Circulation Research

ORIGINAL RESEARCH

Sox17 Deficiency Promotes Pulmonary Arterial Hypertension via HGF (Hepatocyte Grow Factor)/c-Met Signaling

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BACKGROUND: In large-scale genomic studies, *Sox17*, an endothelial-specific transcription factor, has been suggested as a putative causal gene of pulmonary arterial hypertension (PAH); however, its role and molecular mechanisms remain to be elucidated. We investigated the functional impacts and acting mechanisms of impaired Sox17 (SRY-related HMG-box17) pathway in PAH and explored its potential as a therapeutic target.

METHODS: In adult mice, *Sox17* deletion in pulmonary endothelial cells (ECs) induced PAH under hypoxia with high penetrance and severity, but not under normoxia.

RESULTS: Key features of PAH, such as hypermuscularization, EC hyperplasia, and inflammation in lung arterioles, right ventricular hypertrophy, and elevated pulmonary arterial pressure, persisted even after long rest in normoxia. Mechanistically, transcriptomic profiling predicted that the combination of *Sox17* deficiency and hypoxia activated c-Met signaling in lung ECs. HGF (hepatocyte grow factor), a ligand of c-Met, was upregulated in *Sox17*-deficient lung ECs. Pharmacologic inhibition of HGF/c-Met signaling attenuated and reversed the features of PAH in both preventive and therapeutic settings. Similar to findings in animal models, Sox17 levels in lung ECs were repressed in 26.7% of PAH patients (4 of 15), while those were robust in all 14 non-PAH controls. HGF levels in pulmonary arterioles were increased in 86.7% of patients with PAH (13 of 15), but none of the controls showed that pattern.

CONCLUSIONS: The downregulation of Sox17 levels in pulmonary arterioles increases the susceptibility to PAH, particularly when exposed to hypoxia. Our findings suggest the reactive upregulation of HGF/c-Met signaling as a novel druggable target for PAH treatment.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: downregulation ■ inflammation ■ mice ■ penetrance ■ pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a progressive and fatal disease characterized by various pathological phenotypes such as obliterative remodeling and inflammation in the pulmonary vasculature and hypertrophy and dilatation of the right ventricle.[1,2] These phenotypes of PAH are complicated, involving numerous molecular and physiological factors in diverse processes including signal transduction, metabolism, and epigenetics.[1,3–5] Regarding etiology, idiopathic PAH accounts for nearly half of the PAH cases in various reports[1] and ≈25% to 30% of this can be classified as heritable PAH, in which rare mutations have been identified in 17 genes

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Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/CIRCRESAHA.122.320845.
For Sources of Funding and Disclosures, see page XXX.

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ORIGINAL RESEARCH

Novelty and Significance

What Is known?

- Pulmonary arterial hypertension (PAH) can be a fatal disease characterized by various pathological phenotypes such as obliterative remodeling and inflammation in the pulmonary vasculature and hypertrophy and dilatation of the right ventricle.
- *Sox17*, an endothelial-specific transcription factor, was identified as a putative causal gene of pulmonary arterial hypertension.
- Existing animal models mimicking PAH display substantial limitations which have hampered therapeutic advances.

What New Information Does This Article Contribute?

- *Sox17* deficiency and hypoxia jointly induce long-lasting PAH with high penetrance in mice. In addition, Sox17 levels in lung endothelial cells were repressed in patients with PAH.
- Hypoxia may be a second hit that renders the susceptible individuals at further increased risk of PAH, suggesting that reducing exposure to hypoxic conditions, such as cigarette smoking, obstructive sleep apnea, or air pollution, can be implemented as preventive strategies to reduce the burden of PAH.
- Inhibition of HGF (hepatocyte growth factor)s/c-Met signaling successfully treated experimental PAH in both preventive and therapeutic settings, implying that this pathway may serve as a novel druggable target for PAH. Notably, most of patients with PAH showed upregulated HGF levels in pulmonary arterioles.

Summary

Although large-scale genomic studies suggest *Sox17* as a potentially causal gene of PAH, it is unknown how impaired Sox17 pathway contributes to PAH. *Sox17* deficiency coupled with hypoxia promoted long-lasting PAH, as featured by hypermuscularisation, EC hyperplasia, and inflammation in lung arterioles, right ventricular hypertrophy, and elevated pulmonary arterial pressure. Transcriptomic profiling predicted the combination of *Sox17* deficiency and hypoxia activated c-Met signaling in lung ECs. In histologic analysis, HGF, a ligand of c-Met, was upregulated in *Sox17*-deficient lung ECs under hypoxia. Inhibition of HGF/c-Met signaling attenuated experimental PAH in both preventive and therapeutic settings. In line with these observations, we also found repressed Sox17 levels and increased HGF levels in lung ECs of patients with PAH compared with those of control subjects. Therefore, our study uncovers a previously uncharacterized role and the underlying mechanism of Sox 17 in PAH. It also suggests that hypoxia might serve as a second hit in individuals susceptible for PAH and that the reactive upregulation of HGF/c-Met signaling could be a novel druggable target for the treatment of PAH.

Nonstandard Abbreviations and Acronyms

BMPR2	bone morphogenetic protein receptor 2
EC	endothelial cell
GSEA	gene set enrichment analysis
HGF	hepatocyte growth factor
PAH	pulmonary arterial hypertension
RV	right ventricular
Sox17	SRY-related HMG-box17
VSMC	vascular smooth muscle cell

to date.[6,7] Hence, it has been speculated that molecular diversity such as multiple genetic variants may underlie heterogeneous pathologic features, unpredictable disease course, and different treatment responses in patients with PAH.

A recent genomic study based on a large cohort of patients with PAH identified a common risk variant in the *Sox17* (*SRY-related HMG-box17*) locus in 59% of patients and 46% of non-PAH control subjects, raising the possibility of common pathogenic mechanisms shared by a large subset of patients with PAH.[6] This finding suggests that a common risk variant in *Sox17* most likely cooperates with additional hits, including environmental factors such as infections, drugs, and toxins, in the pathogenesis of PAH.[7] Increasing diagnosis of PAH in elderly individuals also suggests a possible role of lifestyle factors in PAH development.[8]

Unlike rare coding variants in a number of genes, including *Sox17* coding region, found in patients with PAH,[10–12] this PAH risk variant is a common non-coding variant in the *Sox17* enhancer region, pointing to the impaired Sox17 (SRY-related HMG-box17) expression as a potential cause of PAH.[6] Sox17 is an endothelial-specific transcription factor that regulates angiogenesis and arterial specification during vascular development.[9,10] Sox17 is also expressed persistently throughout life in endothelial cells (ECs) and plays a crucial role in vascular health during adulthood. For example, Sox17 expression was reduced in intracranial aneurysms of adult patients undergoing microsurgical clipping, whereas it is robustly expressed in normal intracranial arteries from autopsied human subjects.[11] A recent study also suggests that Sox17 is required for pulmonary endothelial

regeneration following vascular injury.[12] These findings suggest the possibility that *Sox17* loss-of-function can induce vascular diseases such as PAH.

Existing models mimicking PAH display substantial limitations, which have hampered therapeutic advances.[13] Specifically, monocrotaline-induced models do not reproduce some important aspects of human PAH, such as progressive vascular and cardiac remodeling, due to too rapid disease progression toward death. In the hypoxia-induced model, another very commonly used PAH model, pulmonary vascular remodeling is at least partly reversible after cessation of hypoxia, in contrast with the irreversible nature of PAH in human subjects. However, some rodent models such as Fisher 344 and genetically susceptible Sprague Dawley rats develop progressive and irreversible PAH phenotypes with high mortality.[14–16] Whereas genetically modified animal models may better recapitulate a subset of human PAH, particularly heritable PAH-associated genetic mutations, it is unclear whether they could represent general mechanisms that are applicable to the majority of PAH patients. Accordingly, developing relevant animal models should be a priority for the scientific community, in an attempt to test potential PAH therapeutics.

In this study, we investigated whether there is a direct involvement of Sox17, in combination with chronic vascular stress such as hypoxia, in the development of PAH. We examined pulmonary vascular and cardiac pathologies in mutant mice lacking endothelial *Sox17* exposed to hypoxia. We also explored whether *Sox17* deficiency in mice can lead to long-lasting PAH through common mechanisms, which would be useful for therapeutic development. We also examined the expression of Sox17 in pulmonary vasculature in PAH patients to obtain clinically translatable evidence showing the contribution of Sox17 to PAH pathogenesis.

METHODS

Data Availability

Expanded methods are available in the Supplemental Material. Please see the Major Resources Table for details. Raw sequencing data and processed transcriptomic data are available at the NCBI GEO under the accession GSE210505.

For reasonable request, data are available through approval and oversight by Korea Advanced Institute of Science and Technology, Seoul National University Hospital, and University of Giessen and Marburg Lung Centre.

Ethics Statements

All animal experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Korea Advanced Institute of Science and Technology (No. KA2019-5) and Seoul National University Hospital (No. 19-0245-S1A2). The experiments also comply with the Guide for the Care and Use of Laboratory Animals (National Research Council, revised in 2011). Human studies were conducted in accordance with the latest Declaration of Helsinki, and the study protocol was reviewed and approved by the Institutional Review Board of Seoul National University Hospital (IRB No. 2003-058-1108) and University of Giessen and Marburg Lung Centre. Written informed consent for proper and ethical use of human tissues was obtained from each individual patient or the patient's next of kin before surgery. In addition, all individual data were stored anonymously.

Statistical Analyses

Continuous and categorical data are presented as mean±SD and numbers with percentage, respectively. We assessed data normality with Shapiro-Wilk test. Parametric analysis was performed for normally distributed data, while non-parametric analysis was used when data were not normally distributed or the sample size was small ($n<6$). Student t test or Mann-Whitney U test was used to compare continuous variables, and the χ^2 test or Fisher exact test was used to compare categorical variables as appropriate. Multiple testing correction was performed in all cases and adjusted P values were presented. Comparison of continuous variables between multiple groups was performed with 1-way ANOVA followed by Scheffé post-hoc test or Kruskal-Wallis test followed by Dunn's multiple comparison test as appropriate. For comparison of categorical variables between multiple groups, Bonferroni correction was performed. Generalized estimating equation models were adopted to analyze clustered data. Statistical analysis was performed with SPSS version 23.0 (SPSS, Inc) and R (v.3.6.3). A 2-sided P 0.05 was considered significant.

RESULTS

Sox17 Deficiency and Hypoxia Jointly Induce Long-Lasting PAH With High Penetrance

Because Sox17 immunoreactivity was detected in lung arterial ECs of adult control mice, we generated conditional *Sox17* knockout mice for its loss-of-function (Figure S1A). Mice with inducible *Sox17* deletion in adult ECs (*Sox17*$^{\Delta EC}$) showed no pathologic features in lung arterioles, indicating that *Sox17* deficiency is not sufficient to drive PAH development spontaneously. We then asked whether loss of *Sox17* can cooperate with chronic hypoxia, which is used to trigger experimental PAH. Sox17 expression was maintained in lung arterial ECs under hypoxic conditions (10% oxygen; Figure S1B). We exposed *Sox17*$^{\Delta EC}$ mice to hypoxia for 3 weeks and evaluated pathologic characteristics related to PAH such as pulmonary vascular remodeling and pulmonary inflammation (Figure 1A). *Sox17*$^{\Delta EC}$ mice exposed to hypoxia (*Sox17*$^{\Delta EC}$/hypoxic mice) showed a prominent increase in wall thickness and the coverage of vascular smooth muscle cells in lung arterioles (Figure 1B and 1C; Figure S1C), recapitulating the hypermuscularized vascular remodeling of PAH. Regarding pulmonary inflammation, the frequency of CD11b$^+$ cells infiltrating the lung was greater in *Sox17*$^{\Delta EC}$/hypoxic mice than in

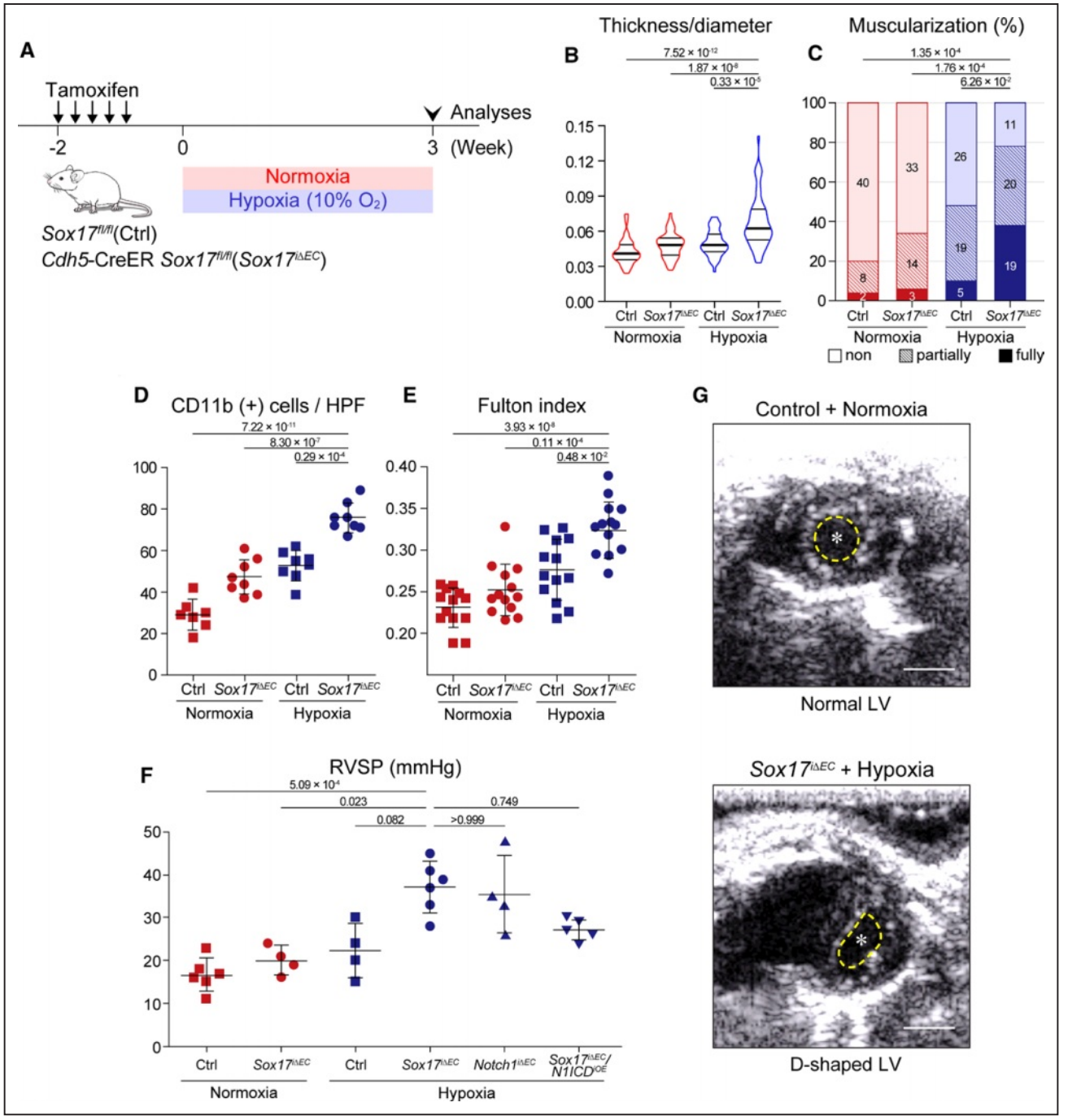


Figure 1. Endothelial *Sox17* deficiency induces severe pulmonary arterial hypertension (PAH) with high penetrance in mice. **A**, Experimental schedule of Sox17 deficiency-induced PAH. Control and *Sox17*$^{i\Delta EC}$ mice were exposed to normoxia or hypoxia for 3 weeks. **B**–**E**, *Sox17*$^{i\Delta EC}$ mice exposed to hypoxia displayed typical pathologic features of PAH as shown by increased wall thickness (**B**) and hypermuscularization of distal pulmonary arterioles (n=50 per group, 5 arterioles in each mice; **C**), increased lung infiltration of CD11b$^+$ cells (number per high power field of lung sections, n=7 for control/normoxic mice and n=8 for other groups; **D**), and RV hypertrophy measured by Fulton index (n=13 per group; **E**). **F**, *Sox17*$^{i\Delta EC}$/hypoxic mice displayed hemodynamic evidence of PAH, including elevated RV systolic pressure, which was attenuated partially by overexpressing *N1ICD* in ECs (n=6 for control/normoxic and *Sox17*$^{i\Delta EC}$/hypoxic mice, n = 5 for *Sox17*$^{i\Delta EC}$/*N1ICD*^{OE}/hypoxic mice, and n=4 for other groups), and (**G**) D-shaped left ventricle (LV), which contrasted to normal shape of the LV in control mice. Asterisks and yellow lines indicate left ventricular cavity and endocardial border, respectively. Data are presented as mean±SD. Generalized estimating equation models (**B** and **C**), 1-way ANOVA with Scheffé post-test (**D** and **E**), and Kruskal-Wallis test with Dunn's post-test (**F**) were performed. Multiple comparison was performed between *Sox17*$^{i\Delta EC}$/hypoxic mice and each other group (**F**). Scale bar, 2 mm (**G**). Ctrl indicates control; HPF, high-power field; LV, left ventricle; and RVSP, RV systolic pressure.

all other groups (Figure 1D). Although not often, inflammatory lesions were found near pulmonary arterioles of *Sox17*$^{\Delta EC}$/hypoxic mice (Figure S1D). Beyond the lung, we also evaluated another important PAH characteristic, the right ventricular (RV) remodeling. *Sox17* deficiency and hypoxia jointly resulted in RV hypertrophy when evaluated by Fulton index (Figure 1E). RV systolic pressure, one of the clinical variables for PAH diagnosis, was also remarkably elevated in *Sox17*$^{\Delta EC}$/hypoxic mice compared with those measured in control/normoxic, *Sox17*$^{\Delta EC}$/normoxic, and control/hypoxic mice (Figure 1F). Cardiac output was also lower in *Sox17*$^{\Delta EC}$/hypoxic mice (median 11.8 mL/min; interquartile range, 11.3–13.1 mL/min) than in control/normoxic mice (median 15.0 mL/min; interquartile range 13.4–16.2 mL/min), although the difference was not statistically significant ($n = 4$ in each group). Two of 6 *Sox17*$^{\Delta EC}$/hypoxic mice had a D-shaped left ventricle on echocardiographic examination as well, indicating severely increased pulmonary arterial pressure while others did not show RV dysfunction (Figure 1G). In contrast, no mice in the other groups (5 controls/normoxic mice, 5 *Sox17*$^{\Delta EC}$/normoxic mice, and 5 control/hypoxic mice) had this feature.

Several experimental PAH models exhibit a mild and transient PAH phenotype.[14,15] In contrast, *Sox17*$^{\Delta EC}$/hypoxic mice maintained increased pulmonary vascular remodeling, inflammation, RV mass, and RV systolic pressure persistently, even 3 weeks after returning to normoxia (Figure S2A through S2F). Altogether, these results indicate that Sox17 normally prevents stress-induced vascular disintegrity in the lung and that *Sox17* deficiency predisposes mice to irreversible hypoxia-induced PAH with high penetrance, suggesting that this mouse model could recapitulate the irreversible disease course of human PAH.

Vasodilators show limited and heterogeneous therapeutic effects in PAH patients.[1,17] We assessed therapeutic responses to 2 standard vasodilators, macitentan (a blocker of endothelin receptor-1) and sildenafil (an inhibitor of phosphodiesterase type 5), in the hypoxia-induced PAH aggravated by *Sox17* loss-of-function. Treatment with macitentan, but not sildenafil, partially decreased wall thickness and vascular smooth muscle cell-coverage of pulmonary arteries, CD11b$^+$ perivascular infiltration, and RV mass (Figure S3). Considering that only partial recovery was achieved by early treatment during hypoxia, it is unlikely that macitentan alone could be effective in subjects with established PAH.

Excessive Proliferation in ECs Underlie Hypoxia-Induced PAH Exacerbated by *Sox17* Deficiency

To understand molecular changes underlying vascular pathology initiated by endothelial *Sox17* deficiency, we performed endothelial-specific transcriptomic profiling using highly endothelial-enriched transcripts from the lung of control and *Sox17*$^{\Delta EC}$/hypoxic mice (Figure 2A through 2D). Principal component analysis and hierarchical clustering of RNA-sequencing results yielded a distinct separation of control from *Sox17*$^{\Delta EC}$/hypoxic groups, verifying the reliability of transcriptomic profiling (Figure 2E and 2F). Among 12 451 genes expressed in pulmonary ECs, 697 genes were differentially expressed > 2-folds ($P<0.05$). In lung ECs from *Sox17*$^{\Delta EC}$/hypoxic mice, 126 genes were upregulated, and 571 genes were downregulated.

Previous studies have suggested hyperproliferation and inflammatory activation as major PAH pathologic events occurring in ECs. In keeping with this, the analysis of differentially expressed genes predicted that the most enriched gene ontology terms were angiogenesis and immune cell infiltration (biological processes) and mitotic cell cycle (the reactome pathways) in the *Sox17*$^{\Delta EC}$/hypoxic group (Figure 3A). Gene set enrichment analysis (GSEA) also predicted significant enrichment of gene sets associated with increased proliferation and inflammation in the *Sox17*$^{\Delta EC}$/hypoxic group compared with the control/normoxic mice (Figure 3B). Inflammatory activation of ECs in *Sox17*$^{\Delta EC}$/hypoxic mice was verified by increased perivascular infiltration of immune cells as described earlier in this work (Figure 1D; Figure S1D). While modest endothelial proliferation was induced by *Sox17*$^{\Delta EC}$ or hypoxia alone, hyperproliferation of ECs was prominent in *Sox17*$^{\Delta EC}$/hypoxic mice, as revealed by an increased number of Ki67$^+$ ECs in pulmonary arterioles (Figure 3C; Figure S4). Furthermore, an abnormally stacked EC layer potentially due to intraluminal EC proliferation was found in lung arterioles of *Sox17*$^{\Delta EC}$/hypoxic mice, though at low frequency (Figure 3D). Notably, other mouse groups showed no abnormal EC layer. Although new blood vessel formation was not found in the lung, angiogenesis predicted by the molecular profiling is compatible with our results because ECs undergo active proliferation in angiogenesis. Although endothelial-mesenchymal transition has been suggested as a plausible etiology for vascular obliteration as well,[18,19] α-SMA and S100a4, conventional mesenchymal markers, were not detected, and gene sets associated with endothelial-mesenchymal transition showed no enrichment in lung arterial ECs from *Sox17*$^{\Delta EC}$/hypoxic mice (Figure 3E and 3F). Although increased glycolysis and mitochondrial abnormalities have been reported in other PAH models,[1,20] our endothelial-specific transcriptomic profiling showed no sign of these features in the PAH subject group (Figure S5).

Sox17 Deficiency Leads to Hypertension-Induced Pulmonary Vascular Pathology in Part via dysregulated Notch Signaling

To understand whether the PAH model lacking *Sox17* in ECs shares a molecular basis with other PAH

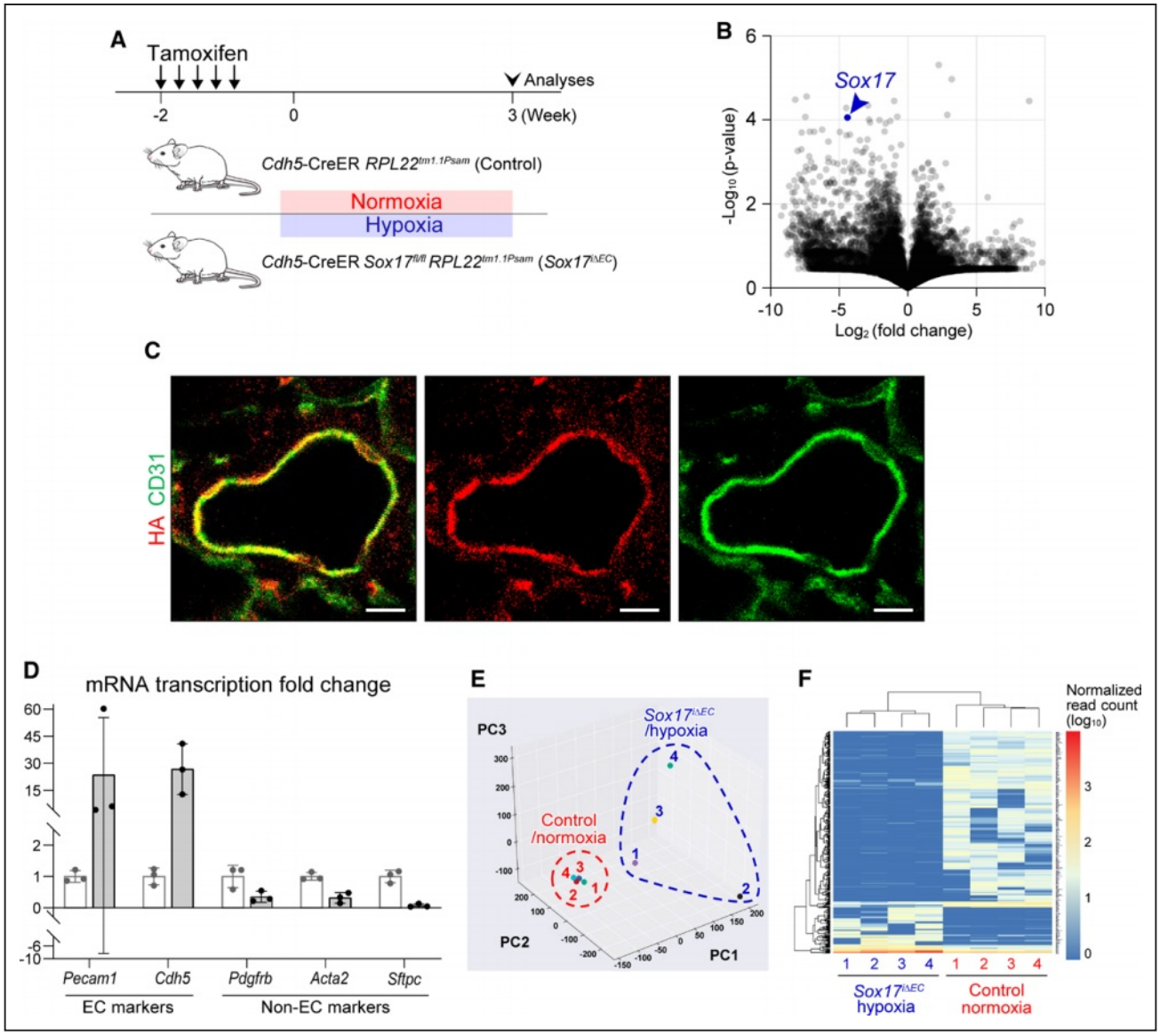
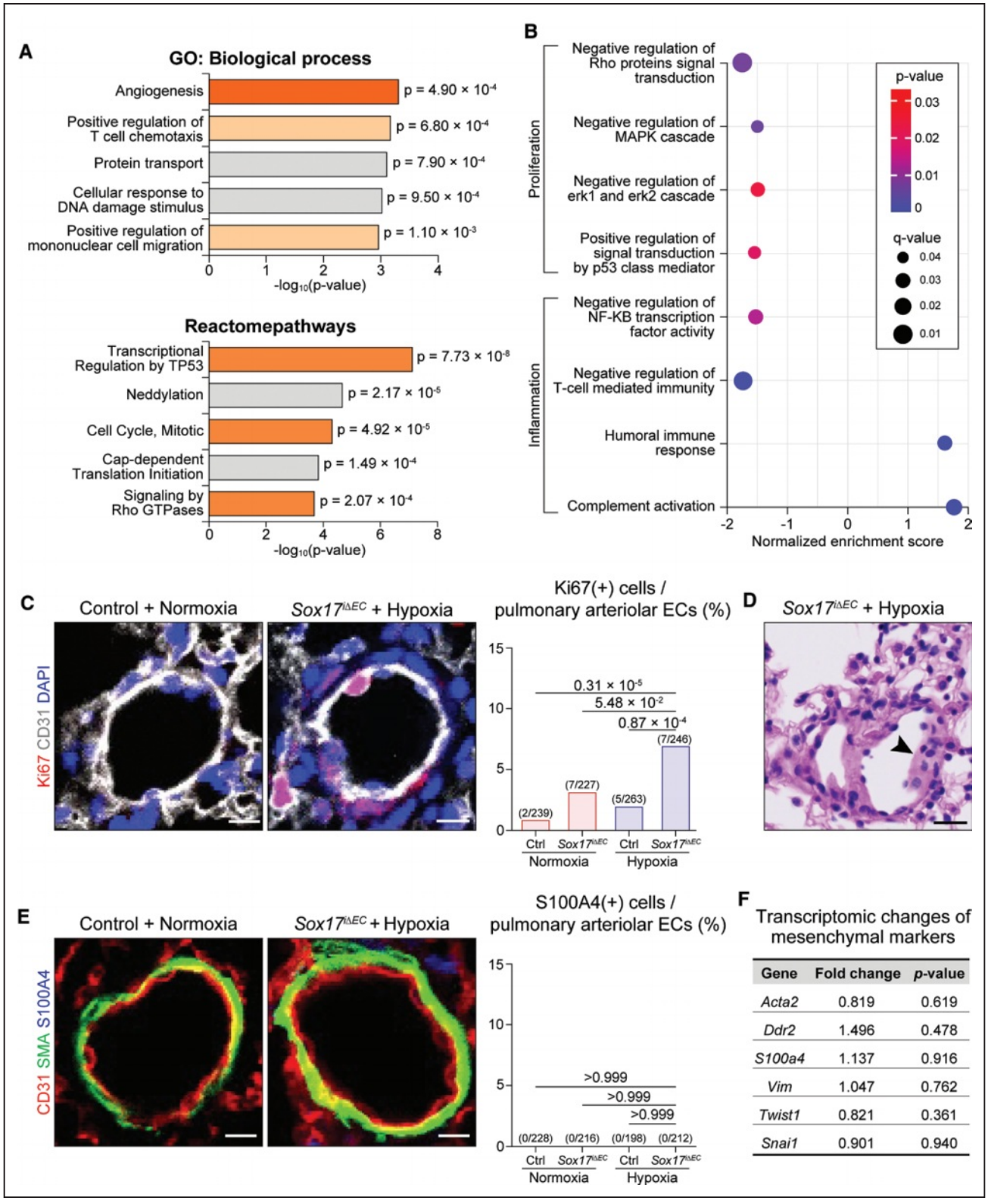


Figure 2. Validation of transcriptomic profiling of pulmonary endothelial cells (ECs).
A, Experimental strategy for isolating endothelial-enriched transcripts in the lung. *Cdh5*-CreER[2]; RPL221,1Psam mice were maintained under normoxia as control group and Cdh5-CreER[2]; Sox17fl/fl RPL221,1Psam mice were exposed to hypoxia as the Sox17iΔEC/hypoxic group following tamoxifen administration, which allowed the synthesis of hemagglutinin (HA)-tagged ribosomes specifically in ECs. Lungs were then harvested. **B**, Volcano plot of differentially expressed genes in lung ECs of control and *Sox17*$^{\Delta EC}$/hypoxic mice. Sox17 highlighted in blue confirms its decreased level. **C**, Immunofluorescence image verifying EC-specific HA immunostaining in the lung. **D**, Fold changes in transcripts of marker genes for EC and other cell types induced by capturing HA-tagged ribosomes. Transcript levels in lung tissue samples for RNA sequencing are validated by quantitative PCR (n=3). Relative levels of transcripts are normalized to those of *Gapdh*. As controls, the averages of samples before the precipitation of HA-tagged ribosomes (left column of each pair) are arbitrarily set as 1. EC markers (*Pecam1, Cdh5*) are enriched, whereas markers of other cell types (*Pdgfrb,* pericytes; *Acta2,* VSMCs; *S*ftpc, pneumocytes) are excluded. Data are presented as mean±SD. **E**, Three-dimensional principal component (PC) analysis and (**F**) unsupervised clustering clearly separate RNA sequencing data from the control (n=4) and *Sox17*$^{\Delta EC}$/hypoxic groups (n=4). Scale bar, 10 μm (**C**).

models, we examined lung EC transcriptional levels of genes in which mutations were identified in PAH patients. While *Eng,* a co-receptor of Bmpr2, and *Cav1*, a critical regulator of Bmpr2 internalization, were significantly repressed in *Sox17*$^{\Delta EC}$/hypoxic mice compared with controls, *Smad4* and *Bmpr2* were also decreased moderately (Figure S6A). In line with this, Gene set enrichment analysis predicted the inactivation of Smad signaling in *Sox17*-deficient lung ECs under hypoxic stress (Figure S6B). However, *Acvrl1*, *Smad9*, *Eif2ak4*, and *Kcnk3* showed no significant changes. These transcriptional alterations suggest that PAH triggered by the combination of *Sox17* deficiency and hypoxia share some molecular mechanisms in ECs with other types of PAH associated with the Bmpr2-Smad pathway.



Gene	Fold change	*p*-value
Acta2	0.819	0.619
Ddr2	1.496	0.478
S100a4	1.137	0.916
Vim	1.047	0.762
Twist1	0.821	0.361
Snai1	0.901	0.940

Figure 3. Loss of *Sox17* promotes abnormal proliferation and inflammatory activation in lung endothelial cells (ECs) under hypoxic stress.

A, The top 5 enriched gene sets by gene ontology (GO) term analysis (top, biological process; bottom, reactome pathways). Gene sets associated with cell proliferation are displayed in dark orange and those associated with inflammation are displayed in light orange. **B**, Dot plot of GSEA results related to cell proliferation and inflammation. *P* are represented by color gradient and the false discovery rate adjusted *P* (ie, *q*-values) are depicted by circle size. **C**, Immunostaining for Ki67, a marker of proliferation, in distal pulmonary arterioles and its quantification in ECs (n=6 mice per group, 5 arterioles from each mouse). Note the significant increase in Ki67 expression in *Sox17*$^{i\Delta EC}$/hypoxic (*Continued*)

Dysregulated Notch1 signaling is implicated in PAH development caused by mutations in *Bmpr2* in mice.[4,21,22] Notably, Notch1 signaling crucial for arterial development and homeostasis is downstream of Sox17.[9,23] Gene set enrichment analysis based on our RNA-sequencing data predicted the inactivation of Notch1 signaling in *Sox17*-deficient lung ECs under hypoxic stress (Figure S7A). In line with this, *Sox17* deletion repressed *Dll4* markedly, but not *Notch1*, and also repressed several Notch downstream targets such as *Hey1*, *Hey2*, and *Hes1* in lung ECs (Figure S8). However, *Dll4* deletion did not alter *Sox17* levels substantially. These findings suggest that *Sox17* deficiency may result in dysregulated Notch pathway via *Dll4* downregulation. We asked whether loss of Notch1 in ECs is involved in the occurrence of hypoxia-induced PAH by excising *Notch1* from adult ECs conditionally (Figure S7B). Most *Notch1*$^{i\Delta EC}$ mice exposed to 10% oxygen for 3 weeks displayed PAH phenotypes, including pulmonary vascular remodeling, lung inflammation, RV hypertrophy (Figure S7C through S7F), and RV systolic pressure (Figure 1F), phenocopying *Sox17*$^{i\Delta EC}$/hypoxic mice. We next studied the effect of endothelial gain of Notch1 signaling on PAH development by overexpressing Notch1 intracellular domain (*N1ICD*) in ECs (*N1ICD*^{iOE}). Gain of Notch1 function partially attenuated wall thickness and vascular smooth muscle cell coverage of pulmonary arterioles and RV mass, but not lung inflammation (Figure S7C through S7F) and RV systolic pressure (Figure 1F), in *Sox17*$^{i\Delta EC}$/hypoxic mice. These findings implicate dysregulation of the Sox17-Notch1 regulatory axis as one mechanism underlying hypoxia-induced vascular pathology. However, there may be complicated mechanisms involved in lung inflammation and cardiac remodeling that exclude enhancement of Notch signaling from potential approaches to effective PAH treatment.

Rescuing PAH Pathogenesis by Regulating the HGF/c-Met Pathway

To find a different pathway through which to target PAH induced by *Sox17* deficiency, we searched for notable signaling pathways predicted by gene set enrichment analysis. We noticed the activation of c-Met signaling, which is well known to promote cell growth in various contexts,[24,25] in lung ECs of *Sox17*$^{i\Delta EC}$/hypoxic mice (Figure 4A). Interestingly, HGF (hepatocyte growth factor), a major ligand of the c-Met receptor, was remarkably upregulated in *Sox17*-deficient pulmonary arterial ECs under hypoxia (Figure 4B). While hypoxia or *Sox17* deficiency individually increased HGF expression in distal pulmonary arterioles, their combination upregulated HGF levels additively (Figure 4C). These findings suggest that complicated regulatory mechanisms control HGF expression and support the hypothesis of c-Met signaling activation in lung ECs of *Sox17*$^{i\Delta EC}$/hypoxic mice. To unveil underlying mechanisms, we examined levels of several transcripts in pulmonary ECs from experimental mouse groups (Figure S9A). As *Hgf* transcriptional levels in lung ECs showed no significant differences among groups, we asked whether microRNAs could regulate HGF expression post-transcriptionally (Figure S9B). Among *Hgf* mRNA-targeting candidates retrieved using microRNA prediction tools, miR-92 and miR-300 were remarkably decreased specifically in *Sox17*-deficient lung ECs under hypoxia, suggesting that reduced microRNA levels may upregulate endothelial HGF levels in PAH models.

To determine whether the c-Met activation is critical for PAH development, we treated *Sox17*$^{i\Delta EC}$/hypoxic mice with crizotinib, a clinically available c-Met inhibitor, from the beginning of hypoxia (Figure 4D). Crizotinib attenuated pulmonary arterial thickening, hypermuscularization, lung infiltration of CD11b^{+} inflammatory cells, and RV hypertrophy significantly (Figure 4E through 4H), verifying that c-Met signaling is critically involved in PAH pathology. When treated in the presence of macitentan, crizotinib reduced the hallmark pathologic features of PAH almost to control levels (Figure 4E through 4H). The combination of crizotinib and macitentan also decreased PAH phenotypes in Sugen 5416/hypoxia mouse model (Figure S10).

Next, in addition to the preventive setting above, we were interested in whether c-Met inhibition could be effective in patients with advanced PAH, which is more clinically relevant. We tested drug treatments in *Sox17*$^{i\Delta EC}$/hypoxic mice with established PAH confirmed by the presence of symptoms (Figure 5A). Considering that the effect of crizotinib monotherapy was similar with macitentan monotherapy in the preventive setting, we focused to investigate the combined effect of c-Met inhibition and vasodilation in the therapeutic setting. *Sox17*$^{i\Delta EC}$/hypoxic mice that received dual treatment with crizotinib and macitentan, but not macitentan monotherapy, appeared to be healthy and showed vigorous activity. In addition, all pathological phenotypes of PAH were rescued markedly by combination treatment with crizotinib and macitentan compared with the modest response to macitentan alone

Figure 3 Continued. mice versus control/normoxic mice. **D**, Histologic image showing vascular obliteration in pulmonary arterioles by abnormal stacking of ECs in *Sox17*$^{i\Delta EC}$/hypoxic mice. **E**, Immunostaining for S100A4 in distal pulmonary arterioles and its quantification in ECs (n=5 mice per group, 5 arterioles from each mouse). Note the lack of S100A4 signals in ECs in both control/normoxic and *Sox17*$^{i\Delta EC}$/hypoxic mice and no between-group difference. **F**, No significant transcriptional changes in mesenchymal markers on the basis of RNA-sequencing data. Data are presented as mean±SD. Numbers in parentheses refer to the numerator and denominator from which the relevant percentages are calculated (**C** and **E**). Generalized estimating equation models were adopted for clustered data (**C** and **E**). Scale bars: 10 μm (**C**, **E**), 20 μm (**D**).

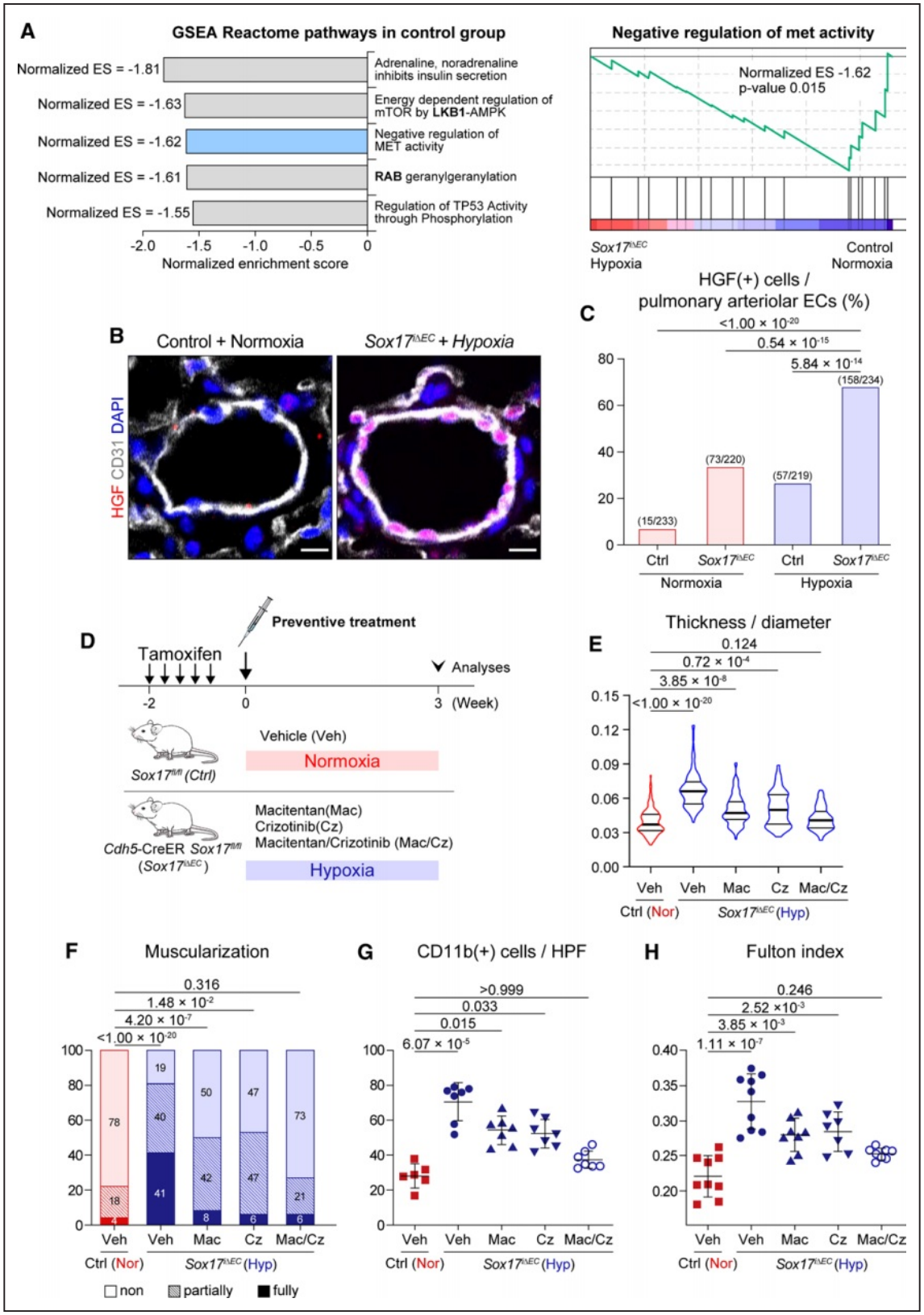


Figure 4. Inhibition of HGF (hepatocyte growth factor)/c-Met signaling prevents the development of pathologic changes promoted by *Sox17* deficiency.

A, The top-ranked reactome pathways by gene set enrichment analysis (GSEA; left) and GSEA plot showing negative enrichment of negative regulation (ie, activation) of c-Met signaling (right) in *Sox17*$^{\Delta EC}$/hypoxic mice. **B**, Representative images of HGF upregulation in distal pulmonary arterioles of *Sox17*$^{\Delta EC}$/hypoxic mice. **C**, The percentage of HGF^{+} endothelial cells (ECs) in distal pulmonary arteriolar ECs in each experimental mouse group (n=6 mice per group, 5 arterioles from each mouse). **D**, Experimental schedule for c-Met inhibition in a preventive setting: (*Continued*)

Figure 4 Continued. following tamoxifen injection, *Sox17*$^{\Delta EC}$ mice treated with vehicle (Veh, 10% DMSO), macitentan (Mac, a vasodilator), crizotinib (Cz, a c-Met inhibitor), or a combination of macitentan and crizotinib (Mac/Cz) during hypoxic exposure for 3 weeks and control mice treated with vehicle under normoxia for the same period. **E**–**H**, Attenuation of PAH phenotypes by c-Met inhibition combined with macitentan comparable to control mice as shown by reduced wall thickness (**E**) and muscularization of distal pulmonary arterioles (n=100 per group, 5 arterioles in each mice; **F**), attenuated lung infiltration by CD11b$^+$ cells (number per high power field, n=6 for control/normoxic mice and n=7 for other groups; **G**), and improved RV hypertrophy (n=9 for control/normoxic and *Sox17*$^{\Delta EC}$/hypoxic groups, n=8 for *Sox17*$^{\Delta EC}$/hypoxic mice treated with macitentan and *Sox17*$^{\Delta EC}$/hypoxic mice treated with macitentan and crizotinib, and n=7 for *Sox17*$^{\Delta EC}$/hypoxic mice treated with crizotinib; **H**) in *Sox17*$^{\Delta EC}$/hypoxic mice. Data are presented as mean±SD. Numbers in parentheses refer to the numerator and denominator from which the relevant percentages are calculated (**C**). Generalized estimating equation models (**C**, **E**, and **F**), 1-way ANOVA with Scheffé post-test (**H**), and Kruskal-Wallis test with Dunn's post-test (**G**) were performed. Scale bars in **B**: 10 μm. ES indicates enrichment score; and HPF, high-power field.

(Figure 5B through 5F). Like crizotininb, capmatinib, another c-Met inhibitor, also rescued mice having established PAH phenotypes effectively (Figure 5B through 5F). Thus, the inhibition of HGF/c-Met signaling showed therapeutic benefit in a clinically relevant PAH model, suggesting the HGF/c-Met pathway as a druggable target for PAH treatment.

Altered Sox17 and HGF Levels in Pulmonary Arterial ECs From Patients With PAH

As a test of clinical relevance, we examined Sox17 expression in the lung specimens from patients with PAH and control subjects. Whereas all 14 control subjects had robust Sox17 immunostaining signals in most pulmonary arterioles, 4 of 15 PAH patients had

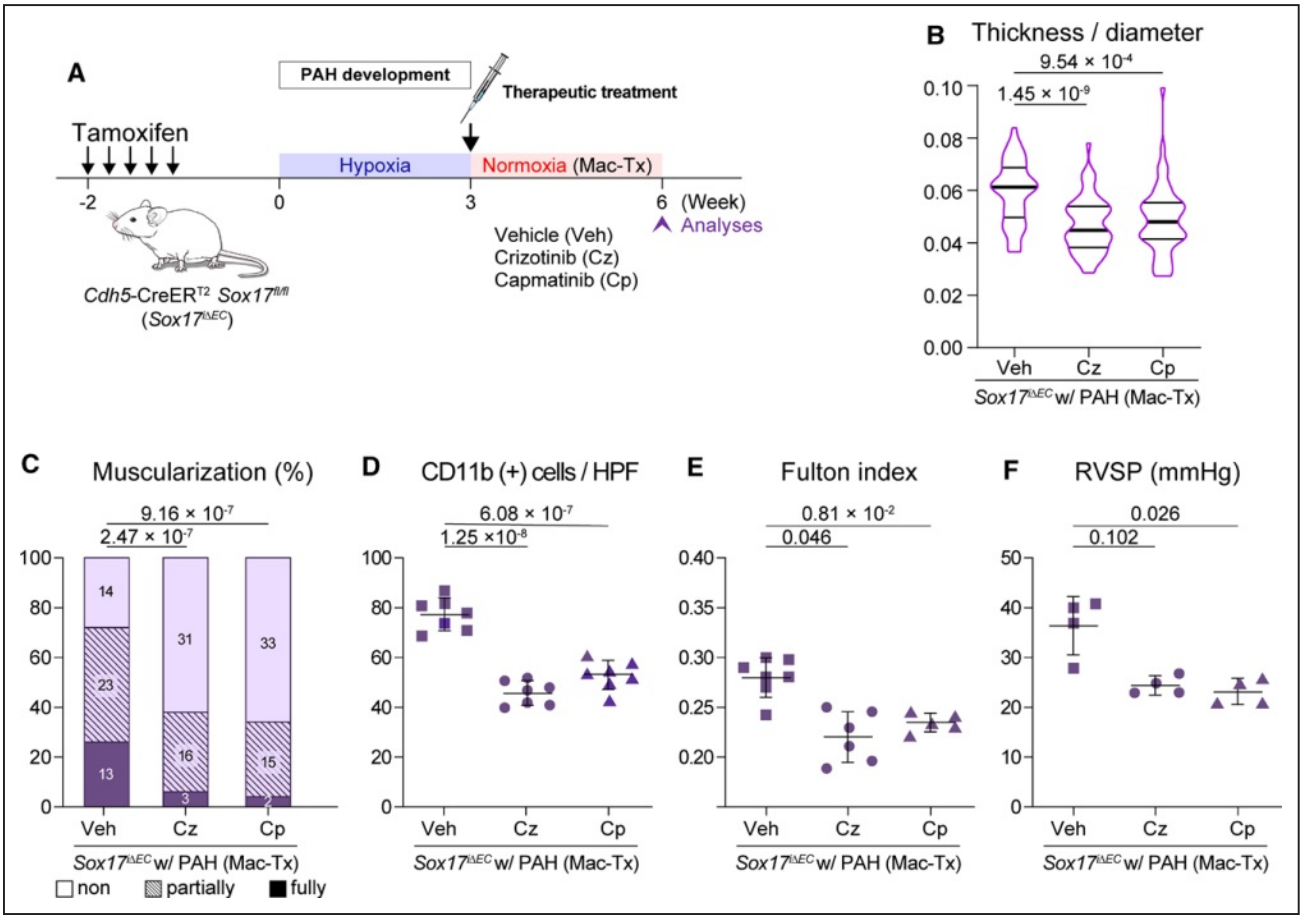


Figure 5. Therapeutic treatment with c-Met inhibitors reverses established pulmonary arterial hypertension (PAH).
A, Experimental schedule for c-Met inhibition in a therapeutic setting: following tamoxifen injection, *Sox17*$^{\Delta EC}$ mice were confirmed to have PAH by echocardiography imaging at the end of 3 weeks of hypoxia and then treated with vehicle (Veh, 10% DMSO), crizotinib (Cz, a c-Met inhibitor), or capmatinib (Cp, a c-Met inhibitor) in the presence of macitentan (Mac-Tx) during the subsequent 3 weeks of normoxia. **B**–**F**, Reversal of PAH phenotypes developed in *Sox17*$^{\Delta EC}$ mice by c-Met inhibition combined with macitentan as shown by reduced wall thickness (**B**) and muscularization of distal pulmonary arterioles (n=50 per group, 5 arterioles in each mice; **C**), decreased lung infiltration by CD11b$^+$ cells (number per high-power field, n=7 per group; **D**), improved RV hypertrophy (n=7 mice treated with macitentan, n=6 mice treated with macitentan and crizotinib, and n=5 mice treated with macitentan and capmatinib; **E**), and reduced RV systolic pressure (n=4 per group; **F**). Data are presented as mean±SD. Generalized estimating equation models (**B** and **C**), 1-way ANOVA with Scheffé post-test (**D**), and Kruskal-Wallis test with Dunn's post-test (**E** and **F**) were performed. HPF indicates high-power field; and PAH, pulmonary arterial hypertension.

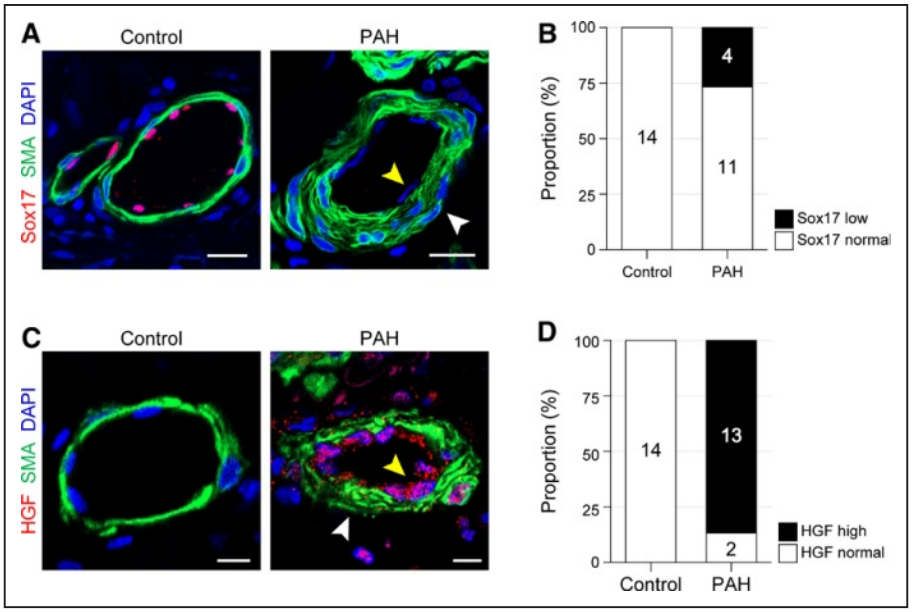


Figure 6. Repressed Sox17 expression and increased HGF (hepatocyte grow factor) expression in pulmonary arterial endothelial cells (ECs) from patients with pulmonary arterial hypertension (PAH) in comparison with control subjects.
A, Immunostaining for Sox17 in lung tissue sections from a control subject and a PAH patient. Note the repressed Sox17 expression in arteriolar ECs (yellow arrow) and hypermuscularization (white arrowhead) in the PAH patient. **B**, Proportions of Sox17 expression levels in pulmonary arteriolar ECs. Note the substantial proportion of low Sox17 expression in the PAH group, contrasting to the control group in which no case of low Sox17 expression was found. **C**, Immunostaining of HGF in lung tissue sections from a control subject and a patient with PAH. Note the increased HGF expression in arteriolar ECs (yellow arrow) and hypermuscularization (white arrowhead) in the patient with PAH. **D**, Proportions of HGF expression levels in pulmonary arteriolar ECs. Note the substantial proportion of high HGF expression in the PAH group, contrasting to the control group in which no case of high HGF expression was found. Numbers in bars indicate absolute numbers of cases. Demographic data for each subject are presented in Table S1. Scale bars in **A** and **C**: 10 μm.

attenuated Sox17 immunoreactivity (Figure 6A and 6B). These findings suggest that repressed Sox17 expression in lung arterial ECs could be related to the occurrence of PAH.

Next, we also examined HGF immunoreactivity in lung tissue sections from PAH patients and control subjects. Consistent with the mouse PAH model, HGF immunoreactivity in pulmonary arterial ECs was readily detectable in 13 of 15 patients with PAH but was barely detected in all 14 non-PAH control subjects (Figure 6C and 6D). Importantly, all 4 patients with PAH with low Sox17 levels showed high HGF immunoreactivity. This finding supports the activation of HGF/c-Met signaling in ECs as a novel mechanism for PAH pathophysiology and suggests that its blockade could be considered as a potential therapeutic option in patients with PAH.

DISCUSSION

In accord with the identification of risk variants in the *Sox17* locus in a large cohort of PAH patients,[6] we found repressed Sox17 expression in pulmonary arterial ECs in a substantial portion of patients with PAH. We demonstrated that *Sox17* deficiency in ECs can result in long-lasting hypoxia-induced PAH in mice, and this action may be mediated via HGF/c-Met signaling, suggesting its potential as a druggable target in PAH (Figure 7).

PAH is a complex disease with diverse pathologic features. An increasing number of heritable genetic mutations have been identified and it has been speculated that they underlie the phenotypic heterogeneity of PAH.[1,5] However, a large proportion of PAH is idiopathic, which means that the exact pathophysiological mechanisms remain unclear.[1,26] In the present work, Sox17 expression in pulmonary arterial ECs was decreased in 4 of 15 patients with PAH (26.7%). These results imply that there are *Sox17*-associated general molecular events shared by patients with PAH, who have been considered to be genetically highly heterogeneous. EC-specific *Sox17* deletion faithfully reproduced PAH pathophysiology in mice, only when combined with hypoxia, indicating the requirement of additional hits to induce PAH. Hypoxia is not only a well-established trigger for experimental PAH in rodents but also is a potential risk factor for PAH in humans, as shown by the high incidence of high-altitude pulmonary hypertension in the Andean population[27] and correlations between PAH and other clinical and environmental conditions leading to hypoxia, including smoking, sleep apnoea syndrome, and air pollution.[28,29] Alternatively, weak risk factors or short duration of exposure can lead to only modest pathologic and hemodynamic changes insufficient to be called PAH, such as raised mean pulmonary arterial pressure below the threshold for diagnosing PAH (ie, 20 mmHg), in a fraction of

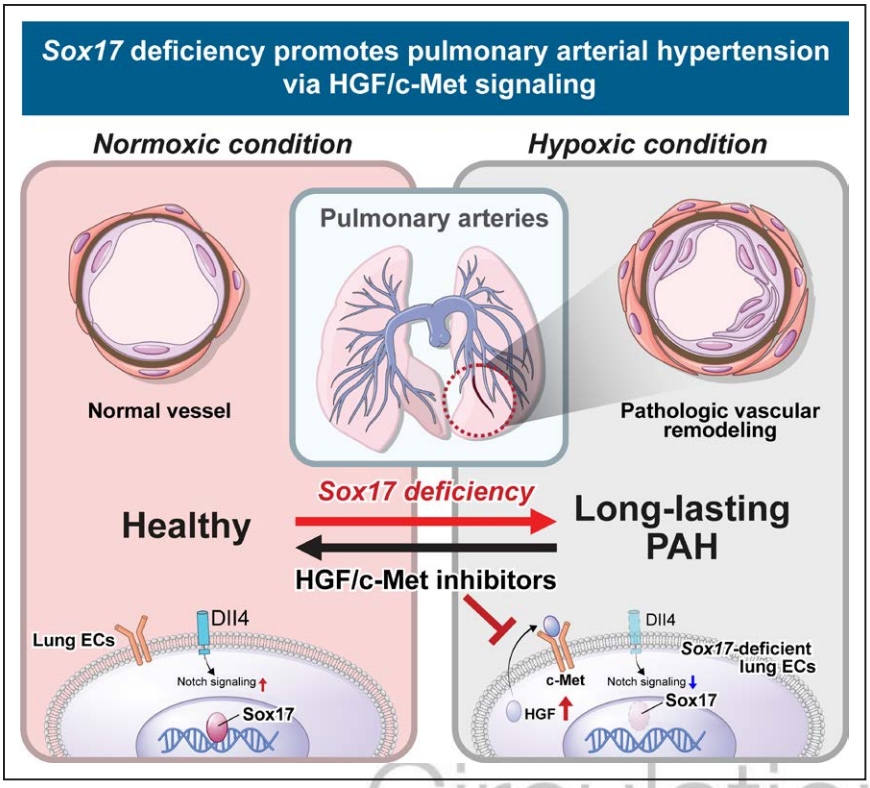


Figure 7. Summarizing illustration. Schematic diagram depicting the role of Sox17 in pulmonary arterial health and its loss leading to pulmonary arterial hypertension via HGF (hepatocyte grow factor) upregulation and impaired Notch signaling. EC indicates endothelial cell; and PAH, pulmonary arterial hypertension.

the population. In the present study, *Sox17*$^{\Delta EC}$/hypoxic mice showed partial luminal narrowing, but not complete occlusion of lung arterioles, which might reflect less severity. They may develop more severe PAH with longer hypoxic stress. Collectively, we speculate that PAH development with a high penetrance in *Sox17* deficiency mice is attributable to the stringent and prolonged hypoxic stress (ie, exposure to 10% oxygen for 3 weeks) on top of low Sox17 levels, partially explaining the gap between the high prevalence of *Sox17* variants and the very low incidence of PAH in humans.

Sox17 is expressed in ECs throughout life and is indispensable for vascular development and vascular health. In this study, Sox17 was robustly expressed in pulmonary arterial ECs in humans and mice. Although inducible *Sox17* deletion from ECs per se resulted in no discernible vascular changes, its combination with hypoxic stress elicited vascular pathology of PAH. This finding is relevant because Sox17 is critical for EC regeneration and restoration of vascular homeostasis against vascular injury, apart from its role in pulmonary vascular development.[12,30] Similarly, Sox17 protects brain arterial ECs from hypertensive stress and thereby restrains intracranial aneurysm formation.[11] Together, these findings are evidence that Sox17 plays an important role in maintaining vascular homeostasis in response to vascular stresses in different contexts.

Notch signaling is also essential for vascular morphogenesis and homeostasis. Recent studies suggest that Notch signaling suppresses PAH development and Notch activation is mediated by the BMPR2 (bone morphogenetic protein receptor 2), whose deficiency is the most well-known genetic factor for PAH.[4] Interestingly, Sox17 is coupled to Notch signaling in several arterial contexts. For example, the Sox17-Notch signaling axis promotes arterial differentiation in nascent vessels[20,23] and contributes to the homeostasis of arterial blood-retinal barrier. In the present work, this signaling axis also protected the endothelial integrity of pulmonary arteries from hypoxic stress, and its perturbation led to PAH development. While the Sox17-Notch signaling axis is a highly conserved arterial genetic program, it seems to have evolved to play an organotypic role.

Endothelial transcriptomic profiling predicted that the combination of *Sox17* deficiency and hypoxia leads to vascular pathology via multiple mechanisms.[31–33] Interestingly, we found that HGF/c-Met signaling, which is fundamental to cell proliferation, was activated in *Sox17*-deficient lung ECs under hypoxia, potentially via microRNA regulation. Although HGF/c-Met signaling has received most attention in cancer proliferation,[24] some studies reported that increased HGF levels were associated with poor prognosis in various cardiovascular disorders.[34,35] These findings on the adverse effect of HGF/c-Met signaling seem to contradict other reports that proposed a protective role of HGF in PAH in monocrotaline-induced rat PAH models.[36] Though frequently used, monocrotaline is toxic to ECs, hepatocytes, and other cell types, and could lead to atypical features which are uncommon in patients with PAH.[37] From a therapeutic perspective, several inhibitors of HGF/c-Met signaling have been proven to be antiproliferative and already

approved in treating multiple cancer types.[38] Considering again the cancer-like features of PAH, HGF/c-Met inhibitors can potentially be repositioned as candidate drugs for PAH. Importantly, HGF expression was increased in pulmonary arterial ECs of not only mouse PAH models but also patients with PAH in the present work. Additionally, pharmacologic inhibition of c-Met signaling substantially attenuated PAH disease pathology, both in preventive and therapeutic protocols.

Although the pathobiology of PAH is complicated by as yet unknown factors, a common feature of PAH is increased pulmonary vascular resistance due to a reduction in the luminal area of pulmonary arterioles. This vascular narrowing is driven by a combination of processes including vasoconstriction, thrombosis, abnormally increased proliferation of ECs and vascular smooth muscle cells, and excessive inflammation.[1] Dysregulated EC proliferation is known to lead to plexiform lesions, a hallmark of severe PAH. Here, we have demonstrated EC hyperproliferation in *Sox17*$^{\Delta EC}$/hypoxic mice, consistent with other PAH animal models[39,40] and patients with PAH.[2,46] Further work will clarify whether our PAH model could have more circulating ECs due to increased EC proliferation, similar to patients with severe PAH.[41] We suggest HGF/c-Met signaling as a mechanism driving EC proliferation in PAH development. The therapeutic potential of c-Met inhibition is thus inspiring because currently available PAH-specific drugs, which have strong pulmonary vasodilating effects, render only limited therapeutic benefits. Therefore, HGF/c-Met signaling could be an effective target for PAH treatment, as it can exert positive effects on all major cell types involved in PAH pathology. Most importantly, we found that enhanced HGF expression in pulmonary arterial ECs was prevalent in patients with PAH, highlighting its clinical relevance.

CONCLUSIONS

The present study demonstrates that the downregulation of Sox17 levels in pulmonary arterioles leads to PAH development, particularly when exposed to hypoxia, suggesting the significant role of Sox 17 in the pathogenesis of PAH. A substantial portion of PAH patients has low Sox17 levels, highlighting the potential translational relevance of this pathway to PAH. Our work also identifies HGF/c-Met signaling as a druggable target in PAH.

ARTICLE INFORMATION

Received January 26, 2022; revision received September 22, 2022; accepted September 23, 2022.

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Acknowledgments

We thank Tae Min Kim (Department of Hemato-oncology in Internal Medicine, Seoul National University Hospital) for insightful advice regarding study design and the interpretation of results, and Moonyoung Rhyoo and Yunmi Lee (KAIST Animal Facility) for maintaining mouse colonies and performing in vitro fertilization of mouse embryos. C. Soon Park, S. Hyun Kim, J. Myung Yang, T. Wook Noh, J.-B. Park, and I. Kim designed the study. C. Soon Park, S. Hyun Kim, J. Myung Yang, T. Wook Noh, Y. Seok Ju, H. Kang, R. Theo Schermuly, J.-H. Park, E. Lee, J.-H. Kim, H. Young Yang, S. Sik Bae, S.-P. Lee, H.J. Park, and J.-B. Park were involved in sample and data collection. C. Soon Park, S. Hyun Kim, S. Sik Bae, J. Han, J.-B. Park, and I. Kim were involved in data analysis and interpretation. C. Soon Park, J.-B. Park, and I. Kim wrote the article, and all authors critically revised the article.

Sources of Funding

This work was supported by the National Research Foundation of Korea of the Ministry of Science and ICT (NRF-2018R1A2A1A05022672 and NRF-2021R1A2C3006538, I. Kim, NRF-2020R1C1C1010890, J-B. Park, and NRF-2019R1C1C1010482, J. Han) and the German Research Foundation (DFG, 268555672-CRC1213 [Collaborative Research Center 1213], Project A08 and A09, R. Theo Schermuly).

Supplemental Material

Expanded Materials and Methods
Figures S1–S13
Tables S1 and S2
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