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# Basigin Mediates Pulmonary Hypertension by Promoting Inflammation and Vascular Smooth Muscle Cell Proliferation

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**Rationale:** Cyclophilin A (CyPA) is secreted from vascular smooth muscle cells (VSMCs) by oxidative stress and promotes VSMC proliferation. However, the role of extracellular CyPA and its receptor Basigin (Bsg, encoded by *Bsg*) in the pathogenesis of pulmonary hypertension (PH) remains to be elucidated.

**Objective:** To determine the role of CyPA/Bsg signaling in the development of PH.

**Methods and Results:** In the pulmonary arteries of patients with PH, immunostaining revealed strong expression of CyPA and Bsg. The pulmonary arteries of *CyPA*<sup>+/-</sup> and *Bsg*<sup>+/-</sup> mice exposed to normoxia did not differ in morphology compared with their littermate controls. In contrast, *CyPA*<sup>+/-</sup> and *Bsg*<sup>+/-</sup> mice exposed to hypoxia for 4 weeks revealed significantly reduced right ventricular systolic pressure, pulmonary artery remodeling, and right ventricular hypertrophy compared with their littermate controls. These features were unaltered by bone marrow reconstitution. To further evaluate the role of vascular Bsg, we harvested pulmonary VSMCs from *Bsg*<sup>+/+</sup> and *Bsg*<sup>+/-</sup> mice. Proliferation was significantly reduced in *Bsg*<sup>+/-</sup> compared with *Bsg*<sup>+/+</sup> VSMCs. Mechanistic studies demonstrated that *Bsg*<sup>+/-</sup> VSMCs revealed reduced extracellular signal-regulated kinase 1/2 activation and less secretion of cytokines/chemokines and growth factors (eg, platelet-derived growth factor-BB). Finally, in the clinical study, plasma CyPA levels in patients with PH were increased in accordance with the severity of pulmonary vascular resistance. Furthermore, event-free curve revealed that high plasma CyPA levels predicted poor outcome in patients with PH.

**Conclusions:** These results indicate the crucial role of extracellular CyPA and vascular Bsg in the pathogenesis of PH. (*Circ Res.* 2014;115:738-750.)

**Key Words:** anoxia ■ hypertension, pulmonary ■ inflammation ■ muscle, smooth, vascular ■ oxidative stress ■ pulmonary circulation

Inflammation contributes to the pathogenesis of pulmonary arterial hypertension (PAH), for which specific therapeutic targets remain elusive.<sup>1,2</sup> PAH is characterized by pulmonary artery vascular remodeling, which involves endothelial cell abnormalities, vascular smooth muscle cell (VSMC) proliferation, and perivascular inflammation.<sup>3-5</sup> Pulmonary vascular remodeling and progressive obliteration of the vessel lumen lead to right ventricular failure and premature death.<sup>6</sup> The key mechanisms of PAH include hypoxia,<sup>7,8</sup> increased local production of proinflammatory cytokines, and loss-of function mutations in bone morphogenetic protein receptor 2,<sup>9</sup> which

affects platelet-derived growth factor (PDGF)-BB signaling.<sup>10</sup> There is a mechanistic link between inflammation and bone morphogenetic protein receptor 2 mutations in PAH.<sup>11</sup> This suggests a potential therapeutic target in the regulation of inflammation in pulmonary vasculature.

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Hypoxia induces activation of nuclear factor of activated T cells (NFAT) and promotes VSMC proliferation.<sup>12</sup> Chronic hypoxic exposure of mice induces vascular remodeling characterized by medial and adventitial thickening of the muscular

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**Nonstandard Abbreviations and Acronyms**

<b>Bsg</b>	basigin
<b>CyPA</b>	cyclophilin A
<b>ERK1/2</b>	extracellular signal-regulated kinase 1/2
<b>IL</b>	interleukin
<b>NFAT</b>	nuclear factor of activated T cells
<b>PAH</b>	pulmonary arterial hypertension
<b>PDGF-BB</b>	platelet-derived growth factor-BB
<b>PH</b>	pulmonary hypertension
<b>RV</b>	right ventricle
<b>RVSP</b>	right ventricle systolic pressure
<b>VSMC</b>	vascular smooth muscle cell

and elastic vessels and muscularization of more distal small vessels.<sup>7</sup> We have demonstrated that pulmonary vascular inflammation plays a crucial role in the development of hypoxia-induced pulmonary hypertension (PH),<sup>13,14</sup> for which Rho-kinase plays a crucial role.<sup>15–17</sup> In addition, Rho-kinase promotes secretion of cyclophilin A (CyPA, encoded by *Ppia*) from VSMCs and extracellular CyPA stimulates VSMC proliferation *in vivo*<sup>18</sup> and *in vitro*.<sup>19,20</sup> CyPA is secreted from VSMC through Rho-kinase activation and vesicle formation.<sup>21</sup> Extracellular CyPA induces endothelial cell adhesion molecule expression,<sup>22</sup> induces apoptosis,<sup>23</sup> and is a chemoattractant for inflammatory cells.<sup>18,24</sup> Basigin (Bsg, also known as CD147 or EMMPRIN, encoded by *Bsg*) is an extracellular CyPA receptor.<sup>25</sup> Importantly, Bsg is an essential receptor for malaria, which disrupts nitric oxide metabolism and causes harmful endothelial activation, including the Rho/Rho-kinase activation.<sup>26</sup> Therefore, we hypothesized that the extracellular CyPA and Bsg signaling may contribute to hypoxia-induced PH.

Here, we report that *CyPA*<sup>+/-</sup> and *Bsg*<sup>+/-</sup> mice exhibit resistance to hypoxia-induced pulmonary vascular remodeling. Consistent with these results, plasma CyPA was significantly increased in patients with PAH and well correlated with the disease severity and long-term survival. Our data suggest that extracellular CyPA and its signaling through Bsg are novel therapeutic targets for PAH.

## Methods

### Animal Experiments

All animal experiments were conducted in accordance with the protocols approved by the Tohoku University Animal Care and Use Committee. Hypoxia-induced PH models were used to assess the effect of CyPA and Bsg deficiency on PH development in mice. Bsg is an important cell-surface molecule involved in early embryogenesis and reproduction.<sup>27,28</sup> Because complete Bsg disruption (*Bsg*<sup>-/-</sup>) in mice results in perinatal lethality, poor pregnancy or abnormal development, we used *Bsg*<sup>+/-</sup> mice in the present study. This is also the same in complete CyPA-deficient mice as to the poor pregnancy.<sup>29</sup> Twelve to 16-week-old male littermate control mice and *Bsg*<sup>+/-</sup> and *CyPA*<sup>+/-</sup> mice on a normal chow diet were exposed to hypoxia or normoxia for 4 weeks as previously described.<sup>13,14</sup> Briefly, hypoxic mice were housed in an acrylic chamber with a nonrecirculating gas mixture of 10% O<sub>2</sub> and 90% N<sub>2</sub> by adsorption-type oxygen concentrator to use exhaust air (Teijin, Japan), whereas normoxic mice were housed in room air (21% O<sub>2</sub>) under a 12-hour light–dark cycle.<sup>13,14</sup> After 4 weeks of exposure to hypoxia (10% O<sub>2</sub>) or normoxia, mice were anesthetized with isoflurane (1.0%). To determine the effect of

CyPA and Bsg deficiency on hypoxia-induced PH development, we measured right ventricle systolic pressure (RVSP), right ventricular hypertrophy, and pulmonary vascular remodeling.<sup>13,14</sup> For right heart catheterization, a 1.2-F pressure catheter (SciSense, Inc., Ontario, Canada) was inserted in the right jugular vein and advanced into the right ventricle (RV) to measure RVSP.<sup>15</sup> All data were analyzed using the PowerLab data acquisition system (AD Instruments) and averaged for 10 sequential beats.

### Statistical Analyses in Animal Experiments

Results are expressed as mean±SEM for all studies except as mentioned in the figure legends. Comparisons of mean values between 2 groups were performed by Welch *t* test. 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, followed by Tukey honestly significant difference multiple comparisons. All reported *P* values are 2-tailed, with a *P* value of <0.05 indicating statistical significance. Analyses were performed in SPSS, version 19.0 (Chicago, IL, USA) and R version 3.0.1.<sup>30</sup>

An expanded Methods section is available in the Online Data Supplement.

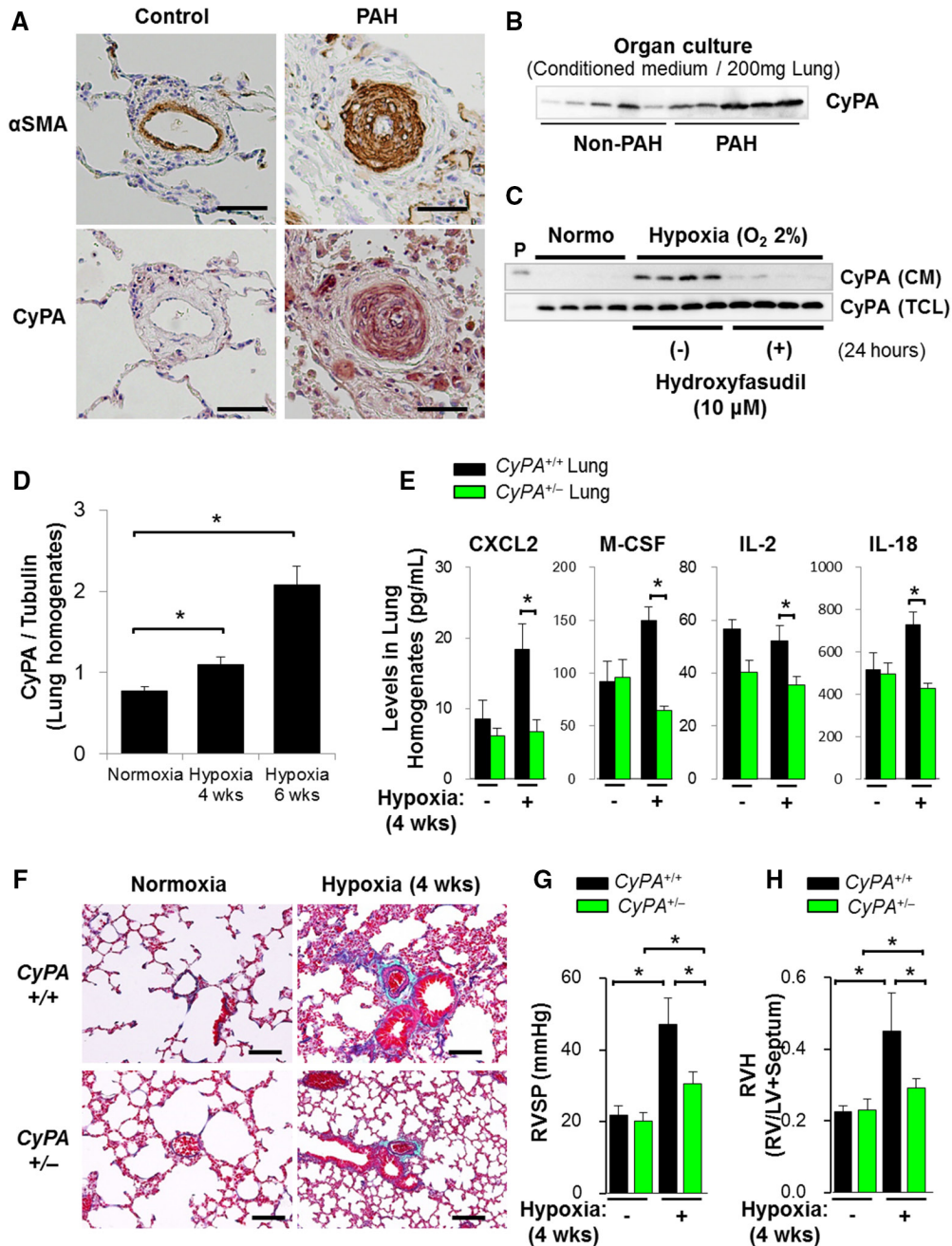
## Results

### Increased CyPA Expression and Secretion in Patients With PAH

To confirm the role of CyPA in pulmonary arteries, we used lung tissues from patients with PAH undergoing lung transplantation. CyPA was strongly expressed in the remodeled pulmonary microvasculature in patients with PAH, especially in the medial layer (Figure 1A). Organ culture experiments revealed increased CyPA secretion (conditioned medium) from the lungs of patients with PAH as comparison with non-PAH controls (Figure 1B). Western blotting showed that hypoxia significantly increased CyPA secretion from VSMCs harvested from patients with PAH (Figure 1C). As we have shown in VSMCs harvested from mouse aorta,<sup>29</sup> a key role for Rho-kinase activity for CyPA secretion was shown by the marked decrease in CyPA secretion with hydroxyfasudil in pulmonary VSMCs from patients with PAH (Figure 1C). In addition, CyPA expression was time dependently increased in the pulmonary microvascular walls in hypoxia-induced PH in wild-type mice (Figure 1D; Online Figure IA). Similarly, intense Bsg expression was noted in pulmonary arteries after hypoxic exposure, suggesting the crucial role of CyPA and Bsg in the pathogenesis of PH. Immunostaining with serial sections showed strong expression of CyPA and Bsg in the remodeled pulmonary arteries of patients with PAH, especially in the medial layer and adventitial inflammatory cells (Online Figure IB). Thus, we used *CyPA*<sup>+/-</sup> and *Bsg*<sup>+/-</sup> mice to examine the role of these molecules in the development of hypoxia-induced PH.

### CyPA Deficiency Ameliorates Hypoxia-Induced PH *In Vivo*

CyPA promotes endothelial cell dysfunction, VSMC proliferation, and inflammatory cell migration.<sup>31</sup> Interestingly, we found hypoxia-induced increase of CyPA expression and inflammatory cells in lungs of wild-type mice (Online Figure I). Thus, we first performed cytokine array to evaluate the levels of cytokines/chemokines and growth factors in *CyPA*<sup>+/-</sup> and littermate controls (*CyPA*<sup>+/+</sup> mice). We found significantly high levels of cytokines in the lung homogenates of *CyPA*<sup>+/+</sup> mice compared with those of *CyPA*<sup>+/-</sup> mice, irrespective of normoxia



**Figure 1. Cyclophilin A (CyPA) deficiency prevents hypoxia-induced pulmonary hypertension (PH).** **A**, Representative photographs showing macroscopic features of distal pulmonary arteries in patients with pulmonary arterial hypertension (PAH) who underwent lung transplantation. The predominant cellular component expressing CyPA in the PAH lung was vascular smooth muscle cells (VSMCs) as evidenced by immunostaining for CyPA and  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) in serial sections. Scale bars, 50  $\mu$ m. **B**, Western blotting of CyPA. Lung organ culture showed secretion of CyPA from the lung in patients with PAH and patients without PH (non-PAH). Conditioned medium (CM) from the lungs (200 mg each) was prepared after 24 hours of culture. **C**, Western blotting showed hypoxia-induced secretion of CyPA from pulmonary VSMCs harvested from patients with PAH, which was completely blocked by hydroxyfasudil (10  $\mu$ mol/L), a selective Rho-kinase inhibitor. **D**, Western blotting showed hypoxia-induced CyPA expression in the lungs from mice after 4 and 6 weeks of hypoxic exposure (n=8 each). \**P*<0.05. **E**, Levels of inflammatory cytokines and growth factors in the lungs from CyPA<sup>+/+</sup> (n=13) and CyPA<sup>+/-</sup> mice (n=8) after 4 weeks of hypoxic exposure. Results are expressed as mean $\pm$ SEM. \**P*<0.05. **F**, Representative distal pulmonary arteries of CyPA<sup>+/-</sup> mice and littermate controls (CyPA<sup>+/+</sup>) exposed to normoxia or hypoxia (10% O<sub>2</sub>) for 4 weeks. Scale bars, 50  $\mu$ m. **G**, Hypoxia-induced PH assessed by right ventricular systolic pressure (RVSP) was significantly attenuated in CyPA<sup>+/-</sup> mice (n=12) as compared with CyPA<sup>+/+</sup> mice (n=10) after 4 weeks of hypoxic exposure. In contrast, there was no difference in RVSP between CyPA<sup>+/+</sup> (n=6) and CyPA<sup>+/-</sup> mice (n=5) after 4 weeks of normoxia. Results are expressed as mean $\pm$ SD. \**P*<0.05. **H**, The increased ratio of right ventricle to left ventricle plus septum weight [RV/(LV+sep)] was attenuated in CyPA<sup>+/-</sup> mice after hypoxic exposure for 4 weeks. Results are expressed as mean $\pm$ SD. \**P*<0.05. CXCL2 indicates chemokine (C-X-C motif) ligand 2; IL, interleukin; M-CSF, macrophage colony-stimulating factor; and TCL, total cell lysates.

or hypoxia for 4 weeks (Online Figure II). After 4 weeks of hypoxia, *CyPA*<sup>+/+</sup> lungs exhibited increased inflammatory cytokines compared with *CyPA*<sup>+/-</sup> lungs, especially in chemokine (C-X-C motif) ligand 2, macrophage colony-stimulating factor, interleukin (IL)-2 and IL-18, all of which contribute to pulmonary vascular remodeling (Figure 1E). The pulmonary arteries of normoxic *CyPA*<sup>+/-</sup> and wild-type (*CyPA*<sup>+/+</sup>) mice did not differ in morphology (Figure 1F). In contrast, mice exposed to hypoxia for 4 weeks exhibited a difference in the medial thickness of pulmonary arteries (Figure 1F). In addition, *CyPA*<sup>+/+</sup> mice exhibited increased RVSP, which was significantly attenuated in *CyPA*<sup>+/-</sup> mice (Figure 1G). The increased ratio of RV to left ventricle plus septum weight [RV/(LV+sep)] was also attenuated in *CyPA*<sup>+/-</sup> mice (Figure 1H), suggesting that CyPA is crucial in hypoxia-induced PH. These results suggest that CyPA is crucial for inflammation, VSMC proliferation, and development of hypoxia-induced PH in mice.

### Bsg Deficiency Ameliorates Hypoxia-Induced PH In Vivo

To further evaluate the role of extracellular CyPA through Bsg signaling, we next performed analysis using *Bsg*<sup>+/-</sup> mice and littermate controls (*Bsg*<sup>+/+</sup> mice). Bsg expression was time dependently increased in the pulmonary microvascular walls in hypoxia-induced PH in wild-type mice (Figure 2A; Online Figure I). The morphology of pulmonary arteries in normoxic *Bsg*<sup>+/-</sup> mice did not differ from those in *Bsg*<sup>+/+</sup> mice (Figure 2B). In contrast, mice exposed to hypoxia for 4 weeks exhibited a significant difference in the medial thickness of pulmonary arteries (Figure 2B). The degree of muscularization was assessed in distal pulmonary arteries with a diameter of 20 to 70  $\mu$ m. As compared with *Bsg*<sup>+/+</sup> mice, *Bsg*<sup>+/-</sup> mice exhibited fewer muscularized distal pulmonary arteries after hypoxic exposure (Figure 2C). Muscularized distal pulmonary arteries exhibited immunoreactivity for  $\alpha$ -smooth muscle actin (Figure 2B). Consistent with these morphological changes, *Bsg*<sup>+/+</sup> mice exhibited increased RVSP, which was attenuated in *Bsg*<sup>+/-</sup> mice (Figure 2D). The increased ratio of RV to left ventricle plus septum weight [RV/(LV+sep)] was also attenuated in *Bsg*<sup>+/-</sup> mice (Figure 2E), suggesting a crucial role of Bsg in hypoxia-induced PH. Importantly, serum levels of growth factors and cytokines/chemokines that promote PH (especially PDGF-BB)<sup>32</sup> were increased in hypoxic *Bsg*<sup>+/+</sup> mice, which was significantly less in *Bsg*<sup>+/-</sup> mice (Figure 2F). These results suggest that Bsg plays a crucial role in hypoxia-induced production and secretion of PDGF-BB and development of pulmonary vascular remodeling.

We then examined hypoxia-induced changes in the number of perivascular inflammatory cells. Gross and histological examination of the lung revealed clear differences in CD45<sup>+</sup> inflammatory cell migration between *Bsg*<sup>+/+</sup> and *Bsg*<sup>+/-</sup> mice (Online Figure III). The increases in perivascular inflammatory cells in *Bsg*<sup>+/+</sup> mice occurred as early as day 1, whereas it remained at low level in *Bsg*<sup>+/-</sup> mice after 4 weeks of hypoxia (Figure 3A and 3B). Consistent with the pathological changes in lung tissues, the levels of cytokines/chemokines, such as macrophage colony-stimulating factor, IL-15, and IL-18, were increased in hypoxic *Bsg*<sup>+/+</sup> lung, which was again less dramatic in *Bsg*<sup>+/-</sup> lung (Figure 3C). Importantly, growth factors such as PDGF-BB and eotaxin, both of which promote PH,<sup>33</sup>

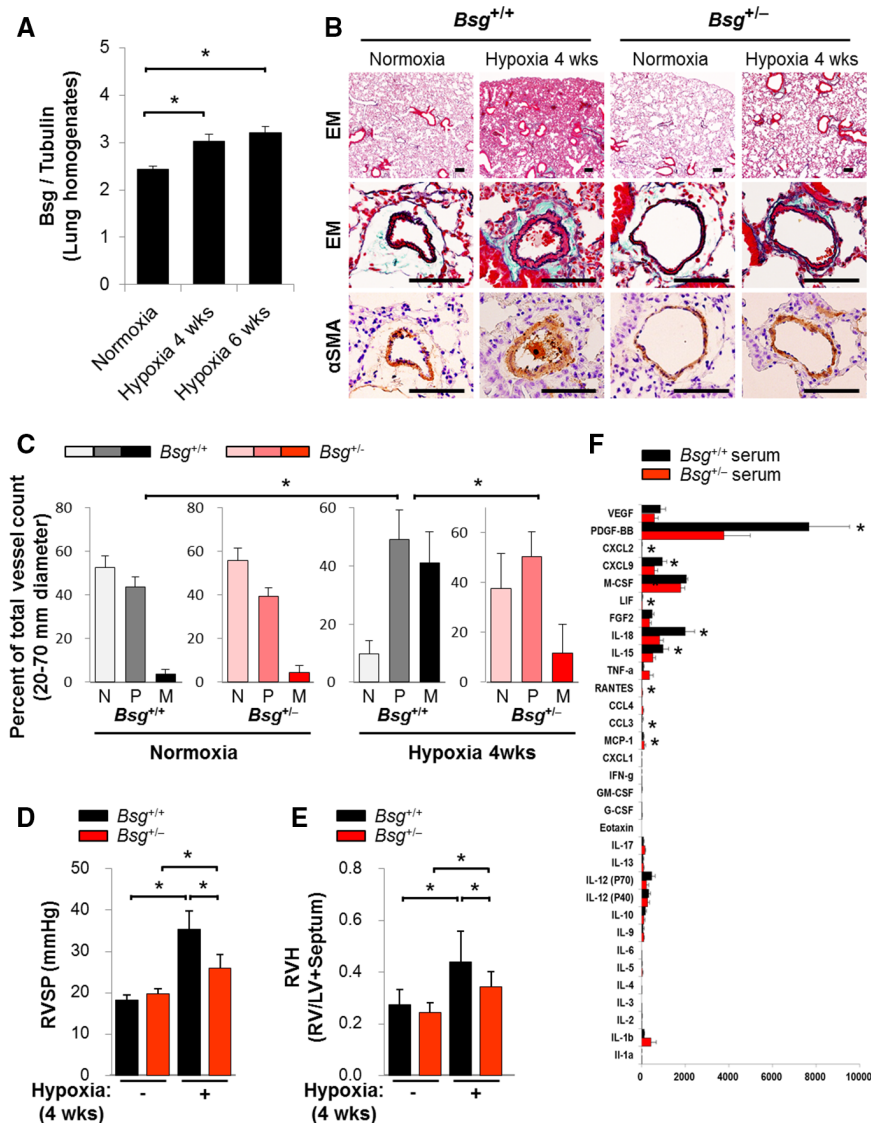
were also induced in *Bsg*<sup>+/+</sup> mice, which were less strongly induced in *Bsg*<sup>+/-</sup> mice (Figure 3D; Online Figure IV).

To confirm whether the expression of CyPA is regulated by Bsg, we next performed immunostaining for CyPA in *Bsg*<sup>+/+</sup> and *Bsg*<sup>+/-</sup> mice. Perivascular expression of CyPA was comparable between normoxic *Bsg*<sup>+/+</sup> and *Bsg*<sup>+/-</sup> lung (Online Figure VA). In contrast, CyPA expression was greater in the muscularized vessels and perivascular inflammatory cells in *Bsg*<sup>+/+</sup> and *Bsg*<sup>+/-</sup> mice after hypoxic exposure (Online Figure VA). Interestingly, the increase in CyPA expression in the pulmonary arteries was less dramatic in *Bsg*<sup>+/-</sup> mice. We further performed Western blotting to compare the expression levels of CyPA between *Bsg*<sup>+/+</sup> and *Bsg*<sup>+/-</sup> lung (Online Figure VB). Bsg expression was increased in the lung in *Bsg*<sup>+/+</sup> and *Bsg*<sup>+/-</sup> mice after hypoxic exposure (Online Figure VB). However, there was no significant difference in CyPA expression between *Bsg*<sup>+/+</sup> and *Bsg*<sup>+/-</sup> mice (Online Figure VB). Then, we performed Western blotting to compare the expression and secretion of CyPA from *Bsg*<sup>+/+</sup> and *Bsg*<sup>+/-</sup> VSMCs (Online Figure VC). Interestingly, the secretion of CyPA was less in *Bsg*<sup>+/-</sup> VSMCs compared with *Bsg*<sup>+/+</sup> VSMCs. Moreover, extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation was less in *Bsg*<sup>+/-</sup> VSMCs compared with *Bsg*<sup>+/+</sup> VSMCs (Online Figure VD).

### Vascular Bsg Is Essential for Hypoxia-induced PH

Extracellular CyPA stimulates migration of inflammatory cells.<sup>24</sup> The chemotactic activities of extracellular CyPA depend on Bsg, which serves as the primary binding site for CyPA on target cells.<sup>25</sup> Inflammation is augmented by cytokines/chemokines and growth factors secreted from perivascular inflammatory cells. To determine whether extracellular CyPA induces secretion of growth factors in a Bsg-dependent manner, we stimulated *Bsg*<sup>+/-</sup> bone marrow cells with human recombinant CyPA ex vivo. Inflammatory cytokine secretion was induced by human recombinant CyPA in *Bsg*<sup>+/+</sup> bone marrow cells (Online Figure VIA); however, the bone marrow from *Bsg*<sup>+/-</sup> mice exhibited a poorer response. This suggests that Bsg plays a crucial role for extracellular CyPA-induced secretion of cytokines/chemokines.

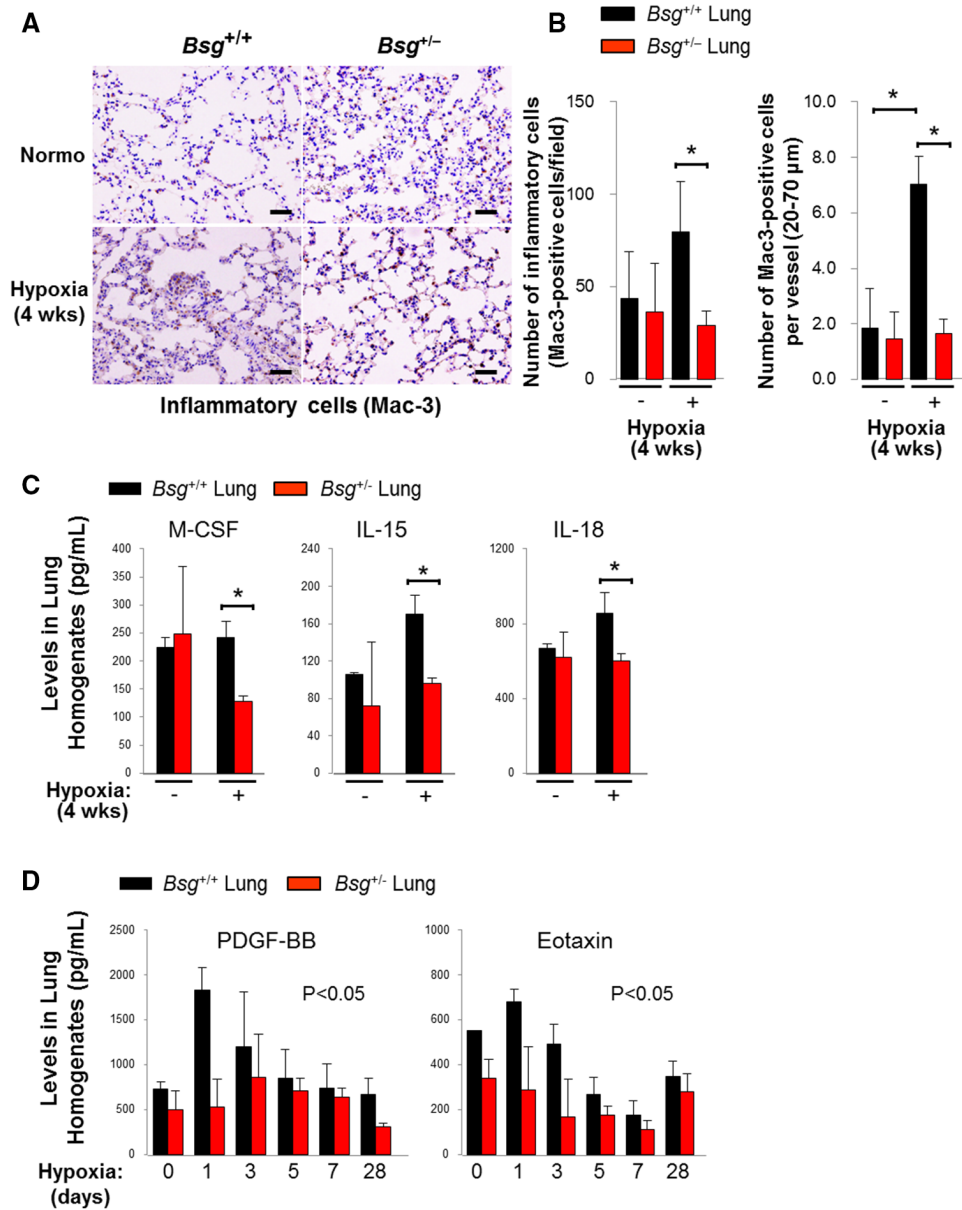
Bsg regulates the survival, proliferation, and adhesion of T-lymphoma cells.<sup>34</sup> Bone marrow-derived cells are involved in the pathogenesis of PH.<sup>13,14,35</sup> Therefore, we hypothesized that Bsg deficiency in bone marrow-derived cells may impair hypoxia-induced PH in *Bsg*<sup>+/-</sup> mice. To test this hypothesis, *Bsg*<sup>+/+</sup> bone marrow cells (GFP<sup>+</sup> [green fluorescent protein]) were transplanted into irradiated *Bsg*<sup>+/+</sup> and *Bsg*<sup>+/-</sup> mice. After reconstitution of the bone marrow, chimeric mice were exposed to normoxia or hypoxia for 4 weeks. GFP expression did not differ in the whole lungs from chimeric mice exposed to normoxia (Figure 4A); however, the number of bone marrow-derived cells (GFP<sup>+</sup> cells) was significantly less in the pulmonary arteries in *Bsg*<sup>+/-</sup> recipient mice as compared with *Bsg*<sup>+/+</sup> recipient mice (Figure 4A). As shown in the 3-dimensional image of a pulmonary artery (Figure 4A), GFP<sup>+</sup> cells adhered to the adventitia after hypoxic exposure. The reduced number of GFP<sup>+</sup> cells in the distal pulmonary arteries of the *Bsg*<sup>+/-</sup> recipient mice suggests that Bsg expressed in the distal pulmonary arteries mediates inflammatory cell recruitment. Consistently, the levels of cytokines/chemokines and growth factors in the lung were significantly lower in *Bsg*<sup>+/-</sup> versus *Bsg*<sup>+/+</sup> recipient mice, regardless of the source



**Figure 2. Basigin (Bsg) deficiency ameliorates hypoxia-induced pulmonary hypertension (PH).** **A**, Western blotting showed hypoxia-induced Bsg expression in the lungs from mice after 4 and 6 weeks of hypoxic exposure ( $n=8$  each).  $*P<0.05$ . **B**, Representative distal pulmonary arteries of *Bsg<sup>-/-</sup>* mice and littermate controls (*Bsg<sup>+/+</sup>*) exposed to normoxia or hypoxia (10%  $O_2$ ) for 4 weeks. Although the morphology of pulmonary arteries was comparable between *Bsg<sup>+/+</sup>* and *Bsg<sup>-/-</sup>* mice under normoxia, a significant difference in the medial thickness of pulmonary arteries was noted when exposed to hypoxia for 4 weeks between the 2 genotypes. The predominant cellular component in the remodeled distal pulmonary arteries was vascular smooth muscle cells as evidenced by immunostaining for  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) in serial sections. Scale bars, 50  $\mu$ m. **C**, Muscularization was assessed in distal pulmonary arteries of a diameter of 20 to 70  $\mu$ m. Under normoxia, there was no difference in muscularization between normoxic *Bsg<sup>+/+</sup>* ( $n=7$ ) and *Bsg<sup>-/-</sup>* mice ( $n=6$ ). However, after chronic hypoxia, *Bsg<sup>-/-</sup>* mice ( $n=15$ ) exhibited less muscularized vessels as compared with *Bsg<sup>+/+</sup>* mice ( $n=9$ ).  $*P<0.05$ . **D**, The development of hypoxia-induced PH assessed by right ventricular systolic pressure (RVSP) was significantly attenuated in *Bsg<sup>-/-</sup>* mice as compared with *Bsg<sup>+/+</sup>* mice after 4 weeks of hypoxic exposure. In contrast, there was no difference in RVSP between the 2 genotypes. Results are expressed as mean $\pm$ SD.  $*P<0.05$ . **E**, After hypoxic exposure for 4 weeks, the increased ratio of right ventricle to left ventricle plus septum weight [RV/(LV+sep)] was significantly attenuated in *Bsg<sup>-/-</sup>* than in *Bsg<sup>+/+</sup>* mice. Results are expressed as mean $\pm$ SD.  $*P<0.05$ . **F**, Serum levels of inflammatory cytokines and growth factors after hypoxic exposure for 7 days were significantly lower in *Bsg<sup>-/-</sup>* mice ( $n=9$ ) than in *Bsg<sup>+/+</sup>* mice ( $n=6$ ). Results are expressed as mean $\pm$ SEM.  $*P<0.05$ . CCL indicates chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; EM, Elastica-Masson; FGF2, fibroblast growth factor 2; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; LIF, leukemia inhibitory factor; M, fully muscularized vessels; M-CSF, macrophage colony-stimulating factor; MCP, monocyte chemoattractant protein; N, nonmuscularized vessels; P, partially muscularized vessels; PDGF-BB, platelet-derived growth factor-BB; RANTES, regulated on activation, normal T cell expressed and secreted; TNF, tumor necrosis factor; and VEGF, vascular endothelial growth factor.

of bone marrow (Online Figure VIB and VIC). These results support the concept that the reduced inflammatory responses in *Bsg<sup>-/-</sup>* mice are because of Bsg deficiency in the recipient lung. Finally, the development of PH assessed by RVSP and right ventricular hypertrophy was consistently less severe in *Bsg<sup>-/-</sup>* than in

*Bsg<sup>+/+</sup>* recipient mice, regardless of the source of bone marrow (Figure 4B). These data suggest that pulmonary vascular Bsg, but not bone marrow-derived Bsg, is critical for the VSMC proliferation and the development of pulmonary vascular remodeling (Figure 4C).



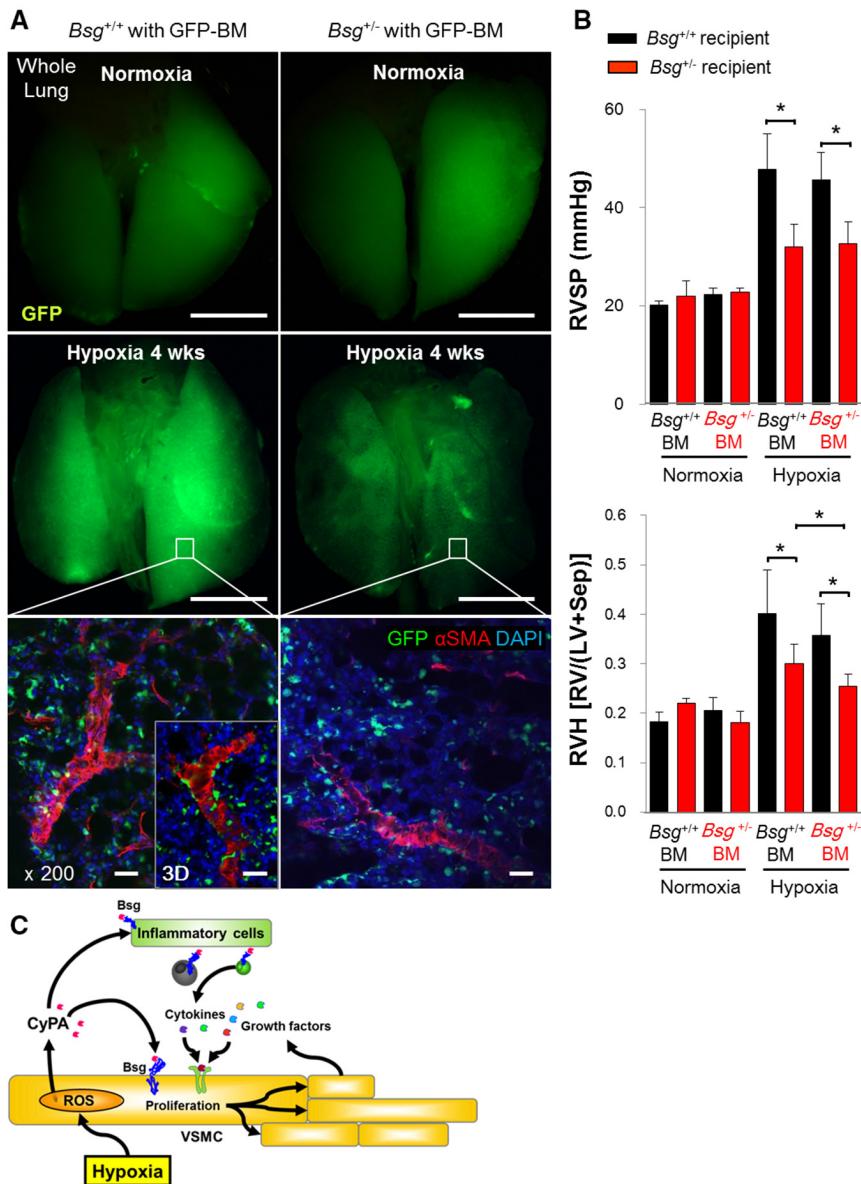
**Figure 3. Basigin (Bsg) deficiency reduces hypoxia-induced inflammatory cell accumulation.** **A**, Representative Mac-3 (CD107b) staining of the lung from *Bsg*<sup>+/+</sup> and *Bsg*<sup>-/-</sup> mice exposed to normoxia or hypoxia (10% O<sub>2</sub>) for 4 weeks. Scale bars, 50 μm. **B**, After hypoxic exposure, the number of Mac-3-positive inflammatory cells in the lung (**left**) or distal pulmonary artery adventitia (**right**) was significantly reduced in *Bsg*<sup>-/-</sup> (n=15) mice than in *Bsg*<sup>+/+</sup> (n=9). In contrast, under normoxia, there was no difference in the cell number between *Bsg*<sup>+/+</sup> (n=7) and *Bsg*<sup>-/-</sup> (n=6). Results are expressed as mean±SD. \*P<0.05. **C**, Levels of inflammatory cytokines (macrophage colony-stimulating factor [M-CSF], interleukin [IL]-15, and IL-18) in the lung homogenates after hypoxic exposure for 4 weeks were significantly lower in *Bsg*<sup>-/-</sup> mice (n=8) than in *Bsg*<sup>+/+</sup> (n=13). Results are expressed as mean±SEM. \*P<0.05. **D**, Time course of serum levels of platelet-derived growth factor-BB (PDGF-BB) and eotaxin in the lung homogenate after hypoxic exposure of *Bsg*<sup>+/+</sup> and *Bsg*<sup>-/-</sup> mice for 4 weeks. Results are expressed as mean±SEM.

### Bsg Regulates Secretion of Cytokines and Growth Factors In Vivo and In Vitro

To confirm the roles of growth factors in circulation and local lung, we harvested pulmonary VSMCs from wild-type mice and stimulated them with serum or lung homogenates from *Bsg*<sup>+/+</sup> or *Bsg*<sup>-/-</sup> mice (Figure 5A). VSMC proliferation was significantly increased by treatment with serum and lung homogenates from hypoxic *Bsg*<sup>+/+</sup> mice as compared with normoxic *Bsg*<sup>+/+</sup> mice (Figure 5A). Moreover, VSMC proliferation was attenuated by the treatment with serum or lung homogenates from *Bsg*<sup>-/-</sup> mice than those from *Bsg*<sup>+/+</sup> mice.

This suggests that the reduced levels of growth factors may contribute to the amelioration of PH in *Bsg*<sup>-/-</sup> mice.

The pathogenesis of PAH in humans is complex, depending on the interactions of genetic predisposition, metabolic problems, and inflammation/infection.<sup>32</sup> Because extracellular CyPA activates ERK1/2 through Bsg,<sup>25</sup> we anticipated decreased ERK1/2 activity in the absence of Bsg in VSMCs. To evaluate the role of Bsg in VSMC proliferation, we harvested pulmonary VSMCs from *Bsg*<sup>+/+</sup> and *Bsg*<sup>-/-</sup> mice.<sup>36</sup> As anticipated, ERK1/2 activity and proliferation were lower in *Bsg*<sup>-/-</sup> VSMCs than in *Bsg*<sup>+/+</sup> VSMCs (Figure 5B). The expression of



**Figure 4. Bone marrow (BM) reconstitution and vascular basigin (Bsg) in pulmonary hypertension (PH).** *Bsg*<sup>+/+</sup> BM cells (GFP<sup>+</sup> [green fluorescent protein]) were transplanted into irradiated *Bsg*<sup>+/+</sup> or *Bsg*<sup>-/-</sup> mice. **A**, Representative pictures of the whole lung (GFP, green) from *Bsg*<sup>+/+</sup> and *Bsg*<sup>-/-</sup> recipient mice with GFP<sup>+</sup> BM exposed to normoxia or hypoxia (10% O<sub>2</sub>) for 4 weeks. Scale bars, 3 mm. Inserts depict  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) staining (Cy3, red) of distal pulmonary arteries from *Bsg*<sup>+/+</sup> and *Bsg*<sup>-/-</sup> recipient mice with GFP<sup>+</sup> BM exposed to hypoxia for 4 weeks. Scale bars, 100  $\mu$ m. **B**, The extent of PH assessed by right ventricular systolic pressure (RVSP) and right ventricular hypertrophy (RVH) was significantly less in the *Bsg*<sup>-/-</sup> recipient mice as compared with *Bsg*<sup>+/+</sup> recipient mice, regardless of the source of BM (*Bsg*<sup>+/+</sup> or *Bsg*<sup>-/-</sup> BM). Normoxia (n=3 each), hypoxia 4 weeks (*Bsg*<sup>+/+</sup> recipient mice with *Bsg*<sup>+/+</sup> BM: n=7; *Bsg*<sup>-/-</sup> recipient mice with *Bsg*<sup>+/+</sup> BM: n=12; *Bsg*<sup>+/+</sup> recipient mice with *Bsg*<sup>-/-</sup> BM: n=8; *Bsg*<sup>-/-</sup> recipient mice with *Bsg*<sup>-/-</sup> BM: n=7). Results are expressed as mean $\pm$ SD. \**P*<0.05. **C**, Hypoxia induces reactive oxygen species (ROS) in the distal pulmonary vascular wall, which promotes cyclophilin A (CyPA) secretion. Extracellular CyPA recruits and stimulates inflammatory cells and promotes secretion of inflammatory cytokines and growth factors. Extracellular CyPA directly stimulates proliferation of vascular smooth muscle cells (VSMCs) through Bsg, promoting additional secretion of cytokines/chemokines and growth factors. The interaction between extracellular CyPA and Bsg in VSMCs may contribute to VSMC proliferation and pulmonary vascular remodeling.

nuclear factor E2-related factor-2 and its downstream heme oxygenase-1, both of which inhibit VSMC proliferation,<sup>37,38</sup> were induced in *Bsg*<sup>-/-</sup> as compared with *Bsg*<sup>+/+</sup> VSMCs (Figure 5C). Integrin  $\alpha$ 3 was inhibited in *Bsg*<sup>-/-</sup> as compared with *Bsg*<sup>+/+</sup> VSMCs under both normoxic and hypoxic conditions (Figure 5C). These data support the impaired inflammatory cell migration in the *Bsg*<sup>-/-</sup> lung.

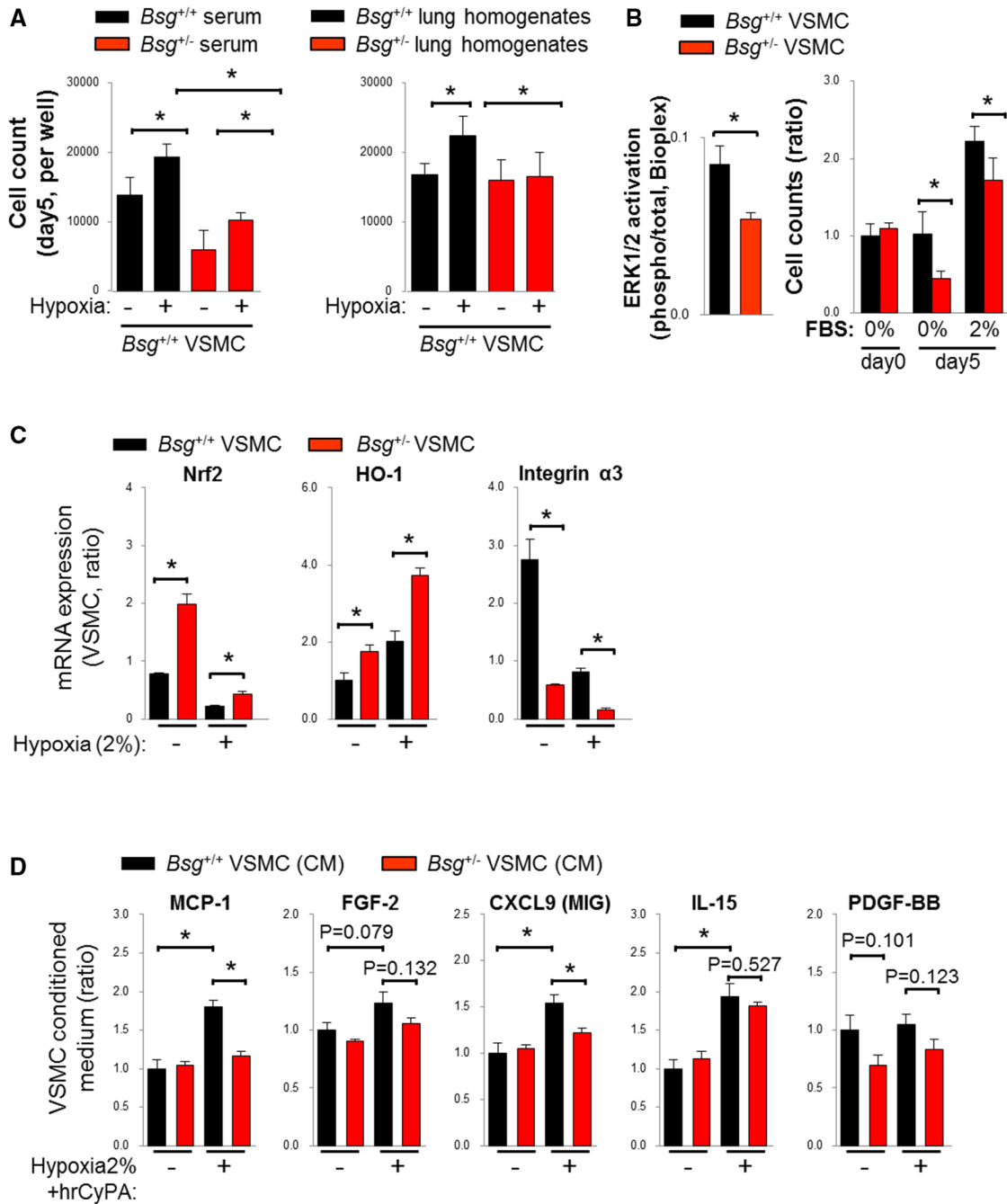
To characterize the mechanisms by which CyPA/Bsg signaling participates in hypoxia-induced VSMC proliferation, we examined the secretion of growth factors from VSMCs in vitro. Stimulation of *Bsg*<sup>+/+</sup> VSMCs with human recombinant CyPA and hypoxia promoted secretion of several cytokines/chemokines and growth factors, especially monocyte chemoattractant protein-1 (MCP-1), fibroblast growth factor 2 (FGF-2), chemokine (C-X-C motif) ligand 9 (monokine induced by gamma interferon [MIG]), and IL-15 (Figure 5D). Furthermore, Bsg deficiency blocked the secretion of these molecules, especially in MCP-1 and chemokine (C-X-C motif) ligand 9 (MIG; Figure 5D). These data suggest that extracellular CyPA mediates

an autocrine/paracrine function in VSMC proliferation partly through Bsg stimulation. However, we do not have a plausible explanation why the secretion of MCP-1 and chemokine (C-X-C motif) ligand 9 was specifically promoted by extracellular CyPA/Bsg signaling. Further analyses will provide us clues to understand the mechanism. Finally, Bsg expression in *Bsg*<sup>+/+</sup> VSMCs was induced by extracellular CyPA and was further augmented by hypoxia (Figure 6A). Thus, extracellular CyPA and VSMC Bsg are crucial for hypoxia-induced inflammation, thereby promoting VSMC proliferation by inducing growth factor secretion and inflammatory cell recruitment (Figure 6B).

### Plasma CyPA Is Associated With the Prognosis of Patients With PAH

Finally, we aimed to confirm the role of extracellular CyPA in the pathogenesis of PAH in humans. We examined human recombinant CyPA-induced secretion of growth factors from VSMCs harvested from the pulmonary arteries of patients with idiopathic PAH. Extracellular CyPA induced secretion of growth factors and

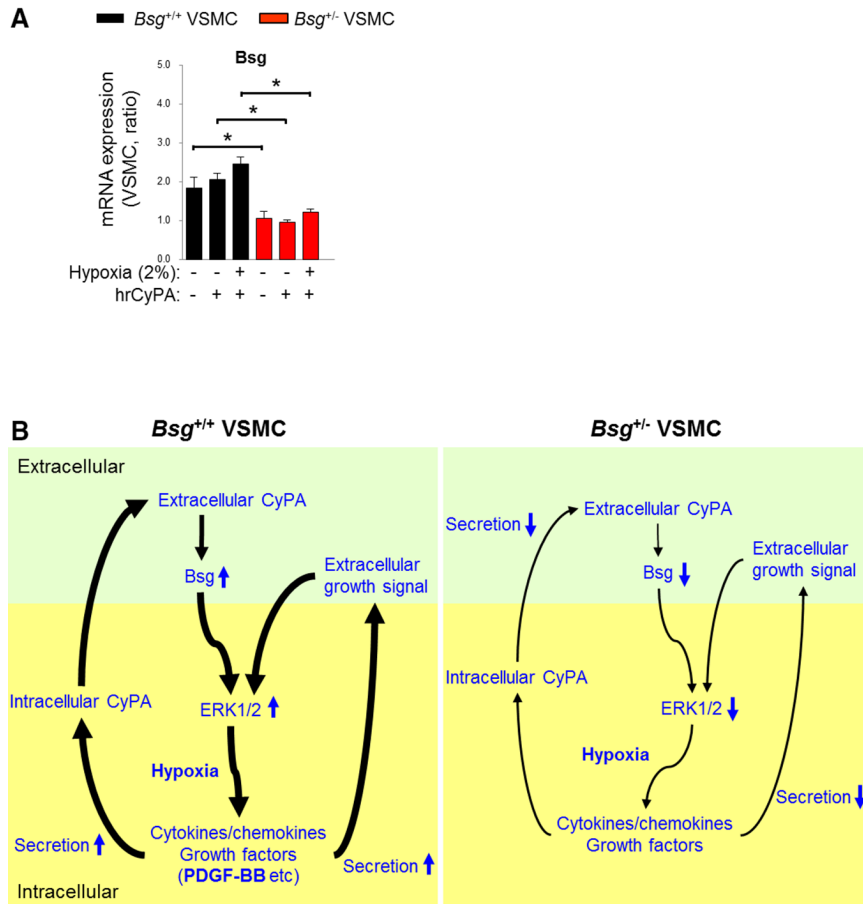




**Figure 5. Basigin (Bsg) is crucial for vascular smooth muscle cell (VSMC) proliferation and secretion of growth factors.** **A**, Stimulation of wild-type VSMCs with serum (left) or lung homogenates (right) prepared from normoxic and hypoxic *Bsg*<sup>+/+</sup> or *Bsg*<sup>-/-</sup> mice (all n=8). Results are expressed as mean±SD. \**P*<0.05. **B**, Graph shows activation of extracellular signal-regulated kinase 1/2 (ERK1/2; p-ERK1/2 per t-ERK1/2 ratio) in VSMCs harvested from *Bsg*<sup>+/+</sup> or *Bsg*<sup>-/-</sup> mice (left). Proliferation was significantly reduced in *Bsg*<sup>-/-</sup> VSMC after culture in medium with FBS (0%), FBS (2%) for 5 days (right). Results are expressed as mean±SD (n=8 samples per group). \**P*<0.05. **C**, mRNA expressions of nuclear factor E2-related factor-2 (Nrf2), heme oxygenase-1 (HO-1), and integrin α3 in VSMCs after 24-hour normoxic and hypoxic conditions (2% O<sub>2</sub>), as determined by quantitative real-time PCR. Results are expressed as mean±SD (n=3 samples per group). \**P*<0.05. **D**, The levels of inflammatory cytokines and growth factors in the conditioned medium (CM) of VSMCs treated with human recombinant cyclophilin A (hrCyPA, 50 nmol/L) and hypoxia (2% O<sub>2</sub>) for 24 hours. Results are expressed as mean±SEM. (n=4 samples per group). \**P*<0.05. CXCL indicates chemokine (C-X-C motif) ligand 2; FBS, fetal bovine serum; FGF, fibroblast growth factor; IL, interleukin; MCP, monocyte chemoattractant protein; and PDGF-BB, platelet-derived growth factor-BB.

chemokines (eg, PDGF-BB, SDF-1 [stromal cell-derived factor], and FGF-2) and inflammatory cytokines (eg, IL-1β, IL-2, and tumor necrosis factor-α) and this effect was enhanced by hypoxia (Figure 7A; Online Figure VII). These results support the notion that extracellular CyPA promotes the secretion of growth factors

from VSMCs in patients with PAH. Thus, we anticipated increased plasma levels of CyPA in patients with PAH. The clinical characteristics and laboratory data of patients with PAH are shown in Online Table I and Online Figure VIII. As anticipated, plasma CyPA levels were elevated in patients with PAH as compared with those without



**Figure 6. Cyclophilin A (CyPA)/basigin (Bsg) signaling promotes extracellular signal-regulated kinase 1/2 (ERK1/2) activation and vascular smooth muscle cell (VSMC) proliferation.** **A**, Bsg mRNA expression in VSMCs after treatment with human recombinant CyPA (hrCyPA, 50 nmol/L) with or without hypoxia (2% O<sub>2</sub>) for 24 hours, as determined by quantitative real-time PCR. Results are expressed as mean±SD (n=3 samples per group). \**P*<0.05. **B**, Extracellular Bsg in VSMCs. Extracellular CyPA and cytokines/chemokines and growth factors activate extracellular growth signals such as ERK1/2. Secretion of cytokines/chemokines and growth factors was reduced in *Bsg*<sup>-/-</sup> VSMCs exposed to extracellular CyPA and hypoxia. PDGF-BB indicates platelet-derived growth factor-BB.

PAH or healthy controls (Figure 7B). Finally, the event-free curve revealed that high plasma CyPA levels (>22 ng/mL) were associated with poor outcome (death or lung transplantation; Figure 7C), suggesting that plasma CyPA is a novel biomarker of disease severity, therapeutic efficacy, and prognosis in patients with PAH.

## Discussion

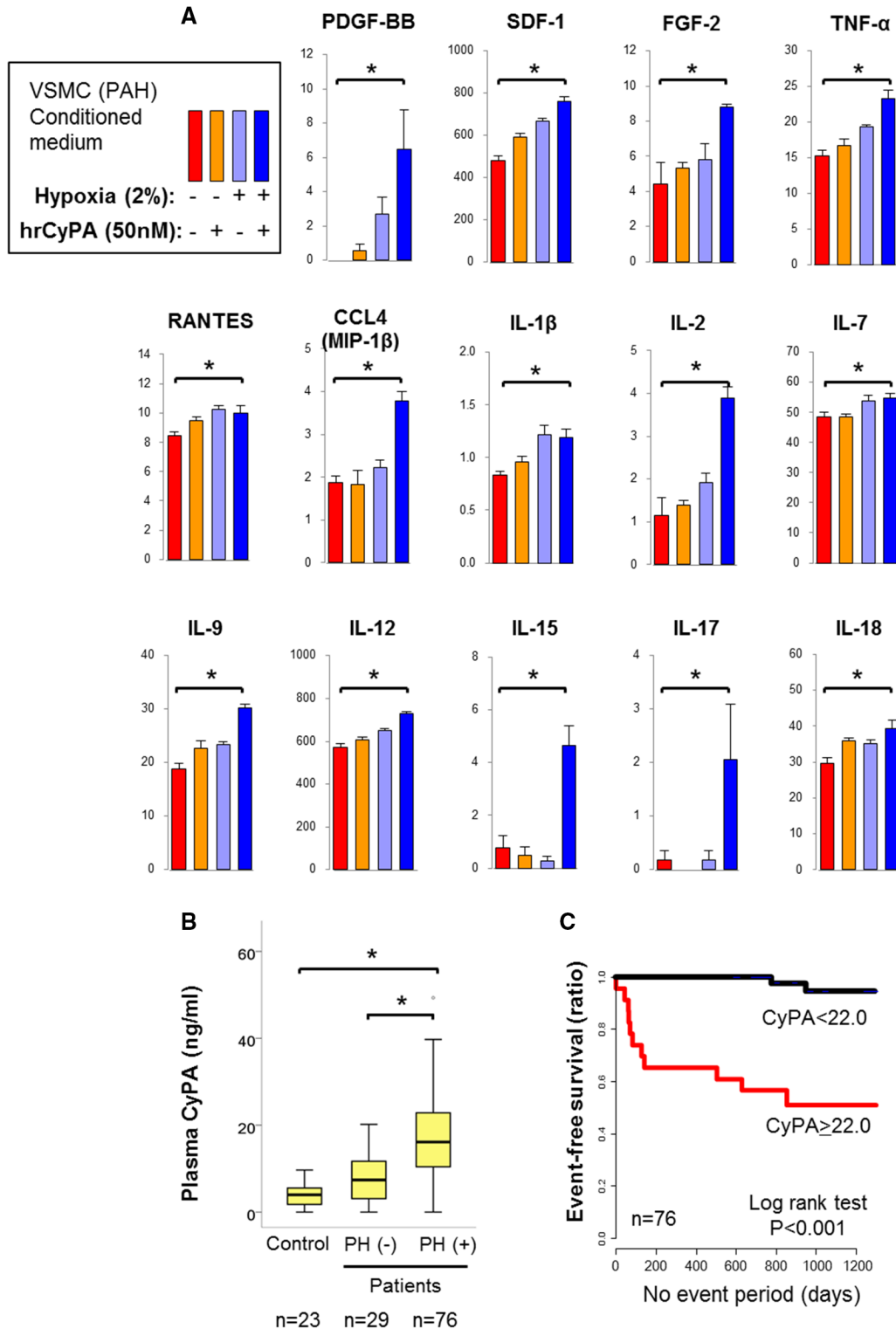
The major finding of this study is that CyPA/Bsg signaling is a novel promoter of PH. We demonstrated that extracellular CyPA and vascular Bsg are crucial for hypoxia-induced PH by inducing growth factor secretion, inflammatory cell recruitment, and VSMC proliferation. The central player in CyPA/Bsg-mediated PH development seems to be cells resident in the vessel wall, especially VSMCs. The development of PH in *Bsg*<sup>+/+</sup> recipient mice did not differ, even after transplantation of *Bsg*<sup>+/+</sup> or *Bsg*<sup>-/-</sup> bone marrow. In addition, PH severity was exacerbated in *Bsg*<sup>+/+</sup> versus *Bsg*<sup>-/-</sup> recipient mice, regardless of the bone marrow source (*Bsg*<sup>+/+</sup> or *Bsg*<sup>-/-</sup>). Based on these findings, we propose that hypoxia induces growth-promoting genes in VSMCs through a CyPA/Bsg-dependent pathway, a novel mechanism for hypoxia-induced PH. These results suggest that extracellular CyPA and vascular Bsg are crucial for PH development and are potential therapeutic target for cardiovascular diseases.

### Vascular Bsg Regulates Pulmonary Vascular Remodeling

We have shown that CyPA is strongly expressed at coronary segments with unstable atherosclerotic plaques and is

increased in the plasma of patients with coronary artery disease<sup>39</sup>; however, the role of extracellular CyPA and its signaling pathway in vivo remained to be examined. In this study, we thus aimed to characterize the role of extracellular CyPA in hypoxia-induced PH and its Bsg-mediated mechanisms.

The present results provide mechanistic insights into CyPA-mediated cardiovascular diseases. Specifically, we propose that vascular Bsg regulates CyPA-mediated pulmonary vascular remodeling and inflammation that leads to PH for the following reasons. First, we demonstrated that growth factor secretion is attenuated in *CyPA*<sup>+/-</sup> and *Bsg*<sup>+/-</sup> mice, resulting in decreased inflammation and vascular remodeling. Second, hypoxia promoted secretion of CyPA from VSMCs, cytokines/chemokines, and growth factors, resulting in Bsg-mediated activation of the ERK1/2 signaling pathway. Third, *Bsg*<sup>-/-</sup> VSMCs exhibited increased expression of nuclear factor E2-related factor-2 and heme oxygenase-1, which inhibit hypoxia-induced PH.<sup>40,41</sup> Consistently, in *Bsg*<sup>-/-</sup> VSMCs, proliferation of VSMCs was inhibited and integrin expression and secretion of growth factors were also inhibited in response to extracellular CyPA and hypoxia. Thus, extracellular CyPA and vascular Bsg cooperatively stimulate recruitment of inflammatory cells to the vessel wall, exacerbating perivascular inflammation and VSMC proliferation. Thus, the cooperative interaction between extracellular CyPA and pulmonary vascular Bsg is critical for the process of hypoxia-induced PH.



**Figure 7. Plasma levels of cyclophilin A (CyPA) predicts prognosis in patients with pulmonary arterial hypertension (PAH).** Extracellular CyPA promotes secretion of growth factor and inflammatory cytokines from pulmonary artery vascular smooth muscle cell (VSMC) from patients with idiopathic pulmonary arterial hypertension. **A**, Growth factors and inflammatory cytokines in conditioned medium from pulmonary artery VSMCs after treatment with human recombinant CyPA (hrCyPA, 50 nmol/L) and hypoxia (2% O<sub>2</sub>) for 48 hours. Results are expressed as mean $\pm$ SD (n=4 samples per group). \*P<0.05. **B**, Plasma levels of CyPA in patients with pulmonary hypertension (PH; n=76), those without PH (n=29) and healthy controls (n=23). Box and whisker plots of plasma levels of CyPA. \*P<0.05. **C**, The Kaplan–Meier curve shows that patients with high plasma CyPA ( $\geq$ 22.0 ng/mL) had more cardiovascular events than those with low plasma CyPA (<22.0 ng/mL). \*P<0.05. CCL indicates chemokine (C-C motif) ligand; FGF, fibroblast growth factor; IL, interleukin; MIP, macrophage inflammatory proteins; PDGF-BB, platelet-derived growth factor-BB; RANTES, regulated on activation, normal T cell expressed and secreted; SDF, stromal cell-derived factor; and TNF, tumor necrosis factor.

## Vascular Bsg Promotes Growth Factor Secretion and Inflammation

We further propose a key role for CyPA/Bsg signaling in pulmonary vascular remodeling. Specifically, we propose that hypoxia-induced secretion of growth factors and cytokines/chemokines requires CyPA/Bsg signaling in the pulmonary vasculature. A recent *in vivo* study showed that Bsg in circulating inflammatory cells functions as a CyPA receptor.<sup>42,43</sup> Consistently, in the present study, Bsg expression was intense in the perivascular inflammatory cells of animal models of PH and patients with PAH. The migration of inflammatory cells to the pulmonary arteries was reduced in *Bsg*<sup>+/-</sup> mice, as was the secretion of inflammatory cytokines/chemokines in the lung. However, against our initial hypothesis, hypoxia-induced migration of GFP<sup>+</sup> cells was significantly reduced in *Bsg*<sup>+/-</sup> versus *Bsg*<sup>+/+</sup> recipient mice even after we transplanted GFP<sup>+</sup> bone marrow cells from the same donor mouse. PH development was dependent on Bsg expression in the recipient mice but not in the bone marrow cells. These results suggest that the main function of Bsg in CyPA-mediated vascular remodeling and PH is not in the inflammatory cells but in the pulmonary vascular Bsg. Bsg induces Rac-1–dependent expression of inflammatory cytokines<sup>44</sup> and promotes VSMC proliferation.<sup>45</sup> These reports support our notion that the secretion of inflammatory cytokines was augmented by cooperative interaction between extracellular CyPA and Bsg in the pulmonary vasculature. Thus, we conclude that vascular CyPA/Bsg signaling is central to the secretion of growth factors, recruitment of inflammatory cells, and pulmonary vascular remodeling.

## Clinical Implications

A key aspect of this study that deserves comment is the strong expression of CyPA and Bsg in the pulmonary arteries of animal models of PH and patients with PAH. We have previously reported that statins and Rho-kinase inhibitors reduce CyPA secretion from VSMCs.<sup>29,46</sup> Rho-kinase is an important therapeutic target in cardiovascular diseases<sup>47</sup> and Rho-kinase inhibition ameliorates PH in animals and humans.<sup>39,48–50</sup> Based on the present study, inhibition of CyPA secretion by Rho-kinase inhibitors may contribute to the therapeutic efficacy of these drugs in PAH.<sup>48,49</sup> Children with severe malaria have been reported to develop PH.<sup>51</sup> The mechanisms of these complications remain elusive. When we consider the role of Bsg as an essential receptor for erythrocyte invasion by *Plasmodium falciparum*,<sup>52</sup> Bsg may contribute to the pathogenesis of PH in patients with severe malaria. Indeed, a recent article demonstrated Bsg expression in VSMCs,<sup>53</sup> a process that may be activated by binding of extracellular CyPA.<sup>25</sup> In addition, Bsg stimulates MMP production.<sup>54</sup> We demonstrated that Bsg is strongly expressed in the pulmonary arteries of patients with PAH. Thus, it is logical to consider that pharmacological agents that prevent the interaction of extracellular CyPA and vascular Bsg could be useful for the treatment of PAH.

Effective management of PAH requires comprehensive prognostic evaluation to determine optimal management strategies.<sup>55</sup> Although several clinical and hemodynamic parameters linked to PAH prognosis have been identified, some are associated with significant limitations such as invasive techniques and subjective measures. Thus, there is a need for a noninvasive biomarker for diagnosis and assessment

of disease prognosis that can predict therapeutic response in patients with PAH. The identification of CyPA as a novel biomarker and mediator of PH associated with inflammation provides insight into the mechanisms of several therapies.

## Potential Bsg-Independent Functions of CyPA and Conclusions

Our previous work implicated an increase in reactive oxygen species signaling in VSMCs by extracellular CyPA.<sup>31</sup> As to the role of reactive oxygen species in hypoxia-induced PH model in mice, PAH in humans and in VSMCs in hypoxia is an issue with significant conflicts and controversies.<sup>56</sup> More studies are needed to address this issue.<sup>57–60</sup> Thus, in the present study, we focused on the Bsg-mediated secretion of CyPA, cytokines/chemokines, and growth factors. In this process, we think that the extracellular CyPA and VSMC Bsg play a crucial role for connecting cell–cell interaction, inflammation, and VSMC proliferation.

Next, we need to discuss as to the Bsg-independent functions of CyPA. Beside the role of extracellular CyPA, there is a potential role of intracellular CyPA on NFAT activation.<sup>61</sup> CyPA has been found as a binding partner of cyclosporine A, which is an immunosuppressive drug in clinical use.<sup>31</sup> It has been established that the CyPA–cyclosporine A complex binds to and inactivates calcineurin, which activates nuclear factor of activated T cells transcription factors.<sup>61</sup> Because this step is important for cytokine/chemokines production and secretion, inhibition of calcineurin by cyclosporine A exerts anti-inflammatory effects. Here, there is strong evidence of an important role of NFAT in PAH in both the PA VSMC and the infiltrating inflammatory cells.<sup>57,62,63</sup> One of the mechanisms for the decreased PH in our models could be the suppression of NFAT signaling.<sup>64</sup> Thus, there is a potential Bsg-independent role of intracellular CyPA on NFAT activation in the development of PAH, and the mechanisms for the decreased PH in our models could be the suppression of NFAT signaling.

Finally, it has been reported already that extracellular CyPA augments endothelial expression of E-selectin.<sup>22</sup> Bsg is also suggested to augment inflammation partly through E-selectin expression on neutrophil.<sup>65</sup> However, in our hypoxia-induced PH model in mice, there was no significant change in E-selectin expression in lung tissues. This suggests the possible importance of Bsg in the specific conditions of hypoxia, which augments inflammation through extracellular CyPA.

In conclusion, the present study suggests that extracellular CyPA and pulmonary vascular Bsg provide a novel therapeutic target for patients with PAH.

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## Disclosures

None.

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## Novelty and Significance

### What Is Known?

- Pulmonary vascular inflammation plays a crucial role in the development of hypoxia-induced pulmonary hypertension (PH), for which Rho-kinase plays a crucial role.
- Rho-kinase promotes secretion of cyclophilin A (CyPA) from vascular smooth muscle cells and extracellular CyPA stimulates endothelial cell adhesion molecule expression, vascular smooth muscle cell proliferation and is a chemoattractant for inflammatory cells.
- Basigin (Bsg) is an essential receptor for malaria, which disrupts nitric oxide metabolism and causes harmful endothelial activation, including the Rho/Rho-kinase activation.

### What New Information Does This Article Contribute?

- CyPA/Bsg signaling is a novel promoter of PH.
- Hypoxia induces growth-promoting genes in vascular smooth muscle cells through a CyPA/Bsg-dependent pathway.
- Extracellular CyPA and vascular Bsg are crucial for PH development and are potential therapeutic target for cardiovascular diseases.

Statins and Rho-kinase inhibitors have been shown to reduce CyPA secretion from vascular smooth muscle cells. Rho-kinase

is an important therapeutic target in cardiovascular diseases and Rho-kinase inhibition ameliorates PH in animals and humans. We demonstrated the strong expression of CyPA and Bsg in the pulmonary arteries of animal models of PH and patients with pulmonary arterial hypertension. Based on this observation, inhibition of CyPA secretion by statins and Rho-kinase inhibitors may contribute to the therapeutic efficacy of these drugs in PH. Children with severe malaria have been reported to develop PH. When we consider the role of Bsg as an essential receptor for erythrocyte invasion by malaria, Bsg may contribute to the pathogenesis of PH in patients with severe malaria. Therefore, pharmacological agents that prevent the interaction of extracellular CyPA and vascular Bsg will be useful for the treatment of PH. Finally, effective management of PH requires comprehensive prognostic evaluation to determine optimal management strategies. Thus, there is a need for a noninvasive biomarker for diagnosis and assessment of disease prognosis. The identification of CyPA as a novel biomarker and mediator of PH will provide tools for the early diagnosis and assessment of PH.

## **Online Supplement**

### **Basigin Mediates Pulmonary Hypertension by Promoting Inflammation and Vascular Smooth Muscle Cell Proliferation**

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**Online Supplement Detailed Methods**

**Online Figures I-VIII**

**Online Figure Legends**

**Online Table I**

**Online Supplement References**

## A detailed, expanded Methods section

### Human Lung Samples

All protocols using human specimens were approved by the Institutional Review Board of Tohoku University, Sendai Japan. Lung tissues were obtained from patients with IPAH at the time of lung transplantation or from control patients at the time of thoracic surgery for lung cancer at a site far from the tumor margins. Before surgery, all patients provided written consent for the use of their lung tissues for IPAH research. For *ex vivo* culture, fresh lung samples obtained during surgery were minced into approximately 200 mg blocks. We maintained equal wet weight of the minced tissue in each well of 6-well plates with Dulbecco's modified Eagle's medium (DMEM). We collected the conditioned medium 24 h after incubation as previously described.<sup>1</sup>

### Isolation of Human Pulmonary Arterial VSMCs

Small pulmonary arteries were also obtained at the time of lung transplantation from patients with IPAH. Pulmonary arterial VSMCs from IPAH patients were isolated from pulmonary arteries smaller than 1.5 mm outer diameter.<sup>2</sup> Pulmonary arterial VSMCs were cultured in DMEM containing 10% FBS at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Passage 4 to 7 VSMCs at 70–80% confluence were used for experiments.

### Preparation of Conditioned Medium

Conditioned medium from VSMCs exposed to normoxia or hypoxia (2% O<sub>2</sub>) in DMEM was collected and filtered to remove cell debris. Likewise, the lungs were incubated for 24 h in DMEM. Collected medium was concentrated 100-fold with an Amicon Ultra filter (Millipore Corporation) to yield concentrated conditioned medium (CM).<sup>3-5</sup>

### Generation and Genotyping of *Ppia*<sup>+/-</sup> and *Bsg*<sup>+/-</sup> Mice

All animal experiments were conducted in accordance with the experimental protocols approved by the Institutional Animal Care and Use Committee of Tohoku University. *Ppia*<sup>+/-</sup> mice were purchased from Jackson Laboratory (Bar Harbor, Maine, stock number 005320). *Bsg*<sup>+/-</sup> mice were produced by Dr. Kadomatsu at Nagoya University (Nagoya, Japan).<sup>6</sup> *Bsg* is an important cell-surface molecule involved in early embryogenesis and reproduction.<sup>7,8</sup> Since complete *Bsg* disruption (*Bsg*<sup>-/-</sup>) in mice results in perinatal lethality, poor pregnancy or abnormal development, we used *Bsg*<sup>+/-</sup> mice in the present study. This is also the same in complete CyPA-deficient mice as to the poor pregnancy.<sup>1</sup> All mice were genotyped by PCR amplification of tail clip samples, and all experiments were performed with male mice using littermate *Ppia*<sup>+/+</sup> or *Bsg*<sup>+/+</sup> as wild-type controls. Animals were housed under a 12-h light and 12-h dark regimen and placed on a normal chow diet. Genotyping primers were as follows: *Ppia*, Common 5'-GCAGTTGTGATTGATCCAGGTCCG-3' and Wild type 5'-CACCTGGAGCACCACTGCCACC-3' and Mutant 5'-CCTGATCGACAAGACCGGCTTCC-3'; *Bsg*, Basigin forward 5'-TGGCCTTCACGCTCTTGAGC-3' and Basigin reverse 5'-GCCTCATCTCTAAGATCACT-3' and Neo forward 5'-CAGCGTCTTGTCATTGGCGA-3' and Neo reverse 5'-GCTCTTCGTCCAGATCATCC-3'.

### Animal Experiments



All animal experiments were conducted in accordance with the protocols approved by the Tohoku University Animal Care and Use Committee. Hypoxia-induced pulmonary hypertension (PH) models were used to assess the effect of Cyclophilin A (CyPA) and Bsg deficiency on PH development in mice. Twelve to 16-week-old male littermate control mice and *Bsg*<sup>+/-</sup> and *Ppia*<sup>+/-</sup> mice on a normal chow diet were exposed to hypoxia or normoxia for 4 weeks as previously described.<sup>9, 10</sup> Briefly, hypoxic mice were housed in an acrylic chamber with a non-recirculating gas mixture of 10% O<sub>2</sub> and 90% N<sub>2</sub> by adsorption-type oxygen concentrator to utilized exhaust air (Teijin, Japan), while normoxic mice were housed in room air (21% O<sub>2</sub>) under a 12-h light-dark cycle.<sup>9, 10</sup> Assessment of PH and histology for mouse type was performed after 4 weeks of hypoxic or normoxic exposure. To determine the effect of CyPA and Bsg deficiency on hypoxia-induced PH development, we measured right ventricle systolic pressure (RVSP), right ventricular hypertrophy (RVH) and pulmonary vascular remodeling.<sup>9, 10</sup>

### **Hemodynamic Measurements**

Twelve to 16-week-old male mice were exposed to hypoxia or normoxia for 4 weeks as previously described.<sup>9, 10</sup> Briefly, hypoxic mice were housed in an acrylic chamber with non-recirculating gas mixture of 10% O<sub>2</sub> and 90% N<sub>2</sub> by adsorption-type oxygen concentrator to utilized exhaust air (Teijin, Japan), while normoxic mice were housed in room air (21% O<sub>2</sub>) under a 12-h light-dark cycle. After 4 weeks of exposure to hypoxia (10% O<sub>2</sub>) or normoxia, mice were anesthetized with isoflurane (1.0%). For right heart catheterization, a 1.2-F pressure catheter (SciSense Inc., Ontario, Canada) was inserted in the right jugular vein and advanced into the right ventricle (RV) to measure RV systolic pressure (RVSP).<sup>9, 10</sup> All data were analyzed using the PowerLab data acquisition system (AD Instruments) and averaged over 10 sequential beats.

### **Histology**

After hemodynamic measurements, animals were anesthetized with isoflurane (1.0%). Pulmonary arteries were perfused with phosphate-buffered saline (PBS) and perfusion-fixed with 10% phosphate-buffered formalin at physiological pressure for 5 min. The whole heart and lungs were harvested, fixed for 24 h, embedded in paraffin, and cross sections (3 μm) were prepared. Paraffin sections were stained with Elastica-Masson (EM) or used for immunostaining. Pulmonary arteries adjacent to an airway distal to the respiratory bronchiole were evaluated as previously reported, with modifications.<sup>9, 10</sup> Briefly, arteries were considered fully muscularized if they had a distinct double elastic lamina visible throughout the diameter of the vessel cross section. The arteries were considered partially muscularized if they had a distinct double elastic lamina visible for at least half the diameter. The percentage of vessels with double elastic lamina was calculated as the number of muscularized vessels per total number of vessels counted. In each section, a total of 60–80 vessels were examined by use of a computer-assisted imaging system (BX51, Olympus, Tokyo, Japan). This analysis was performed in the small vessels with external diameters of 20~70 μm.

### **Assessment of Right Ventricular Hypertrophy**

After right ventricular pressure was recorded, the animals were exsanguinated and the lungs and heart were isolated and fixed with 10% phosphate-buffered formalin. Formaldehyde-fixed dry hearts were dissected and the right ventricular wall was removed from the left ventricle and septum. The ratio of the right ventricle to the left ventricle plus septum weight [RV/(LV+S)] was calculated to determine the extent of right ventricular hypertrophy.

## **Immunohistochemistry**

Lung tissues were obtained from patients with IPAH at the time of lung transplantation. Before surgery, all patients provided written consent for the use of their lung tissues for IPAH research. Ethics approval was obtained from the Ethics Committee of Tohoku University Graduate School of Medicine. CyPA immunostaining has been described elsewhere.<sup>4, 5, 11</sup> In brief, formaldehyde-fixed sections were incubated with primary antibody overnight at 4°C.<sup>12</sup> To detect CyPA, peroxidase-conjugated streptavidin (1:1000 dilution; Jackson Immuno, 016-030-084) was counterstained with hematoxylin. The primary antibodies were CyPA polyclonal (1:1000 dilution; BIOMOL Research Laboratories, Inc.), Bsg (1:400 dilution; R&D systems, AF972 for human tissue and AF772 for mouse tissue),  $\alpha$ -smooth muscle actin ( $\alpha$ SMA, clone 1A4, 1:400 dilution; Sigma-Aldrich, A5691), CD31 (PECAM-1, 1:400 dilution; BD Pharmingen), leukocyte common antigen, CD45 (clone Ly-5, 1:100 dilution; BD Pharmingen, #550539), and Mac-3 (CD107b, 1:400 dilution; BD Pharmingen, #553322). As a negative control, species- and isotype-matched IgG were used in place of the primary antibody. Slides were viewed with a microscope (BX51, Olympus, Tokyo, Japan) equipped with a digital camera and analyzed by DP Controller and DP Manager Software (Olympus, Tokyo, Japan).

## **Monocrotaline**

Adult male Sprague-Dawley rats (6 weeks of age, 150~170g body weight) were used in the present study. All procedures were performed according to the protocols approved by the Institutional Committee for Use and Care of Laboratory Animals of Tohoku University. Rats were housed under climate controlled conditions on a 12:12-hour light-dark cycle with access to chow and water. Animals were allowed 1 week to adjust to the new environment. Monocrotaline (Sigma-Aldrich Co, St. Louis, MO, USA) was dissolved in 1N HCl, and the pH was adjusted to 7.4 with 1N NaOH. The solution was administered as a single subcutaneous injection (60 mg/kg) in a volume of 3 ml/kg. Control, age-matched rats received an equal volume of isotonic saline.

## **Bone Marrow Transplantation**

Bone marrow transplantation was performed as previously described.<sup>1, 9, 10, 13, 14</sup> Briefly, recipient mice were lethally irradiated and received an intravenous injection of  $5 \times 10^6$  donor GFP<sup>+</sup> bone marrow cells suspended in 100  $\mu$ l calcium- and magnesium-free PBS with 2% fetal bovine serum (FBS). After transplantation, the mice were placed on a regular chow diet for 6 weeks followed by hypoxic exposure (10% O<sub>2</sub>) for 4 weeks. Transgenic mice ubiquitously expressing green fluorescent protein (GFP) were obtained from Jackson Laboratory.

## **Bone Marrow-Derived Cell Recruitment Assays**

Whole lung imaging was performed 4 weeks after exposure to hypoxia (10% O<sub>2</sub>) or normoxia. Animals were anesthetized with isoflurane (1.0%) and pulmonary arteries were perfused with PBS and perfusion-fixed with 4% phosphate-buffered paraformaldehyde at physiological pressure for 5 min. Whole lungs were viewed with an entity fluorescence microscope (Leica, MZ16FA) equipped with a digital camera. The whole heart and lungs were harvested, fixed for 6 h, embedded in OCT (Tissue-Tek; Miles Inc., Elkhart, Illinois, USA) and snap-frozen, and cross-sections (20  $\mu$ m) were prepared. Migrating GFP<sup>+</sup> cells and pulmonary microvasculature were analyzed by labeling with primary mAbs against  $\alpha$ SMA and confocal microscopy (Zeiss, LSM780).

### **Harvest of Mouse Pulmonary Artery VSMCs**

Mouse pulmonary artery VSMCs were cultured from each group of 23–26 g male mice and maintained in DMEM containing 10% FBS at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air as previously described.<sup>15</sup> Passage 4 to 7 VSMCs at 70–80% confluence were used for experiments.

### **Measurement of Cytokine/Chemokine and Growth Factors by Bioplex System**

Cytokine/chemokine secretion from VSMCs or whole lung and their levels in serum were measured with a Bioplex system according to the manufacturer's instructions (Bio-Rad, Tokyo, Japan). We measured cytokines in conditioned medium from VSMCs (100-mm dish, 10 mL DMEM). To analyze the levels of cytokines/chemokines in the lung tissues, pulmonary arteries were perfused with PBS and the circulating blood was completely removed. Left lung tissues were homogenized with Tissue Protein Extraction Reagent (500 µL, T-PER, Pierce, #78510), centrifuged (4°C, 2500 g, 20 min), and clear supernatants were standardized for total protein content using the BCA Protein Assay kit (Pierce).<sup>13</sup> Mouse cytokines/chemokines and growth factors were measured with commercially available kits (Bio-Rad, 9-Plex, MD0-00000EL and 23-Plex, M60-009RDPD). Human cytokines/chemokines and growth factors were measured with commercially available kits (Bio-Rad, 27-Plex, #M50-0KCAF0Y and 21-Plex, #MF0-005KMII). Each experiment was performed in duplicate.

### **Western blotting**

VSMCs were treated with human recombinant Cyclophilin A (50 nM, Prospec, US) with or without human recombinant PDGF-BB (20 ng/mL; R&D systems, RSD-220-BB-010-10UG) in normoxic and hypoxic (O<sub>2</sub> 2%) conditions. VSMCs were washed twice with cold PBS and harvested on ice in Cell Lysis Buffer (Cell Signaling Technology, #9803) with protease inhibitor cocktail (Sigma, P8340). Total cell lysates were loaded on SDS-PAGE, electrotransferred to a nitrocellulose membrane, and blocked for 1 h at room temperature in 5% BSA in Tris-Buffered Saline with Tween 20 (TBST). The primary antibodies were CyPA (1:1000 dilution; BIOMOL Research Laboratories, Inc.), epidermal growth factor receptor (EGFR) (D38B1, 1:1000 dilution; Cell Signaling, #4267S), Bsg (1:1000 dilution; R&D systems, AF972 for human tissue and AF772 for mouse tissue), phospho-ERK1/2 (1:1000 dilution; Cell Signaling, #4370L), total-ERK1/2 (1:1000 dilution; Cell Signaling, #4695S), and  $\alpha$ -tubulin (1:5000 dilution; Sigma-Aldrich, T5168). Proteins were visualized by the enhanced chemiluminescence system (ECL Western Blotting Detection Kit, GE Healthcare). Densitometric analysis was performed with Image J Software (NIH).

### **RNA Isolation and Real-time PCR**

Isolation of total RNA from mouse VSMCs or lung tissues was performed using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol. Total RNA was converted to cDNA using PrimeScript RT Master Mix (Takara). Primers for murine *Bsg* (Assay ID: Mm01144228\_g1), *BMPR2* (Assay ID: Mm00432134\_m1), *Nox2* (*Cybb*, Assay ID: Mm01287743\_m1), *p47phox* (Assay ID: Mm00447921\_m1), *Nfe2l2* (*Nrf2*, Assay ID: Mm00477784\_m1), *Hmox1* (Assay ID: Mm00516005\_m1), *Ctgf* (Assay ID: Mm01192932\_g1), *Rhoa* (Assay ID: Mm00834507\_g1), and *Gapdh* (Assay ID: Mm99999915\_g1) were purchased from Life Technologies (TaqMan assays, Applied Biosystems, US). Quantitative real-time PCR on the CFX 96 Real-Time PCR Detection System (Bio-Rad) was performed using SsoFast Probes Supermix (Bio-Rad) with TaqMan Probes for murine samples. The Ct value determined

by CFX Manager Software (version 2.0, Bio-Rad) for all samples was normalized to housekeeping gene *Gapdh* and the relative fold change was computed by the  $\Delta\Delta C_t$  method.

### **Cell Proliferation Assay**

Mouse pulmonary arterial VSMCs were seeded in 24-well plates (200,000 cells/well) in DMEM with 10% FBS. The next day, VSMCs were starved for 24 h, then stimulated with 0% FBS, 2% FBS, or human recombinant Cyclophilin A (50 nM) with or without PDGF-BB (20 ng/mL) for up to 5 days. Medium was changed on day 3 and cells were counted on day 5.

### **Statistical Analyses in Animal Experiments**

Results are expressed as mean  $\pm$  standard error of the mean (s.e.m.) for all studies except as mentioned in the figure legends. Comparisons of means between 2 groups were performed by Welch's *t*-test. Comparisons of mean responses associated with two of the two main effects of the different genotypes and the severity of pulmonary vascular remodeling were performed by two-way analysis of variance (ANOVA), followed by Tukey's HSD (honestly significant difference) multiple comparisons. All reported P values are 2-tailed, with a P value of less than 0.05 indicating statistical significance. Analyses were performed in SPSS, version 19.0 (Chicago, IL, USA) and R version 3.0.1.<sup>16</sup>

### **Clinical Study**

We conducted an observational study of the prognostic value of CyPA in patients with symptoms or signs of PH who were referred to Tohoku University Hospital in Sendai for right heart catheter examination from November 2007 through January 2012. If patients underwent catheter examination more than once, our analysis was based only on data obtained from the first examination. Patients with pulmonary arterial hypertension (PAH) were enrolled. Patients with cancer were excluded. A total of 105 consecutive patients with signs of PH on echocardiography or background diseases such as collagen diseases or congenital heart diseases were enrolled. Patients with chronic thromboembolic pulmonary hypertension (CTEPH) were excluded. The Ethical Review Board of our institution approved the study and written informed consent was obtained from all participants.

### **Right Heart Catheter Examination**

At baseline, right heart catheter examination was performed with recordings on a data system. Two experienced cardiologists, blinded to the patients' CyPA plasma levels, evaluated mean pulmonary arterial pressure (mean PAP) and made a diagnosis of PH when mean PAP was  $\geq 25$  mmHg<sup>17</sup>. PVR was calculated as the TPPG (defined as mPAP-PCWP, mmHg) divided by cardiac output (CO, L/min). The relationship between plasma CyPA and the presence or absence of PH was analyzed. As a control, we evaluated the plasma levels of CyPA in patients who underwent coronary angiography and were diagnosed with chest pain syndrome because of the absence of organic stenosis and coronary vasospasm.

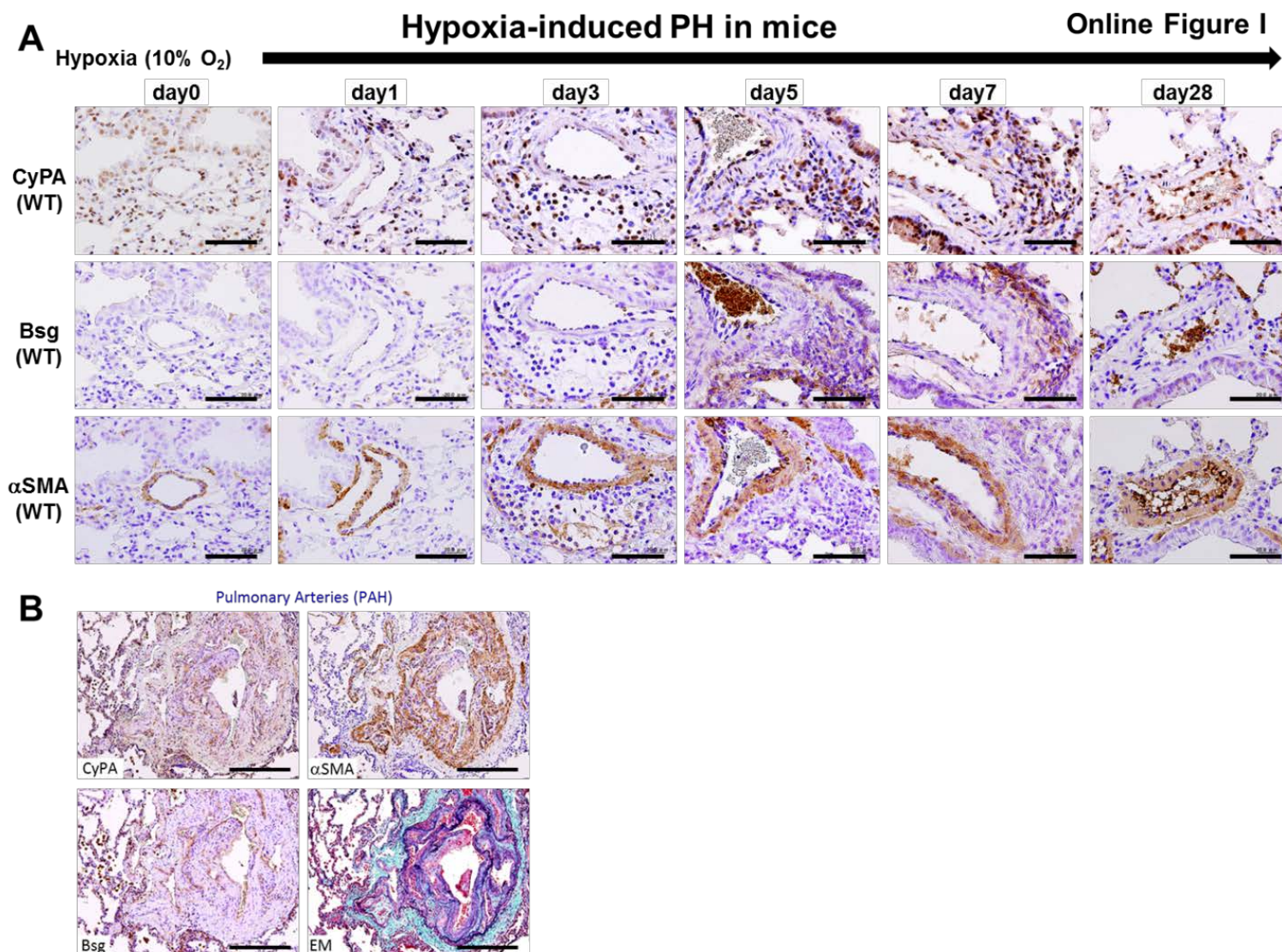
### **Baseline Measurements**

Information on vital status and data were obtained from our department's database by means of a computerized search performed on July 1, 2013.<sup>18</sup> No patients were lost to follow-up. Fasting blood samples from the vein of patients who were resting in the supine position were drawn for measurement of CyPA before catheter examination. Plasma samples were collected using EDTA and centrifuged for 10 min at 2,500 g within 30 min of collection; aliquots were stored at -80°C.

CyPA was measured by immunoassay based on the sandwich technique (Human Cyclophilin A ELISA Kit, CSB-E09920h, Cusabio). Across the entire analysis in our laboratory, duplicate measures of plasma CyPA were highly correlated ( $r = 0.92$ ).<sup>19</sup>

### **Statistical Analyses in the Clinical Study**

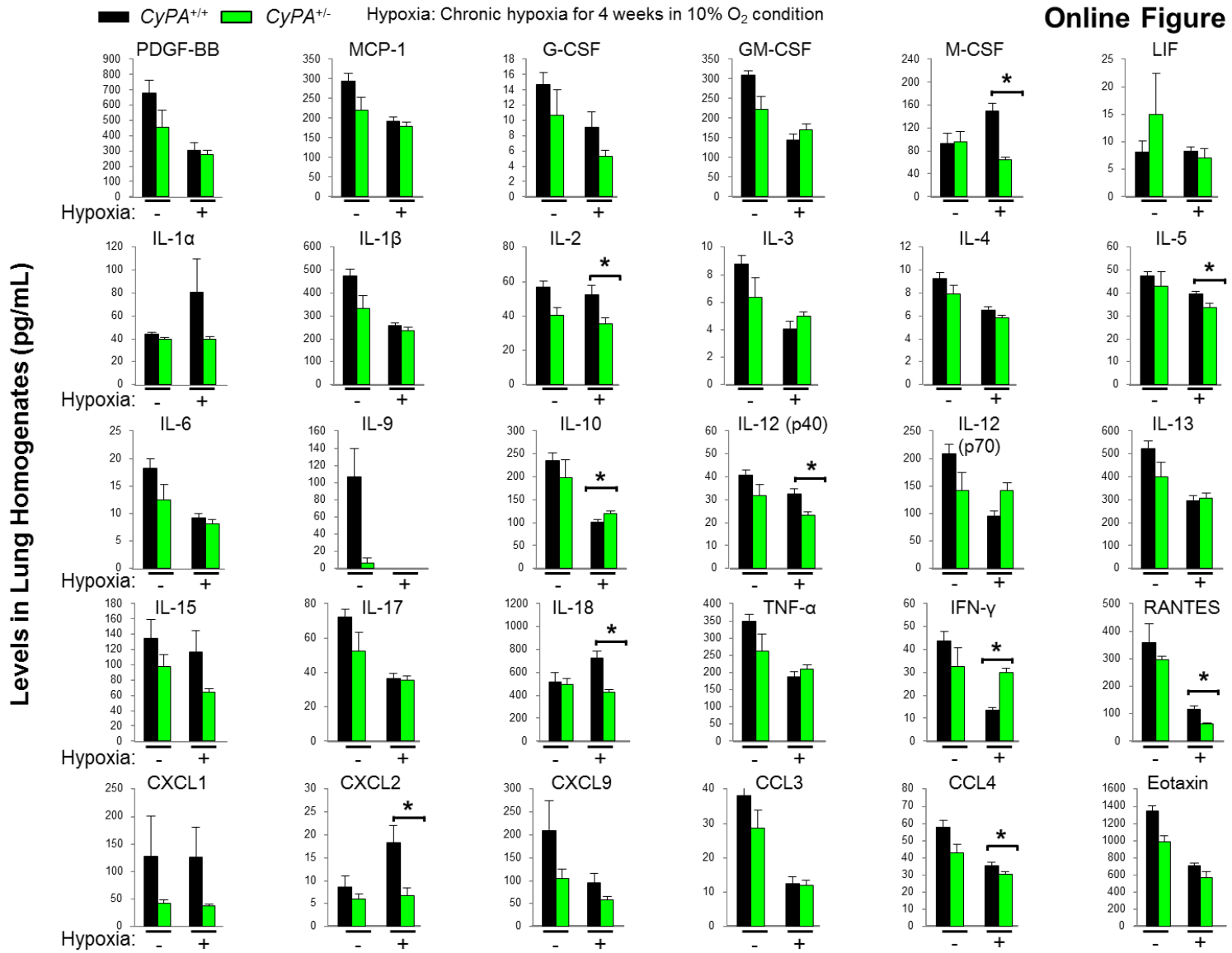
Plasma levels of CyPA were presented as mean  $\pm$  standard error of the mean (s.e.m.), or medians and interquartile range (IQR) for variables with skewed distributions. CyPA analyses were performed in subgroups defined according to the increase (worsening) or decrease (improvement) of PVR after medical treatment. A Welch's *t*-test was used for comparisons between groups. Event (death or lung transplantation)-free survival curves were generated for both groups (CyPA  $\geq 22.0$  ng/mL vs. CyPA  $< 22.0$  ng/mL). All reported P values are 2-tailed, with a P value of less than 0.05 indicating statistical significance. Analyses were performed in SPSS, version 19.0 (Chicago, IL, USA) and R version 3.0.1.<sup>16</sup>



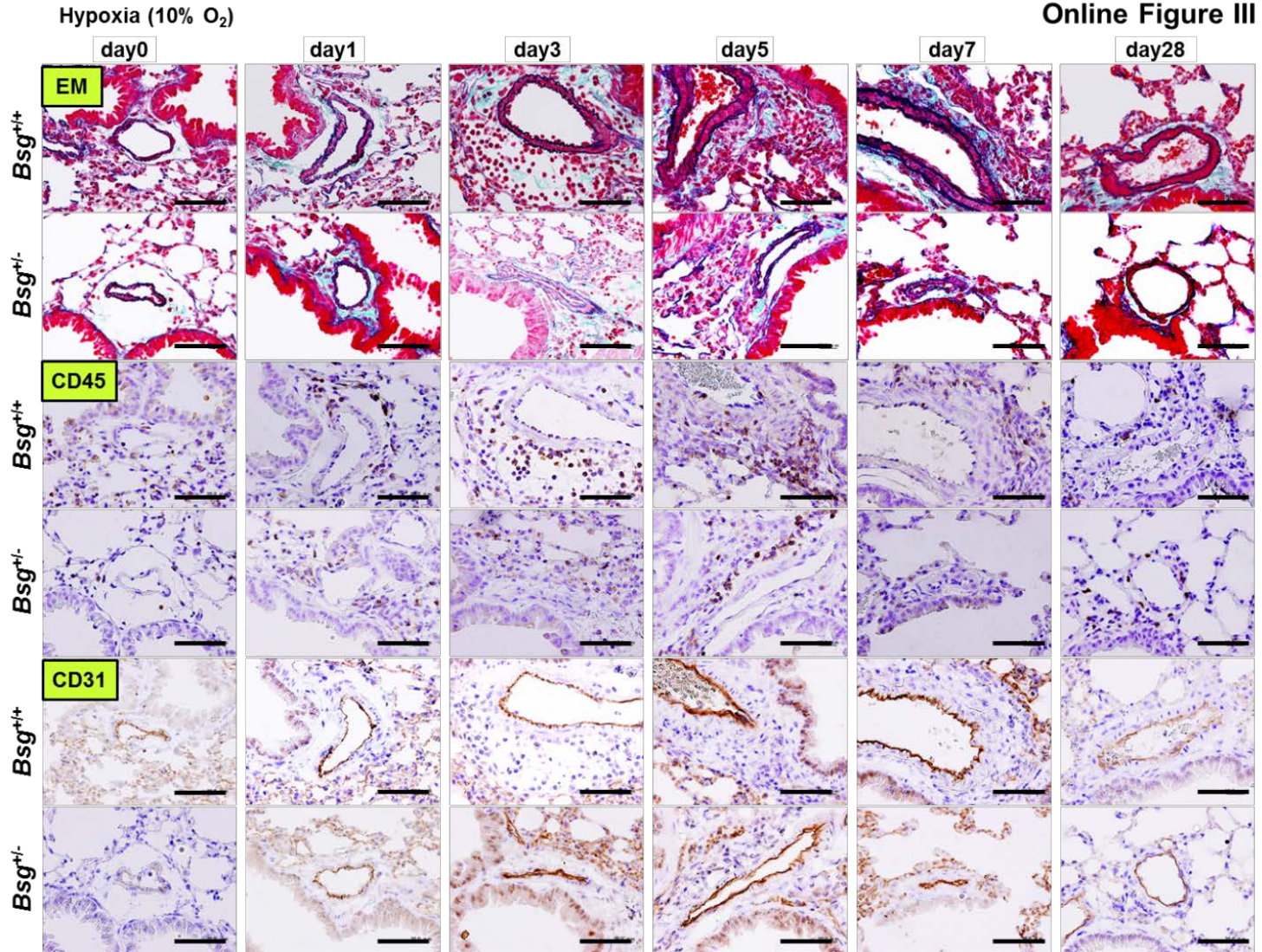
**Online Figure I. The expression of CyPA and Bsg in the distal pulmonary arteries from mice with hypoxia-induced PH**

(A) Representative photographs showing macroscopic features of pulmonary arteries in wild-type (WT) mice exposed to hypoxia (10% O<sub>2</sub>) for 0, 1, 3, 5, 7, and 28 days. Perivascular inflammatory cells and VSMCs in the hypoxic lung predominantly expressed CyPA as evidenced by immunostaining for CyPA and  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) in serial sections. Potent Bsg expression was noted in the perivascular and intravascular inflammatory cells and erythrocytes.

(B) Representative photographs showing macroscopic features of remodeled pulmonary arteries in patients with pulmonary arterial hypertension (PAH). CyPA, cyclophilin A. Bsg, Basigin. EM, elastica-Masson staining. Scale bars, 50  $\mu$ m.

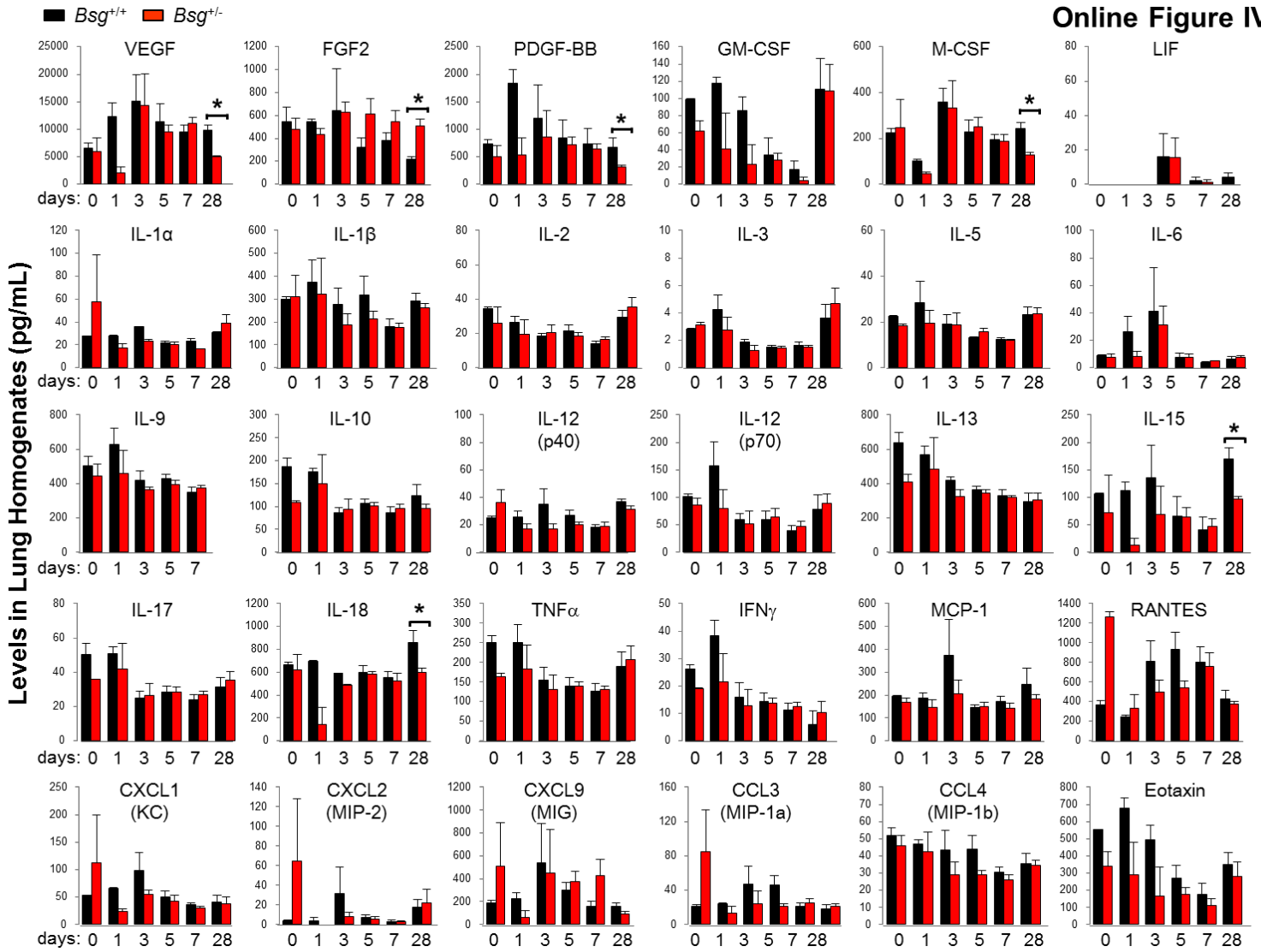


**Online Figure II. Inflammatory cytokines and growth factors in the lungs of *CyPA*<sup>+/-</sup> mice**  
 The levels of inflammatory cytokines and growth factors in the lungs of *CyPA*<sup>+/-</sup> mice and littermate controls (*CyPA*<sup>+/+</sup>) after for 4 weeks of hypoxia (10% O<sub>2</sub>) or normoxia. Results are expressed as mean ± SEM. \*P<0.05.



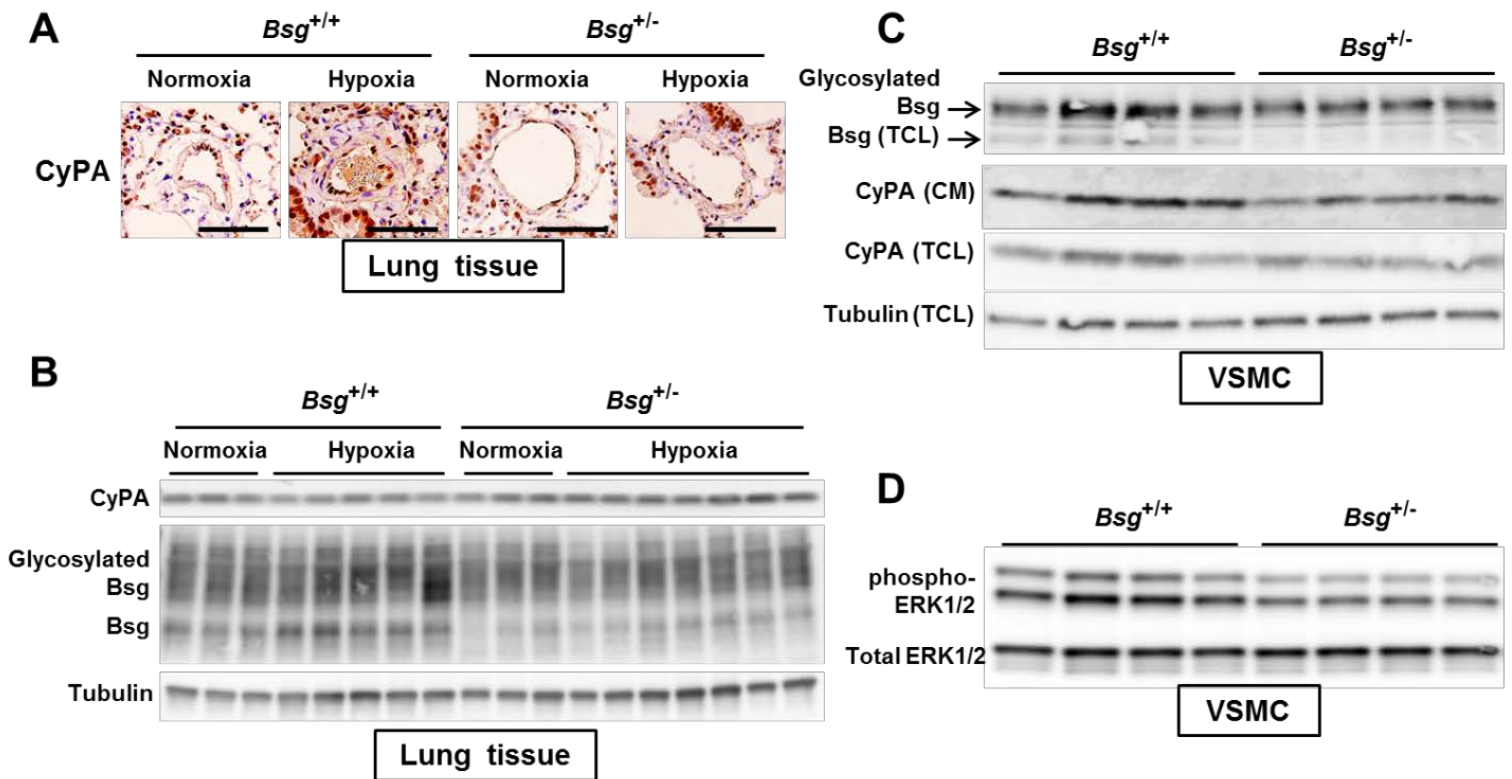
**Online Figure III. Migration of perivascular CD45<sup>+</sup> inflammatory cells in *Bsg*<sup>+/-</sup> mice**  
 Pulmonary arteries from *Bsg*<sup>+/+</sup> and *Bsg*<sup>+/-</sup> mice after hypoxic exposure (10% O<sub>2</sub>) for 0, 1, 3, 5, 7, and 28 days. EM, Elastica Masson staining. Scale bars, 50 μm.





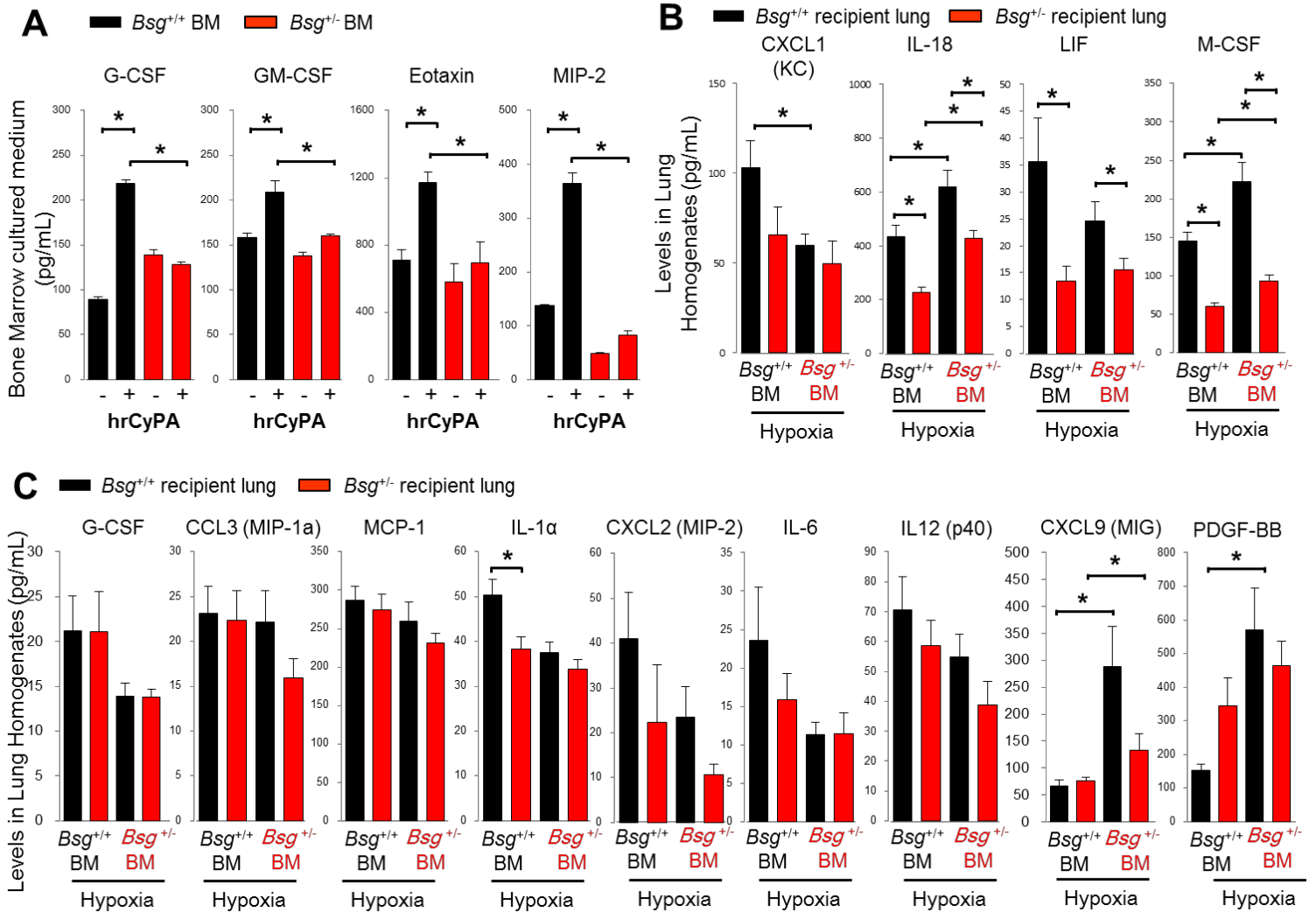
**Online Figure IV. Time-course of the levels of cytokines/chemokines and growth factors in lung homogenates during chronic exposure to hypoxia**

Cytokines/chemokines and growth factors secreted from the lungs of *Bsg*<sup>+/+</sup> and *Bsg*<sup>+/-</sup> mice after hypoxic exposure (10% O<sub>2</sub>) for 0, 1, 3, 5, 7, and 28 days. \*P<0.05.



### Online Figure V. CyPA expression and secretion is regulated by Bsg

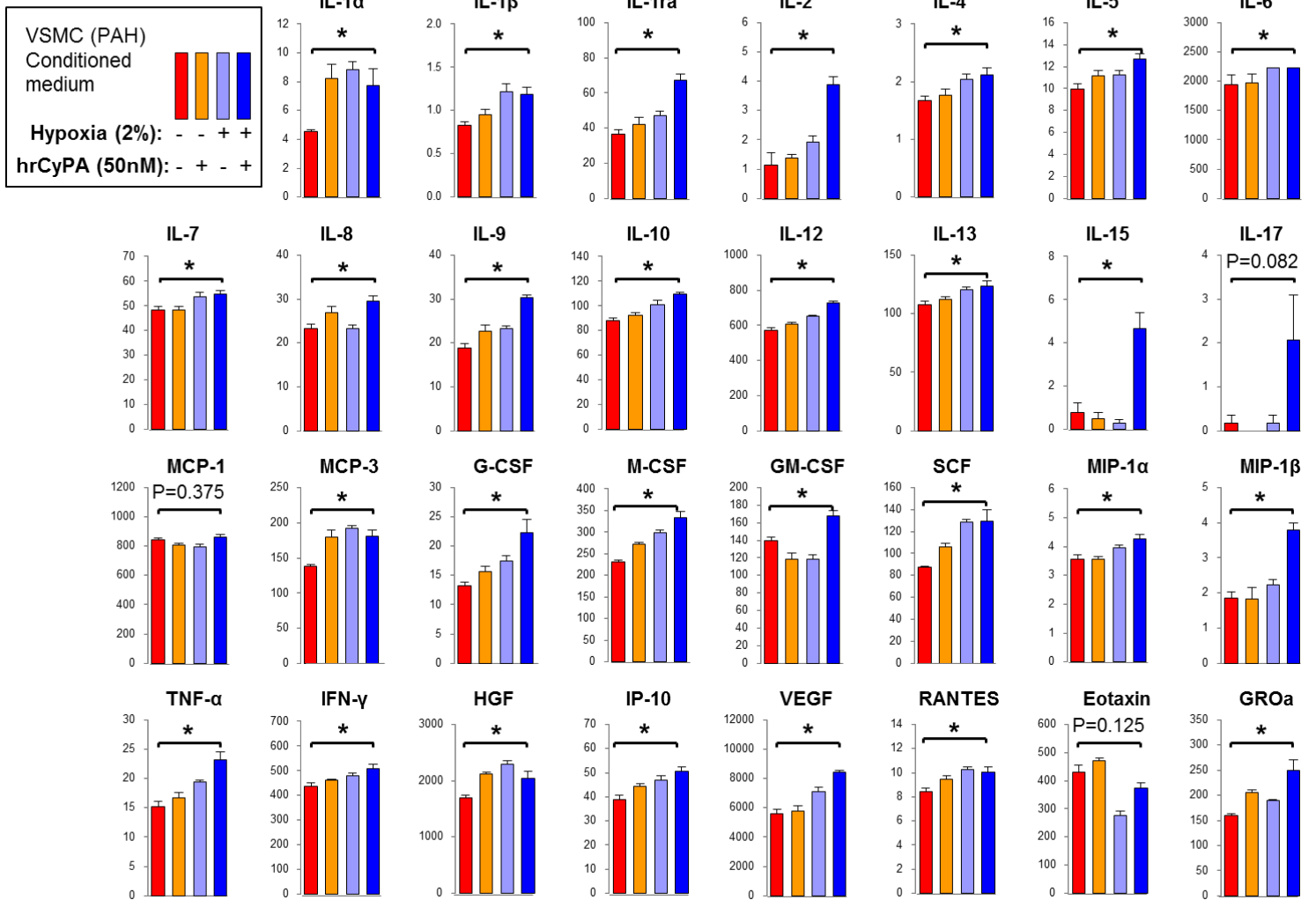
(A) Representative immunostaining for CyPA in *Bsg*<sup>+/+</sup> and *Bsg*<sup>+/-</sup> lung in normoxic and hypoxic conditions (4 wks). (B) Western blotting of CyPA in *Bsg*<sup>+/+</sup> and *Bsg*<sup>+/-</sup> lung tissues in normoxic and hypoxic conditions (4 wks) (n=3~6). (C) Western blotting of CyPA and Bsg in *Bsg*<sup>+/+</sup> and *Bsg*<sup>+/-</sup> VSMCs (n=4). (D) Western blotting of phosphorylated and total ERK1/2 in *Bsg*<sup>+/+</sup> and *Bsg*<sup>+/-</sup> VSMCs (n=4).



**Online Figure VI. Bsg and extracellular CyPA-induced secretion of cytokines in bone marrow cells**

To determine whether extracellular CyPA induces secretion of cytokines/chemokines and growth factors in a Bsg-dependent manner, we stimulated bone marrow cells with human recombinant CyPA (hrCyPA) *ex vivo*. (A) Inflammatory cytokine secretion was induced by hrCyPA in *Bsg*<sup>+/+</sup>-bone marrow cells; however, the induction was less dramatic in the bone marrow of *Bsg*<sup>+/-</sup> mice. \*P<0.05. (B, C) Cytokines/chemokines and growth factors in the chimeric lungs of *Bsg*<sup>+/+</sup> recipient mice and *Bsg*<sup>+/-</sup> recipient mice (n=7~10). Results are expressed as mean  $\pm$  SEM. \*P<0.05.

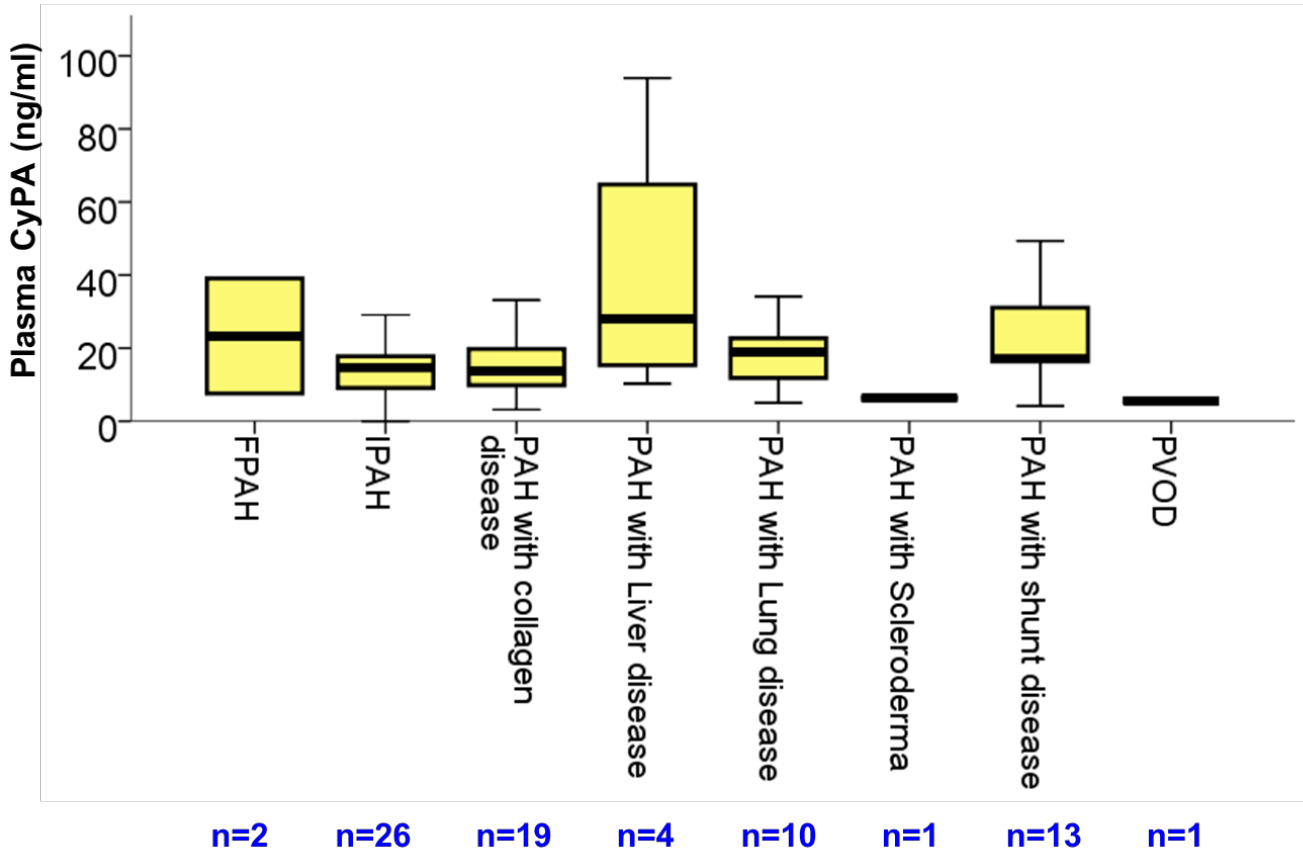
## Online Figure VII



### Online Figure VII. Secretion of inflammatory cytokines and growth factors by distal pulmonary artery VSMCs from patients with PAH

Extracellular CyPA and hypoxia (2% O<sub>2</sub>) induced secretion of cytokines/chemokines and growth factors from pulmonary artery VSMCs. Results are expressed as mean ± SEM. \*P<0.05.

CyPA levels according to the diagnosis within the PAH group



**Online Figure VIII. CyPA levels according to the diagnosis within the PAH group.**

Plasma levels of CyPA in patients with familial PAH (FPAH, n=2), idiopathic PAH (IPAH, n=26), PAH with collagen diseases (n=19), liver diseases (n=4), lung diseases (n=10), scleroderma (n=1), shunt diseases (n=13) and pulmonary veno-occlusive disease (PVOD, n=1). Box-and-whisker plots of plasma levels of CyPA.

## Online Table I

	Healthy control	PH (-)	PH (+)
Age (years)	56±12	55±17	51±15
Female (%)	57	76	74
Body mass index (kg/m <sup>2</sup> )	24.0±2.7	21.7±3.2	21.9±4.0
WHO class			
I/II (%)	100.0	89.2	78.0
III (%)	0.0	10.8	15.6
IV (%)	0.0	0.0	6.4
Mean RAP (mmHg)	-	4.8±2.8	5.7±3.3
Mean PAP (mmHg)	-	17.2±4.2	39.8±14.0
Mean PCWP (mmHg)	-	7.3±2.8	8.7±2.9
Cardiac index (L/min/m <sup>2</sup> )	-	3.4±0.9	3.0±0.6
PVR (dyne·sec·cm <sup>-5</sup> )	-	156±92	637±462
SvO <sub>2</sub> (%)	-	76.1±7.9	67.8±8.2

Baseline characteristics of patients with PH

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