonary arterial hypertension. Int J Mol Sci. 2021;22:1–11.
15. Lee KE, Simon MC. SnapShot: hypoxia-inducible factors. Cell. 2015;163:1288.

16. Cahill E, Costello CM, Rowan SC, Harkin S, Howell K, Leonard MO, Southwood M, Cummins EP, Fitzpatrick SF, Taylor CT, Morrell NW, Martin F, McLoughlin P. Gremlin plays a key role in the pathogenesis of pulmonary hypertension. Circulation. 2012;125:920–30.
17. Parikh VN, Jin RC, Rabello S, Gulbahce N, White K, Hale A, Cottrill KA, Shaik RS, Waxman AB, Zhang YY, Maron BA, Hartner JC, Fujiwara Y, Orkin SH, Haley KJ, Barabási AL, Loscalzo J, Chan SY. MicroRNA-21 integrates pathogenic signaling to control pulmonary hypertension: results of a network bioinformatics approach. Circulation. 2012;125:1520–32.
18. Spiekerkoetter E, Tian X, Cai J, Hopper RK, Sudheendra D, Li CG, El-Bizri N, Sawada H, Haghighat R, Chan R, Haghighat L, de Jesus Perez V, Wang L, Reddy S, Zhao M, Bernstein D, Solow-Cordero DE, Beachy PA, Wandless TJ, Ten Dijke P, Rabinovitch M. FK506 activates BMPR2, rescues endothelial dysfunction, and reverses pulmonary hypertension. J Clin Invest. 2013;123:3600–13.
19. Spiekerkoetter E, Sung YK, Sudheendra D, Bill M, Aldred MA, van de Veerdonk MC, Vonk Noordegraaf A, Long-Boyle J, Dash R, Yang PC, Lawrie A, Swift AJ, Rabinovitch M, Zamanian RT. Low-dose FK506 (Tacrolimus) in end-stage pulmonary arterial hypertension. Am J Respir Crit Care Med. 2015;192:254–7.

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