

EP3 receptor deficiency attenuates pulmonary hypertension through suppression of Rho/TGF- β 1 signaling

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Pulmonary arterial hypertension (PAH) is commonly associated with chronic hypoxemia in disorders such as chronic obstructive pulmonary disease (COPD). Prostacyclin analogs are widely used in the management of PAH patients; however, clinical efficacy and long-term tolerability of some prostacyclin analogs may be compromised by concomitant activation of the E-prostanoid 3 (EP3) receptor. Here, we found that EP3 expression is upregulated in pulmonary arterial smooth muscle cells (PASCs) and human distal pulmonary arteries (PAs) in response to hypoxia. Either pharmacological inhibition of EP3 or *Ep3* deletion attenuated both hypoxia and monocrotaline-induced pulmonary hypertension and restrained extracellular matrix accumulation in PAs in rodent models. In a murine PAH model, *Ep3* deletion in SMCs, but not endothelial cells, retarded PA medial thickness. Knockdown of EP3 α and EP3 β , but not EP3 γ , isoforms diminished hypoxia-induced TGF- β 1 activation. Expression of either EP3 α or EP3 β in EP3-deficient PASCs restored TGF- β 1 activation in response to hypoxia. EP3 α/β activation in PASCs increased RhoA-dependent membrane type 1 extracellular matrix metalloproteinase (MMP) translocation to the cell surface, subsequently activating pro-MMP-2 and promoting TGF- β 1 signaling. Activation or disruption of EP3 did not influence PASC proliferation. Together, our results indicate that EP3 activation facilitates hypoxia-induced vascular remodeling and pulmonary hypertension in mice and suggest EP3 inhibition as a potential therapeutic strategy for pulmonary hypertension.

Introduction

Pulmonary arterial hypertension (PAH), a rare but often fatal disease characterized by an average pulmonary arterial (PA) pressure of greater than 25 mmHg, contributes to unacceptably high morbidity and mortality of adult and pediatric patients with lung diseases (1). PA remodeling is the pathogenic hallmark of all forms of pulmonary hypertension. Deposition of extracellular matrix (ECM), such as fibronectin and collagen, and proliferation, migration, and hypertrophy of vascular smooth muscle cells (VSMCs) result in PA hypertrophy and muscularization, leading to increased pulmonary vascular resistance in PAH (2). Current therapies such as vasodilators, endothelin receptor antagonists, and phosphodiesterase inhibitors mainly aim to relieve symptoms without significant improvements in overall

prognosis; therefore, these therapies do little to ameliorate the underlying vascular remodeling in PAH.

COX-derived prostaglandins (PGs) play an essential role in the maintenance of pulmonary vascular tone and modulation of pulmonary vascular remodeling in response to inflammatory stimulations through activation of their specific receptors (3). COX-1 is ubiquitously expressed in lung tissue, while COX-2 can be induced in the smooth muscle layer of pulmonary blood vessels by chronic hypoxia (4, 5). Disruption or knockdown of COX-2 exacerbates hypoxia- and monocrotaline-induced (MCT-induced) pulmonary hypertension and enhances the contractility of VSMCs (6, 7). In vitro, both thromboxane A₂ (TxA₂) and low concentrations of PGE₂ evoke contraction of PAs through the thromboxane (TP) and E prostanoid 3 (EP3) receptors, respectively (8–10), and the nitric oxide synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME) could augment the contractile function of PGE₂ (11). In contrast, prostacyclin analogs (prostaglandin I₂, PGI₂) induce PA relaxation, inhibit platelet aggregation, and ameliorate PA remodeling through suppression of pulmonary arterial

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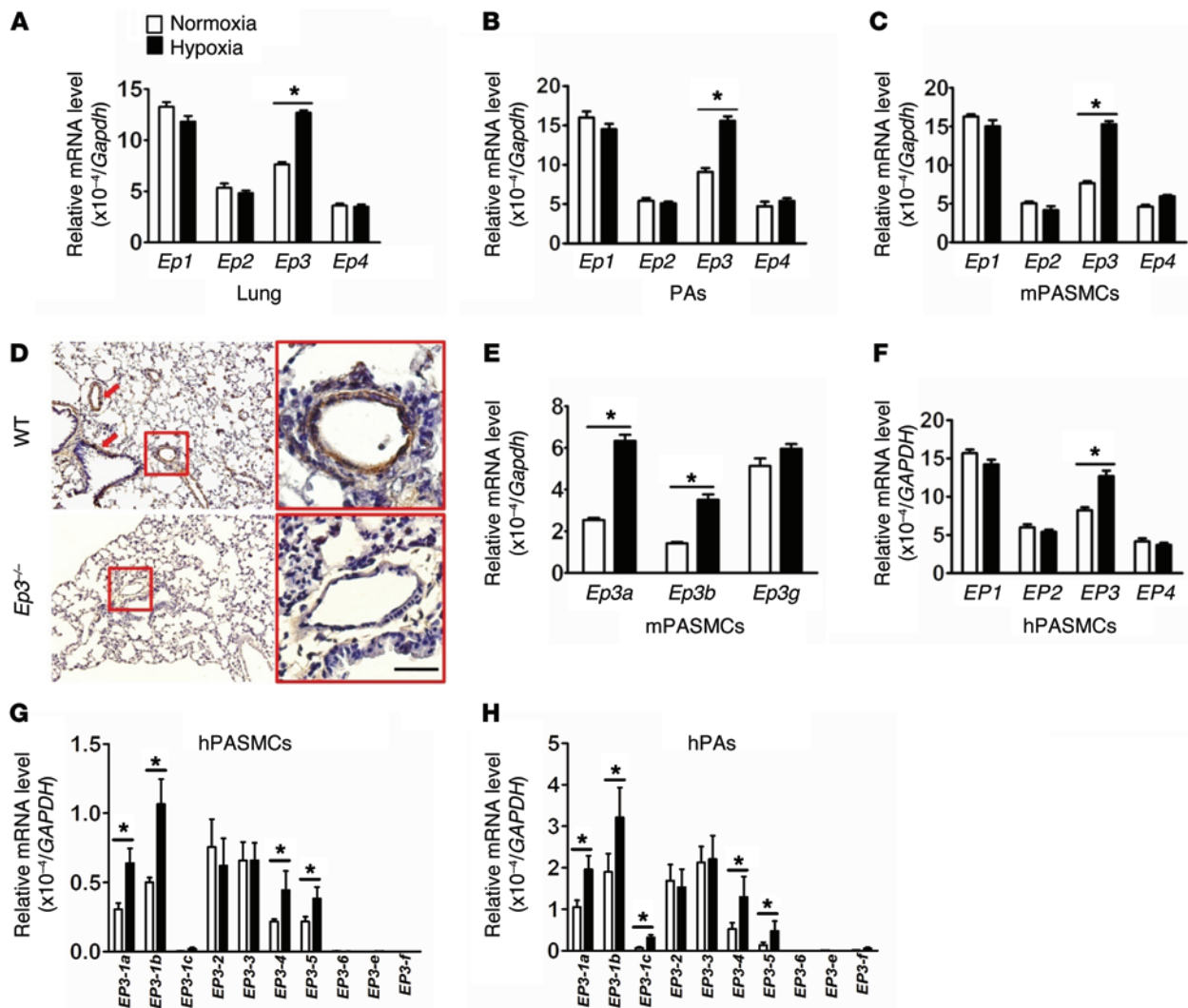


Figure 1. EP3 expression is upregulated in PASMCs in response to hypoxia. (A) Relative mRNA levels of *Ep1-4* in lung tissues from mice exposed to chronic hypoxia ($n = 6$). (B) Relative mRNA levels of *Ep1-4* in PAs of hypoxia-treated mice ($n = 6$). (C) Relative mRNA levels of *Ep1-4* in primary mPASMCs exposed to 1% O_2 ($n = 6$). (D) *Ep3* in situ hybridization in lung tissues from WT and *Ep3*^{-/-} mice. Red arrows indicate strong staining. Scale bar: 20 μ m (E) Relative mRNA levels of *Ep3* isoforms in mPASMCs treated with normoxia or hypoxia ($n = 6$). (F) Relative mRNA levels of *EP1-4* in primary hPASMCs exposed to 1% O_2 ($n = 6$). * $P < 0.05$ versus normoxia. (G) Relative mRNA levels of *EP3* isoforms in hPASMCs exposed to hypoxia. (H) Relative mRNA levels of *EP3* isoforms in human PAs exposed to hypoxia. hPASMCs and human PAs in culture were exposed to 1% O_2 for 24 hours before submission to real-time quantitative RT-PCR analysis ($n = 6$ per group). * $P < 0.05$ versus normoxia by 2-tailed Student's *t* test.

smooth muscle cell (PASMC) proliferation (12, 13). Additionally, enhanced secretion of TxA_2 and reduced secretion of PGI_2 were observed in patients with both primary and secondary pulmonary hypertension (14, 15). Blockade of TP-mediated signaling significantly suppresses the hypoxia-induced hyperactivity of the PA response to phenylephrine (16). Disruption of the I prostanoid (IP) receptor in mice results in more severe pulmonary hypertension and vascular remodeling after chronic exposure to hypoxia (17). In contrast, activation of the IP receptor by overexpression of PGI_2 synthase protects against the development of hypoxia-induced PAH in rodents (18, 19). Therefore, restoration and activation of IP signaling using analogs of PGI_2 , such as iloprost, treprostinil, and beraprost, represent effective strategies in the treatment of PAH (20). However, the clinical efficacy and safety of these analogs may be altered because of their heterogeneous affinities for

the various PG receptors. For example, in addition to the IP receptor, iloprost, treprostinil, and beraprost differentially bind to the EP receptors, including EP3 (21–26). Likewise, diminished relaxation of PAs in response to iloprost, treprostinil, and beraprost was reported in MCT-induced PAH in rats via a mechanism that involves stimulation of the contractile EP3 receptor (27, 28). Furthermore, the EP3 receptor appears to be functionally upregulated in the PAs of MCT-treated rats (28), indicating that EP3 receptor may be involved in the pathogenesis of PAH.

In this report, we demonstrate that PGE_2 generation and EP3 expression increased in both rodent and human PASMCs, in human PAs in response to hypoxia, and in PAs from mouse models of hypoxia-induced PAH. Disruption of *Ep3* attenuated both hypoxia-induced and hypoxia plus SU5416-induced (HySu-induced) PAH and pulmonary vascular remodeling in mice through

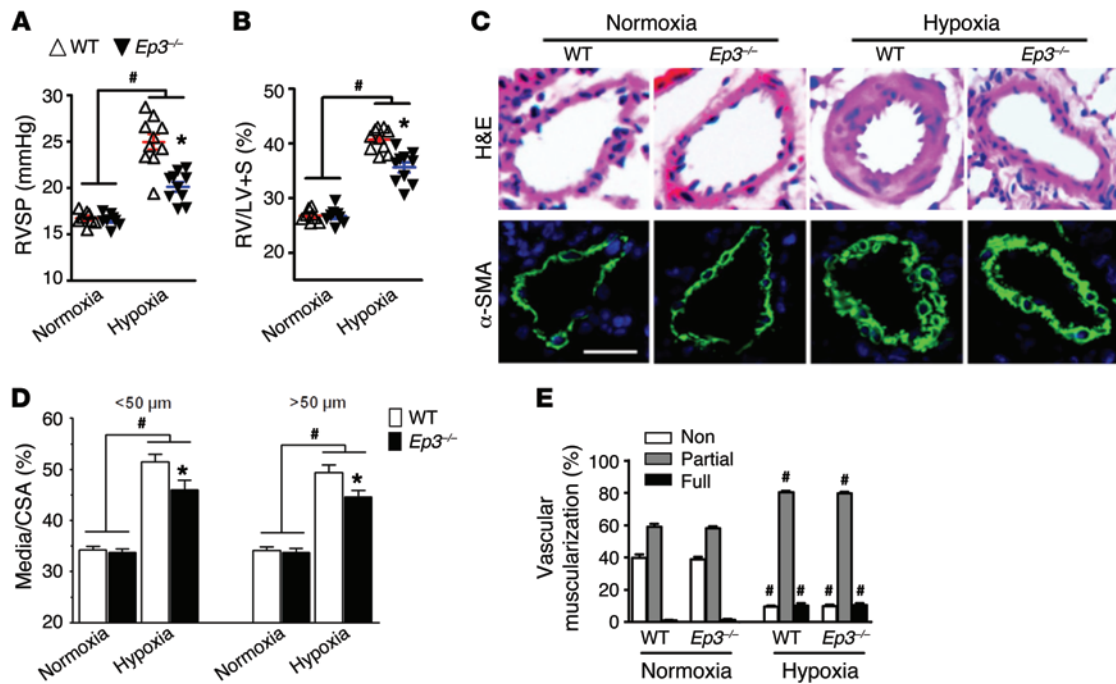


Figure 2. Absence of *Ep3* attenuates pulmonary hypertension and pulmonary vascular remodeling in chronic hypoxia-induced PAH in mice. (A) RVSP in *Ep3*^{-/-} and WT controls after exposure to hypoxia. (B) Ratio of RV/LV+S in *Ep3*^{-/-} and WT control mice after exposure to hypoxia. (C) Representative images of H&E staining and α -SMA (green) immunostaining of lung tissues from hypoxia-treated *Ep3*^{-/-} and WT mice. Scale bar: 20 μ m. (D) Quantification of the ratio of vascular medial thickness to total vessel size (Media/cross-sectional area [CSA]) for the hypoxia model. (E) Proportion of non-, partially, or fully muscularized pulmonary arterioles (20–50 μ m in diameter) from hypoxia-treated mice. (A–E) $n = 8$ –10 mice. * $P < 0.05$ versus WT and # $P < 0.05$ versus normoxic controls by 2-way ANOVA with Bonferroni's post-hoc analysis.

suppression of Rho-dependent extracellular MMP-2/TGF- β 1 signaling. More important, EP3 antagonist treatment attenuated the development of pulmonary hypertension in the MCT rat model. These observations indicate that EP3 is a potential therapeutic target for the management of PAH.

Results

EP3 is upregulated in PAs in multiple experimental PAH animal models. COX-2 expression is increased in lung tissues from PAH patients and in PAs from hypoxia-induced PAH mouse models (4, 5). As such, we also observed the substantial induction of all PG products, including PGE₂ (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI7656DS1) in lungs in response to chronic hypoxia. All PGE₂ receptors (*EPI*-4) were abundantly expressed in the lung (Figure 1A), PAs (Figure 1B), and cultured murine PSMCs (mPSMCs) (Figure 1C). Interestingly, *Ep3* expression was strikingly elevated in lungs, in PAs from chronic hypoxia-treated mice (Figure 1, A and B), and in cultured mPSMCs exposed to hypoxia (Figure 1C). In situ hybridization showed strong *Ep3* expression in vessel wall and tunica muscularis bronchiorum in the lungs (Figure 1D). All 3 mouse *Ep3* splice variants (*a*, *b*, and *g*) were coexpressed in mPSMCs, and only *Ep3a* and *Ep3b* levels significantly increased in PSMCs under hypoxic conditions (Figure 1E). Similarly, we observed EP3 upregulation induced by hypoxia in human PSMCs (hPSMCs) and human PA (hPA) tissue (Figure 1, F–H). Ten splice mRNAs and 8 isoforms of human EP3 have been identified (29, 30). Only *EP3-1a*, *EP3-1b*, *EP3-1c*, *EP3-4*, and *EP3-5* were significantly upregulated in hPAs in response to hypoxia (Figure 1, G and H).

In addition, we also detected significant induction of *EP3* expression in PDGF-BB-treated hPSMCs (Supplemental Figure 2). Taken together, *EP3* expression in PAs is upregulated in response to hypoxia, indicating that the PGE₂/EP3 axis might be involved in the progression of PAH.

Deletion of EP3 attenuates the development of both hypoxia- and HySu-induced PAH in mice. Next, we tested whether disruption of *Ep3* would modulate the progression of PAH in different murine models. After a 3-week exposure to chronic hypoxia (10% O₂), mice developed significant elevation in right ventricular systolic pressure (RVSP) (Figure 2A) and in the ratio of the weight of the free right ventricular wall to the weight of the left ventricular wall plus the septum (RV/LV+S) (Figure 2B) compared with what was observed in normoxic controls. Intriguingly, *Ep3*-deficient (*Ep3*^{-/-}) mice displayed a significant reduction in RVSP (20.14 \pm 0.50 mmHg vs. 24.98 \pm 0.84 mmHg, $P < 0.05$) (Figure 2A) and in RV/LV+S (35.60 \pm 0.92% vs. 40.67 \pm 0.64%, $P < 0.05$) (Figure 2B) compared with that in WT controls. Moreover, *Ep3* deletion attenuated pulmonary vascular remodeling induced by hypoxia through a reduction of pulmonary vascular wall thickness (Figure 2, C and D) and muscularization (Figure 2E). We then tested another classical experimental PAH model induced by HySu with more intense pulmonary vascular remodeling in *Ep3*^{-/-} mice. Consistent with the hypoxia model, *Ep3*-deficient mice displayed similar protection against the development of PAH and PA remodeling in the HySu-induced PAH model (Figure 3, A–E). However, we did not detect any significant difference in hypoxia-induced inflammatory cell infiltration around the PAs between *Ep3*^{-/-} and WT mice (Supplemental Figure 3).

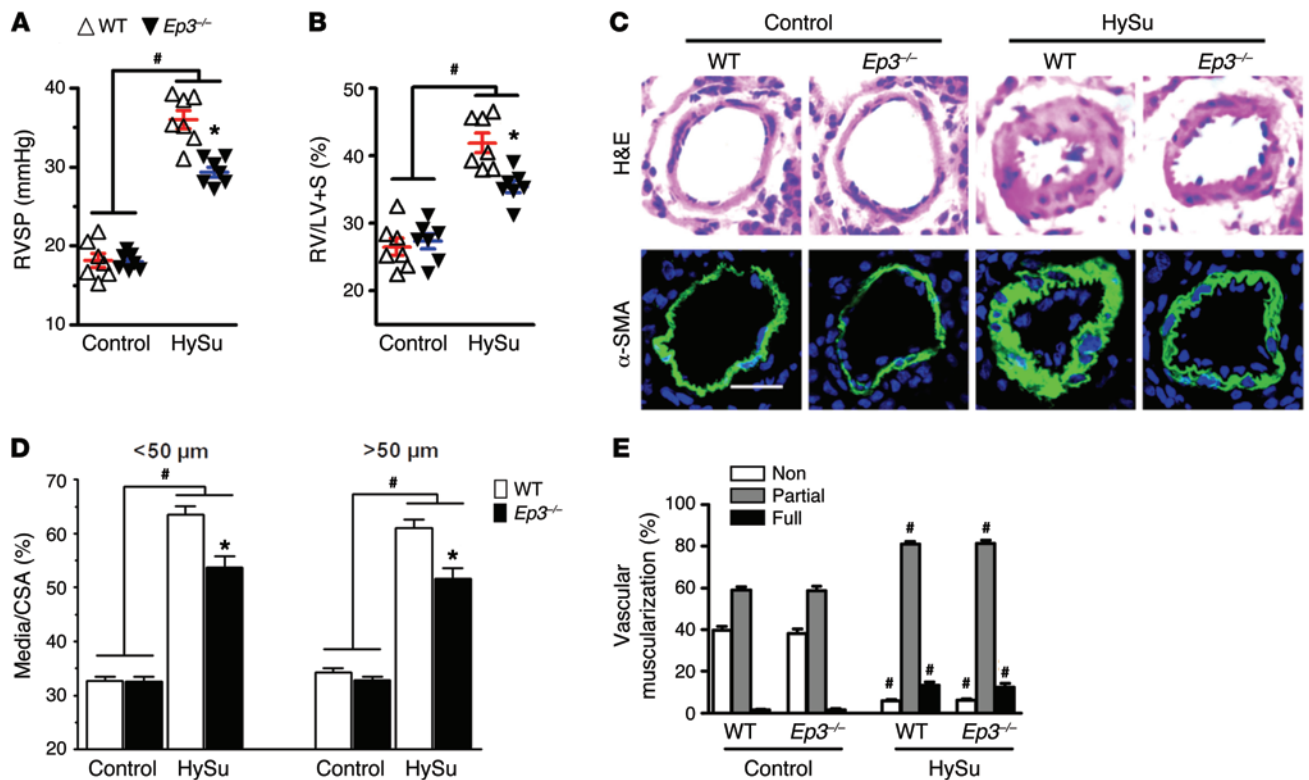


Figure 3. Disruption of EP3 ameliorates PAH and pulmonary vascular remodeling in HySu-induced PAH in mice. (A) RVSP in $Ep3^{-/-}$ and WT mice after HySu treatment. (B) RV/LV+S in $Ep3^{-/-}$ and WT mice after HySu treatment. (C) Representative images of H&E staining and α -SMA (green) immunostaining of lung sections from HySu-treated $Ep3^{-/-}$ and WT mice. Scale bar: 20 μ m. (D) Quantification of the ratio of vascular medial thickness to total vessel size for the HySu treatment model. (E) Proportion of non-, partially, or full muscularized pulmonary arterioles (20–50 μ m in diameter) from HySu-treated mice. (A–E) $n = 7$ mice. * $P < 0.05$ versus WT and # $P < 0.05$ versus control by 2-way ANOVA with Bonferroni's post-hoc analysis.

To further dissect the role of the EP3 receptor in endothelial cells (ECs) or VSMCs in the pathogenesis of PAH, EC $Ep3^{-/-}$ ($Ep3^{fl/fl}$ *Tie2-Cre*, Supplemental Figure 4A) and VSMC $Ep3^{-/-}$ ($Ep3^{fl/fl}$ *Sm22-Cre* Supplemental Figure 4A) mice were subjected to HySu treatment. Again, $Ep3$ mRNA was expressed in both ECs and VSMCs of the flox control mice, but was diminished in ECs from $Ep3^{fl/fl}$ *Tie2-Cre* mice and in VSMCs from $Ep3^{fl/fl}$ *Sm22-Cre* mice (Supplemental Figure 4B). We observed that $Ep3$ ablation in VSMCs, but not in ECs, ameliorated PAH development in mice by reducing RVSP, RV/LV+S, and PA remodeling (Supplemental Figure 4, C–F).

Pharmacological blockade of EP3 suppresses progression of MCT-induced pulmonary hypertension and pulmonary vascular remodeling in rats. We then explored the therapeutic effect of the selective EP3 inhibitor L-798106 on pulmonary vascular remodeling in a model of established pulmonary hypertension induced by MCT in rats. As shown in Supplemental Figure 5A, L-798106 reaches peak plasma concentration after 1 hour when administered as a single peritoneal injection. It exhibits linear proportional pharmacokinetics over the effective and safe dose range (30–1,000 nM) (31, 32), and the elimination half-life ($t_{1/2}$) is approximately 8 hours (Supplemental Figure 5A). A dose of 200 mg/kg L-798106 was administered to the MCT-treated rats twice a day at the beginning of the third week after MCT treatment (Supplemental Figure 5B). As anticipated, MCT-treated rats displayed much higher RVSP, RV/LV+S ratios, and thickness of pulmonary vascular walls 3 weeks after injection, indicating that MCT-in-

duced PAH models were established (Figure 4, A–F). Analogous to the genetic deficiency of $Ep3$ (Figures 2 and 3), L-798106 administration significantly reduced the augmented RVSP, RV/LV+S ratio, and pulmonary vascular wall thickness in MCT-treated rats (Figure 4, A–F). These results indicate that a selective EP3 inhibitor can alleviate the progression of pulmonary vascular remodeling of established pulmonary hypertension.

EP3 mediates accumulation of ECM proteins in PAs through activation of TGF- β 1 signaling in response to hypoxia. Pathological lesions in PAH patients, including neomuscularization and fibrotic changes (ECM deposition), predominantly affect the distal pulmonary arterioles (33). Immunostaining for α smooth muscle actin (α -SMA) demonstrated that there was no significant difference in neomuscularization of distal pulmonary arterioles, as calculated by α -SMA⁺ cells, between $Ep3^{-/-}$ mice and their littermate controls after exposure to hypoxia (Figure 2 and Supplemental Figure 6A). We then explored whether the EP3 receptor mediates PASM proliferation in response to hypoxia. As shown in Supplemental Figure 6, B–D, neither genetic deletion nor knockdown nor pharmacological activation of the EP3 receptor significantly influenced the proliferation of mPASCs or hPASCs. Consistently, in hypoxia- and MCT-induced PAH models, PCNA⁺ PASCs were not altered by $Ep3$ deletion or inhibition (Supplemental Figure 6, E–H). However, the accumulation of ECM proteins (collagen I, fibronectin, and tenascin C) induced by chronic hypoxic stress was significantly reduced in

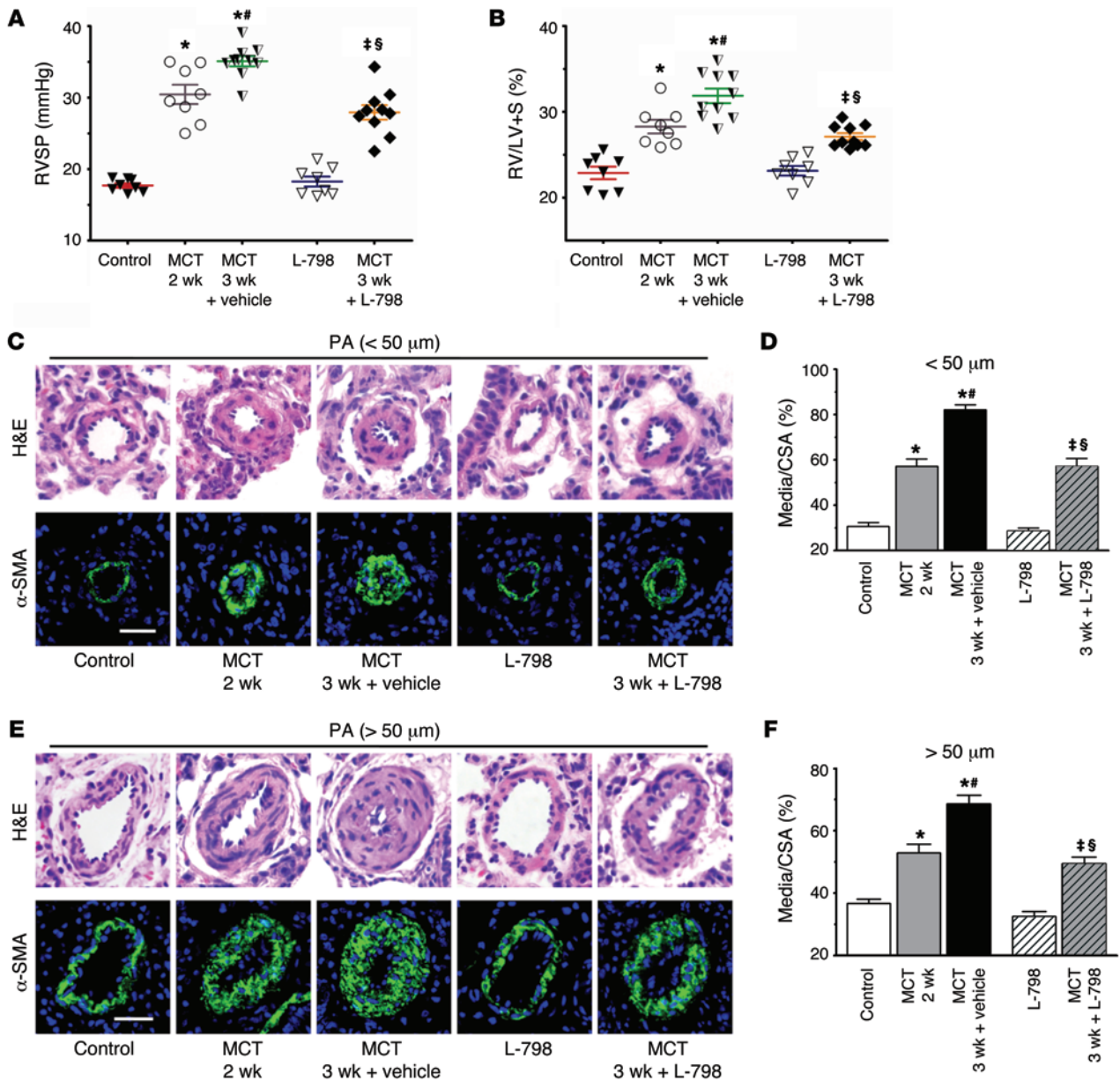


Figure 4. Inhibition of EP3 alleviates progression of MCT-induced PAH in rats. (A and B) Effect of L-798106 administration on RVSP and RV/LV+S of MCT-induced PAH rats. (C) Representative images of H&E staining and α -SMA (green) immunostaining of PAs (20–50 μ m in diameter). (D) Quantification of vascular medial thickness for images in C. (E) Representative images of H&E staining and α -SMA immunostaining of PAs (51–100 μ m in diameter). (F) Quantification of vascular medial thickness for images in E. Scale bars: 20 μ m. $n = 8$ –10/group. * $P < 0.05$ versus control; ** $P < 0.05$ versus MCT treatment after 2 weeks; § $P < 0.05$ versus L-798106; § $P < 0.05$ versus MCT treatment for 3 weeks plus vehicle. Data were calculated by 2-tailed Student's t test or 1-way ANOVA with Bonferroni's post-hoc analysis as appropriate. L-798, L-798106.

PAs of *Ep3*-deficient mice (Figure 5, A–C), and this reduction was reflected at the mRNA transcriptional level in lung tissues (Figure 5, D–G) and by Masson's trichrome staining in hypoxia- and MCT-treated animals (Supplemental Figure 7). Likewise, we observed a significant reduction of both mRNA and protein expression levels of fibronectin, collagen I, and tenascin C in *Ep3*^{-/-} mPASCs under hypoxic conditions compared with levels in WT mPASCs (Supplemental Figure 8).

Since a notable reduction of ECM deposition was observed in PAs from *Ep3*^{-/-} PAH mice, we next determined whether TGF- β 1 signaling is altered in *Ep3*^{-/-} PASCs in response to

hypoxia. Both bone morphogenetic proteins (BMPs) and TGF- β isoforms that mediate signaling are believed to be involved in PA remodeling in PAH (34). Consistent with previous reports (35, 36), we observed that hypoxia suppressed the expression of the inhibitor of differentiation 1 (ID1) (Supplemental Figure 9A), a downstream target of the BMP receptor 2 pathway, and hypoxia induced the expression of PAI-1, a target of TGF- β 1 signaling (Supplemental Figure 9B), in mPASCs. Meanwhile, *Ep3* deletion attenuated hypoxia-stimulated PAI-1 expression without significant influence on ID1 expression, indicating that the EP3 receptor may modulate TGF- β 1 signaling in mPASCs.

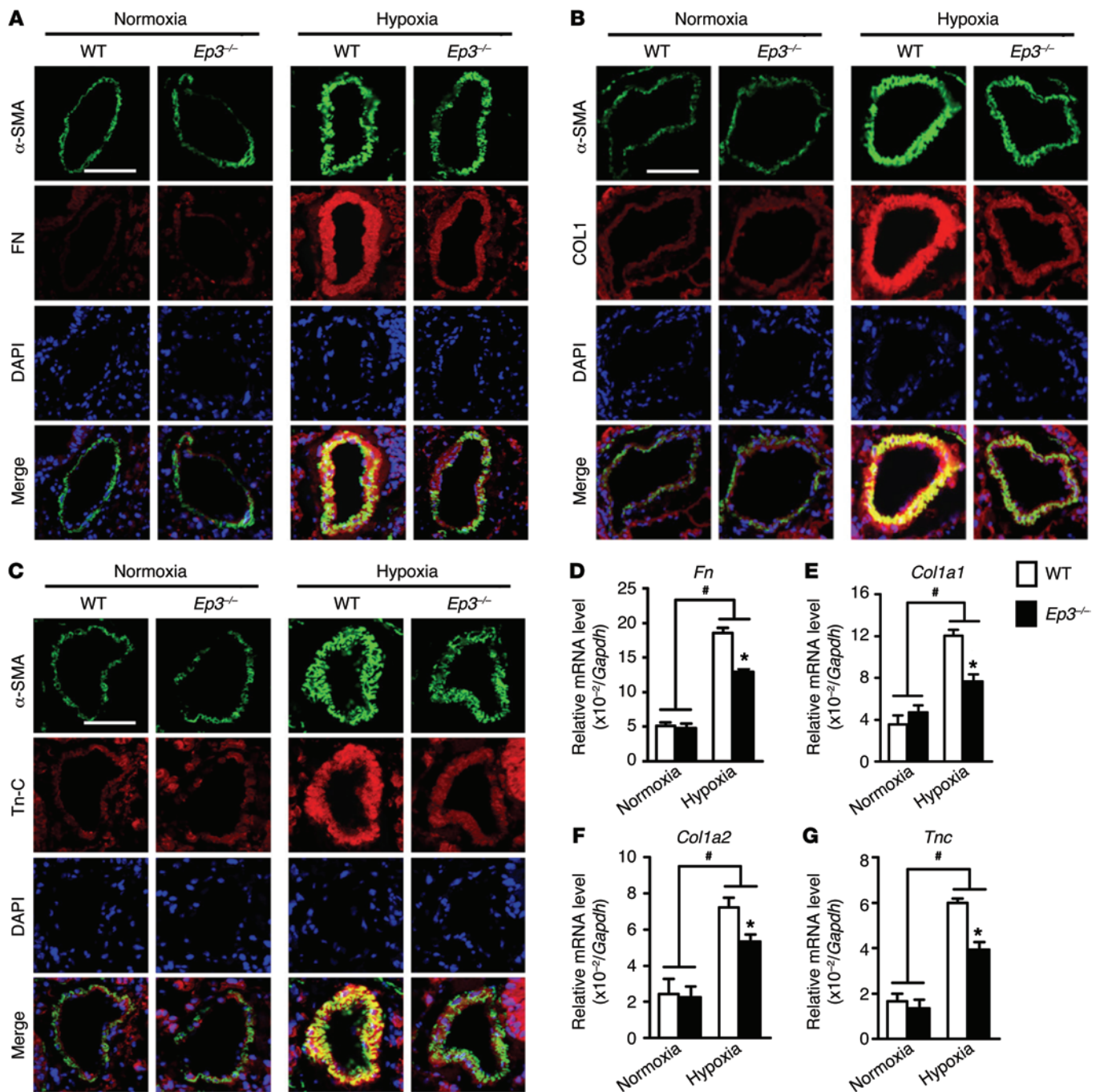


Figure 5. *EP3* mediates accumulation of ECM proteins in PAs in response to hypoxia. (A–C) Immunofluorescence staining (red) of fibronectin (FN), collagen I (COL1), and tenascin C (Tn-C). Slides were double stained with α -SMA (green) and counterstained with DAPI (blue) for nuclei. Scale bars: 20 μ m. (D–G) Relative mRNA levels of *Fn*, collagen 1 α 1 (*Col1a1*), collagen 1 α 2 (*Col1a2*), and Tn-C (*Tnc*) in lungs from normoxia- or hypoxia-treated *Ep3*^{-/-} and WT mice ($n = 4$). * $P < 0.05$ versus WT and # $P < 0.05$ versus normoxia by 2-way ANOVA with Bonferroni's post-hoc analysis.

ELISA and Western blot analyses showed that the levels of culture-active TGF- β 1 and phosphorylation of SMAD2/3 were depressed in hypoxia-stimulated *Ep3*^{-/-} PASCs (Figure 6, A–D). PDGF-BB can activate TGF- β 1 signaling and drive collagen synthesis in PASCs (36). We then examined the effect of *Ep3* deletion on PDGF-BB-induced TGF- β 1 signaling in mPASCs. Again, *Ep3* disruption suppressed the induction of active TGF- β 1 in culture medium, suppressed cellular phospho-

rylation of SMAD2 stimulated by PDGF-BB in mPASCs (Supplemental Figure 10, A and B), and restrained both protein and mRNA expression of the TGF- β 1 downstream targets fibronectin, collagen I, and tenascin C (Supplemental Figure 10, C–G). Accordingly, in PAs from chronic hypoxia-treated *Ep3*^{-/-} mice, SMAD2 phosphorylation levels also decreased (Figure 6E), further supporting the conclusion that EP3-mediated TGF- β 1 activation contributes to PA remodeling in PAH.

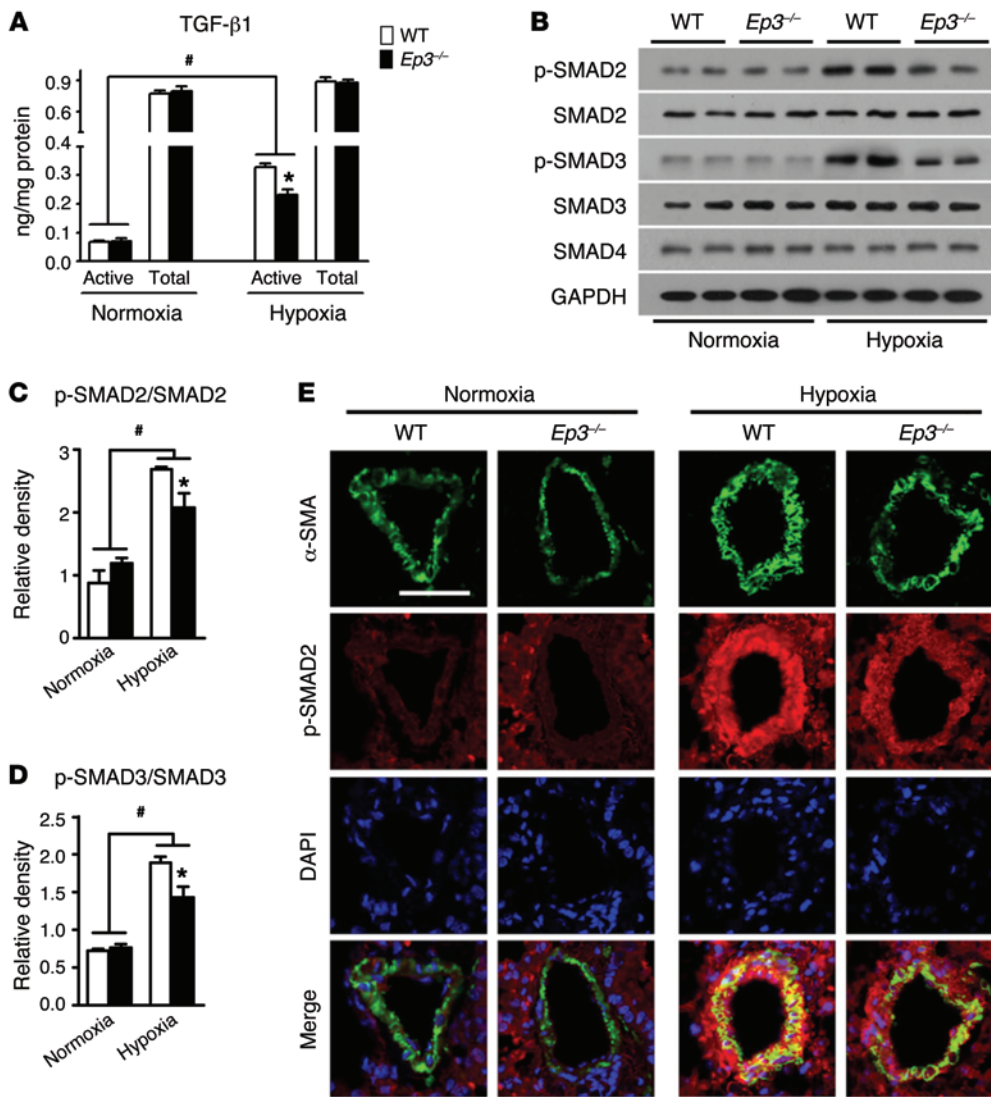


Figure 6. *Ep3* deficiency suppresses TGF- β 1 signaling pathway in hypoxia-treated PASCs and PAs. (A) Content of active TGF- β 1 and total TGF- β 1 in *Ep3*^{-/-} and WT PASC medium after normoxia or hypoxia treatment. (B) Western blot analysis of phosphorylated (p-) SMAD2/3 in *Ep3*^{-/-} and WT PASCs after normoxia or hypoxia treatment. (C and D) Quantification of SMAD2 and SMAD3 phosphorylation ($n = 6$). * $P < 0.05$ versus WT and # $P < 0.05$ versus normoxia by 2-way ANOVA with Bonferroni's post-hoc analysis. (E) Immunofluorescence staining of p-SMAD2 (red) and α -SMA (green) of lung sections from normoxia- or hypoxia-treated *Ep3*^{-/-} and WT mice. Scale bar: 20 μ m.

Both *Ep3a* and *Ep3b*, but not *Ep3g*, mediate activation of TGF- β 1 signaling in the PASC response to hypoxia. By using common *Ep3* siRNA (si*Ep3*) (31), *Ep3* expression was reduced by 75% in primary mPASCs, resulting in an approximately 40% to 50% reduction in transcription of fibronectin and collagen 1, suppression of latent TGF- β 1 activation, and suppression of SMAD2 phosphorylation in the PASC response to hypoxia (Figure 7, A-C). Since all 3 *Ep3* splice variants are expressed in PAs and primary mPASCs (Figure 1E), we next evaluated the EP3 variant that mediates TGF- β 1 signaling activation triggered by hypoxia. PASCs from WT mice were subjected to transfection with siRNA specifically designed for each variant. We observed that knockdown of *Ep3a* and *Ep3b* reduced active TGF- β 1 content in the culture medium and intracellular phosphorylation of SMAD2 and suppressed hypoxia-induced expression of fibronectin and collagen I at both mRNA and protein levels in PASCs (Figure 8, A-F). However, silencing of *Ep3g* did not significantly influence TGF- β 1 signaling in PASCs (Figure 8, A-D, and G).

Next, we sought to determine whether reexpression or overexpression of *Ep3* variants could restore the activation of TGF- β 1 signaling in PASCs. Primary PASCs isolated from *Ep3*^{-/-} mice

were transfected with murine *Ep3a*, *Ep3b*, and *Ep3g* cDNAs that were HA tagged at the extracellular N terminus. Overexpression efficiency was determined by real-time PCR 24 hours after transfection (Supplemental Figure 11A). Reexpression of *Ep3a* and *Ep3b* in PASCs rescued hypoxia-induced activation of TGF- β 1 signaling, as evidenced by increased phosphorylation of SMAD2 and upregulation of fibronectin and collagen I (Supplemental Figure 11, B-G), whereas *Ep3g* overexpression showed very little influence on TGF- β 1 signaling in PASCs (Supplemental Figure 11, B-E, and H). Taken together, these results indicate that both *Ep3a* and *Ep3b*, but not *Ep3g*, mediate activation of TGF- β 1 signaling in response to hypoxia.

Ep3a/b mediates RhoA/Rho-associated kinase-dependent activation of MMP-2. TGF- β 1 is synthesized and secreted into the ECM as an inactive latent precursor that is composed of an N-terminal latency-associated peptide (LAP) and a C-terminal mature TGF- β 1. Proteolytic cleavage of LAP is required for activation of TGF- β 1. Many factors such as an acidic microenvironment, thrombospondin 1 (Tsp-1), integrin, and MMP-2/9 can activate TGF- β 1 (37-39). Interestingly, MMP-2 expression in cultured mPASCs and PAs was depressed in *Ep3*^{-/-} mice under hypoxic

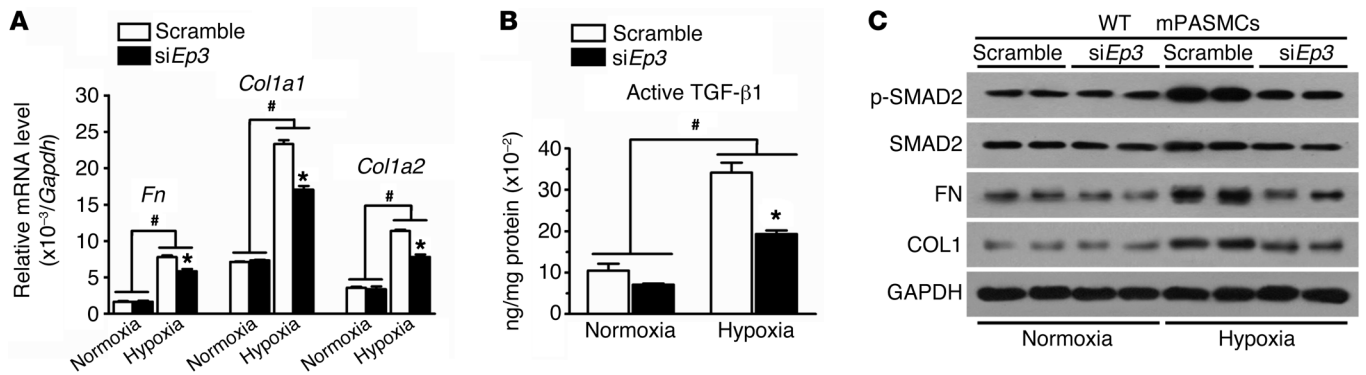


Figure 7. Knockdown of *Ep3* attenuates hypoxia-induced TGF-β1 activation in PASCs. (A) Effect of *Ep3* knockdown on mRNA levels of *Fn*, *Col1a1*, and *Col1a2* in normoxia- or hypoxia-treated PASCs. (B) Effect of *Ep3* knockdown on active TGF-β1 content in culture medium of normoxia- or hypoxia-treated PASCs (n = 4). *P < 0.05 versus scramble and #P < 0.05 versus normoxia by 2-way ANOVA with Bonferroni's post-hoc analysis. (C) Effect of *Ep3* knockdown on TGF-β1 signaling in normoxia- or hypoxia-treated PASCs.

stress (Supplemental Figure 12, A and B). Furthermore, we were unable to detect significant alternations of expression of other TGF-β1 activators (Tsp-1, αV integrin, and MMP-9) in *Ep3*^{-/-} cells or controls (Supplemental Figure 12). A gelatin zymographic assay also showed reduced MMP-2 activity in the culture of hypoxia-treated *Ep3*^{-/-} PASCs (1.68 ± 0.07 AU versus WT, 2.47 ± 0.12 AU; P < 0.05) (Supplemental Figure 13A and Figure 9A) and in the PAs of *Ep3*^{-/-} mice subjected to chronic hypoxia (2.24 ± 0.10 AU versus WT, 3.09 ± 0.12 AU; P < 0.05) (Supplemental Figure 13B and Figure 9B). Accordingly, overexpression of *Ep3a* and *Ep3b* significantly elevated hypoxia-induced MMP-2 activation in the culture of mPASCs (*Ep3a*, 3.970.26 AU; *Ep3b*, 3.87 ± 0.12 AU; vs. vector, 2.21 ± 0.06 AU; P < 0.05) (Supplemental Figure 13C and

Figure 9C), whereas overexpression of *Ep3g* in PASCs failed to significantly alter MMP-2 activity. Thus, activation of both *Ep3a* and *Ep3b* variants promotes gene transcription and secretion of MMP-2 in PAs in response to a chronically hypoxic environment.

The EP3 receptor modulates multiple intracellular signaling pathways by coupling different types of heterotrimeric G proteins, including G_{os}, G_{ai}, and G_{ai2/13} (29). To further examine the underlying mechanisms involved in the regulation of EP3-mediated TGF-β1 signaling in PASCs, the following treatments were applied to PASCs overexpressing EP3α/β: wortmannin, a phosphatidylinositol 3-kinase inhibitor; pertussis toxin (PTX), a G_{ai} protein blocker; H-89, an inhibitor of G_{os} protein downstream cAMP-dependent protein kinase (PKA); and Y-27632, a

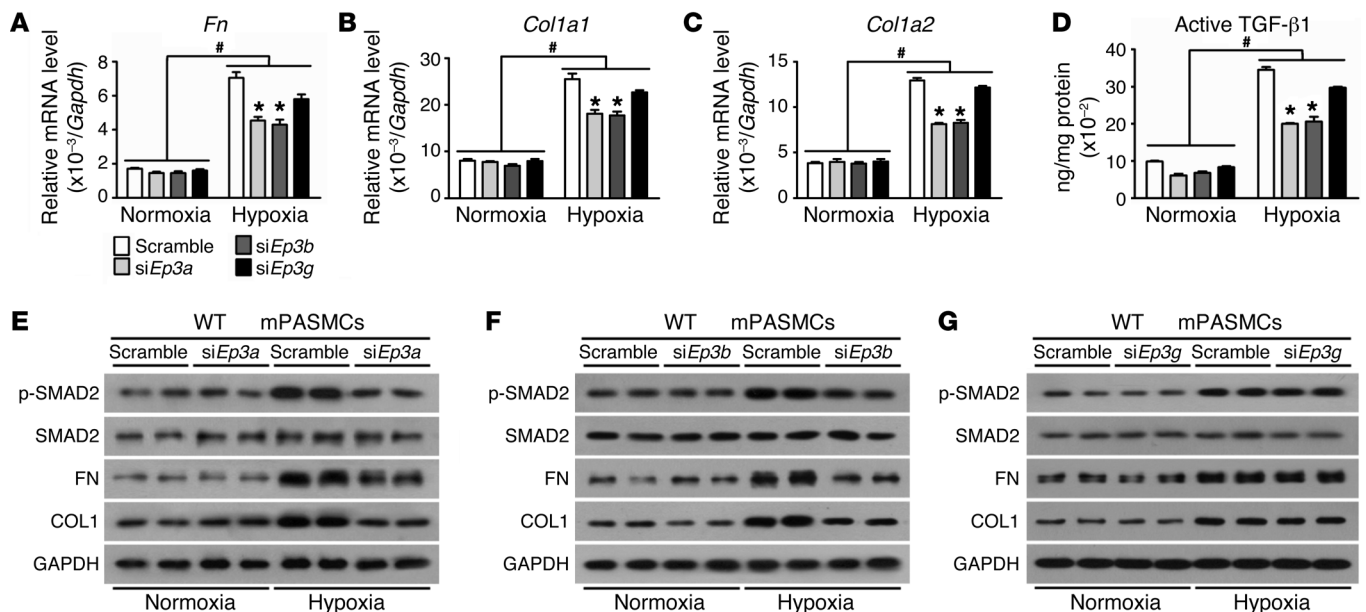


Figure 8. Knockdown of *Ep3a/b* variants attenuates hypoxia-induced TGF-β1 activation in PASCs. (A–C) Effect of single *Ep3a/b/g* variant knockdown on mRNA expression of *Fn*, *Col1a1*, and *Col1a2* in normoxia- or hypoxia-treated PASCs. (D) Effect of single *Ep3a/b/g* variant knockdown on active TGF-β1 content in culture medium of normoxia- or hypoxia-treated PASCs (n = 4). *P < 0.05 versus scramble and #P < 0.05 versus normoxia by 2-way ANOVA with Bonferroni's post-hoc analysis. (E–G) Western blot analysis of p-SMAD2, FN, and collagen I protein in PASCs transfected with siRNA specifically designed for *Ep3a* (E), *Ep3b* (F), and *Ep3g* (G), respectively.

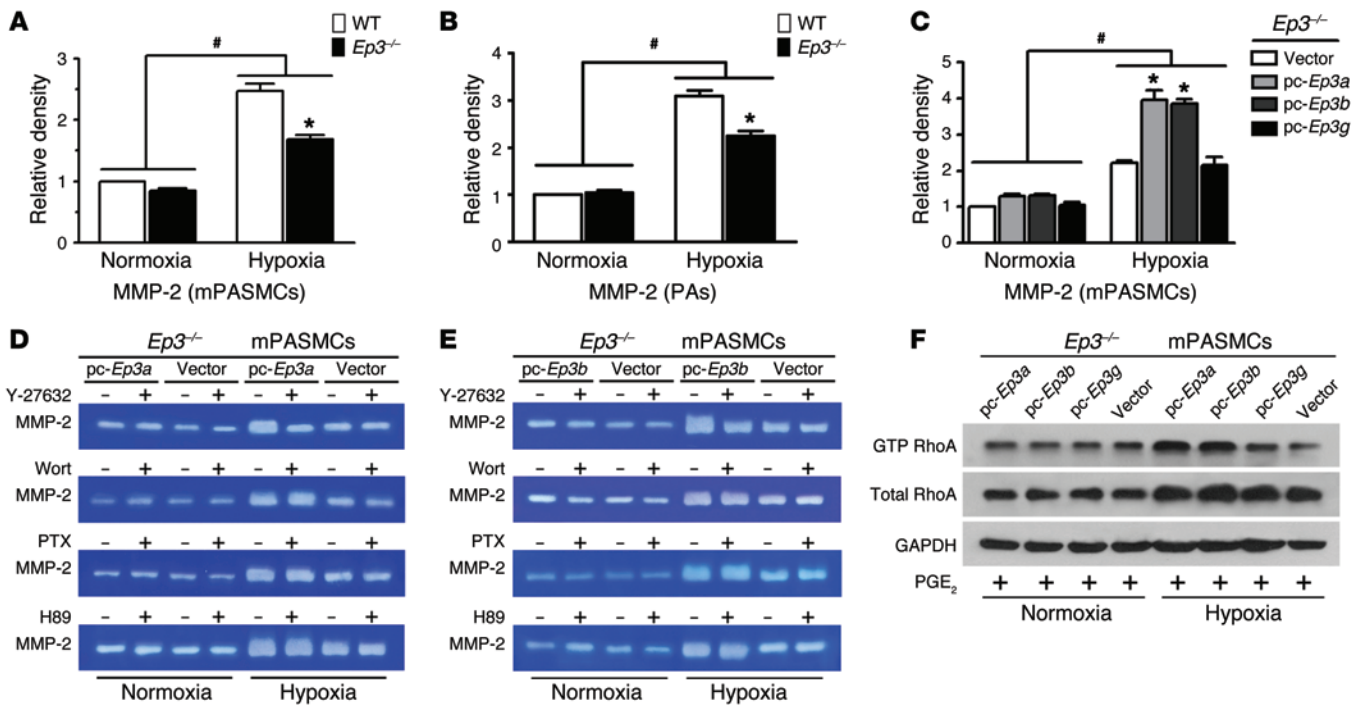


Figure 9. *Ep3a/b* variants mediate RhoA/ROCK-dependent activation of extracellular MMP-2 in cultured mPASCs. (A) Culture MMP-2 activity of PASCs from *Ep3^{-/-}* and WT mice. (B) MMP-2 activity in PAs from *Ep3^{-/-}* and WT mice after normoxia or hypoxia treatment ($n = 6$). $*P < 0.05$ versus WT and $*P < 0.05$ versus normoxia by 2-way ANOVA with Bonferroni's post-hoc analysis. (C) Culture MMP-2 activity in *Ep3*-deficient PASCs reexpressing *Ep3a*, *Ep3b*, or *Ep3g* ($n = 3$). $*P < 0.05$ versus vector and $*P < 0.05$ versus normoxia by 2-way ANOVA with Bonferroni's post-hoc analysis. Effect of Y-27632, wortmannin (Wort), PTX, and H89 on culture MMP-2 activity in *Ep3*-deficient PASCs with forced expression of *Ep3a* (D) or *Ep3b* (E). (F) Western blot analysis of active RhoA in *Ep3*-deficient PASCs with forced expression of *Ep3a*, *Ep3b*, or *Ep3g* in the presence of PGE_2 . pc, pcDNA3.1 vector.

Rho-associated kinase (ROCK) inhibitor. The elevated activity of MMP-2, induced by overexpression of both *Ep3a* and *Ep3b* in PASCs under hypoxic conditions, was greatly decreased by pretreatment with the ROCK inhibitor Y-27632 (Figure 9, D and E), while no significant effects were detected using wortmannin, PTX, or H-89 (Figure 9, D and E). Meanwhile, active RhoA levels were notably increased in *Ep3a/b*-overexpressing PASCs compared with those in controls (Figure 9F), which further supports the notion that *Ep3a/b*-mediated activation of MMP-2 is RhoA/ROCK dependent.

Ep3a/b modulates hypoxia-induced MMP-2/TGF- β 1 activation by facilitating membrane translocation of MT1-MMP via Rho/ROCK-dependent actin remodeling. Activation of RhoA/ROCK signaling is known to be involved in the regulation of actin cytoskeletal remodeling (40), which has recently been recognized to mediate the trafficking of membrane type 1-MMP (MT1-MMP) to the cell surface (41–43). MT1-MMP is a transmembrane protease that cleaves extracellular pro-MMP-2. On the basis of the reduced extracellular MMP-2 activity and reduced Rho/ROCK signaling observed in *Ep3^{-/-}* PASCs under hypoxic conditions, we hypothesized that EP3 mediates intracellular MT1-MMP movement toward the membrane and subsequently activates the MMP-2/TGF- β 1 signaling pathway by modulating Rho/ROCK activity in PASCs. Upon hypoxic stimulation, MT1-MMP protein moved to the cell membrane (Supplemental Figure 14A). *Ep3^{-/-}* PASCs displayed much less membrane MT1-MMP but more MT1-MMP in the cytoplasm in response to hypoxia compared with that in

WT controls (Supplemental Figure 14, A and B). In contrast, complementation of *Ep3a* and *Ep3b*, but not *Ep3g*, rescued the levels of membrane-localized MT1-MMP in mPASCs in response to hypoxia (Supplemental Figure 14, C and D). Thus, these results suggest that PGE_2 -EP3 α/β invokes MMP-2 activation through the regulation of MT1-MMP membrane translocation.

Next, we sought to determine whether movement of MT1-MMP regulated by the EP3 receptor is dependent on RhoA/ROCK-mediated actin polymerization. Consistent with reduced RhoA activity (Figure 9F), polymerization of F-actin was also decreased in *Ep3^{-/-}* PASCs exposed to hypoxia, as evidenced by rhodamine phalloidin staining (Figure 10A). Reexpression of either *Ep3a* or *Ep3b* in *Ep3^{-/-}* PASCs restored polymerization of F-actin (Figure 10B), which could be attenuated by the specific Rho inhibitor C3 transferase (C3), the ROCK inhibitor Y-27632, and the actin polymerization inhibitor latrunculin B (LB) (Figure 10B). We further measured and quantified the relative levels of monomeric (globular) versus polymeric (filamentous) forms of actin in PASCs under hypoxic conditions. In agreement with immunofluorescence observations, reexpression of *Ep3a/b* in PASCs led to an augmented ratio of F-actin to G-actin, which, likewise, was also suppressed by pretreatment with C3, Y-27632, or LB (Supplemental Figure 15 and Figure 10C). These results indicate that *Ep3a/b* mediates a significant shift toward filamentous stress fiber formation (F-actin polymerization). Similarly, induction of MT1-MMP protein, phosphorylation of SMAD2, and expression of fibronectin and collagen I in PASCs, all of

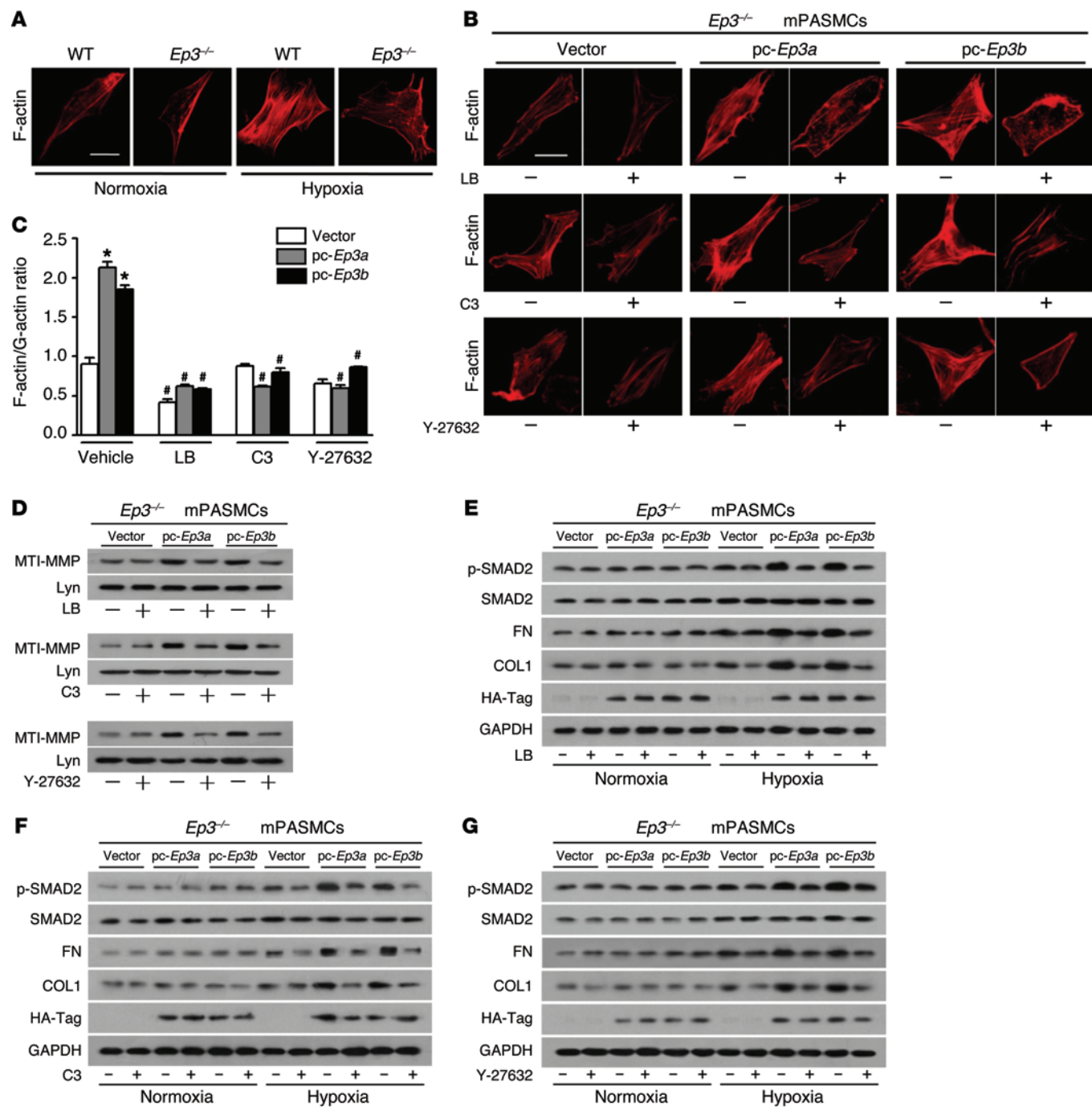


Figure 10. *Ep3a/b* variants mediate membrane localization of MT1-MMP through RhoA/ROCK-dependent cytoskeletal remodeling. (A) Rhodamine phalloidin staining of F-actin in *Ep3*^{-/-} and WT PASCs after normoxia or hypoxia exposure. (B) Effect of LB, C3, and Y-27632 on F-actin polymerization in *Ep3*-deficient PASCs with forced expression of *Ep3a* or *Ep3b*. (C) Quantification of F-actin/G-actin ratio in hypoxia-treated, *Ep3a/b*-complemented PASCs in the presence or absence of LB, C3, or Y-27632 (*n* = 3). **P* < 0.05 versus vector and #*P* < 0.05 versus vehicle by 2-way ANOVA with Bonferroni's post-hoc analysis. (D) Immunoblot analysis of MT1-MMP membrane expression in *Ep3a/b*-overexpressing PASCs with or without pretreatment with LB, C3, or Y-27632. (E-G) Immunoblot analyses of p-SMAD2, fibronectin, and collagen I in *Ep3a/b*-complemented PASCs with or without pretreatment with LB, C3, or Y-27632.

which were provoked by *Ep3a/b* overexpression, were completely blunted by pretreatment with C3, Y-27632, or LB (Figure 10, D-G). Thus, these data indicate that *Ep3a/b* variants regulate MT1-MMP movement toward the membrane to activate MMP-2/TGF-β1 signaling in PASCs through Rho/ROCK-dependent cytoskeletal remodeling.

Ep3a/b variants are coupled via G_{α12} to activate Rho/ROCK/MMP-2/TGF-β1 signaling in PASCs in response to hypoxia. Evidence indicates that EP3-mediated Rho/ROCK activation occurs through coupling to G_{α12/13} (44, 45). To determine whether the G protein interaction occurs in PASCs, HA-tagged *Ep3a* or *Ep3b* was introduced into cultured PASCs. Coimmunoprecipita-

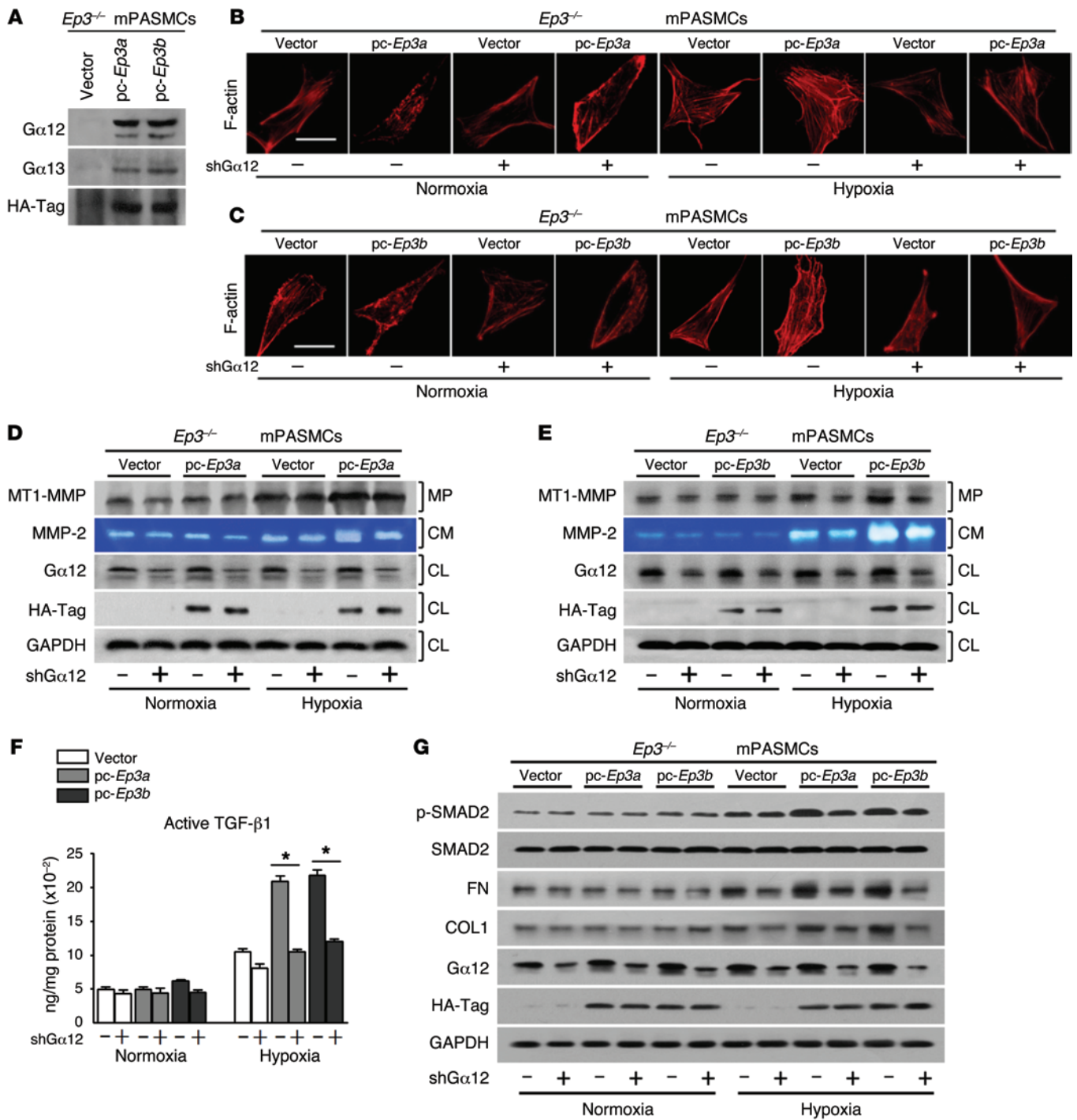


Figure 11. *Ep3a/b* mediates ROCK/MT1-MMP/MMP-2/TGF-β1 signaling in PASCs via $G_{\alpha 12}$ upon hypoxic stress. (A) Western blot analysis of *Ep3a/b* binding to $G_{\alpha 12}$ or $G_{\alpha 13}$. (B and C) Effect of $G_{\alpha 12}$ knockdown on F-actin polymerization in *Ep3*-deficient PASCs with reexpression of *Ep3a* or *Ep3b*. (D and E) Effect of $G_{\alpha 12}$ knockdown on MT1-MMP membrane localization and MMP-2 activity in the culture medium (CM) of *Ep3a*- or *Ep3b*-reexpressing PASCs. MP, membrane protein; CL, cell lysate. (F) Effect of $G_{\alpha 12}$ knockdown on active TGF-β1 content in culture medium of *Ep3a/b*-reexpressing PASCs ($n = 4$). * $P < 0.05$ by 2-tailed Student's *t* test. (G) Effect of $G_{\alpha 12}$ knockdown on *Ep3a/b*-mediated TGF-β1 signaling in PASCs.

tion using an HA-Tag antibody displayed both $G_{\alpha 12}$ and $G_{\alpha 13}$ in the HA-EP3 immunocomplex, with a greater abundance of $G_{\alpha 12}$ than of $G_{\alpha 13}$ (Figure 11A). Then, shRNAs, designed to specifically knock down the expression of $G_{\alpha 12}$ or $G_{\alpha 13}$, were transfected into *Ep3a*- and *Ep3b*-overexpressing mPASCs to determine which of the 2 G proteins transduces EP3-mediated Rho/MMP-2/TGF-β1

signaling. As shown in Figure 11, silencing $G_{\alpha 12}$ significantly suppressed the following processes: induction of F-actin polymerization (Figure 11, B and C); augmentation of MT1-MMP membrane levels (Figure 11, D and E); upregulation of extracellular MMP-2 activity (Figure 11, D and E); enhanced cleavage of latent TGF-β1 in culture (Figure 11F); and boosted expression of fibronectin and

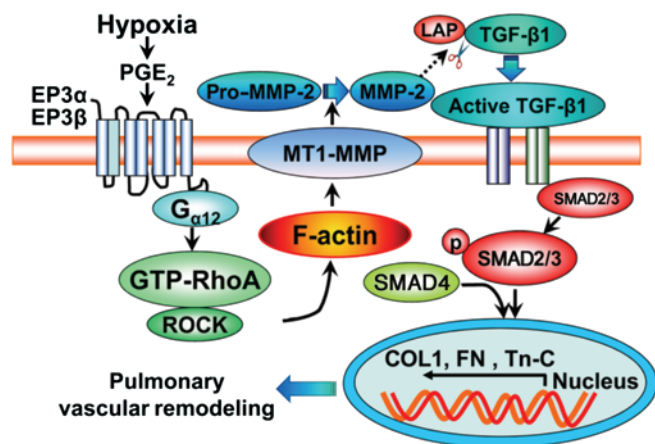


Figure 12. Schematic diagram of *Ep3a/b*-mediated PA remodeling through Rho-dependent MT1-MMP/MMP-2/TGF- β 1 signaling.

collagen I (Figure 11G) in mPASCs caused by *Ep3a/b* overexpression under hypoxic stimulatory conditions. However, we did not observe a significant effect of $G_{\alpha 13}$ silencing on EP3-mediated TGF- β 1 signaling (Supplemental Figure 16). Therefore, these results suggest that *Ep3a/b* variants mediate Rho-dependent TGF- β 1 signaling in PASCs in response to hypoxia via coupling to $G_{\alpha 12}$ (Figure 12). In primary cultured hPASCs, Rho-dependent TGF- β 1 signaling was also suppressed by inhibition of the EP3 receptor (Supplemental Figure 17).

Discussion

The EP3 receptor can mediate vasoconstriction of human arteries including PAs (46). In this study, we found that *EP3* expression was upregulated in human and mouse PASCs in response to hypoxia and was significantly elevated in the PAs of hypoxia-induced PAH mouse models. Furthermore, we demonstrated that the EP3 receptor was involved in the development of PAH in 3 rodent models through both pharmacological inhibition and genetic deletion approaches. In addition, activation of the EP3 receptor, including both *Ep3a* and *Ep3b* splice variants, boosted hypoxia-stimulated TGF- β 1 signaling and fibrotic protein expression in PASCs and PAs by increasing extracellular MMP-2 activity. More interestingly, EP3 α/β -mediated MMP-2/TGF- β 1 activation was attributed to localization of MT1-MMP toward the cell membrane through Rho/ROCK-dependent actin cytoskeletal remodeling. Therefore, inhibition of the EP3 receptor confers protection against PAH through suppression of Rho/ROCK-dependent TGF- β 1 signaling.

PGs, the metabolic products of arachidonic acid via phospholipase A₂ (PLA₂) and COX catalysis, are implicated in the regulation of vascular homeostasis, including pulmonary vascular tone. Pharmacological inhibition and genetic deletion of cytosolic PLA₂ attenuate hypoxia-induced pulmonary vasoconstriction and PA resistance in mice (47, 48). Moreover, COX-2 can be induced greatly by hypoxia in PAs and PASCs (7). Both nonselective and COX-2-selective NSAIDs suppress hypoxic vasoconstriction in PAs (47); however, disruption of the *Cox2* gene exaggerates hypoxia-induced pulmonary hypertension and vascular remodeling

in rodents (7), indicating that COX-2-derived PGs may be involved in the development of PAH. Patients with PAH produce fewer vasodilators but excessive vasoconstrictors (33). For instance, reduced circulating levels of the vasodilator PGI₂ and elevated levels of vasoconstrictive thromboxane were observed in PAH patients (14). We found that the vasoconstrictive EP3 receptor, which also mediates the contraction of PAs (9, 10), was strikingly upregulated in human and murine PASCs in response to hypoxia in vitro and in PAs in rodent PAH models. Interestingly, we found that 5 of 10 different human EP3 splice variants (*EP3-1a*, *EP3-1b*, *EP3-1c*, *EP3-4*, and *EP3-5*) in PAs were markedly elevated in response to hypoxia. Others failed to detect the EP3 upregulation in cultured PASCs from MCT-PAH rats, probably due to the different experimental approaches adopted (49). Moreover, the EP3 receptor is also involved in the mediation of pulmonary vascular contraction induced by isoprostanes, which are oxidative metabolites of the polyunsaturated fatty acids that are found to be markedly elevated in PAH patients (50, 51). We were unable to detect any significant influences of EP3 receptor activation, inhibition, or genetic deletion on PASC proliferation in vitro or in vivo and observed no notable impact of EP3 ablation on PASC hypertrophy in PAH animals. Indeed, disruption or inhibition of the EP3 receptor attenuates overdeposition of collagen and thickening of the smooth muscle layer in pulmonary arterioles and attenuates the increase in RVSP and the thickness of the right ventricular wall in both hypoxic and MCT-induced PAH models. These observations suggest that elevated expression of the EP3 receptor in PAs contributes to pulmonary vascular remodeling in PAH.

The increased activation of TGF- β 1 signaling in PAs was reported in idiopathic PAH patients (52) and in different PAH animal models (53, 54), and inhibition of the TGF- β 1 receptor activin receptor-like kinase 1 prevents the development of PAH (55). Similarly, we observed elevated TGF- β 1 signaling in the PAs of hypoxia-induced PAH models. Interestingly, inhibition or loss of the EP3 receptor suppressed hypoxia-induced activation of TGF- β 1 signaling in PASCs and PAs, implying that targeting the EP3 receptor confers protection against PAH by suppressing TGF- β 1 signaling. Although 3 *EP3* splice isoforms are coexpressed in vasculature, only *Ep3a* and *Ep3b*, not *Ep3g*, seem to be involved in the mediation of hypoxia-induced TGF- β 1 activation in PASCs and maintenance of cell polarity, as previously described (31). These phenomena are perhaps due to the unique C terminus of EP3 γ , which is crucial for its activation of the GTP-binding protein and cellular distribution (56, 57).

The dissociation or activation of TGF- β from LAP is a critical regulatory event (58). We found that extracellular MMP-2 activity was notably reduced in PAs from hypoxia-stressed *Ep3*^{-/-} mice and in the culture of *Ep3*^{-/-} PASCs under hypoxic conditions and that reexpression of *Ep3a/b* boosted MMP-2 activity in PASC culture, which is in agreement with TGF- β 1-signaling alterations. These observations indicate that *Ep3a/b*-mediated extracellular MMP-2 activation cleaves LAP and promotes TGF- β 1 signaling upon hypoxic stress. Moreover, TGF- β 1 signaling may also regulate MMP-2 transcription (59), as reduced transcription of MMP-2 was detected in *Ep3*^{-/-} PASCs. Interestingly, blockade of $G_{\alpha 12}$ downstream ROCK (60) or knockdown of $G_{\alpha 12}$ reduced the exaggerated MMP-2 activation caused by overexpression of *Ep3a/b* in PASCs, further supporting the idea that the *Ep3a/b*-triggered MMP-2/TGF- β 1 cascade

in response to hypoxic stress is dependent on activation of the $G_{\alpha 12}$ /RhoA pathway. Similarly, MMP-2-mediated TGF- β 1 activation and subsequent matrix-associated protein deposition are also implicated in arterial stiffness in aged arteries (61). Moreover, PGE₂/EP3-mediated arterial contraction seems to require ROCK activation (62), which may also contribute to the development of PAH.

Rho GTPase is a master regulator controlling cytoskeletal-dependent functions such as cytokinesis, actin cytoskeletal organization, cell adhesion, and migration (63). *Ep3* deficiency suppressed hypoxia-induced GTP-RhoA levels in PSMCs, while overexpression of *Ep3a/b* by transfection augmented RhoA activity upon hypoxia exposure. Accordingly, overexpression of *Ep3a/b* increased F-actin polymerization and subsequent MT1-MMP movement toward the cell membrane, which was inhibited by the actin polymerization inhibitor LB. These observations indicate that EP3 α/β -mediated actin cytoskeletal remodeling regulates intracellular MT1-MMP transportation through activation of Rho GTPase. CD44, a hyaluronic acid receptor, is believed to interact with the hemopexin-like domain of MT1-MMP (41) and to link MT1-MMP with actin filaments through direct CD44 binding to F-actin (41, 43). As such, leptin mediates myocardial matrix remodeling by regulating the cell-surface location of MT1-MMP in cardiac fibroblasts via Rho GTPase-mediated actin polymerization, which facilitates the development of heart failure in obesity (43, 64). Thus, EP3 α/β activation, which stimulates the MMP-2/TGF- β 1-signaling cascade in the PSMC response to hypoxic stress, induces Rho/ROCK-dependent actin cytoskeletal reorganization, an event that may be implicated in PAH pathogenesis. Consequently, long-term treatment with ROCK inhibitors markedly reduces PA pressure and ameliorates PA remodeling in MCT- and hypoxia-induced PAH in rodents (65–67). In humans with PAH, intake of ROCK inhibitors also leads to significant improvement of pulmonary hemodynamics (68).

In summary, we showed that the vasoconstrictive receptor EP3 is upregulated in PAs and PSMCs in response to hypoxia. Additionally, we demonstrated that pharmacological inhibition or genetic deletion of *Ep3* retards the progress of both hypoxia- and MCT-induced PAH in rodents through suppression of the Rho/ROCK-dependent MMP-2/TGF- β 1 signaling pathway. Thus, blockade of the EP3 receptor may be a promising strategy for the management of PAH.

Methods

Further information can be found in the Supplemental Methods.

Animals. All the mice were maintained on a C57BL6 genetic background. WT littermates were generated as experimental controls from EP3 receptor heterozygous matings. VSMC- and EC-specific *Ep3*-KO mice were generated by crossing *Ep3^{fl/fl}* mice (69) with *Sm22-Cre* and *Tie2-Cre* transgenic mice, respectively.

Chronic hypoxia-induced pulmonary hypertension model in mice. Eight- to 10-week-old male *Ep3*-KO (*Ep3^{-/-}*) mice and their age- and weight-matched WT littermates were exposed to room air (normoxia) or 10% oxygen (hypoxia) in a ventilated chamber, as described previously (36). All animals had access to standard mouse chow and water, and they were subjected to an alternating 12-hour light/12-hour dark cycle under controlled temperature conditions. The chamber was opened twice a week for 10 to 15 minutes for cleaning and supplementing food and water. After 3 weeks, pulmonary hypertension and pulmonary vascular remodeling were assessed.

SU5416 combined with chronic hypoxia-induced pulmonary hypertension model in mice. Eight- to 10-week-old *Ep3^{-/-}*, VSMC-*Ep3^{-/-}*, and EC-*Ep3^{-/-}* male mice and age-matched controls received a single weekly s.c. injection of the VEGFR inhibitor SU5416 (20 mg/kg), which was suspended in carboxymethylcellulose (CMC) solution (0.5% [w/v] carboxymethylcellulose sodium, 0.9% [w/v] sodium chloride, 0.4% [v/v] polysorbate 80, and 0.9% [v/v] benzyl alcohol in deionized water). Control mice received vehicle instead. The animals were exposed to chronic hypoxia (10% O₂) in a ventilated chamber for 3 weeks (70). At the end of the treatment period, mice were anesthetized, and hemodynamic changes were assessed.

MCT-induced pulmonary hypertension model in rats. Eight-week-old male Sprague-Dawley rats were injected once s.c. with or without MCT (60 mg/kg). At the beginning of the third week, MCT-injected rats received L-798106 (200 mg/kg) or vehicle twice a day.

Statistics. All data are expressed as the mean \pm SEM. Data were analyzed using GraphPad Prism software, version 5.0 (GraphPad Software). The 2-tailed unpaired Student's *t* test was performed to compare 2 data sets. Multiple comparisons were tested with ANOVA followed by Bonferroni's post test. A *P* value of less than 0.05 was considered statistically significant.

Study approval. All animals were maintained and handled in accordance with the guidelines approved by the IACUC of the Institute for Nutritional Sciences, Chinese Academy of Sciences. Human lung sections ($-1 \times 1 \times 1$ cm) were obtained from patients undergoing lobectomy or pneumonectomy for bronchial carcinoma. Lung tissue distant from the tumor was selected to isolate distal PAs. All the lungs were judged by the pathologist to have no vascular pathological changes under microscopy. PA sections were cut and incubated in DMEM under normoxic or hypoxic conditions for 24 hours, then subjected to RNA extraction. Written informed consent was obtained from all subjects prior to their participation in this study. Approval for these studies was obtained from the ethics committee of Ruijin Hospital, which is affiliated with Shanghai Jiaotong University.

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