Osteoprotegerin Disruption Attenuates HySu-Induced Pulmonary Hypertension Through Integrin αβ3/FAK/ AKT Pathway Suppression

Daile Jia, MD*; Qian Zhu, MD*; Huan Liu, MD; Caojian Zuo, MD; Yuhu He, MD; Guilin Chen, BSc; Ankang Lu, MD, PhD

Background—Pulmonary arterial remodeling characterized by increased vascular smooth muscle proliferation is commonly seen in life-threatening disease, pulmonary arterial hypertension (PAH). Clinical studies have suggested a correlation between osteoprotegerin serum levels and PAH severity. Here, we aimed to investigate vascular osteoprotegerin expression and its effects on the signal transduction pathways mediating its activity.

Methods and Results—Serum osteoprotegerin levels were significantly elevated in patients with PAH and correlated with disease severity as determined by the World Health Organization (WHO) functional classifications and 6-minute walking distance tests. Similarly, increased osteoprotegerin expression was observed in the pulmonary arteries of hypoxia plus SU5416– and monocrotaline-induced PAH animal models. Moreover, osteoprotegerin disruption attenuated hypoxia plus SU5416–induced PAH progression by reducing pulmonary vascular remodeling, whereas lentiviral osteoprotegerin reconstitution exacerbated PAH by increasing pulmonary arterial smooth muscle cell proliferation. Furthermore, pathway analysis revealed that osteoprotegerin induced pulmonary arterial smooth muscle cell proliferation by interacting with integrin αβ3, to elicit downstream focal adhesion kinase and AKT pathway activation.

Conclusions—Osteoprotegerin facilitates PAH pathogenesis by regulating pulmonary arterial smooth muscle cell proliferation, suggesting that it may be a potential biomarker and therapeutic target in this disease.

(Circ Cardiovasc Genet. 2017;10:e001591. DOI: 10.1161/CIRCGENETICS.116.001591.)

Key words: biomarkers • cell proliferation • osteoprotegerin • pulmonary hypertension • pulmonary vascular smooth muscle cells

Pulmonary arterial hypertension (PAH) is a progressive cardiopulmonary disease characterized by pulmonary artery remodeling. During this process, endothelial, fibroblast, inflammatory, and vascular smooth muscle cells undergo activation and proliferation, leading to enhanced pulmonary vascular resistance and pressure, heart failure, and death. Despite an improved understanding of the disease development and treatment in recent decades, the mechanisms associated with pulmonary arterial hypertension (PAH) pathogenesis remain poorly understood. Moreover, current therapies mainly target endothelial function, which fails to fully reverse vascular remodeling and improve long-term survival; thus, the discovery of new molecular targets or signaling pathways therapeutically capable of inhibiting remodeling of pulmonary arteries is needed.

Osteoprotegerin is a member of the tumor necrosis factor receptor superfamily and expressed in a variety of tissues, including heart and lung. Osteoprotegerin was initially identified for its roles in bone metabolism and cell death through competitive inhibition of receptor activator of nuclear factor-κB ligand binding to its cognate receptor, as well as blocking the ligation of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) with its membrane-associated death receptors, respectively. However, recent clinical and experimental data have also linked osteoprotegerin to carcinogenesis and a range of cardiovascular disorders, including PAH. Notably, clinical data showed that osteoprotegerin serum levels were higher in idiopathic pulmonary hypertension and associated with the indicators of disease severity and prognosis, and also indicated that osteoprotegerin expression was increased in both concentric and plexiform lesions in remodeled pulmonary arteries.

See Clinical Perspective

Received January 27, 2016; accepted November 29, 2016.
From the Department of Cardiology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China.
*Drs Jia and Zhu contributed equally to this work.


Correspondence to Ankang Lu, MD, PhD, Department of Cardiology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, 197 Ruijin Rd, Shanghai 200025, China. E-mail Ivankang@medmail.com.cn

© 2017 American Heart Association, Inc.
This study included 76 subjects separated into 2 groups. The PAH and controls groups consisted of 31 and 45 participants, respectively. PAH was diagnosed based on mean pulmonary artery pressure of $\geq 25$ mm Hg with a normal pulmonary capillary wedge pressure of $\leq 15$ mm Hg, as confirmed by right heart catheterization at rest.\(^\text{19}\) Because of the heterogeneous role of osteoprotegerin in the pathogenesis of various diseases, patients with comorbidities—such as infectious diseases, severe kidney failure, acute coronary syndrome diseases, heart failure, left heart diseases, diabetes mellitus, ongoing liver disease, and malignancies—were excluded from our analysis. Basal characteristics and PAH-associated symptoms for the study population are shown in Table. All subjects provided

### Methods

#### Clinical Subjects

This study included 76 subjects separated into 2 groups. The PAH and controls groups consisted of 31 and 45 participants, respectively. PAH was diagnosed based on mean pulmonary artery pressure of $\geq 25$ mm Hg with a normal pulmonary capillary wedge pressure of $\leq 15$ mm Hg, as confirmed by right heart catheterization at rest.\(^\text{19}\) Because of the heterogeneous role of osteoprotegerin in the pathogenesis of various diseases, patients with comorbidities—such as infectious diseases, severe kidney failure, acute coronary syndrome diseases, heart failure, left heart diseases, diabetes mellitus, ongoing liver disease, and malignancies—were excluded from our analysis. Basal characteristics and PAH-associated symptoms for the study population are shown in Table. All subjects provided
written informed consent, and the study was approved by the Local Research Ethics Committee of Ruijin Hospital at Shanghai Jiao Tong University.

RNA Extraction and Real-Time Polymerase Chain Reaction
Total RNA were extracted from lung tissues and pulmonary artery homogenates, hPASMCs, and mPASMCs with Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was carried out using a reverse transcriptase kit (Transgene, Strasbourg, France) according to the manufacturer’s instructions. The resulting cDNA was amplified with 40 cycles of real-time polymerase chain reaction (RT-PCR) using primers described in the Data Supplement. All PCR products were verified by electrophoresis (Figure 1A).

Mouse models, hemodynamic measurement, Western blotting, osteoprotegerin measurement, siRNA-mediated gene silencing, lung histology, immunostaining, cell culture, cell proliferation assay, and immunoprecipitations and immunoblotting.

Mouse models, hemodynamic measurement, Western blotting, osteoprotegerin measurement, siRNA-mediated gene silencing, lung histology, immunostaining, cell culture, cell proliferation assay, and immunoprecipitations and immunoblotting are described in more detail in the Data Supplement.

Statistical Analysis
The data are presented as the means±SEM. Chi-square testing and Pearson correlation analysis were performed to assess discrete variables and correlations. For 2-group comparisons of serum osteoprotegerin levels in human only, 2-tailed Student’s t test was performed. Differences between the 2 groups were assessed by using Kruskal–Wallis test. P<0.05 was considered to be statistically significant.

Results
Serum Osteoprotegerin Concentrations Were Increased in Patients With PAH and Correlated With Disease Severity
Of the 76 patients assessed for eligibility between March 2010 and September 2015, 31 met the PAH inclusion criteria and 45 volunteers were recruited as controls. Basal characteristics and PAH-associated symptoms for the study population are shown in Table. The control population showed no significant difference in age or sex distribution relative to the PAH group. Osteoprotegerin serum levels were significantly higher in patients from the PAH group as compared to the controls (1.74±0.16 pmol/L versus 4.09±0.41 pmol/L, P<0.0001; Figure 1B) and positively correlated with mean pulmonary artery pressure (R²=0.4058, P<0.0001; Figure 1C) and pulmonary vascular resistance (R²=0.3422, P=0.0005; Figure I in the Data Supplement). In addition, the modified WHO functional classification and 6-minute walking test (6MWT) are two independent predictors of survival and severity for patients with PAH.19–21Consistently, increased serum osteoprotegerin levels were observed in PAH patients with WHO class III or IV relative to those with class I or II (3.00±0.40 pmol/L versus 4.88±0.57 pmol/L, P=0.019; Figure 1D). Moreover, the osteoprotegerin levels of patients exhibiting reduced exercise capacity (6MWT ≤440 m) were also significantly higher than in those with a 6MWT>440 m (4.76±0.48 pmol/L versus 2.43±0.42 pmol/L, P=0.0068; Figure 1E).19,22 In addition, after a mean of 24 months of...
follow-up, clinical improvement was observed in 14 patients with PAH (11 idiopathic pulmonary hypertension and 3 congenital heart disease-related PAH) treated with prostacyclin analogs (9/14, 7 idiopathic pulmonary hypertension and 2 congenital heart disease) or bosentan (5/14, 4 idiopathic pulmonary hypertension and 1 congenital heart disease). These patients responded with decreased WHO functional classifications (2.9; 95% CI, 2.51–3.35 to 2.0; 95% CI, 1.3761–2.39, \( P=0.0018 \)) and an increased 6MWT (Figure 1F). Notably, treated patients also exhibited decreased serum osteoprotegerin levels during a second evaluation (Figure 1G).

### Osteoprotegerin Is Upregulated in the Pulmonary Arteries of HySu-Induced PAH Mice

The combination of SU5416 and chronic hypoxia (HySu) induces PAH in mice, which mimics the complex remodeling processes and cytokine profiles observed in human patients. Using this model, we found that osteoprotegerin expression was strikingly elevated in lungs and pulmonary arteries (PAs) from HySu-induced mice compared with normotensive lung tissues (Figure 2A). Moreover, osteoprotegerin mRNA levels were significantly upregulated in cultured mPASMCs and hPASMCs exposed to hypoxia (Figure 2B). Immunofluorescence analysis of murine lung tissue revealed high osteoprotegerin levels in remodeled vessel walls in the lungs, but was substantially lower in control lung tissue (Figure 2C; Figure II in the Data Supplement). Similar results were also observed in pulmonary arteries from monocrotaline-induced PAH rats (Figure 2D; Figure III in the Data Supplement). Furthermore, serum osteoprotegerin was strikingly higher after HySu treatment than in normoxic controls (2.62±0.42 pmol/L versus 5.26±0.60 pmol/L, \( P=0.0070 \); Figure 2E) and correlated with right ventricular systolic pressure (\( R^2=0.4074 \), \( P=0.0078 \); Figure 2F) and the ratio of the right ventricular wall to the left ventricular wall plus the septum (RV/LV+S; \( R^2=0.3483 \), \( P=0.0161 \); Figure 2G). Taken together, these data suggest that osteoprotegerin is upregulated in PAs after HySu treatment and may facilitate PAH progression.

### Absence of Osteoprotegerin Attenuates the Development of HySu-Induced PAH in Mice

Next, we investigated whether osteoprotegerin disruption would modulate PAH progression in HySu-induced PAH. As expected, wild-type (WT) mice subjected to HySu treatment >3 weeks developed significant elevations in right ventricular systolic pressure (19.75±0.75 mm Hg versus 35.75±1.10 mm Hg, \( P=0.0009 \); Figure 3A) and in the RV/LV+S (23.94±0.90% versus 39.54±1.21%, \( P=0.0002 \); Figure 3B) as compared to normoxic controls. No differences in systemic arterial pressure were observed under normoxic conditions between OPG −/− and WT animals (Figure IV in the Data Supplement). In contrast, OPG −/− mice showed a significant reduction in right ventricular systolic pressure.
Osteoprotegerin in Pulmonary Hypertension

Jia et al

Effect of Osteoprotegerin on PASMC Proliferation In Vivo and In Vitro

As abnormal PASMC proliferation was the major underlying cause of pulmonary vascular remodeling in PAH and osteoprotegerin expression was mostly upregulated in PASMCs, we monitored osteoprotegerin-mediated PASMC proliferation in vivo and in vitro by proliferating cell nuclear antigen immunostaining. Notably, proliferating cell nuclear antigen expression was significantly higher in PASMCs from Hypoxia-induced PAH mice as compared to normoxic controls, but strikingly reduced in OPG−/− counterparts (Figure 4A and 4B). Moreover, α-smooth muscle actin immunostaining revealed a significant difference in pulmonary arteriolar neomuscularization, as estimated by α-smooth muscle actin+ cells, between Hypoxia-treated OPG−/− and WT mice (Figures 3D and 4C), but no differences in cellular apoptosis as determined by terminal deoxynucleotidyl transferase dUTP nick end-labeling staining (Figure V in the Data Supplement). Collectively, these data indicate that osteoprotegerin disruption limits pulmonary vascular remodeling by inhibiting PASMC proliferation. Under normoxic conditions in vitro, both mPASMCs and hPASMCs were serum starved for 24 hours before stimulation with recombinant osteoprotegerin (rOPG). Proliferation was measured by cell counting kit (CCK-8) with 10 ng/mL of platelet-derived growth factor-BB as a positive control. After rOPG stimulation, cell proliferation was significantly increased when compared with PASMCs lacking rOPG stimulation, maximal at 50 ng/mL (Figure 4D and 4E). However, in hypoxic conditions following different durations (0, 24, 48, and 72 hours) of exposure to 1% O2/5% CO2, proliferation of PASMCs isolated from OPG−/− mice was significantly reduced when compared with WT controls (Figure 4F).

Osteoprotegerin Reconstitution in Lung Tissues Exacerbates Hypoxia-Induced PAH in OPG−/− Mice

To further determine the effect of osteoprotegerin on PAH pathogenesis, we reconstituted osteoprotegerin expression

Figure 3. Osteoprotegerin (OPG) disruption attenuates hypoxia plus SU5416 (Hypoxia)-induced pulmonary arterial hypertension development in model mice. A, RVSP and (B) right ventricular wall to the left ventricular wall plus the septum in wild-type (WT) and OPG−/− mice after Hypoxia treatment. C, The degree of musculartization (nonmuscularized, partially muscularized, and fully muscularized vessels, 20–70 μm in diameter) of small pulmonary vessels from Hypoxia-induced WT and OPG−/− mice. D, Representative hematoxylin and eosin staining and α-smooth muscle actin (α-SMA; green) immunostaining of lung sections from Hypoxia-induced WT and OPG−/− mice. Scale bar, 20 μm. E, Quantification of the ratio of medial area to cross-sectional area (ie, Medial/cross-sectional area) in Hypoxia-induced model mice. Data are presented as the means±SEM (n=8; *P<0.05 vs WT and #P<0.05 vs control).
in OPG−/− mice with lentivirus. The transfection efficiency was examined by immunofluorescence and RT-PCR. The immunofluorescence results demonstrated that osteoprotegerin expression was observed in bronchiolar epithelial cells, alveolar cells, and pulmonary arteries in lenti-osteoprotegerin–treated lungs compared to lenti-green fluorescent protein controls (Figure 5A). Furthermore, the RT-PCR analysis demonstrated strikingly increased levels of osteoprotegerin mRNA in the lenti-osteoprotegerin–treated lungs compared with lenti-green fluorescent protein controls (Figure 5B). Interestingly, lenti-osteoprotegerin mouse showed a significant increase in right ventricular systolic pressure (36.63±1.32 mm Hg versus 30.63±1.36 mm Hg, P=0.0135; Figure 5C), RV/LV+S (38.80±1.47% versus 31.57±1.07%, P=0.0030; Figure 5D), medial wall thickness (Figure 5E and 5F), and muscularization (Figure 5G) compared to controls after 3 weeks of HySu exposure. Moreover, PASMC proliferating cell nuclear antigen expression was strikingly higher in HySu/lenti-osteoprotegerin mice versus HySu/ lenti-green fluorescent protein controls (Figure 5H and 5I). Thus, these results suggest that osteoprotegerin promotes pulmonary vascular remodeling.

Osteoprotegerin-Mediated PASMC Proliferation Occurs Through Integrin αβ3/FAK/AKT Signaling

To determine the repertoire of integrins expressed in mPASMCs and PAs, RT-PCR was performed using primers designed to integrins α1, αv, αv, αv, β3, and β3. Compared to normotensive controls, α1, αv, and β3 levels were strikingly elevated in cultured hypoxic mPASMCs and PAs from HySu-treated mice, whereas αv and β3 were decreased. No significant differences were observed in α1, αv, and β3 expressions (Figure 6A and 6B). To determine whether osteoprotegerin could directly interact with integrins, mPASMCs cultured under normoxic and hypoxic conditions for 48 hours were subjected to anti-osteoprotegerin immunoprecipitation analysis. Notably, these results demonstrated that osteoprotegerin specifically interacts with integrin αv and β3, but not with αv and αv, under hypoxic conditions (Figure 6C). Integrin engagement has previously been shown to trigger focal adhesion kinase (FAK), extracellular signal–regulated kinase 1/2, and AKT activation to promote the proliferation of various cancer and endothelial cells.14,15 To determine whether these pathways are activated in pulmonary hypertension, we examined FAK, extracellular signal–regulated kinase 1/2, and AKT phosphorylation in cultured mPASMCs. Interestingly, exposure to hypoxia (1% O2) for 48 hours increased the phosphorylation of all 3 effectors (Figure 6D and 6E); however, both FAK and AKT phosphorylation were decreased in hypoxic OPG−/− PASMCs, whereas extracellular signal–regulated kinase 1/2 phosphorylation remained unchanged (Figure 6D and 6E). These observations support that osteoprotegerin promotes PASMC proliferation through integrin αβ3/FAK/AKT pathway activation.

To determine whether osteoprotegerin-mediated PASMCs proliferation is the integrin αβ3 dependent, we examined the effect αβ3 siRNA on the phosphorylation of FAK and AKT and cell proliferation. Primary PASMCs were transfected with αβ3 siRNA, resulting in a 69.1% and 64.6% decrease in αβ3 integrin mRNA expression, respectively (Figure 7A). Importantly, integrin αβ3 knockdown PASMCs showed decreased rOPG-induced FAK and AKT phosphorylation (Figure 7B and 7C) and cell proliferation (Figure 7D) after osteoprotegerin stimulation. Next, we examined whether osteoprotegerin stimulation could rescue the activation of FAK/AKT signaling in PASMCs. Primary PASMCs isolated from OPG−/− mice were stimulated with 50 ng/mL rOPG, and the phosphorylation of FAK and AKT were significantly elevated, further supporting the notion that osteoprotegerin mediated the activation of FAK/AKT signaling in PASMCs (Figure 7E). Moreover, osteoprotegerin stimulation strikingly...
increased PASMCs proliferation, which was significantly suppressed by pretreatment with FAK siRNA or AKT inhibitor triciribine (Figure 7F and 7G).

**Discussion**

This study demonstrated that serum osteoprotegerin levels positively correlated with disease severity in patients with PAH. Notably, osteoprotegerin expression was upregulated in hPASMCs and mPASMCs in response to hypoxia, as well as in the pulmonary arteries of HySu-induced PAH mice and monocrotaline-induced PAH rats. Moreover, osteoprotegerin disruption attenuates PASMC proliferation and subsequent PAH progression and could be restored by osteoprotegerin reconstitution. Ultimately, these effects were mediated by osteoprotegerin-induced FAK/AKT pathway activation through a direct interaction between integrin $\alpha_\beta_3$ and osteoprotegerin. Collectively, these observations indicate that osteoprotegerin plays a critical role in pulmonary vascular remodeling in PAH and is, therefore, a putative therapeutic target for this disease.

Osteoprotegerin is known for its predominant function in bone metabolism. Previous data demonstrate that elevated osteoprotegerin levels are associated with osteopetrosis, whereas its inhibition leads to osteoporosis. Moreover, osteoprotegerin can bind to TRAIL and alter its effects in sensitive cells to promote cell proliferation. Furthermore, accumulating evidence suggests that osteoprotegerin regulates vascular calcification and coronary artery disease. We found that osteoprotegerin was significantly upregulated in PAs in HySu-induced PAH mice and monocrotaline-induced PAH rat. Indeed, osteoprotegerin secretion could be elevated in PASMCs by reduced bone morphogenic proteins receptor 2 (BMR-R2) expression and increased expression of
5-hydroxytryptamine and interleukin-1,
which play critical roles in PAH pathogenesis. The theorized role of osteoprotegerin in PAH was based on clinical data and experimental evidence without specific interventions, where direct proof from genetic models is lacking. Our results address this gap, as osteoprotegerin disruption inhibited PASMC proliferation and mitigated disease severity in HySu-induced PAH animal model. Moreover, osteoprotegerin reconstitution exacerbates pulmonary vascular remodeling, including medial wall thickening and PA muscularization. Elevated osteoprotegerin levels have been previously observed in various diseases; thus, patients with comorbidities were excluded from the sample population to avoid any unintended artifacts in the study data. Notably, we found that patients with PAH displayed increased serum osteoprotegerin concentrations, which correlated with disease severity as determined by WHO functional classifications and 6MWT. However, because of the rarity of PAH and the financial issues that patients with PAH face, we only recruited 14 patients with follow-up evaluation ≈ 24 months after receiving treatment. Moreover, all patients showed stable or improved disease after prostacyclin analogs or bosentan therapy, which prevented an analysis of osteoprotegerin levels in nonresponders. Furthermore, the functional significance of osteoprotegerin in PAH pathophysiology should be evaluated in a larger population of patients with specific morbidities.

TRAIL is expressed in vascular smooth muscle cells (VSMCs), and it mediates proliferation by binding to its receptors, DR4 and DCR1. Notably, the expression of TRAIL and its receptors are markedly enhanced in PASMCs from patients with PAH and animal models and facilitate disease progression. These findings suggest that osteoprotegerin may interact with other novel signaling pathways to promote PASMC proliferation rather than solely function as a TRAIL decoy receptor. Many studies, including those of the pulmonary vasculature, indicate that αvβ3 integrin activation promotes SMC proliferation, migration, and survival. Similarly, we observed upregulated αvβ3 integrin in mPASMCS and PAs, where its interaction with osteoprotegerin induced focal adhesion kinase (FAK), AKT, and ERK, respectively. Protein levels are expressed as fold change over normoxic WT (n=4). Data are presented as the means±SEM.*P<0.05 vs normoxia and **P<0.01 vs normoxia.

In conclusion, our study showed that osteoprotegerin is upregulated in pulmonary arteries of HySu-
monocrotaline-induced PAH and hypoxic PASMC cultures. In addition, we demonstrated that osteoprotegerin disruption hindered HySu-induced PAH progression in mice through \( \alpha_\beta_3/FAK/AKT \) pathway suppression, suggesting that osteoprotegerin blockade may be a promising target for PAH therapy.

**Sources of Funding**

This work was supported by the National Natural Foundation of China (81570038).

**Disclosures**

None.

**References**


interaction between osteoprotegerin and integrin
vascular smooth muscle cell proliferation and mitigated the increased right ventricular systolic pressure and ventricular wall biomarker and therapeutic target in this disease. Taken together, our data suggest that osteoprotegerin facilitates PAH pathogenesis and thus may be a potential CLINICAL PERSPECTIVE
Osteoprotegerin was initially identified as a secreted decoy receptor for receptor activator of nuclear factor-κB ligand and tumor necrosis factor-related apoptosis-inducing ligand to regulate bone metabolism and cell survival, respectively. However, recent clinical and experimental data have also linked osteoprotegerin to a range of cardiovascular disorders, including pulmonary hypertension (PAH). Here, we found that serum osteoprotegerin levels were increased in patients with PAH and correlated with disease severity. Moreover, pulmonary arteries from hypoxia plus SU5416– and monocrotaline-induced PAH animal models also showed elevated osteoprotegerin expression. Notably, OPG−/− mice exhibited decreased pulmonary vascular smooth muscle cell proliferation and mitigated the increased right ventricular systolic pressure and ventricular wall thickening observed in wild-type hypoxia plus SU5416–induced PAH controls. Lentinival osteoprotegerin reconstitution enhanced pulmonary vascular remodeling characterized by pulmonary arterial smooth muscle cell proliferation, medial wall thickening, and pulmonary artery muscularization. Pathway analysis revealed that these effects resulted from a direct interaction between osteoprotegerin and integrin αvβ3, which subsequently triggered focal adhesion kinase/AKT pathway activation. Taken together, our data suggest that osteoprotegerin facilitates PAH pathogenesis and thus may be a potential biomarker and therapeutic target in this disease.
Osteoprotegerin Disruption Attenuates HySu-Induced Pulmonary Hypertension Through Integrin αvβ3/FAK/AKT Pathway Suppression
Daile Jia, Qian Zhu, Huan Liu, Caojian Zuo, Yuhu He, Guilin Chen and Ankang Lu

_Circ Cardiovasc Genet_. 2017;10:
doi: 10.1161/CIRCGENETICS.116.001591
_Circulation: Cardiovascular Genetics_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2017 American Heart Association, Inc. All rights reserved.
Print ISSN: 1942-325X. Online ISSN: 1942-3268

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circgenetics.ahajournals.org/content/10/1/e001591

Data Supplement (unedited) at:
http://circgenetics.ahajournals.org/content/suppl/2017/01/11/CIRCGENETICS.116.001591.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation: Cardiovascular Genetics_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation: Cardiovascular Genetics_ is online at:
http://circgenetics.ahajournals.org//subscriptions/
Supplemental Methods

PCR primer sequences
human OPG 3′ → 5′ – GTGTGCAATGCAAGGAAGG, 5′ → 3′ – CACACTCCAAATCCAGGAG; human GAPDH 3′ → 5′ – CCCTATTGACCTCAACTACATGGT, 5′ → 3′ – GAGGGGCCATCCACAGTCTTCTG; mouse OPG 3′ → 5′ – CAGCATCGCTCTGTTCTGTGA, 5′ → 3′ – CTGGGTTTTGCTGAGTCTCA; mouse integrin α1 3′ → 5′ – CCTTCCCTCGGATGTGAGTCA, 5′ → 3′ – GAGGGGCCATCCACAGTCTTCTG; mouse integrin α2 3′ → 5′ – TGTCTGGCGTAATGAGTCTCA, 5′ → 3′ – CCTCTTCGCTACTCGGTC; mouse integrin α3 3′ → 5′ – CCGTGTTAGTATAGTCTACCC; mouse integrin α4 3′ → 5′ – GATGCTGTTTGCTGACTCGG; mouse integrin α5 3′ → 5′ – CTTTCCCTCGGAGTTTTACCG, 5′ → 3′ – GCTGTCAAAATGAGTGGTGAC; mouse integrin α6 3′ → 5′ – CTGCTGTGGAAGCTGGGATTC, 5′ → 3′ – CTCCTCCTTGAACTGAGTCG; mouse integrin α7 3′ → 5′ – GAGGGGCCATCCACAGTCTTCTG.

Hypoxia plus SU5416 (HySu)-induced pulmonary arterial hypertension (PAH) mouse model and lenti-OPG infection
Eight-to-ten-week-old OPG+/− male mice (Shanghai Biomodel Organism Co., Ltd, Shanghai, China) and age/weight-matched controls were exposed to normoxia(room air,21%O₂) or hypoxia(10%O₂) in a
ventilated chamber. The VEGFR2 antagonist SU5416 (20mg/kg) or vehicle control were injected subcutaneously once a week in the hypoxia and normoxia groups, respectively. For the monocrotaline (MCT)-induced PAH model, 8-week-old male Sprague-Dawley rats received a subcutaneous injection of MCT (60mg/kg) or vehicle control. For gene delivery into the lungs, 1-2 mice were anesthetized with pentobarbital (30 mg/kg) and the trachea was exposed through a midline incision. Lenti-OPG or lenti-GFP control viral particles (3×10^8 transducing units [TU] in 30µL of PBS) were injected into the trachea followed by an injection of air. Seven days after lentiviral infection, mice were exposed to HySu for 3 weeks and then examined for right ventricular systolic pressure (RVSP) and pulmonary vascular remodeling. All animals had access to standard mouse chow and water, and were maintained under controlled temperature conditions with 12-h light/dark cycles. All animal care and experimental procedures were conducted in accordance with international guidelines for the care and use of laboratory animals.

**Hemodynamic measurements**

Right ventricular pressure was measured as previously described. 3-4 Briefly, mice were anaesthetized with an intraperitoneal injection of pentobarbital (30 mg/kg), ventilated through a transtracheal catheter, and placed in a supine position on a heating platform. Zero the instrument before taking hemodynamic measurements. After a median sternotomy, a 1.4-F micro-tip pressure transducer catheter (Millar Instruments, Houston, TX, USA) was placed through the right ventricle apex. Right ventricular pressure was continuously recorded using a PowerLab data acquisition system (ADInstruments, Sydney, Australia) until the right ventricular pressure was stable and the effect of fluid inertia was minimized. Pulmonary circulation was flushed with PBS, and the heart and lungs were removed. The right ventricle (RV) was carefully dissected and weighed, and right ventricular hypertrophy was calculated by the weight ratio of RV to the left ventricle (LV) plus septum (RV/LV+S). Mean systemic arterial pressure (SBP) was monitored non-invasively using the tail-cuff method.

**Western blotting**

Pulmonary arterial smooth muscle cell (PASMC) lysates were prepared in RIPA buffer and centrifuged at 12000¥g for 12 min. A Pierce BCA Protein Assay Kit (Pierce, Rockford, IL) was used to quantify total
protein. Equal quantities of proteins were boiled and resolved by 10% SDS-PAGE, transferred onto nitrocellulose membranes, and then incubated with 5% skimmed milk for 2h. Blots were probed overnight at 4 °C with primary antibodies as follows: osteoprotegerin(OPG) antibody(ab73400;Abcam, Cambridge, UK), FAK antibody (3285; Cell Signaling Technology, Danvers, MA, USA), phospho-FAK antibody (3283; Cell Signaling Technology), AKT antibody (9272; Cell Signaling Technology), phospho-AKT antibody (4060; Cell Signaling Technology), p44/42 MAPK (ERK1/2) antibody (4696; Cell Signaling Technology), phospho-p44/42 MAPK (ERK1/2) antibody (9101; Cell Signaling Technology), and GAPDH antibody (5174; Cell Signaling Technology).

**OPG measurement**

Venous blood samples were obtained during hospitalization. After centrifugation (2000 rpm) for 20 min, the serum was collected and stored at −80°C. OPG concentration was measured immediately after thawing by enzyme-linked immunosorbent assays (ELISAs) with commercially available kits (R&D Systems, Minneapolis, MN, USA).

**siRNA-mediated gene silencing**

Small interfering RNA (siRNA) experiments were carried out with pooled siRNAs targeting mouse integrin αv, integrin β3, FAK, or fatty acid synthase (FASN) (Santa Cruz, Dallas, TX, USA). A scrambled siRNA was used as a negative control. Cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

**Lung histology**

The lower lobes of the left lungs were fixed with 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for morphological analysis. Pulmonary vascular remodeling was assessed by the percentage of muscularization and vascular medial wall thickness—calculated as a ratio of the medial area to cross sectional area (i.e., Medial/CSA)—in ImageJ. Other lung sections were collected for immunostaining and RNA or protein extraction.

**Immunostaining**
Paraffin-embedded lung tissue sections were deparaffinized, rehydrated, and then incubated with primary antibodies against α-smooth muscle actin (α-SMA; A5228; Sigma-Aldrich, St. Louis, MO, USA), OPG (ab73400; Abcam), or proliferating cell nuclear antigen (PCNA; 13110; Cell Signaling Technology) overnight at 4°C. After incubation with the applicable secondary antibodies, slides were then counterstained with nuclear 4',6-diamidino-2-phenylindole, sealed with anti-fade reagent, and then visualized by laser-scanning confocal microscopy (Carl Zeiss, Oberkochen, Germany).

Cell culture

Human PASMCs were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). Primary mPASMCs were isolated from peripheral small pulmonary artery explants of 6–8-week-old mice. Briefly, proximal pulmonary arteries (PAs) were carefully dissected and the adhering fat and connective was removed under a dissecting microscope. Arteries were then cut into rings (1–2mm²) and explanted in culture dishes. The cells were cultured in Dulbecco's Modified Eagle Medium/F-12 (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 100U/mL penicillin, 0.1mg/mL streptomycin, 2mM L-glutamine, and 20% fetal bovine serum in a humidified incubator (Thermo Fisher Scientific) at 37°C with 5% CO₂. The smooth muscles were positively stained for smooth muscle actin by immunofluorescence staining. For the experiment, PASMCs seeded in culture dishes were maintained in hypoxic (1%O₂/5%CO₂) or normoxic (21%O₂/5%CO₂) conditions.

Cell proliferation assay

Cell proliferation was analyzed with a cell counting kit (CCK-8; Beyotime, Shanghai, China) following the manufacturer’s instructions. For this, cell suspensions (100μL/well) were seeded in a 96-well plate, and 10 μL CCK-8 reagent was added to each well. After a 2–4 h incubation, absorbance was measured at 450 nm with a microplate reader and used to represent cell viability.

Immunoprecipitation and immunoblotting

For OPG immunoprecipitation, PASMCs were cultured under normoxic or hypoxic for 48h and lysed as described above. Lysates (1mg) were incubated with 5μg OPG antibody (sc-8468; Santa Cruz) at 4°C for 3h, followed by incubation with Protein A/G Agarose (Life Technologies, Carlsbad, CA, USA) overnight.
at 4°C. Immunoprecipitates were washed extensively and then subjected to immunoblotting with antibodies against osteoprotegerin (OPG) (ab73400; Abcam), integrin α1(ab181434; Abcam), integrin α8(sc-25713; Santa Cruz), integrin αv(ab179475; Abcam), or integrin β3(ab38460; Abcam).

Supplementary References

**Supplemental Figure 1.** Correlation between serum osteoprotegerin (OPG) and pulmonary vascular resistance (PVR) in pulmonary hypertension (PAH) patients (n=31).
Supplemental Figure 2. Immunofluorescence staining of \( \alpha \)-SMA (green), OPG (red), and nuclei (DAPI; blue) in mouse lung tissues. Scale bar: 50\( \mu \)m.
Supplemental Figure 3. Immunofluorescence staining of α-SMA(green), OPG(red), and nuclei (DAPI; blue) in rat lung tissues. Scale bar: 50μm.
Supplemental Figure 4. Systemic arterial pressure (SBP) in OPG−/− and WT mice with hypoxia plus SU5416 (HySu)-induced PAH (n = 8).
Supplemental Figure 5. (A) Immunofluorescence of terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL; red) and alpha-smooth muscle actin (α-SMA; green) in the lungs of HySu-induced PAH mice. Scale bar: 20μm. (B) Quantification of TUNEL-positive cells in pulmonary arteries (PAs) in (A). At least 10 fields/mouse (n = 8). Values are expressed as a percentage relative to WT PAs.
Supplemental Figure 6. Effect of fatty acid synthase (FASN) siRNA knockdown on PASMC proliferation following OPG stimulation. PASMCs were transfected with FASN siRNA or scramble and then incubated with OPG (50 ng/ml) for 48 h after OPG stimulation (50 ng/mL).