

ORIGINAL RESEARCH

ADAMTS8 Promotes the Development of Pulmonary Arterial Hypertension and Right Ventricular Failure

A Possible Novel Therapeutic Target

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RATIONALE: Pulmonary arterial hypertension (PAH) is characterized by pulmonary vascular remodeling with aberrant pulmonary artery smooth muscle cells (PASMCs) proliferation, endothelial dysfunction, and extracellular matrix remodeling.

OBJECTIVE: Right ventricular (RV) failure is an important prognostic factor in PAH. Thus, we need to elucidate a novel therapeutic target in both PAH and RV failure.

METHODS AND RESULTS: We performed microarray analysis in PASMCs from patients with PAH (PAH-PASMCs) and controls. We found a ADAMTS8 (disintegrin and metalloproteinase with thrombospondin motifs 8), a secreted protein specifically expressed in the lung and the heart, was upregulated in PAH-PASMCs and the lung in hypoxia-induced pulmonary hypertension (PH) in mice. To elucidate the role of ADAMTS8 in PH, we used vascular smooth muscle cell-specific ADAMTS8-knockout mice (ADAMTS8^{ASM22}). Hypoxia-induced PH was attenuated in ADAMTS8^{ASM22} mice compared with controls. ADAMTS8 overexpression increased PASC proliferation with downregulation of AMPK (AMP-activated protein kinase). In contrast, deletion of ADAMTS8 reduced PASC proliferation with AMPK upregulation. Moreover, deletion of ADAMTS8 reduced mitochondrial fragmentation under hypoxia in vivo and in vitro. Indeed, PASMCs harvested from ADAMTS8^{ASM22} mice demonstrated that phosphorylated DRP-1 (dynamin-related protein 1) at Ser637 was significantly upregulated with higher expression of profusion genes (Mfn1 and Mfn2) and improved mitochondrial function. Moreover, recombinant ADAMTS8 induced endothelial dysfunction and matrix metalloproteinase activation in an autocrine/paracrine manner. Next, to elucidate the role of ADAMTS8 in RV function, we developed a cardiomyocyte-specific ADAMTS8 knockout mice (ADAMTS8^{AmMHC}). ADAMTS8^{AmMHC} mice showed ameliorated RV failure in response to chronic hypoxia. In addition, ADAMTS8^{AmMHC} mice showed enhanced angiogenesis and reduced RV ischemia and fibrosis. Finally, high-throughput screening revealed that mebendazole, which is used for treatment of parasite infections, reduced ADAMTS8 expression and cell proliferation in PAH-PASMCs and ameliorated PH and RV failure in PH rodent models.

CONCLUSIONS: These results indicate that ADAMTS8 is a novel therapeutic target in PAH.

VISUAL OVERVIEW: An online [visual overview](#) is available for this article.

Key Words: cell proliferation ■ hypertension, pulmonary ■ hypoxia ■ inflammation ■ pulmonary artery

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Novelty and Significance

What Is Known?

- Pulmonary arterial hypertension (PAH) is characterized by pulmonary vascular remodeling with aberrant pulmonary artery smooth muscle cell (PASMC) proliferation, endothelial dysfunction, and extracellular matrix remodeling.
- Right ventricular (RV) function is an important prognostic factor in PAH.

What New Information Does This Article Contribute?

- ADAMTS8 (A disintegrin and metalloproteinase with thrombospondin motifs 8), a secreted protein, was upregulated in PASMCs from PAH patients (PAH-PASMCs).
- ADAMTS8 promoted proliferation of PASMCs, extracellular matrix remodeling, and endothelial dysfunction in an autocrine/paracrine manner.
- The depletion of ADAMTS8 improved pulmonary hypertension (PH) and RV dysfunction in rodent models of PH.
- Mebendazole, an anthelmintic drug, reduced ADAMTS8 expression in PASMCs and their proliferation and ameliorated PH and RV dysfunction in rodent models of PH.

This is the first study that demonstrates the pathogenic role of ADAMTS8 in PAH. Based on the findings of the present study, the upregulation of ADAMTS8 in PASMCs from PAH patients (PAH-PASMCs) contributed to the aberrant cell proliferation of PASMCs

and extracellular matrix remodeling. Mechanistic analysis demonstrated ADAMTS8 induced numerous intracellular signals to promote cell proliferation of PASMCs accompanied by activation of extracellular matrix metalloproteinases and NOX4-mediated reactive oxygen species production. Moreover, ADAMTS8 secreted from adjacent PASMCs induced endothelial dysfunction in an autocrine/paracrine manner through downregulation of VEGFR2 (vascular endothelial growth factor receptor 2)/AMPK (AMP-activated protein kinase) signaling. Additionally, we demonstrated the depletion of ADAMTS8 attenuated pulmonary hypertension (PH) and RV failure in rodent animal models of PH. These findings suggested that these integrated effects caused by the upregulation of ADAMTS8 in PASMCs may promote the pathogenesis of PAH. Next, using high-throughput screening, we discovered that mebendazole, an anthelmintic drug, reduced ADAMTS8 expression in PASMCs and their proliferation. Finally, we demonstrated that mebendazole ameliorated PH and RV dysfunction in rodent models of PH with downregulation of ADAMTS8. Based on these findings, we identify ADAMTS8 as a therapeutic target for PAH and propose a possibility of drug repositioning in mebendazole as PAH treatment.

Nonstandard Abbreviations and Acronyms

αSMA	α-smooth muscle actin
ACC	acetyl-CoA carboxylase
ADAMTS8	a disintegrin and metalloproteinase with thrombospondin motifs 8
AMPK	AMP-activated protein kinase
ANF	atrial natriuretic factor
ARRIVE	Animal Research: Reporting of In Vivo Experiments
BNP	brain natriuretic peptide
Bsg	basigin
CM	conditioned medium
CyPA	cyclophilin A
DCF	7-dichlorodihydrofluorescein
DRP-1	dynamins-related protein 1
ECAR	extracellular acidification rate
ECM	extracellular matrix
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
FBS	fetal bovine serum
FOXO1	forkhead box protein O1
GLUT4	glucose transporter type 4
GOT	glutamate oxaloacetate transaminase
GPT	glutamate pyruvate transaminase

GWAS	genome-wide association study
HIF-1α	hypoxia-inducible factor-1α
hrADAMTS8	human recombinant ADAMTS8
MMP	matrix metalloproteinase
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
OCR	oxygen consumption rate
PA	pulmonary artery
PAEC	pulmonary artery endothelial cell
PAH	pulmonary arterial hypertension
PASMC	pulmonary artery smooth muscle cell
PDGF	platelet-derived growth factor
PGC-1α	peroxisome proliferator-activated receptor γ coactivator-1α
PH	pulmonary hypertension
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
RUNX2	runt-related transcription factor 2
RV	right ventricular
RVH	right ventricular hypertrophy
RVSP	right ventricle systolic pressure
TGF-β	transforming growth factor-β
TSP-1	thrombospondin type 1
VEGF	vascular endothelial growth factor
VEGFR2	VEGF receptor 2

Structural remodeling of pulmonary vessels is an important feature of pulmonary arterial hypertension (PAH) reflecting distal arterial muscularization and matrix remodeling.¹ PAH is a complex and progressive cardiopulmonary disorder with poor prognosis and no curative options. The pathogenesis of PAH involves many cell types (eg, endothelial cells, vascular smooth muscle cells, and immune cells) and multiple factors (eg, genetic background, DNA damage, hypoxia, and inflammation) that together affect many signaling pathways. Although many approaches to treatment of PAH have been developed over the past decades, the prognosis still remains poor.² Although several experimental studies have shown beneficial effects of several drugs with different mechanisms of action, the currently approved medication for PAH mainly focuses on dilating the remodeled pulmonary vessels.¹ Considering the complexity of the pathogenesis of PAH, it is reasonable to expect that diverse signaling pathways are involved, necessitating the development of comprehensive and integrative theories of the underlying mechanisms.

Right ventricular (RV) dysfunction is an important prognostic factor in PAH.³ Indeed, RV failure is the main cause of death in PAH patients.⁴ In addition, postcapillary pulmonary hypertension (PH) is a major complication of left ventricular (LV) failure. RV dysfunction accompanied with elevated pulmonary artery (PA) pressure worsens the prognosis of LV failure dramatically.⁵ Although insufficient angiogenesis in the setting of increased PA pressure induces RV ischemia and dysfunction, the detailed mechanism of the impaired RV angiogenesis in patients with PH remains elusive.⁶ Thus, RV dysfunction has emerged as an important research priority in the cardiopulmonary research field. Here, we hypothesized that unidentified pathogenic proteins strongly expressed in pulmonary artery smooth muscle cells of PAH patients (PAH-PASMCs) could promote PA remodeling and induce RV failure.

To identify novel pathogenic proteins, we performed microarray analyses using PAH-PASMCs and found significant upregulation of ADAMTS8 (a disintegrin and metalloproteinase with thrombospondin motifs 8; encoded by the *ADAMTS8* gene) compared with control PASMCs. We further found that ADAMTS8, a secreted protein, was highly expressed in the lung of PAH patients, mice with hypoxia-induced PH, and rats with Sugen/hypoxia-induced PH. ADAMTS family proteins (ADAMTSs) are structurally and functionally similar to MMPs (matrix metalloproteinases) and ADAMs (a disintegrin and metalloproteinases).⁷ Unlike ADAMs, which are membrane-anchored proteins, ADAMTSs are secreted proteinases binding to extracellular matrix (ECM).⁸ Various ADAMTSs have been shown to regulate cell proliferation, adhesion, migration, and intracellular signaling.^{7,8} Importantly, recent large-scale (GWASs) genome-wide association studies demonstrated that

ADAMTS7 is a novel locus for coronary atherosclerosis.⁹ ADAMTS7 is ubiquitously expressed in many tissues, especially in the aortic walls. In contrast, ADAMTS8 is highly expressed in the lung and the heart.¹⁰ It is also known that ADAMTS8 plays a crucial role in antiangiogenic responses.¹⁰ In particular, ADAMTS8 regulates functional responses in cardiac tissues after myocardial infarction in rats.¹¹ However, the role of ADAMTS8 in the development of PAH remains to be elucidated.

To determine whether ADAMTS8 participates in the pathogenesis of PAH, we used a multidisciplinary translational approach. Here, we report that ADAMTS8 promotes proliferation of PASMCs, ECM remodeling, and endothelial dysfunction in an autocrine/paracrine manner. Using mice with PASMC-specific ADAMTS8 deficiency, we demonstrated a pathogenic role of ADAMTS8 in the development of hypoxia-induced PH. Additionally, using mice with cardiac-specific ADAMTS8 deficiency, we demonstrated a pathogenic role of ADAMTS8 in the development of RV dysfunction in hypoxia-induced PH. Finally, we discovered that mebendazole, an anthelmintic drug, reduces ADAMTS8 expression in PASMCs and their proliferation and ameliorates PH and RV dysfunction in rodent models of PH. Thus, our data suggest that ADAMTS8 could be a novel and feasible therapeutic target of PAH.

METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request. All experiments were performed in accordance with human and animal ethical guidelines in Tohoku University and Laval University. Additional detailed methods are available in the [Online Data Supplement](#).

Human Lung Samples

Lung tissues were obtained from patients at the time of lung transplantation or surgery for lung cancer at a site far from the tumor margins. All patients provided written informed consent for the use of their lung tissues for the present study.

Animal Experiments

All animal experiments were performed in accordance with the protocols approved by the Tohoku University Animal Care and Use Committee (No. 2015-Kodo-008) based on the ARRIVE trial (Animal Research: Reporting of In Vivo Experiments) guideline. We performed all experiments with thorough randomization.

Statistical Analyses

All results are shown as mean \pm SEM. Comparisons of means between 2 groups were performed by unpaired Student *t* test or 1-way ANOVA with interaction terms, followed by Tukey honestly significant difference for multiple comparisons. Comparisons of mean responses associated with the 2 main effects of the different genotypes and the severity of pulmonary

vascular remodeling were performed by 2-way ANOVA with interaction terms, followed by Tukey honestly significant difference for multiple comparisons. Statistical significance was evaluated with JMP 12 (SAS Institute, Inc, Cary) or R version 3.3.2 (<http://www.R-project.org/>). The time-dependent data were analyzed by repeated-measures linear mixed-effect model with lmer 1.1-12 and lmerTest 2.0-33 packages of R. The ratio of fully muscularized vessels was analyzed by the Poisson regression with the offset equals to the sum of total vessels with multcomp 1.4-6 package or R. All reported *P* are 2-tailed, with a *P* < 0.05 indicating statistical significance.

RESULTS

Screening for Novel Therapeutic Targets in PAH

To find novel therapeutic targets of PAH, we established cell libraries of primary cultured PSMCs from PAH patients undergoing lung transplantation and performed gene expression microarray analysis (Figure 1A). Among a total of 26 083 genes analyzed, the microarray analysis showed significant changes in 1858 genes, which were upregulated or downregulated in PAH-PSMCs compared with control PSMCs (Figure 1B, SAM *t* test *P* < 0.05 and absolute values of logarithm of fold changes > 1.0). We used the following selection criteria to identify relevant genes among the genes that showed significant changes in the microarray analysis; (1) upregulation in PAH-PSMCs, (2) expression in the lung and the heart, (3) encoding of a secretory protein, and (4) encoding of a vascular regulatory protein. After rigorous analyses of the microarray data and previous publications, we finally selected ADAMTS8 from other genes (interleukin 6 receptor and clusterin) which met these criteria (Figure 1B). As ADAMTS8 has been reported as a secreted protein specifically expressed in the lung and the heart.¹⁰ The structure of ADAMTS8 shares a common component with the structure of TSP-1 (thrombospondin type 1 motif; Figure 1C). To confirm that ADAMTS8 is expressed in distal pulmonary arteries, we used lung tissues from PAH patients. Immunostaining showed that ADAMTS8 was strongly expressed in the thickened walls of distal pulmonary arteries in patients with from PAH (Figure 1D). Moreover, ADAMTS8 in α SMA (α -smooth muscle actin)-positive areas of distal pulmonary arteries was upregulated in PAH patients (Figure 1E; Online Figure I). In agreement, Western blot showed that the amount of ADAMTS8 was significantly increased in the lungs of PAH patients compared with those of controls (Figure 1F). Moreover, real-time PCR (RT-PCR) showed that the *ADAMTS8* gene was expressed more strongly in PAH-PSMCs than in control PSMCs (Figure 1G). Furthermore, protein levels of ADAMTS8 were significantly increased in PAH-PSMCs compared with control PSMCs (Figure 1H). On the contrary, there was no significant difference in the expression level of

cells (PAECs) and PAH-PAECs (Online Figure IIA). Furthermore, ADAMTS8 was equally expressed in both PAH-PAECs and PAH-PSMCs (Online Figure IIB). Importantly, ADAMTS8 expression was specific to the lung and the heart, and hypoxia increased the ADAMTS8 protein level in the lung of wild-type mice (Figures 1I and 1J). In contrast, hypoxia reduced the ADAMTS8 protein level in the heart of wild-type mice (Figure 1K). These results suggest that ADAMTS8 in PSMCs may be involved in the development of PAH.

Targeted Deletion of ADAMTS8 in PSMCs Ameliorates Hypoxia-Induced PH

To evaluate the specific role of ADAMTS8 in PSMCs, we developed a smooth muscle-specific ADAMTS8 knockout mouse (*ADAMTS8^{lox/lox}/Sm22 α -cre^{+/-}*; *ADAMTS8^{ΔSM22 α}* ; Figure 2A; Online Figure III). *ADAMTS8^{ΔSM22 α}* and control (*ADAMTS8^{lox/lox}/Sm22 α -cre^{-/-}*) mice showed normal growth under physiological conditions. Systolic blood pressure, diastolic blood pressure, heart rate, and body weight, as well as cardiac function assessed by echocardiography, were comparable between the 2 genotypes at baseline (Figure 2B; Online Figure IV). Indeed, it has been reported that *SM22 α* is also expressed in myeloid cells, including neutrophils, monocytes, and macrophages, in addition to smooth muscle cells.¹² It is reported that ADAMTS8 is expressed in macrophage.¹³ It is also known that the myeloid cells play a crucial role in tissue homeostasis.^{14,15} There was no difference in blood levels of total bilirubin, GOT (glutamate oxaloacetate transaminase), GPT (glutamate pyruvate transaminase), creatinine, or blood urea nitrogen (Online Figure V). Furthermore, ADAMTS8 levels in the lungs were significantly lower in *ADAMTS8^{ΔSM22 α}* mice than in control mice (Online Figure VIA). The morphology of pulmonary arteries in normoxic *ADAMTS8^{ΔSM22 α}* mice did not differ from that of normoxic control mice (Figure 2C). In contrast, a significant difference in the medial thickness of pulmonary arteries was noted after the animals were subjected to hypoxia for 4 weeks (Figure 2C). In particular, compared with control mice, *ADAMTS8^{ΔSM22 α}* mice showed fewer muscularized distal pulmonary arteries in response to hypoxia (Figure 2C). Muscularized distal pulmonary arteries exhibited immunoreactivity to α SMA (Figure 2C). Consistent with these morphological changes, control mice showed increased right ventricular systolic pressure (RVSP), which was attenuated in *ADAMTS8^{ΔSM22 α}* mice (Figure 2D). By contrast, systemic blood pressure was comparable between the 2 genotypes (Online Figure VIB). The increased ratio of right ventricle to left ventricle plus septum weight (right ventricular hypertrophy [RVH]) was also attenuated in *ADAMTS8^{ΔSM22 α}* mice (Figure 2E), suggesting a crucial role for ADAMTS8 in hypoxia-induced PH. These results indicate that ADAMTS8

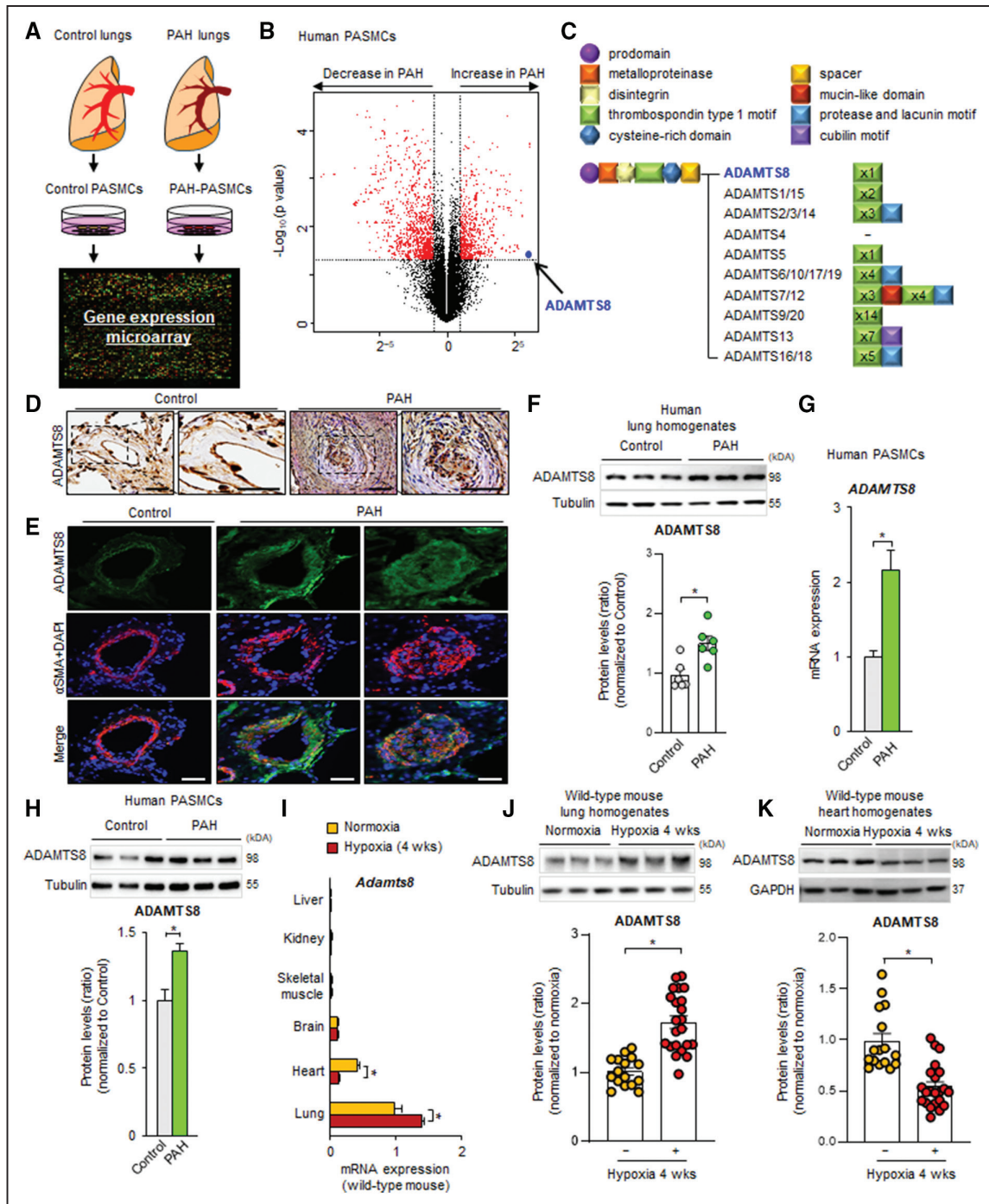


Figure 1. Screening for novel therapeutic targets in pulmonary arterial hypertension (PAH).

A, Primary culture of pulmonary artery smooth muscle cells (PASCs) from patients with pulmonary arterial hypertension (PAH). **B**, Volcano plots of gene expression variations in PAH-PASCs and control PASCs. The blue plot represents a probe for *ADAMTS8*. Dashed lines represent an adjusted P of 0.05 and ± 1 -fold change. Blue plots represent probes for *ADAMTS8* (a disintegrin and metalloproteinase with thrombospondin motifs 8); **C**, Domain structures of the ADAMTS family members. **D**, Representative results of immunostaining of distal pulmonary arteries of PAH patients who underwent lung transplantation. Scale bars, 50 μm . **E**, Representative results of immunofluorescence of distal pulmonary arteries of control and PAH patients. The smooth muscle layer is visualized by αSMA (Alexa Fluor-563, red). Double-immunostaining for *ADAMTS8* (Alexa Fluor-488, green) and αSMA (red). Scale bars, 50 μm . **F**, Representative Western blot and quantification of *ADAMTS8* and tubulin in the lungs of control patients and those with PAH ($n=6$ each). **G**, Real-time polymerase chain reaction (RT-PCR) analyses of *ADAMTS8* in PAH-PASCs and control PASCs ($n=5$ each). **H**, Representative Western Blot and the results of densitometric analysis of *ADAMTS8* in PAH-PASCs and control PASCs ($n=5$ each). **I**, RT-PCR analyses of *Adams8* in tissues from normoxic and hypoxic (10% O_2 , 4 wk) mice ($n=4-8$). **J** and **K**, Representative Western blot and results of densitometric analysis of *ADAMTS8* in homogenates of the lung (**J**) and the heart (**K**) from normoxic and hypoxic (10% O_2 , 4 wk) mice ($n=16-22$). Results are expressed as mean \pm SEM. * $P < 0.05$. The normality assumption was tested by Shapiro-Wilk normality test. Comparisons of means between 2 groups were performed by unpaired Student t test for normally distributed samples or Mann-Whitney U -test for not normally distributed samples.

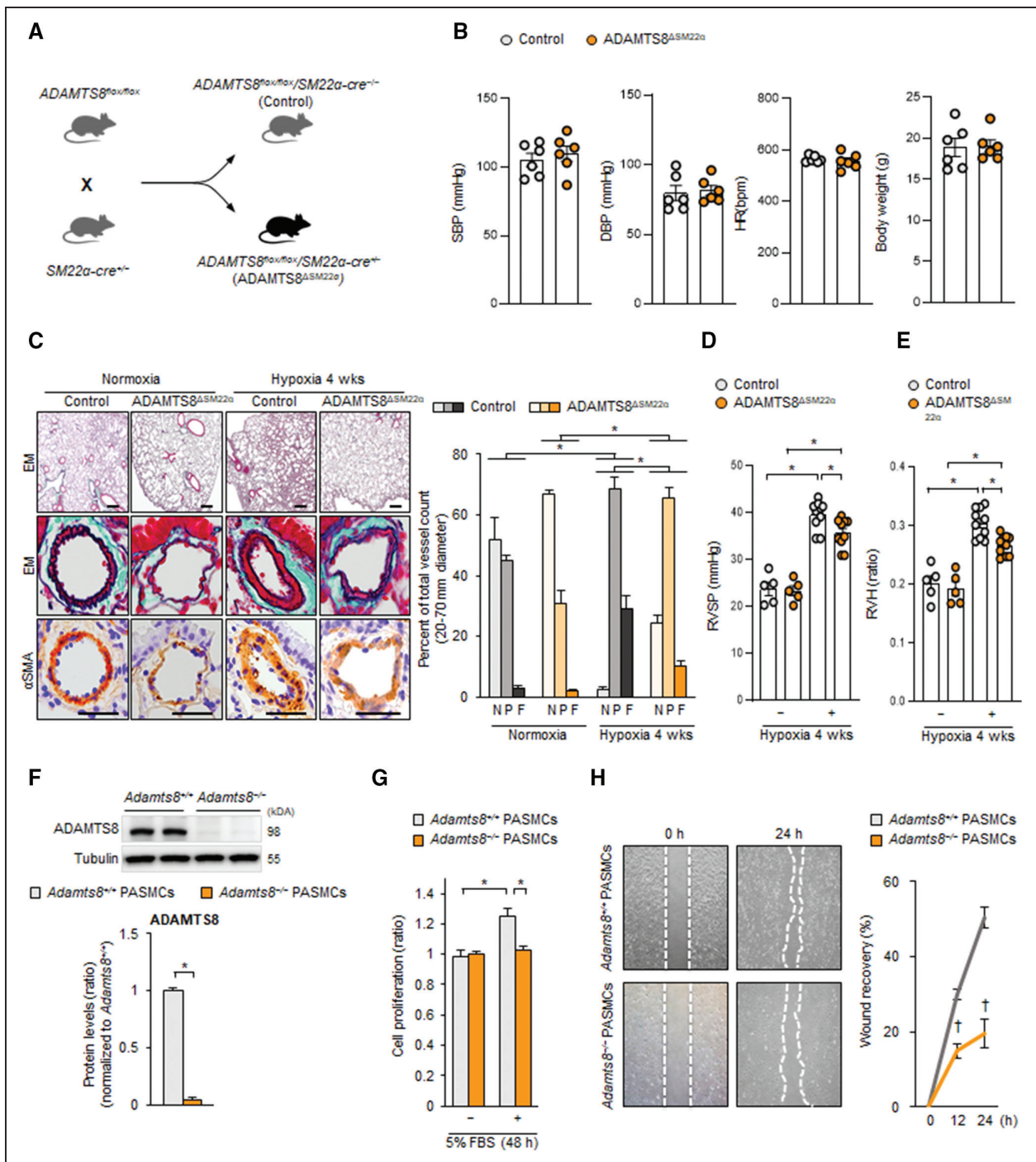


Figure 2. Pulmonary artery smooth muscle cell (PASC)-specific deletion of ADAMTS8 (a disintegrin and metalloproteinase with thrombospondin motifs 8) ameliorates hypoxia-induced pulmonary hypertension (PH).

A, Schematic outline for generating vascular smooth muscle cell-specific ADAMTS8-knockout (ADAMTS8^{ASM22α}) mice. **B**, Systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR), and body weight in 8-wk-old ADAMTS8^{ASM22α} and control mice under normoxia ($n=6$ each). **C**, Representative results of Elastica-Masson (EM) and immunostaining for α SMA (α -smooth muscle actin) of distal pulmonary arteries subjected to normoxia or hypoxia (10% O₂) for 4 wks. Muscularization of the distal pulmonary arteries in ADAMTS8^{ASM22α} and control mice subjected to normoxia ($n=5$ each) or hypoxia (10% O₂) for 4 wk ($n=10$ each). Scale bars, 50 μ m. **D** and **E**, Right ventricular systolic pressure (RVSP) and right ventricular hypertrophy (RVH) in ADAMTS8^{ASM22α} and control mice subjected to normoxia ($n=5$ each) or hypoxia (10% O₂) for 4 wk ($n=10$ each). **F**, Representative Western blots and quantification of ADAMTS8 protein levels in PASCs from ADAMTS8^{ASM22α} and control mice (Adams8^{-/-} PASCs vs Adams8^{+/+} PASCs, $n=4$ each). **G**, Proliferation of Adams8^{+/+} and Adams8^{-/-} PASCs for 48 h ($n=8$ each). **H**, Representative pictures from wound healing assay in Adams8^{+/+} and Adams8^{-/-} PASCs ($n=6$ each). $\dagger P<0.05$ compared with Adams8^{+/+} PASCs. Results are expressed as mean \pm SEM. * $P<0.05$. The normality assumption was tested by Shapiro-Wilk normality test. Comparisons of means between 2 groups were performed by unpaired Student t test for normally distributed samples or Mann-Whitney U -test for not normally distributed samples. For multiple comparisons, 2-way ANOVA followed by the Tukey HSD (honestly significant difference) method or the Dunnett method for multiple comparison, as appropriate.

in PSMCs plays a crucial role in the development of hypoxia-induced PH.

To further examine the possible role of ADAMTS8 in the development of PH in vivo, we performed mechanistic experiments using PSMCs in vitro. To evaluate the role of ADAMTS8 in PSMCs, we harvested PSMCs from ADAMTS8^{ASM22α} (*Adamts8*^{-/-} PSMCs) and control mice (*Adamts8*^{+/+} PSMCs). ADAMTS8 levels in primary cultured PSMCs were significantly lower in ADAMTS8^{ASM22α} mice than in control mice (Figure 2F). Interestingly, *Adamts8*^{-/-} PSMCs showed reduced proliferation compared with *Adamts8*^{+/+} PSMCs in response to 5% fetal bovine serum (FBS) (Figure 2G). Additionally, a wound healing assay showed reduced cell migration in *Adamts8*^{-/-} PSMCs compared with *Adamts8*^{+/+} PSMCs (Figure 2H). These results demonstrate that ADAMTS8 promotes PSMC proliferation and migration.

ADAMTS8 Affects Cell Cycle Regulatory Genes in PSMCs

In light of the crucial role of ADAMTS8 as a secretory protein, we then evaluated the role of ADAMTS8 in intracellular signaling and cell cycle regulation using PSMCs and the hrADAMTS8 (human recombinant ADAMTS8) protein. Western blot analysis showed that *Adamts8*^{-/-} PSMCs, compared with *Adamts8*^{+/+} PSMCs, had increased phosphorylation of AMPK (AMP-activated protein kinase)/acetyl-CoA carboxylase (ACC) signaling, and reduced Bcl-2/Bax ratio, which resulted in decreased PCNA expression (Figure 3A). In line with this observation, the depletion of ADAMTS8 showed the upregulation of the caspase-3 and the increased apoptosis in PSMCs (Online Figure VIIA and VIIB). Moreover, the knockdown of ADAMTS8 increased AMPK phosphorylation and downregulated BCL-2/Bax ratio and PCNA levels in control and PAH-PSMCs (Online Figure VIIC). Treatment of human PSMCs with hrADAMTS8 promoted cell proliferation in a dose-dependent manner (Figure 3B). Moreover, Western blot analysis showed that hrADAMTS8 treatment reduced phosphorylation of AMPK/ACC signaling (Figure 3C) and increased the Bcl-2/Bax ratio (Figure 3D). To further examine the role of excess of ADAMTS8 in PSMCs, we overexpressed ADAMTS8 in human PSMCs using an ADAMTS8-encoding plasmid. Constitutive production of ADAMTS8 in PSMCs induced a 6-fold increase in the ADAMTS8 level compared with a control plasmid, leading to increased expression of NADPH (nicotinamide adenine dinucleotide phosphate) oxidase 4 (*NOX4*) and decreased expression of *CDKN1B* (a cell cycle regulator),¹⁶ *BAX* (an apoptosis-related gene),¹⁷ and *APLN*,¹⁸ all of which were associated with the pathogenetic vascular cell phenotypes of PAH in the previous studies (Figure 3E). Moreover, the upregulation of ADAMTS8 in PSMCs reduced phosphorylation of

AMPK and increased the Bcl-2/Bax ratio and cell proliferation (Online Figure VIII). In contrast, a knockdown of ADAMTS8 using siRNA decreased the expression of *NOX4* and increased the expression of *CDKN1B*,¹⁹ *BAX*,¹⁷ *BCL2L11* (Bim),¹⁷ and *APLN*,²⁰ all of which were related to the reduced cell proliferation of PSMCs (Figure 3F). Moreover, ADAMTS8 siRNA markedly reduced proliferation of human PSMCs compared with control siRNA (Figure 3G). These results demonstrate that ADAMTS8 affects intracellular signaling and cell cycle regulatory genes in PSMCs.

ADAMTS8-Mediated Mitochondrial Dysfunction in PSMCs

Considering the importance of ADAMTS8 in PSMC proliferation and migration, we next focused on the role of ADAMTS8 in the ability of intracellular metabolism to tolerate the hyperproliferative status. Recent studies have shown an emerging role of reactive oxygen species (ROS) and mitochondrial function in PSMC proliferation.²¹ To examine the role of ADAMTS8 as a modulator of ROS and mitochondrial function in PSMCs, we used *Adamts8*^{+/+} and *Adamts8*^{-/-} PSMCs. 2,7-dichlorodihydrofluorescein (DCF) staining showed significantly lower levels of ROS in *Adamts8*^{-/-} PSMCs than in *Adamts8*^{+/+} PSMCs under hypoxia (Figure 4A). CellROX staining also showed significantly lower levels of ROS in *Adamts8*^{-/-} PSMCs than in *Adamts8*^{+/+} PSMCs under hypoxia (Figure 4B). In contrast, MitoSOX staining showed significantly higher levels of mitochondrial ROS in *Adamts8*^{-/-} PSMCs than in *Adamts8*^{+/+} PSMCs under the same condition (Figure 4C). Additionally, DCF and CellROX stainings showed significantly lower levels of ROS in control and PAH-PSMCs transfected with ADAMTS8 siRNA under both normoxia and hypoxia (Online Figure IXA and IXB). MitoSOX staining showed significantly higher levels of mitochondrial ROS in control and PAH-PSMCs transfected with ADAMTS8 siRNA under the same condition (Online Figure IXC). DHE staining of the lung produced consistent results, showing that hypoxic exposure increased ROS levels within autofluorescence of the elastic lamina in the distal pulmonary arteries in both genotypes (Figure 4D). However, the levels of ROS in pulmonary arteries were significantly less in ADAMTS8^{ASM22α} mice compared with control mice (Figure 4D). Recent studies have identified a mechanistic link between mitochondrial ROS production, mitochondrial morphology, and quality control in vitro.²² Thus, we next examined mitochondrial morphology and networks using MitoTracker and transmission electron microscopy in *Adamts8*^{+/+} and *Adamts8*^{-/-} PSMCs. Interestingly, *Adamts8*^{-/-} PSMCs showed less mitochondrial fragmentation than *Adamts8*^{+/+} PSMCs after hypoxia (Figure 4E).

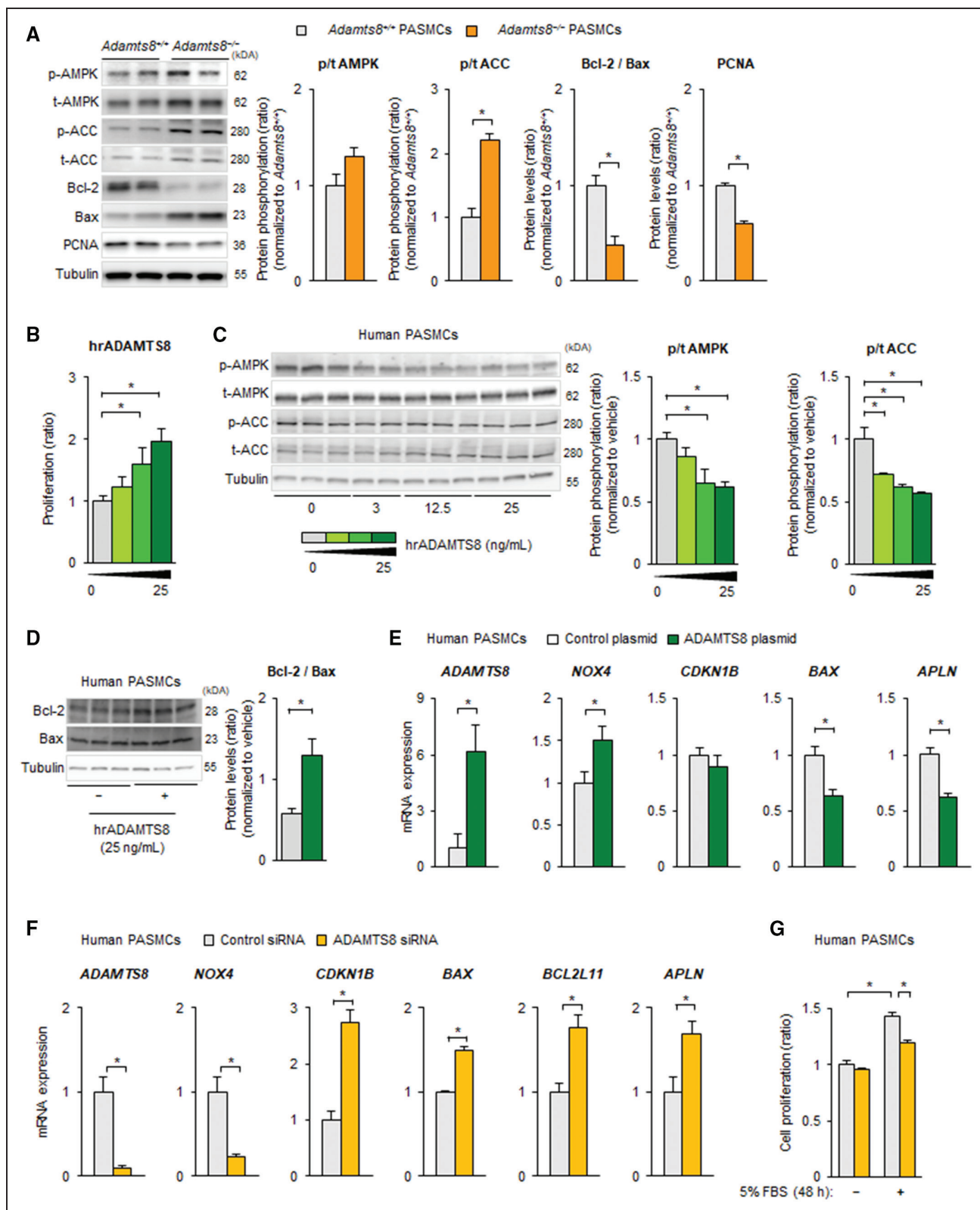


Figure 3. ADAMTS8 (a disintegrin and metalloproteinase with thrombospondin motifs 8) promotes pulmonary artery smooth muscle cell (PASMC) proliferation.

A, Representative Western blots and quantification of phosphorylated AMPK (AMP-activated protein kinase) at Thr17 (p-AMPK), total AMPK (t-AMPK), ACC (acetyl-CoA carboxylase) phosphorylated at Ser79 (p-ACC), total ACC (t-ACC), Bcl-2, Bax, and proliferating cell nuclear antigen (PCNA) in PASMCs from ADAMTS8^{ASM22α} and control mice (*Adamts8*^{-/-} PASMCs vs *Adamts8*^{+/+} PASMCs, n=6 each). **B**, Proliferation of human PASMCs treated with hrADAMTS8 (human recombinant ADAMTS8) for 48 h (n=8 each). **C**, Representative Western blots and quantification of p-AMPK, t-AMPK, p-ACC, and t-ACC in human PASMCs treated with hrADAMTS8 for 24 h (n=3 each). (Continued)

Thus, we next examined these molecules in the context of mitochondrial fission and fusion in *Adamts8^{+/+}* and *Adamts8^{-/-}* PSMCs. Interestingly, we found that protein level of DRP-1 (dynamin-related protein 1) and its phosphorylation level at Ser637 were significantly upregulated in *Adamts8^{-/-}* PSMCs compared with *Adamts8^{+/+}* PSMCs (Figure 4F). RT-PCR showed that profusion genes (*Mfn1* and *Mfn2*) were significantly upregulated in *Adamts8^{-/-}* PSMCs compared with *Adamts8^{+/+}* PSMCs (Figure 4G). In contrast, expression of profission genes (*Fis1* and *Mff*) was comparable in *Adamts8^{+/+}* and *Adamts8^{-/-}* PSMCs (Figure 4H). In agreement, transmission electron microscopy showed less mitochondrial fragmentation in *Adamts8^{-/-}* PSMCs compared with *Adamts8^{+/+}* PSMCs (Online Figure X). Additionally, we found less mitochondrial fragmentation in pulmonary arterial walls of ADAMTS8^{ΔSM22α} mice after 4 weeks of exposure to hypoxia (10% O₂) compared with control mice (Figure 4I). Moreover, the suppression of ADAMTS8 showed less mitochondrial fragmentation in addition to the upregulated phosphorylated DRP-1 at Ser637 and profusion genes in control and PAH-PSMCs after hypoxia (Online Figure IXD through IXF). These results suggest that ADAMTS8 is one of the mechanistic explanations for the increased mitochondrial fragmentation in PAH-PSMCs. Using a Seahorse XF24-3 apparatus, which provides information on mitochondrial function through real-time measurements of oxygen consumption rate (OCR; a marker of oxidative phosphorylation) and extracellular acidification rate (ECAR; a surrogate for glycolysis), we examined hypoxia-induced responses in *Adamts8^{+/+}* and *Adamts8^{-/-}* PSMCs (Online Figure XIA). OCR reflects the mitochondrial respiration rate and energy production and ECAR reflects the rate of glycolysis in PSMCs. Here, we observed significantly higher levels of OCR/ECAR ratio and maximal OCR in *Adamts8^{-/-}* PSMCs compared with *Adamts8^{+/+}* PSMCs (Online Figure XIB). Moreover, hypoxic exposure reduced OCR and OCR/ECAR ratio compared with normoxia in both *Adamts8^{+/+}* and *Adamts8^{-/-}* PSMCs (Online Figure XIB). These results suggest that ADAMTS8 is one of the mechanistic explanations for the mitochondrial dysfunction in PAH-PSMCs. Finally, *Adamts8^{-/-}* PSMCs showed significantly lower levels of NADPH oxidase activity than *Adamts8^{+/+}* PSMCs after hypoxia (Figure 4J). These results demonstrate that the balance

between mitochondrial fission and fusion is regulated by ADAMTS8 in PSMCs (Online Figure XII).

ADAMTS8 Activates MMPs and Perivascular Inflammation

ADAMTSs are secreted extracellular enzymes that degrade ECM. Degradation of ECM in turn affects other ECM enzymes. Thus, we next examined whether ADAMTS8 could influence other ECM enzymes, such as MMPs, CyPA (cyclophilin A), and its receptor Bsg (basigin).^{23,24} Bsg is also known as extracellular MMP inducer, which binds to extracellular CyPA and activates MMPs.²⁵ In situ zymography showed that exogenous hrADAMTS8 treatment significantly activated MMPs in human PSMCs in a dose-dependent manner (Figure 5A). Western blotting also showed that MMP-2 and MMP-9 in PSMCs were upregulated after hrADAMTS8 treatment (Online Figure XIIB). hrADAMTS8 also upregulated MMP-12 and MMP13 (Online Figure XIIB). Additionally, Western blot showed that hrADAMTS8 treatment increased the secretion of CyPA and Bsg, both of which activate extracellular MMPs and vascular remodeling, in a dose-dependent manner (Figure 5B). To further evaluate the role of ADAMTS8 as a modulator of MMP activity in PSMCs, we used *Adamts8^{+/+}* and *Adamts8^{-/-}* PSMCs. Importantly, MMP activity assessed with DQ gelatin was significantly lower in *Adamts8^{-/-}* PSMCs than in *Adamts8^{+/+}* PSMCs under hypoxia (Figure 5C). This result was confirmed by in situ zymography (DQ gelatin), which demonstrated that hypoxia-induced MMP activation in the lung was significantly reduced in ADAMTS8^{ΔSM22α} mice compared with control mice (Figure 5D). Moreover, MMP-2 induction by hypoxia was significantly weaker in the lungs of ADAMTS8^{ΔSM22α} mice compared with control mice (Figure 5E). Since activation of MMPs promotes pulmonary vascular inflammation, we next examined the role of ADAMTS8 in hypoxia-induced inflammatory cell migration. As expected, hypoxia exacerbated perivascular inflammation in control mice, whereas ADAMTS8^{ΔSM22α} mice showed reduced accumulation of perivascular Mac-3⁺ inflammatory cells in the lung (Online Figure XIV). Thus, ADAMTS8 activates MMPs and exacerbates perivascular inflammation in an autocrine/paracrine manner (Online Figure XV).

Figure 3 Continued. D, Representative Western blots and quantification of Bcl-2 and Bax in human PSMCs treated with hrADAMTS8 (25 ng/mL) for 24 h ($n=3$ each). **E**, Real-time polymerase chain reaction (RT-PCR) analyses of *ADAMTS8*, *NOX4*, *CDKN1B*, *BAX*, and *APLN* mRNA in human PSMCs treated with human *ADAMTS8* plasmid DNA or control plasmid DNA for 48 h ($n=6$ each). Average expression values are normalized to *GAPDH* mRNA. **F**, RT-PCR analyses of *ADAMTS8*, *BAX*, *NOX4*, *CDKB1B*, *BCL2L11*, and *APLN* mRNA in human PSMCs treated with control siRNA or ADAMTS8 siRNA for 48 h ($n=6$ each). Average expression values are normalized to *GAPDH* mRNA. **G**, Proliferation of human PSMCs treated with control siRNA or ADAMTS8 siRNA for 48 h ($n=8$ each). Results are expressed as mean \pm SEM. * $P<0.05$. The normality assumption was tested by Shapiro-Wilk normality test. Comparisons of means between 2 groups were performed by unpaired Student *t* test for normally distributed samples or Mann-Whitney *U*-test for not normally distributed samples. For multiple comparisons, 1-way ANOVA for normally distributed samples followed by the Tukey honestly significant difference method or the Dunnett method for multiple comparison, as appropriate. The multiple comparison for not normally distributed samples was performed with Kruskal-Wallis test. F indicates fully muscularized vessels; N, nonmuscularized vessels; and P, partially muscularized vessels.

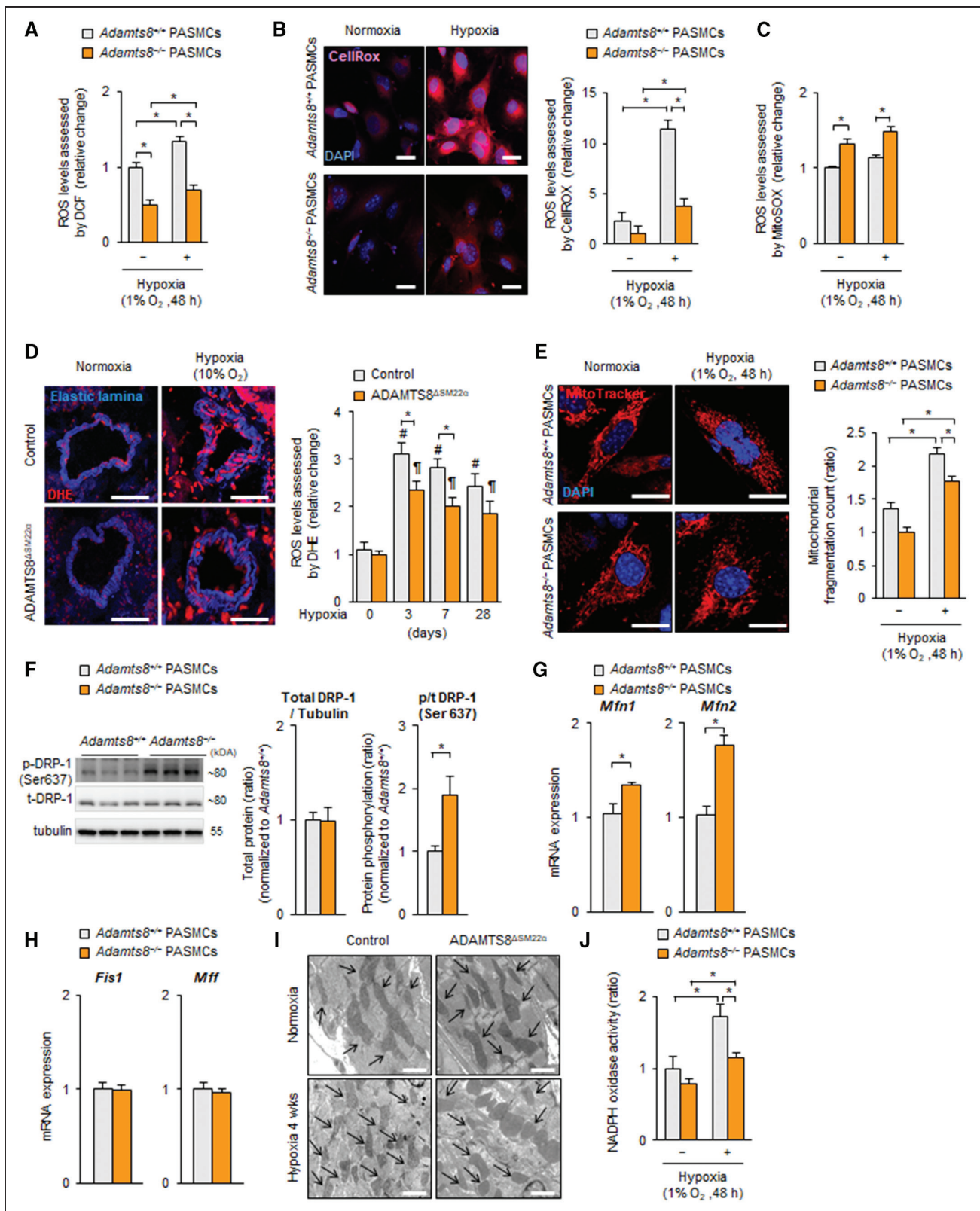


Figure 4. ADAMTS8 (a disintegrin and metalloproteinase with thrombospondin motifs 8)-mediated mitochondrial dysfunction in pulmonary artery smooth muscle cells (PASCs).

A, Quantification of 2,7-dichlorodihydrofluorescein (DCF) fluorescence intensity in *Adamts8*^{+/+} and *Adamts8*^{-/-} PASCs after exposure to normoxia (21% O₂) or hypoxia (1% O₂) for 48 h (n=8 each). **B**, Representative images of CellROX staining and quantitative analysis of CellROX fluorescence intensity in *Adamts8*^{+/+} and *Adamts8*^{-/-} PASCs under normoxia (21% O₂) or hypoxia (1% O₂) for 48 h (n=4 each). Scale bars, 25 μm. **C**, Quantification of MitoSOX fluorescence intensity in *Adamts8*^{+/+} and *Adamts8*^{-/-} PASCs under normoxia (21% O₂). (Continued)

ADAMTS8-Mediated Interaction Between PSMCs and PAECs

We have recently demonstrated the crucial roles of the interaction between PSMCs and PAECs in the development of PH.²⁶ In that study, we demonstrated the crucial role of endothelial AMPK/ACC signaling in pulmonary vascular homeostasis. Thus, we next assessed the effects of PSMC-derived ADAMTS8 on paracrine signaling of PAECs. Interestingly, hrADAMTS8 treatment significantly inhibited VEGF (vascular endothelial growth factor)-induced phosphorylation of VEGFR2 (VEGF receptor 2) in human PAECs (Figure 6A). Additionally, hrADAMTS8 treatment suppressed AICAR-induced phosphorylation of AMPK/ACC in human PAECs (Figure 6B). Consistent with this result, hrADAMTS8 treatment significantly reduced PAEC proliferation in a dose-dependent manner (Figure 6C). Next, we prepared conditioned medium (CM) from human PSMCs transfected with an *ADAMTS8*-encoding plasmid (ADAMTS8-CM) or a control plasmid (control-CM) (Figure 6D). ADAMTS8-CM significantly inhibited VEGF-induced phosphorylation of VEGFR2 in human PAECs (Online Figure XVIA). Treatment of human PAECs with ADAMTS8-CM significantly suppressed AMPK phosphorylation (Figure 6E), tube formation (Figure 6F), and cell proliferation (Online Figure XVIB) in PAECs compared with treatment with control CM. Moreover, ADAMTS8-CM significantly increased ROS production in PAECs compared with control-CM (Figure 6G). In support of this result, RT-PCR analysis showed that ADAMTS8-CM increased expression of *BAX*, a proapoptotic factor, and reduced expression of *CCND1*, a cell-cycle promoter, and *APLN* (Apelin) in human PAECs compared with control-CM (Figure 6H). ADAMTS8-CM significantly decreased the Bcl-2/Bax ratio in addition to the tendency of suppressed apelin in PAECs treated with ADAMTS8-CM (Online Figure XVIC). Furthermore, we also treated PAECs with CM from PAH-PSMCs (PAH-CM) (Online Figure XVID). We found that treatment of PAH-CM on PAECs significantly suppressed AMPK

phosphorylation and tube formation compared with that of CM from control PSMCs (Online Figure XVIIE and XVIIIF). Moreover, PAH-CM significantly increased ROS production in PAECs compared with CM from control PSMCs (Online Figure XVIG). qRT-PCR showed that PAH-CM upregulated *BAX* and suppressed *CCND1* and *APLN* (Online Figure XVIIH). Hence, these data suggested that the secretion of ADAMTS8 from PSMCs might be associated with the interaction between PAECs and PSMCs in the PAH pathogenesis. (Figure 6I).

Targeted Deletion of ADAMTS8 in Cardiomyocytes Ameliorates RV Failure in PH

Since ADAMTS8 is also expressed in the heart, we developed a cardiomyocyte-specific ADAMTS8 knockout mouse (*ADAMTS8^{lox/lox}/αMHC-cre^{+/-}*; *ADAMTS8^{ΔαMHC}*; Figure 7A). *ADAMTS8^{ΔαMHC}* and control (*ADAMTS8^{lox/lox}/αMHC-cre^{-/-}*) mice showed normal growth under physiological conditions. Systolic blood pressure, diastolic blood pressure, heart rate, and body weight, as well as cardiac function assessed by echocardiography, were comparable between the 2 genotypes at baseline (Online Figure XVII). ADAMTS8 levels in the heart were significantly lower in *ADAMTS8^{ΔαMHC}* mice than in control mice (Figure 7B). To further examine the role of myocardial ADAMTS8 in response to elevated PA pressure, we used hypoxia to induce PH in *ADAMTS8^{ΔαMHC}* mice. After 4 weeks of hypoxia, there was no significant difference in RVSP or vascular remodeling between the 2 genotypes, whereas RVH was significantly reduced in *ADAMTS8^{ΔαMHC}* mice compared with control mice (Figure 7C; Online Figure XVIII). Importantly, chronic hypoxia reduced the walking distance, evaluated using a treadmill, in control mice, and this effect was significantly ameliorated in *ADAMTS8^{ΔαMHC}* mice (Figure 7D). Furthermore, echocardiography showed that *ADAMTS8^{ΔαMHC}* mice had a significantly higher Act/ET ratio, increased tricuspid annular plane systolic excursion, increased cardiac output, and reduced RVID compared with control

Figure 4 Continued. or hypoxia (1% O₂) for 48 h (n=8 each). **D**, Left, representative images of dihydroethidium (DHE) staining of pulmonary arteries from *ADAMTS8^{ΔSM22α}* and control mice after hypoxic exposure (10% O₂) for 28 d. Right, quantification of DHE fluorescence intensity within autofluorescence of the elastic lamina in the distal pulmonary arteries from *ADAMTS8^{ΔSM22α}* and control mice after hypoxic exposure (10% O₂) for 0, 3, 7, and 28 d (n=5 each). Scale bars, 50 μm. #P<0.05 compared with normoxic control mice. †P<0.05 compared with normoxic *ADAMTS8^{ΔSM22α}* mice. **E**, Representative images of *Adamts8^{+/+}* and *Adamts8^{-/-}* PSMCs after normoxia (21% O₂) or hypoxia (1% O₂) for 48 h (n=4 each) labeled for mitochondria. Nuclei were counterstained using DAPI. Scale bars, 20 μm. **F**, Representative Western blots and quantification of DRP-1 (dynamidin-related protein 1; p-DRP-1) phosphorylated at Ser637 and total DRP-1 (t-DRP-1) in *Adamts8^{+/+}* and *Adamts8^{-/-}* PSMCs (n=6 each). **G**, Real-time polymerase chain reaction (RT-PCR) analyses of *Mfn1* and *Mfn2* mRNA in *Adamts8^{+/+}* and *Adamts8^{-/-}* PSMCs (n=6 each). Average expression values are normalized to *Gapdh* mRNA. **H**, RT-PCR analyses of *Fis1* and *Mff* mRNA in *Adamts8^{+/+}* and *Adamts8^{-/-}* PSMCs (n=6 each). Average expression values are normalized to *Gapdh* mRNA. **I**, Representative transmission electron microscopy images of mitochondria in smooth muscle layers of pulmonary arteries from *ADAMTS8^{ΔSM22α}* and control mice subjected to normoxia (21% O₂) or hypoxia (10% O₂) for 4 wk. The arrows indicate mitochondria. Scale bars, 1 μm. **J**, Quantification of NADPH (nicotinamide adenine dinucleotide phosphate) oxidase activity in *Adamts8^{+/+}* and *Adamts8^{-/-}* PSMCs after exposure to normoxia (21% O₂) or hypoxia (1% O₂) for 48 h (n=8 each). Results are expressed as mean±SEM. *P<0.05. The normality assumption was tested by Shapiro-Wilk Normality Test. Comparisons of means between 2 groups were performed by unpaired Student *t* test for normally distributed samples or Mann-Whitney *U*-test for not normally distributed samples. For multiple comparisons, 1-way ANOVA for normally distributed samples followed by the Tukey HSD (honestly significant difference) method or the Dunnett method for multiple comparison, as appropriate. The multiple comparison for not normally distributed samples was performed with Kruskal-Wallis test. ROS indicates reactive oxygen species.

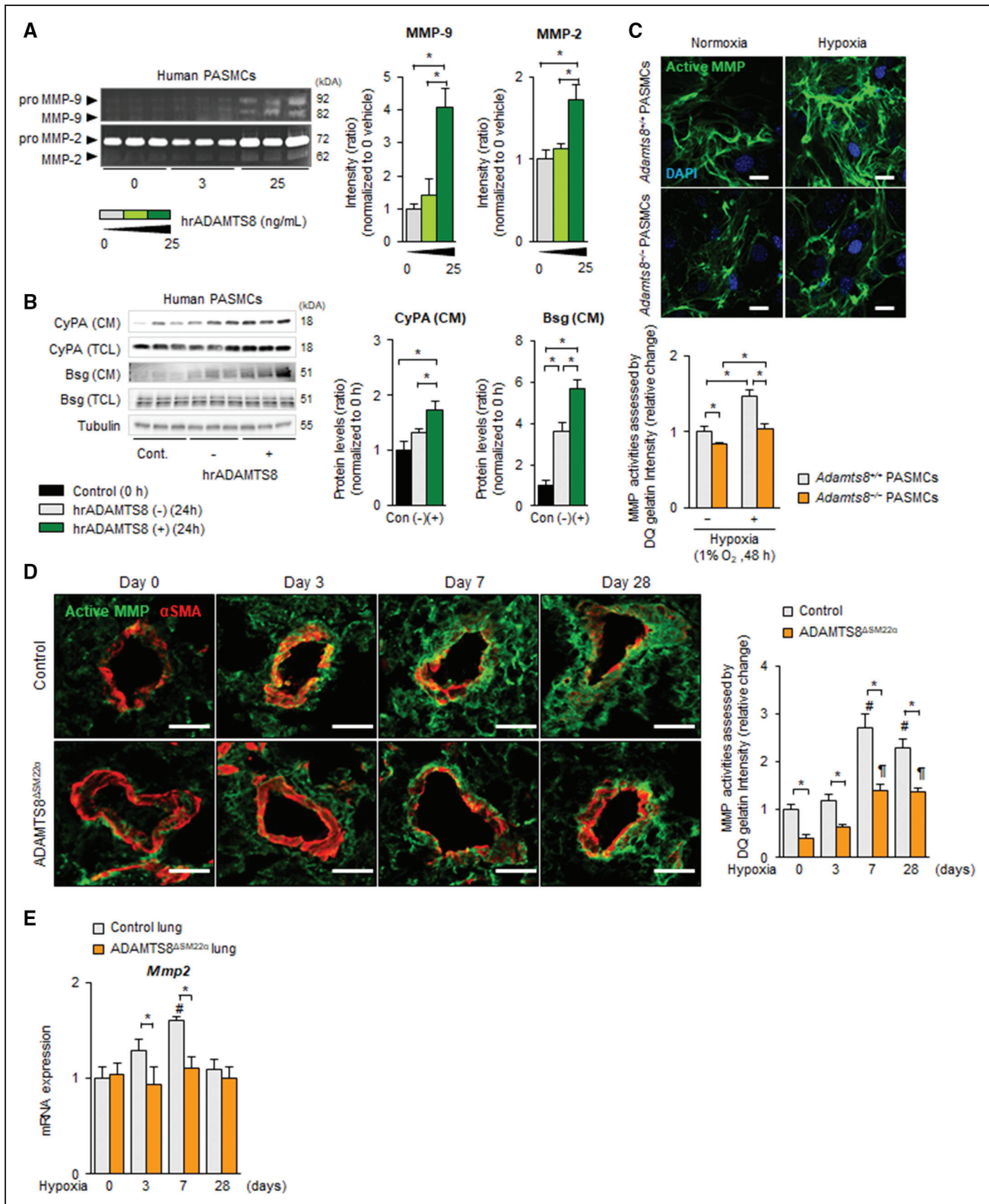


Figure 5. ADAMTS8 (a disintegrin and metalloproteinase with thrombospondin motifs 8) promotes MMP (matrix metalloproteinase) activation.

A, Gelatin zymography detection of pro-MMP-2, MMP-2, pro MMP-9, and MMP-9 in conditioned medium (CM) from human pulmonary artery smooth muscle cells (PASMCs) treated with hrADAMTS8 (human recombinant ADAMTS8) for 24 h ($n=3$ each). **B**, Representative Western blots and quantification of cyclophilin A (CyPA) and basigin (Bsg) in CM and total cell lysate (TCL) of human PASMCs treated with hrADAMTS8 (25 ng/mL) for 24 h ($n=3$ each). **C**, Representative images of in situ zymography (DQ gelatin) in *Adamts8*^{+/+} and *Adamts8*^{-/-} PASMCs after exposure to normoxia (21% O₂) or hypoxia (1% O₂) for 48 h. Nuclei were counter-stained using DAPI. (Continued)

mice after hypoxia for 4 weeks (Figure 7E; Online Figure XIX). Moreover, sirius red staining showed significantly less RV fibrosis in ADAMTS8^{Δα}MHC mice than in control mice after chronic hypoxia (Figure 7F). Although immunostaining for CD31 showed that hypoxia increased capillary length and decreased capillary density, ADAMTS8^{Δα}MHC mice demonstrated reduced capillary length and higher capillary density in RVs than in RVs of control mice after hypoxic exposure (Figure 7G; Online Figure XX). Consistently, staining with a hypoxic probe showed that ADAMTS8^{Δα}MHC mice had a smaller hypoxic area than control mice after hypoxia (Figure 7H). Finally, RT-PCR analysis showed significantly reduced expression of ANF (atrial natriuretic factor), BNP (brain natriuretic peptide), collagen 3a (Col3a), and GLUT4 (glucose transporter type 4) in RVs of ADAMTS8^{Δα}MHC mice than in RVs of control mice after chronic hypoxia (Figure 7I). These results demonstrate that ADAMTS8 in cardiomyocytes promotes the development of cardiac hypertrophy, fibrosis, and RV dysfunction in response to elevated RV pressure.

Mebendazole Downregulates ADAMTS8 and Ameliorates PH and RV Failure

Given a key role of ADAMTS8 in pulmonary vascular remodeling and RV failure discovered in the present experiments above, we finally aimed to identify a therapeutic agent that can downregulate ADAMTS8. An *in silico* screening using the Life Science Knowledge Bank software (<http://www.lskb.w-fusionus.com/>) identified several compounds as possible inhibitors of ADAMTS8. However, all these compounds were MMP inhibitors, which were shown to have serious side effects in pre-clinical trials.²⁷ Next, we used the public chemical library of the Drug Discovery Initiative (<http://www.ddi.u-tokyo.ac.jp/en/>), a collection of 3336 clinically used compounds and derivatives. A high-throughput screening identified 3 compounds that downregulate ADAMTS8 expression and PAH-PASMC proliferation (Figure 8A). Among them, we focused on mebendazole (Figure 8B), which is used for treatment of parasite infections and has antiproliferative effects against cancer cells.²⁸ We found that mebendazole treatment suppressed ADAMTS8 expression (Figure 8C) and proliferation of PAH-PAMSCs

in a dose-dependent manner (Figure 8D). Mebendazole also suppressed control PASMC proliferation with the suppression of ADAMTS8. These data were consistent after ADAMTS8 in control PASMCs was overexpressed by ADAMTS8 plasmid (Online Figure XXIA and XXIB). Furthermore, mebendazole treatment demonstrated downregulation of PAH-PASMC proliferation after the knockdown of ADAMTS8 by siRNA (Online Figure XXIC and XXID). We then examined the effect of administration of mebendazole in hypoxia-induced PH in mice. Daily administration of mebendazole for 3 weeks had no effect on body weight, heart rate, or blood pressure compared with vehicle controls (Online Figure XXIIA). Importantly, ADAMTS8 expression in the lung and the heart was significantly attenuated by mebendazole treatment (Online Figure XXIIB and XXIIC). Moreover, mebendazole significantly suppressed muscularization of distal pulmonary arteries after hypoxic exposure (Online Figure XXIID) and significantly reduced RVSP and RVH compared with vehicle control (Online Figure XXIIIE). Furthermore, mebendazole significantly reduced expression of ANF, Col3a, and GLUT4 in the RV (Online Figure XXIIF). To further confirm the therapeutic potential of mebendazole in PAH, we used a rat model of PH induced by Sugen/hypoxia.²⁹ In this model, the levels of ADAMTS8 in the lung and RV were significantly elevated, which was inhibited by mebendazole treatment when started even after the development of PH (treatment protocol; Figure 8E). To directly demonstrate the therapeutic effect of ADAMTS8 inhibition *in vivo*, rats were nebulized with ADAMTS8 siRNA after the establishment of PH (treatment protocol). Nebulization and inhalation of ADAMTS8 siRNA tended to reduce the protein levels of ADAMTS8 in the lungs of Sugen/hypoxia-induced PH rat model (Online Figure XXIIIA and XXIIIB). Moreover, ADAMTS8 inhibition ameliorated PH and RV failure in rats *in vivo* (Online Figure XXIIIC and XXIIID). Furthermore, mebendazole treatment significantly reduced RVSP, RVH (Figure 8F) and the wall thickness of distal pulmonary arteries compared with vehicle control treatment (Figure 8G). Echocardiography showed that mebendazole treatment increased AcT, tricuspid annular plane systolic excursion, cardiac output, and LVDd in addition to reduced RVID compared with vehicle control treatment (Online Figure XXIV). Moreover, mebendazole treatment significantly

Figure 5 Continued. Scale bars, 20 μ m. Quantification of DQ gelatin fluorescence intensity in *Adamts8*^{+/+} and *Adamts8*^{-/-} PASMCs under normoxia (21% O₂) or hypoxia (1% O₂) for 48 h (*n*=8 each). **D**, Left, representative results of *in situ* zymography and immunostaining for α SMA (α -smooth muscle actin) of the distal pulmonary arteries in ADAMTS8^{ASM22 α} and control mice subjected to normoxia or hypoxia (10% O₂) for 0, 3, 7, and 28 d. Right, quantitation of MMP activity in the α SMA-positive area in distal pulmonary arteries of ADAMTS8^{ASM22 α} control mice subjected to hypoxia (10% O₂) for 28 d (*n*=5 each). Scale bars, 25 μ m. #*P*<0.05 compared with normoxic control mice. †*P*<0.05 compared with normoxic ADAMTS8^{ASM22 α} mice. **E**, Real-time polymerase chain reaction (RT-PCR) analyses of *Mmp-2* mRNA in lung homogenates from ADAMTS8^{ASM22} and control mice subjected to normoxia (*n*=4 each) or hypoxia (10% O₂) for 0, 3, 7, and 28 d (*n*=8 each). Average expression levels are normalized to *Gapdh* mRNA. #*P*<0.05 compared with normoxic control mice. †*P*<0.05 compared with normoxic ADAMTS8^{ASM22 α} mice. Results are expressed as mean \pm SEM. **P*<0.05. The normality assumption was tested by Shapiro-Wilk Normality Test. Comparisons of means between 2 groups were performed by unpaired Student *t* test for normally distributed samples or Mann-Whitney *U* test for not normally distributed samples. For multiple comparisons, 1-way ANOVA for normally distributed samples followed by the Tukey honestly significant difference method or the Dunnett method for multiple comparison, as appropriate. The multiple comparison for not normally distributed samples was performed with Kruskal-Wallis test.

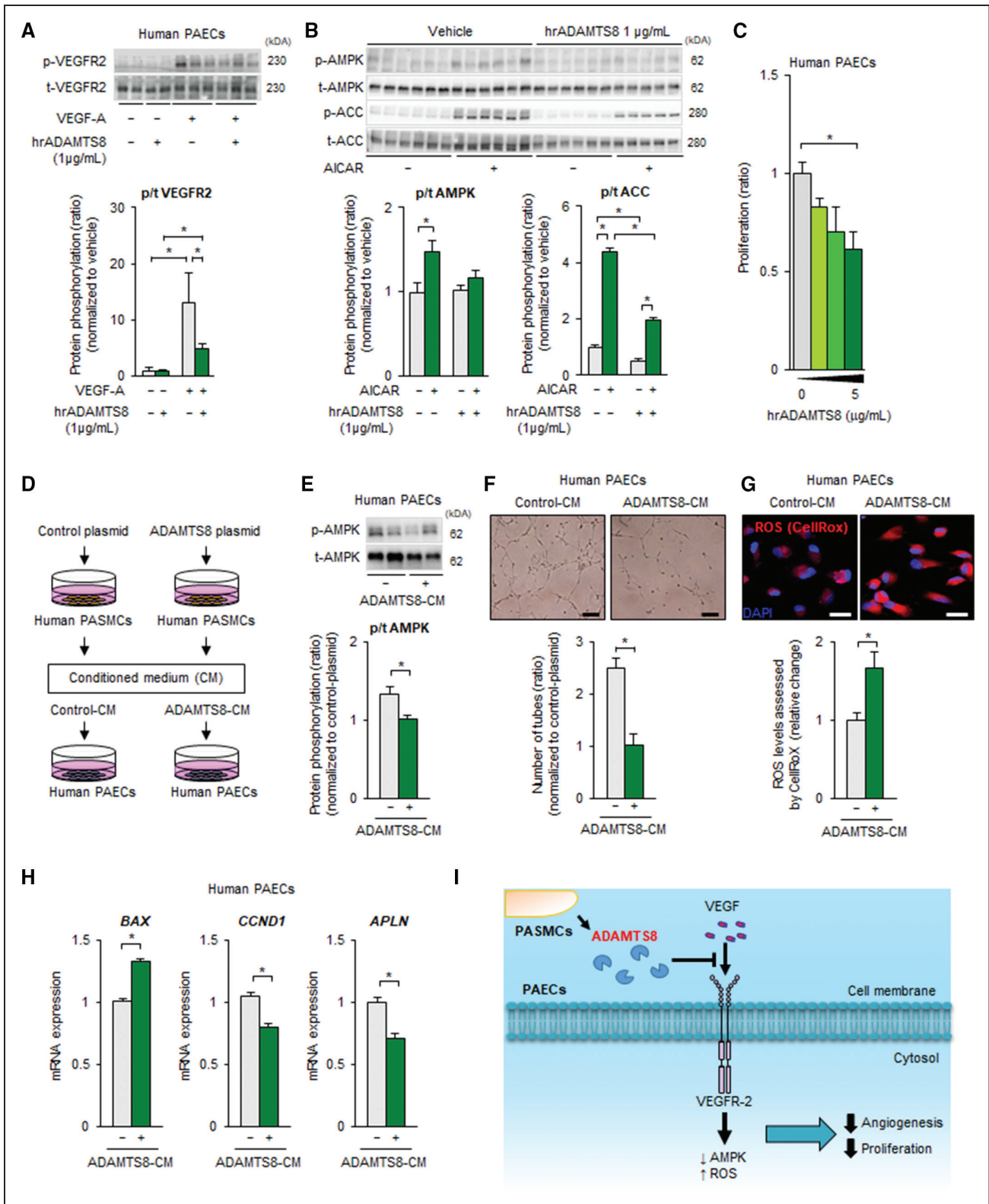


Figure 6. ADAMTS8 (a disintegrin and metalloproteinase with thrombospondin motifs 8)-mediated interaction between pulmonary artery smooth muscle cells (PASMCs) and pulmonary artery endothelial cells (PAECs).

A, Representative Western blots and quantification of VEGF (vascular endothelial growth factor) receptor 2 phosphorylated at Tyr951 (p-VEGFR2) and total VEGF receptor 2 (t-VEGFR2) in human PAECs treated with hrADAMTS8 (human recombinant ADAMTS8; µg/mL, 15 min) followed by VEGF-A stimulation (25 ng/mL, 15 min, n=3 each). **B**, Representative Western Blots and quantification of AMPK (AMP-activated protein kinase) phosphorylated at Thr17 (p-AMPK), t-AMPK (total AMPK), ACC phosphorylated at Ser79 (p-ACC), and total ACC (acetyl-CoA carboxylase; t-ACC) in human PAECs treated with hrADAMTS8 (1 µg/mL) for 24 h followed by AICAR stimulation for 2 h (Continued)

increased capillary density in RV (Figure 8H), reduced RV fibrosis (Figure 8I), and increased walking distance (Figure 8J). These results demonstrate that mebendazole suppresses ADAMTS8 expression in the lung and RV and ameliorates PH and RV failure (Figure 8K).

DISCUSSION

The present study demonstrates that upregulation of ADAMTS8 in PSMCs contributes to the pathogenesis of PAH, which involves proliferation and migration of PSMCs, enhanced MMP activity, and mitochondrial dysfunction. The present study also proposes ADAMTS8 inhibition in PSMCs as a novel strategy to prevent the development of PH. These conclusions are based on the following findings: (1) ADAMTS8 was upregulated in PAH-PSMCs, (2) knockdown of ADAMTS8 in PSMCs attenuated the development of hypoxia-induced PH in mice, (3) knockdown of ADAMTS8 reduced PSMC proliferation and migration, (4) ADAMTS8 reduced VEGF-induced PAEC proliferation and ameliorated endothelial function, (5) knockdown of ADAMTS8 in cardiomyocytes ameliorated the development of cardiac hypertrophy, fibrosis, and RV failure in response to elevated PA pressure, and (6) mebendazole treatment reduced ADAMTS8 expression in the lung and the RV and ameliorated PH and RV failure in rodents.

ADAMTS8 As a Novel Pathogenic Protein in PAH

We utilized a translational multidisciplinary approach to find a novel pathogenic protein linking diverse signaling pathways that promote the development of PAH. ADAMTS8 was selected as a key protein involved in the pathogenesis of PAH based on analysis of 1858 genes that were upregulated or downregulated in PAH-PSMCS. ADAMTSs are secreted proteins characterized by presence of an MMP domain and a variable number of TSP-1. Numerous studies have suggested a crucial role of MMP and TSP-1 in vascular homeostasis and vasculopathy, including PH.³⁰ Furthermore, various ADAMTSs have been shown to regulate cell proliferation, adhesion, migration, and intracellular signaling.^{7,8} For example, ADAMTS1 deficiency induces

thoracic aortic aneurysms and dissections in mice, while it is downregulated in the aorta of patients with marfan syndrome.³¹ Moreover, the GWAS study demonstrated that ADAMTS7 is a novel locus for coronary artery disease in humans.⁹ In support of this finding, ADAMTS7 deficiency suppressed neointimal formation after wire injury in mice and downregulated migration of vascular smooth muscle cells.³² It is also known that ADAMTS8 plays a crucial role in antiangiogenic responses.¹⁰ Unlike ADAMTS1 and 7, both of which are ubiquitously expressed in various tissues, ADAMTS8 is specifically expressed in the lung and the heart.¹⁰

The molecular mechanisms in organogenesis and cellular differentiation is regulated by epigenetics in addition to genetics.³³ Additionally, the expression of ADAMTS8 in cancer cells was regulated by epigenetic modification.³⁴ The epigenetic modification may be involved in the selective tissue distribution of ADAMTS8. Consequently, it would be speculated that ADAMTS8 is implicated in the homeostasis of pulmonary vascular system rather than that of systemic vasculature. Indeed, ADAMTS8^{ΔSM22α} mice did not show any phenotype in the vascular system except the attenuation of vascular remodeling in lung in response to hypoxia. Although we were unable to detect ADAMTS8 in the plasma in both PAH patients and controls, previous studies reported that ADAMTS8 is autocatalytically and proteolytically cleaved just after secretion.^{10,35} Moreover, other ADAMTS family proteins are autocatalytically and proteolytically cleaved within its spacer region by matrix metalloproteinases.^{36,37} Thus, ADAMTS8 protein could be quickly degraded after the secretion in the local pulmonary vascular bed. Taken together, it is conceivable that the selective tissue distribution of ADAMTS8 may result in no apparent phenotypic changes in the vascular system except for the lung. At the same time, for the measurement of plasma levels of ADAMTS8, we may need further improvement of the method such as preparation and preservation after blood sampling. Although the analyses of circulating ADAMTS8 requires further investigation, our results raise the hypothesis that ADAMTS8 plays a key role in the pathogenesis of PAH. According to the high-throughput transcription factor functional studies from the transfac database (<http://gene-regulation.com/pub/databases>).

Figure 6 Continued. (1 mmol/L, $n=6$ each). **C**, Proliferation of human PAECs during treatment with hrADAMTS8 (0.025, 1, and 5 $\mu\text{g}/\text{mL}$) for 48 h ($n=8$ each). **D**, Treatment of human PAECs with conditioned medium (CM) from control plasmid-transfected PSMCs (Control-CM) or ADAMTS8 plasmid-transfected PSMCs (ADAMTS8-CM). **E**, Representative Western blots and quantification of p-AMPK and total AMPK in human PAECs treated with Control-CM or ADAMTS8-CM for 24 h ($n=6$ each). **F**, Representative pictures from the tube formation assay and quantitative analysis in human PAECs treated with control-CM or ADAMTS8-CM for 8 h ($n=6$ each). Scale bars, 100 μm . **G**, Representative images of CellROX and quantitative analysis of CellROX fluorescence intensity in human PAECs treated with Control-CM or ADAMTS8-CM for 24 h ($n=4$ each). Scale bars, 25 μm . **H**, Real-time polymerase chain reaction (RT-PCR) analyses of *BAX*, *CCND1*, and *APLN* mRNA in human PAECs treated with Control-CM or ADAMTS8-CM for 24 h ($n=6$ each). Average expression levels are normalized to *GAPDH* mRNA. **I**, Schematic representation of molecular mechanisms of ADAMTS8-mediated interaction between PSMCs and PAECs. Results are expressed as mean \pm SEM. * $P<0.05$. The normality assumption was tested by Shapiro-Wilk Normality Test. Comparisons of means between 2 groups were performed by unpaired Student *t* test for normally distributed samples or Mann-Whitney *U*-test for not normally distributed samples. For multiple comparisons, 1-way ANOVA for normally distributed samples followed by the Tukey honestly significant difference method or the Dunnett method for multiple comparison, as appropriate. The multiple comparison for not normally distributed samples was performed with Kruskal-Wallis test. CCND1 indicates cyclin D1.

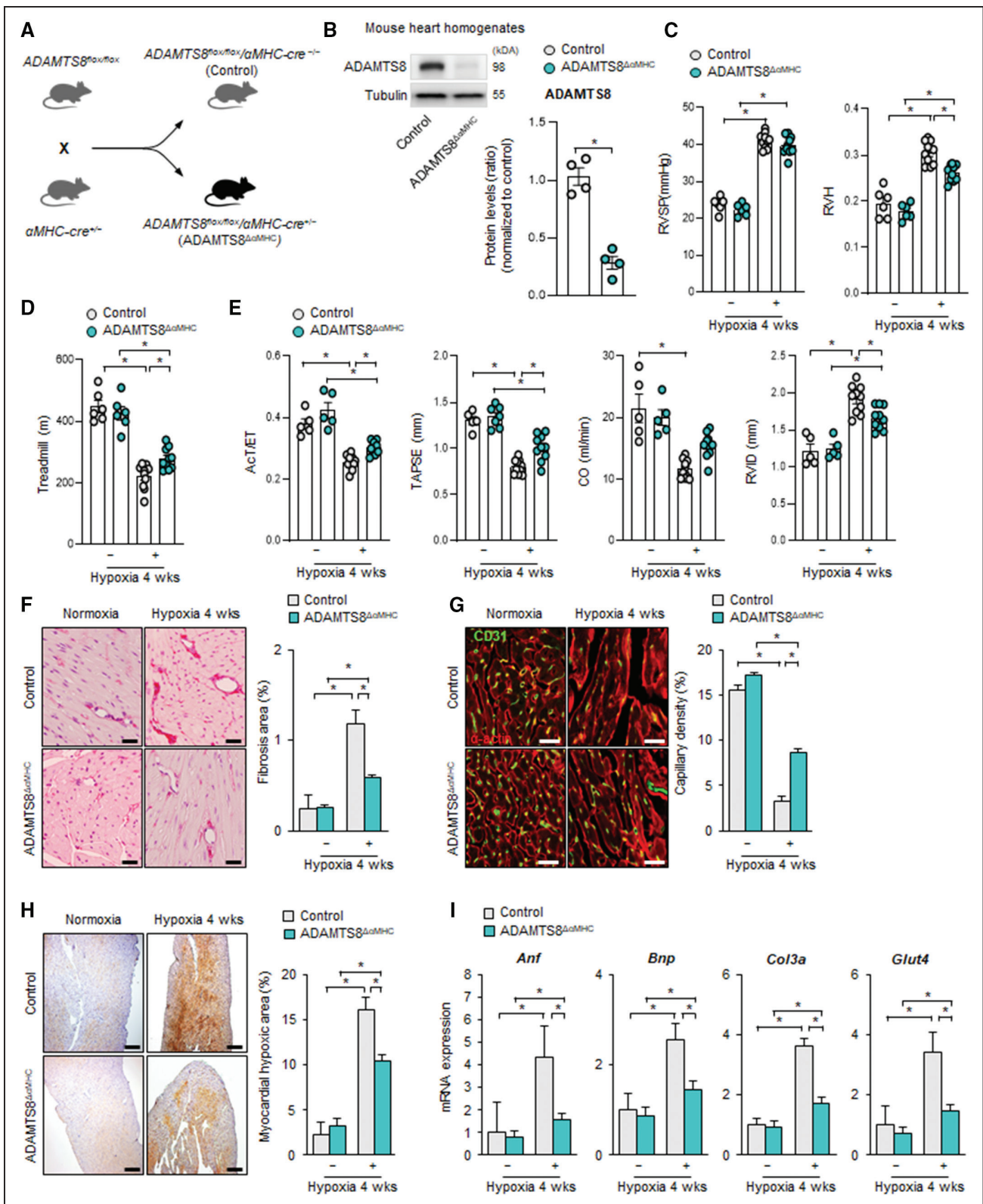


Figure 7. Cardiomyocyte-specific deletion of ADAMTS8 (a disintegrin and metalloproteinase with thrombospondin motifs 8) ameliorates right ventricular (RV) failure.

A, Schematic outline for generating cardiomyocyte-specific ADAMTS8 knockout ($ADAMTS8^{\Delta aMHC}$) mice. **B**, Quantification of protein levels of ADAMTS8 in the hearts of $ADAMTS8^{\Delta aMHC}$ and control mice ($n=4$ each). **C**, RV systolic pressure (RVSP) and RV hypertrophy (RVH) in $ADAMTS8^{\Delta aMHC}$ and control mice subjected to normoxia ($n=6$ each) or hypoxia (10% O_2) for 4 wk ($n=10$ each). **D**, Walking distance, assessed by a treadmill test, in $ADAMTS8^{\Delta aMHC}$ and control mice subjected to normoxia ($n=6$ each) or hypoxia (10% O_2) for 4 wk ($n=10$ each). (Continued)

html) and genecards database (<http://www.genecards.org/>), ADAMTS8 expression is potentially regulated by binding of several transcription factors, such as FOXO1 (forkhead box protein O1), RUNX2 (runt-related transcription factor 2), and estrogen receptor 1 (ER1), to its promoter region. Previous studies have demonstrated that these transcription factors also regulate PAH-PASMC proliferation. Thus, it is possible that ADAMTS8 expression is upregulated in PAH-PASMCs, at least in part, by these transcription factors.

ADAMTS8 Induces ROS Production and MMP Activation

ADAMTS8 degrades proteoglycans, which are key components of ECM.³⁵ ECM affects cellular behavior in physiological and pathological processes and provides structural support. ECM can sequester and locally release growth factors, such as EGF (epidermal growth factor) and TGF- β (transforming growth factor- β).³⁸ Thus, ECM remodeling through proteolytic degradation can release these growth factors, affecting cell proliferation and migration.³⁸ In the present study, exogenous hrADAMTS8 treatment promoted PASMC proliferation and upregulated several ECM enzymes, including MMP-2, MMP-9, MMP-12, MMP-13, CyPA, and Bsg, all of which play crucial roles in the pathogenesis of PAH.^{24,30,39,40} In contrast, ADAMTS8 deficiency downregulated PASMC proliferation in vitro and ameliorated pulmonary vascular remodeling in vivo, which was accompanied by significant downregulation of MMPs in the lung. These results indicate that ADAMTS8 promotes ECM remodeling, PASMC proliferation, and development of PAH.

In addition to the ADAMTS8-induced ECM remodeling, we demonstrated that ADAMTS8 changed the intracellular metabolism in PASMCs, including AMPK signaling, apoptosis signaling, ROS levels, and mitochondrial function. ROS serve as important intracellular and intercellular messengers in a variety of signaling pathways that promote smooth muscle cell proliferation, migration, expression of proinflammatory mediators, and ECM remodeling. NOX4-induced ROS production activates HIF-1 α (hypoxia-inducible factor-1 α)⁴¹ and HIF-2 α in PASMCs.⁴² HIFs suppress mitochondria-dependent

apoptosis and increase cell proliferation, which are hallmarks of PAH.⁴³ Moreover, NOX4 is upregulated in PASMCs by hypoxia, as well as in the lungs of PAH patients.⁴⁴ Interestingly, NOX4-dependent activation of mTORC2 (mammalian target of rapamycin complex 2) promotes proliferative, apoptosis-resistant phenotypes of PAH-PASMC via downregulation of AMPK signaling.¹⁷ It is well known that AMPK regulates metabolism, which results in the suppression of anabolism to minimize ATP consumption and the acceleration of catabolism to stimulate ATP production.⁴⁵ Furthermore, the majority of ATP production in cells is regulated by TCA cycles. As expected, in addition to the upregulation of AMPK signaling, *Adamts8*^{-/-} PASMCs showed upregulated basal OCR, which suggested increased ATP production. Moreover, genetic ablation of the AMPK in cancer cells promotes metabolic shift to glycolysis.⁴⁶ The suppression of AMPK signal and metabolic abnormality (ie, suppression of mitochondrial glucose oxidation and increased glycolysis) are implicated in PAH pathogenesis including PASMCs.^{17,47} These previous reports support the upregulation of OCR/ECAR (metabolic shift from glycolysis to mitochondrial oxidation) in *Adamts8*^{-/-} PASMCs, demonstrating increased AMPK signaling. In addition, previous reports showed that downregulation of AMPK promoted cell proliferation in cancer cells and PASMC from PAH patients.^{17,48} Activation of AMPK inhibits PDGF (platelet-derived growth factor)-induced PASMC proliferation.⁴⁹ Indeed, *Adamts8*^{-/-} PASMCs also showed reduced proliferation in response to 5% FBS. Thus, we consider that the upregulation of metabolism (increased ATP production and metabolic shift to mitochondrial glucose oxidation) and the downregulation of cell proliferation may be associated with the increased AMPK activity after the depletion of ADAMTS8 in PASMCs. AMPK also promotes mitochondrial biogenesis by activation of PGC-1 α (peroxisome proliferator-activated receptor γ coactivator-1 α).⁵⁰ PGC-1 α is related to mitochondrial dynamics since it promotes MFN2, and PGC-1 α -mediated downregulation of MFN2 contributes to the mitochondrial fragmentation and excessive proliferation of PAH-PASMCs.⁵¹ All these previous studies support our present findings that ADAMTS8 enhances NOX4-mediated ROS production and PASMC proliferation and downregulates

Figure 7 Continued. **E**, Quantitative analyses of the parameters of RV function in ADAMTS8^{ΔMHC} and control mice subjected to normoxia ($n=6$ each) or hypoxia (10% O₂) for 4 wk ($n=10$ each). **F**, Representative pictures from sirius red staining and quantitative analysis of interstitial fibrotic area in RVs of ADAMTS8^{ΔMHC} and control mice subjected to normoxia or hypoxia (10% O₂) for 4 wk ($n=6$ each). Scale bars, 25 μ m. **G**, Representative immunostaining pictures of RVs and quantitative analysis of capillary density in ADAMTS8^{ΔMHC} and control mice subjected to normoxia or hypoxia (10% O₂) for 4 wk ($n=6$ each). Scale bars, 25 μ m. **H**, Representative results of immunostaining for pimonidazole for detection of myocardial hypoxia in RVs. Quantitative analysis of the myocardial hypoxic area in RVs from ADAMTS8^{ΔMHC} and control mice subjected to normoxia or hypoxia (10% O₂) for 4 wk ($n=6$ each). Scale bars, 100 μ m. **I**, Real-time polymerase chain reaction (RT-PCR) analyses of *Anf*, *Bnp*, *Col3a*, and *Glut4* mRNA in RVs from ADAMTS8^{ΔMHC} and control mice subjected to normoxia ($n=6$ each) or hypoxia (10% O₂) for 4 wk ($n=12$ each). Average expression levels are normalized to *Gapdh* mRNA. Results are expressed as mean \pm SEM. * $P<0.05$. The normality assumption was tested by Shapiro-Wilk Normality Test. Comparisons of means between 2 groups were performed by unpaired Student *t* test for normally distributed samples or Mann-Whitney *U*-test for not normally distributed samples. For multiple comparisons, 2-way ANOVA for normally distributed samples followed by the Tukey HSD (honestly significant difference) method. Act, acceleration time; CO, cardiac output; ET, ejection time; RVID, RV internal diameter; and TAPSE, tricuspid annular plane systolic excursion.

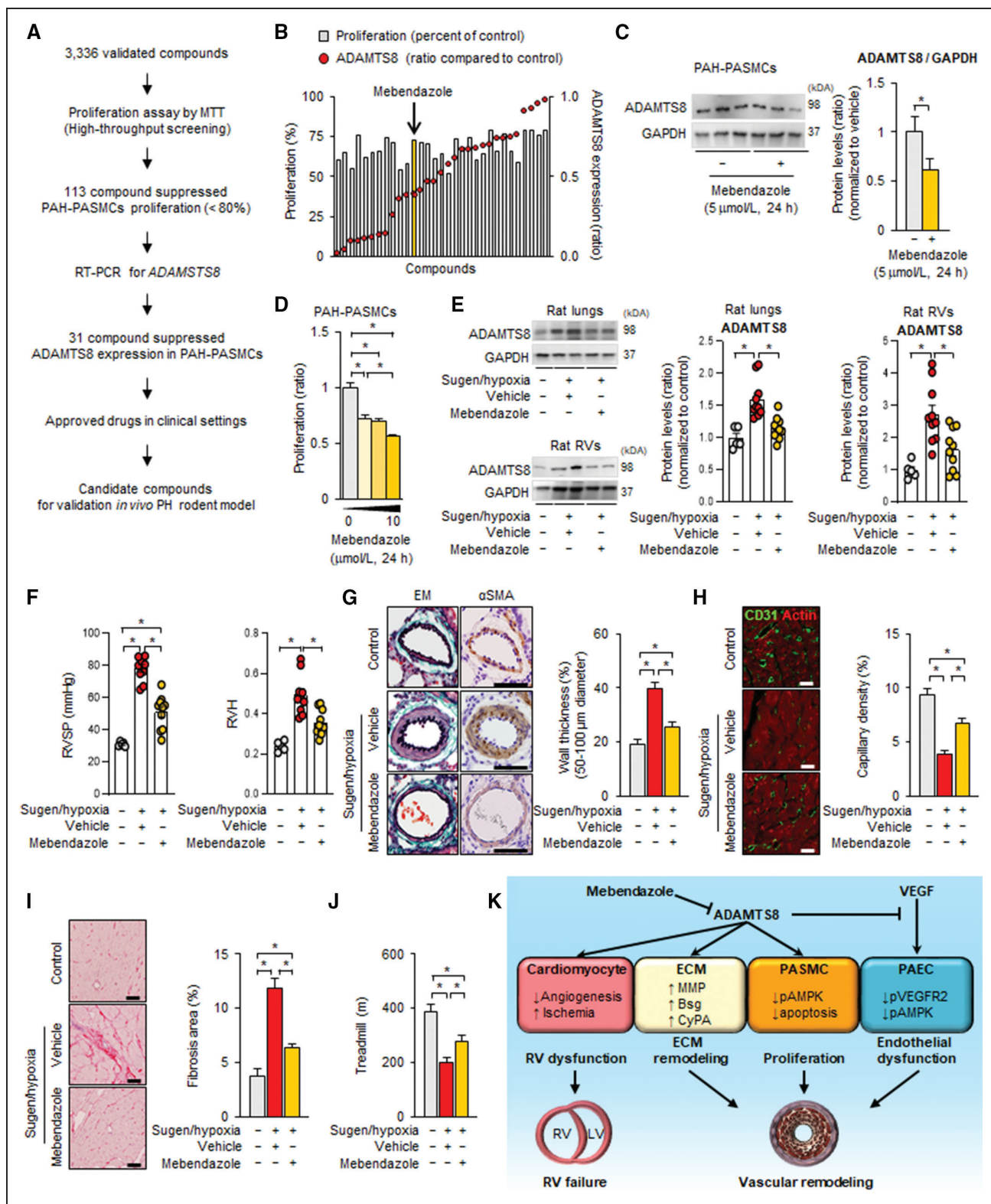


Figure 8. Mebendazole ameliorates pulmonary hypertension (PH) in animal models in vivo.

A, Schematic outline of high-throughput screening (HTS) to identify compounds that suppress *ADAMTS8* expression and proliferation in pulmonary artery smooth muscle cells (PASMCs) from patients with pulmonary arterial hypertension (PAH-PASMCs). First, we treated PAH-PASMCs with 3336 compounds (5 μmol/L each) for 48 h and performed a proliferation assay (MTT assay). We identified 113 compounds that suppressed PAH-PASMC proliferation by >20%. We then treated PAH-PASMCs with these 113 compounds (5 μmol/L each) for 4 h and measured *ADAMTS8* expression by RT-PCR using extracted total RNA, resulting in identification of 31 compounds that suppress *ADAMTS8* mRNA expression. Drugs approved in clinical settings were selected for further analysis. Finally, we examined the effects of 3 compounds in mice with hypoxia-induced PH for validation. **B**, Results for the 31 compounds that suppress PAH-PASMC proliferation (*Continued*)

AMPK and apoptosis signaling. Moreover, genetic deletion of ADAMTS8 in PSMCs reduced hypoxia-mediated mitochondrial fragmentation. Thus, ADAMTS8 induces numerous intracellular signals by activation of extracellular MMPs and resultant NOX4-mediated ROS production in PAH-PASMCs.

ADAMTS8 Causes Endothelial Dysfunction in an Autocrine/Paracrine Manner

An initial loss of PAECs as a result of environmental stresses (eg, hypoxia, inflammation, and SU5416) is recognized as the first step of vascular remodeling in the pathogenesis of PAH.^{26,29} In the present study, exogenous hrADAMTS8 downregulated the VEGF/VEGFR2 pathway in PAECs and impaired endothelial function. These results were consistent with the previous studies demonstrating that ADAMTS8 downregulates VEGF-induced angiogenesis and cell proliferation in human dermal ECs.¹⁰ ADAMTS1 has antiangiogenic properties since it binds to VEGF-A and negatively modulates VEGF function in ECs via TSP-1 motifs. Thus, it is possible that ADAMTS8 downregulates the VEGF/VEGFR2 pathway using its TSP-1 motifs. We also demonstrated that hrADAMTS8 and ADAMTS8-CM downregulated AMPK signalling in PAECs. Our data are consistent with previous reports demonstrating that AMPK in PAECs is regulated by VEGF signaling.⁵² Moreover, ADAMTS8-CM significantly increased ROS levels in PAECs and inhibited angiogenesis via increased expression of *BAX* and reduced expression of *CCND1*. It is known that AMPK plays an important role in endothelial function and vascular homeostasis through several mechanisms, including eNOS (endothelial nitric oxide synthase), antiapoptotic effect, and ROS regulation in PAECs.²⁶ Taken together, these data indicate that ADAMTS8 secreted from adjacent PSMCs induces endothelial dysfunction in an autocrine/paracrine manner through downregulation of

the VEGFR2/AMPK signaling. Our data suggest that the upregulation of ADAMTS8 in PSMCs could exacerbate the phenotype of PAH-PASMC, endothelial dysfunction, and extracellular matrix remodeling in an autocrine/paracrine manner. Hence, these integrated effects caused by the upregulation of ADAMTS8 in PSMCs will promote the pathogenesis of PAH.

ADAMTS8 Promotes RV Fibrosis and Failure

To explore the role of ADAMTS8 in the RV, we used α MHC-Cre-mediated ADAMTS8-knockout. According to the validated protein expression studies from the Human Protein Atlas portal (www.proteinatlas.org), α MHC is also expressed in skeletal muscle in addition to higher expression in the heart. There was no study which examined the role of α MHC in skeletal muscle. Additionally, there was no research showing the expression of ADAMTS8 in skeletal muscle in humans. We demonstrated that ADAMTS8 ^{α MHC} mice showed normal cardiac function and excise capacity under normoxia. These data suggest that there was no obvious influence on skeletal muscle in response to α MHC-Cre-mediated ADAMTS8-depletion under normoxia. Thus, these data indicate that ADAMTS8 ^{α MHC} mice did not show any phenotypic change under physiological conditions. The hypoxic ADAMTS8 ^{α MHC} mice showed improved RV function and reduced RVH compared with hypoxic control mice, whereas vascular remodeling and RVSP were comparable between the 2 genotypes in response to hypoxia. Indeed, by using a PA banding model, it was clearly demonstrated that both pharmacological and genetic approaches reduced RVH and improved RV function without any effects on RVSP.^{53,54} Thus, our present findings indicate that loss of ADAMTS8 in cardiomyocytes had no effect on vascular remodeling and PH, but diminished RVH and improved RV function. Furthermore, ADAMTS8 ^{α MHC} mice showed reduced capillary length

Figure 8 Continued. (gray bars) and ADAMTS8 gene expression (red plots). **C**, Representative Western blots and quantification of ADAMTS8 (a disintegrin and metalloproteinase with thrombospondin motifs 8) in PAH-PASMCs treated with mebendazole (5 μ mol/L) for 48 h ($n=6$ each). **D**, Proliferation of PAH-PASMCs cultured in 5% fetal bovine serum with mebendazole (0.5, 5, or 10 μ mol/L) for 24 h (vehicle, $n=16$; mebendazole, $n=8$ each). **E**, Representative Western blot and quantification of ADAMTS8 protein levels in the lung and RVs of rats subjected to chronic hypoxia for 21 d in combination with administration of a VEGF receptor blocker SU5416 (Sugen/hypoxia rats) followed by treatment with vehicle or mebendazole for 14 d after induction of PAH. **F**, Right ventricular (RV) systolic pressure (RVSP) and RV hypertrophy (RVH) in Sugden/hypoxia rats as a result of treatment with vehicle or mebendazole for 14 d after induction of PAH. **G**, Left, representative results of Elastica-Masson (EM) and immunostaining for α SMA (α -smooth muscle actin) of distal pulmonary arteries in the lungs from Sugden/hypoxia rats treated with vehicle or mebendazole for 14 d after induction of PAH. Right, quantitative analysis of medial wall thickness of pulmonary arteries with a diameter of 50 to 100 μ m. Scale bars, 50 μ m. **H**, Left, representative images of immunostaining of RVs from Sugden/hypoxia rats treated with vehicle or mebendazole for 14 d after induction of PAH. Right, quantitative analysis of capillary density in RVs. Scale bars, 25 μ m. **I**, Left, representative pictures of sirius red staining of the RVs from Sugden/hypoxia rats treated with vehicle or mebendazole for 14 d after induction of PAH. Right, quantitative analysis of the interstitial fibrotic area in the RVs. Scale bars, 25 μ m. **J**, Walking distance in Sugden/hypoxia rats treated with vehicle or mebendazole. **K**, Schematic representation of molecular mechanisms of ADAMTS8-mediated pulmonary vascular remodeling and RV dysfunction. $n=5$ for control rats (rats that did not receive SU5416 injection or were subjected to chronic hypoxia), $n=10$ for vehicle or mebendazole-treated rats per group. Results are expressed as mean \pm SEM. * $P<0.05$. The normality assumption was tested by Shapiro-Wilk Normality Test. Comparisons of means between 2 groups were performed by unpaired Student *t* test for normally distributed samples or Mann-Whitney *U*-test for not normally distributed samples. For multiple comparisons, 1-way ANOVA for normally distributed samples followed by the Tukey honestly significant difference method. The multiple comparison for not normally distributed samples was performed with Kruskal-Wallis test. MTT indicates 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

compared with control mice after hypoxic exposure. These results are consistent with the previous studies demonstrating that capillary length in the RV is highly correlated with RV volume in animal models of PH and PAH patients.^{55–57} Consistently, ADAMTS8^{Δ α MHC} mice showed increased capillary density after hypoxia. This may suggest a passive change in intercapillary distance due to reduced RV volume. Interestingly, ADAMTS8^{Δ α MHC} mice also showed less myocardial ischemia and GLUT4 expression in the RV. Increased expression of GLUT4 in the RV suggests a metabolic switch to glycolysis, which is a strong indicator of RV dysfunction in animal models of PH and patients with PAH.^{58,59} Here, ADAMTS8 inhibits VEGF-induced angiogenesis *in vitro*.¹⁰ Additionally, VEGF in RV was upregulated after hypoxia-induced PH in mice.⁶⁰ Moreover, VEGF-induced angiogenesis improved RV function in monocrotaline-induced PH and Sugen/hypoxia-induced PH in rats.^{61,62} In contrast, insufficient angiogenesis in the setting of RVH induces ischemia and fibrosis in RV.⁶¹ Therefore, downregulation of ADAMTS8 can exert protective effects on the RV as well. Accordingly, it is speculated that the downregulation of ADAMTS8 in the RV from wild-type mice showed the cardioprotective effect against RV dysfunction in hypoxia-induced PH model. Consequently, it is conceivable that in the cardiomyocyte-specific ADAMTS8-knockout mice, the complete depletion of ADAMTS8 in cardiomyocyte prevented its pathogenic effects on fibrotic change in the RV and resulted in the attenuation of RV failure in hypoxia-induced PH.

Mebendazole in Drug Repositioning

The traditional approach to drug discovery involves identification and validation of new molecular entities, which is a time-consuming and costly process. More than 80% of new compounds ultimately fail in human studies even if they show beneficial effects in preclinical studies. The high failure rates and costs involved in the development of new drugs led to an increased interest in drug repositioning, the process of identifying new indications for existing drugs. Using a high-throughput screening of 3336 compounds, including drugs and bioactive compounds, and further validation studies, we identified mebendazole as a potent ADAMTS8 inhibitor. Mebendazole is the most widely used drug for treating patients with helminth infestation owing to high efficacy, few side effects, and low cost. These characteristics can be beneficial if mebendazole is to be used as a novel agent for PAH therapy as an ADAMTS8 inhibitor. Additionally, we found that mebendazole ameliorated hypoxia-induced PH in mice and Sugen/hypoxia-induced PH in rats by downregulating ADAMTS8 in the lungs and the RV. Recent studies reported that mebendazole downregulates cell proliferation in several cancers.²⁸ Mebendazole is currently in clinical trials for cancer (NCT01729260

and NCT01837862), and no toxicity has been reported so far. Thus, mebendazole may be a promising agent, and a possibility of drug repositioning for use in patients with PAH needs to be explored.

Study Limitations

The present study has several limitations. First, ADAMTS8 was associated with cancer development in the previous study.⁶³ Moreover, there were several reports that demonstrated that PAH-PASMCs share several features with cancer cells, including hyperproliferation and resistance to apoptosis.^{4,59,64} Thus, ADAMTS8 could be regulated by the underlying pathological mechanism that gives cancer-like phenotype in PAH-PASMCs. Second, we used SM22 α as a driver for the tissue-specific expression in the vascular smooth muscle cells in the present study; however, it is known that SM22 α is expressed not only in the vascular smooth muscle cells but also in the myeloid cells. It is also reported that ADAMTS8 is expressed in macrophages.¹³ Myeloid cells, including neutrophils, monocytes, and macrophages, promote inflammation and exacerbate vascular remodeling in the pathogenesis of PAH. Given that the SM22 α is expressed in these cells, we need to consider the possible involvement of hematopoietic subsets, including neutrophils, monocytes, and macrophages, in the phenotype of SM22 α -Cre-mediated ADAMTS8-knockout mice under hypoxia. Third, we did not use tamoxifen-inducible models for time-specific ADAMTS8 knockout. However, ADAMTS8^{ΔSM22 α} and ADAMTS8^{Δ α MHC} mice showed normal growth and normal phenotypes under normoxia. There are no data suggesting that SM22 α or α MHC-Cre-mediated ADAMTS8-depletion created lethal phenotypes in embryos or pups. Thus, these data suggest that the role of ADAMTS8 in hypoxia-induced PH is sufficiently elucidated without tamoxifen induction model. Finally, the beneficial effects of mebendazole may involve several mechanisms other than downregulation of ADAMTS8. Mebendazole regulates several intracellular molecules, including those involved in hedgehog signaling.⁶⁵ Additionally, mebendazole suppressed vascular smooth muscle cell proliferation and attenuated neointimal formation following arterial injury in mice.⁶⁶ Thus, further study is needed to elucidate the mechanism of the therapeutic effects of mebendazole.

Clinical Implication and Conclusions

Despite the rapid progress in the therapy of PAH in the last decade, there is still no cure for this devastating disease. This is largely attributable to the fact that various cell types and diverse signaling pathways induce vascular remodeling and RV dysfunction in PAH.⁶ It is now widely accepted that epigenetic modifications are key factors in cancer and PAH following environmental stimuli (eg, hypoxia

and infection).⁶⁴ DNA methylation is a common epigenetic modification. In particular, previous studies reported methylation of the *ADAMTS8* gene in cancer cells.⁶⁷ Furthermore, epigenetic modification can be modulated by SNPs, as has been clearly illustrated in a recent study on *ADAMTS7* in coronary atherosclerosis.⁶⁸ Thus, genome screening for the *ADAMTS8* gene and *ADAMTS8*-associated genes can be beneficial for PAH patients.

In conclusion, the present study demonstrates that *ADAMTS8* promotes vascular remodeling and RV failure in PAH patients and thus could be a novel therapeutic target of the disorder, for which mebendazole may be a therapeutic agent.

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Disclosures

None.

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