Transgelin as a therapeutic target to prevent hypoxic pulmonary hypertension

Rui Feng Zhang,1,2* Lihong Shi,1,2* Lin Zhou,3 Gensheng Zhang,4 Xiaohong Wu,1,2 Fangchun Shao,1,2 Guofeng Ma,1,2 and Kejing Ying1,2

1 Department of Respiratory Medicine, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, China; 2 Key Laboratory of Biotherapy in Zhejiang Province, Biomedical Research Center, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, China; 3 Department of Gastroenterology, the First Affiliated Hospital of Zhejiang University, Hangzhou, China; and 4 Department of Critical Care medicine, the Second Affiliated Hospital of Zhejiang University, Hangzhou, China

Transgelin as a therapeutic target to prevent hypoxic pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 306: L574–L583, 2014. First published January 24, 2014; doi:10.1152/ajplung.00327.2013.—We previously observed that transgelin was preferentially expressed in human pulmonary smooth muscle cells (PAMSCs) under hypoxia and that the upregulation of transgelin was independent of hypoxia-inducible factor 1α (HIF-1α). Reduced transgelin expression was accompanied by significantly impaired migration ability in vitro. However, the regulation mechanism of transgelin and its function in preventing hypoxic pulmonary hypertension (HPH) was unclear. In the present study, RNA interference with hypoxia-inducible factor 2α (HIF-2α) was employed in human PAMSCs. Transgelin expression was diminished in HIF-2α-siRNA-treated cells at both the mRNA and protein levels under hypoxia. However, HIF-2α did not transactivate the transgelin promoter directly. TGF-β1 concentration in human PAMSCs culture medium was higher under hypoxia, and the accumulated TGF-β1 under hypoxia was regulated by HIF-2α. Furthermore, luciferase and chromatin immunoprecipitation assays indicated that TGF-β1/Smad3 could bind to the transgelin promoter, resulting in increased transgelin expression. In addition to nonintact cellular migration, inhibition of transgelin expression resulted in impaired proliferation in vitro under hypoxia. A lentiviral vector used to inhibit transgelin expression was constructed and intratracheally instilled in rats 3 wk prior to hypoxia treatment. Our final results indicated that inhibition of transgelin expression locally could attenuate increased right ventricular systolic pressure and its associated cardiac and pulmonary vascular remodeling under hypoxia. Our findings indicate that transgelin could play an important role in the pathogenesis of pulmonary vascular remodeling under hypoxia.

Transgelin, which is also known as SM22α, WS3–10, and mouse p27, is a shape change-sensitive 22- to 25-kDa actin-binding protein of the calponin family that is localized to the cytoskeleton (30). It is considered a smooth muscle cell lineage-restricted protein, and it is expressed abundantly and exclusively in visceral and vascular smooth muscle cells postnatally. In addition, it is one of the earliest markers of smooth muscle differentiation (2). For many years, the function of transgelin was unclear. However, more recently, several functions of transgelin have been elucidated, including organization of actin distribution, inhibition of the phenotypic modulation of smooth muscle cells from contractile to synthetic/proliferative cells, and regulation of calcium-independent smooth muscle cell contraction, proliferation, cell migration, and tumor suppression (2, 6, 8, 11, 15, 33).

Hypoxic pulmonary hypertension (HPH) is one of the most significant subtypes of pulmonary hypertension (PH) (24). It is a serious threat to patients (9). Pulmonary vascular remodeling plays a more crucial role in HPH pathogenesis. The key pathological findings of pulmonary vascular remodeling are increased thickening of pulmonary vessel walls and muscularization of small arteries (34). Similar pathologic changes, including pulmonary smooth muscle hypertrophy and proliferation, have been found in laboratory animals (34, 36). In addition, many studies have shown that hypoxia stimulates pulmonary arterial smooth muscle cells (PASMCs) proliferation and migration in vitro, which could be important mechanisms of pulmonary vessel remodeling associated with HPH (25).

To study the cellular and molecular mechanisms of PASMCs in regulating the remodeling of lung vessels, we compared the differences in protein expression in human PASMCs under normoxia and hypoxia using two-dimensional difference gel electrophoresis (2-DE DIGE) in combination with MALDI-TOF/TOF MS/MS in our previous study (38). We found that one actin-associated protein, transgelin, was preferentially expressed in human PAMSCs under hypoxia compared with normoxia (38). We also found that reduced transgelin expression in human PASMCs was accompanied by significantly impaired migration ability in vitro under hypoxia (38). This result indicates that transgelin could play an important role in the pathogenesis of pulmonary vascular remodeling under hypoxia.

* R. Zhang and L. Shi contributed equally to this work.

Address for reprint requests and other correspondence: K. Ying, Dept. of Respiratory Medicine, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang Univ., 3 East Qingchun Rd., Hangzhou, China (e-mail: yingkejing@163.com).

Key Laboratory of Biotherapy in Zhejiang Province, Biomedical Research Center, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, China; 2 Department of Gastroenterology, the First Affiliated Hospital of Zhejiang University, Hangzhou, China; and 3 Department of Critical Care medicine, the Second Affiliated Hospital of Zhejiang University, Hangzhou, China

Submitted 13 November 2013; accepted in final form 13 January 2014

Hypoxic pulmonary hypertension (HPH) is one of the most significant subtypes of pulmonary hypertension (PH) (24). It is a serious threat to patients (9). Pulmonary vascular remodeling plays a more crucial role in HPH pathogenesis. The key pathological findings of pulmonary vascular remodeling are increased thickening of pulmonary vessel walls and muscularization of small arteries (34). Similar pathologic changes, including pulmonary smooth muscle hypertrophy and proliferation, have been found in laboratory animals (34, 36). In addition, many studies have shown that hypoxia stimulates

It is widely known that hypoxia-inducible factor 1α (HIF-1α) is a central transcriptional factor in the primary hypoxia-driven signaling pathways. HIF-1α is selectively stabilized under hypoxia and dimerizes with its constitutive subunit, hypoxia-inducible factor 1β (HIF-1β), to form the heterodimeric transcription factor HIF-1. During hypoxia, HIF-1 translocates into the nucleus, binds to hypoxia-responsive elements (HREs) in downstream genes, and activates the expression of various genes (14, 28). Our previous results showed that upregulation of transgelin occurred independently of HIF-1α (38). Unlike HIF-1α, HIF-2α regulates a unique set of genes, and most genes regulated by HIF-2α overlap with those induced by HIF-1α (1). HIF-2α seems to play a more minor role in hypoxia-driven signaling pathways than HIF-1α. During hypoxia, more genes are known to be regulated by HIF-1α than
by HIF-2α. Thus any unique contribution of HIF-2α remains largely unknown (1). In recent years, some studies have shown that HIF-2α is more important than HIF-1α under certain conditions (13, 14, 27). Ahmad and colleagues (1) found that the adenosine A2A receptor was a unique angiogenic target of HIF-2α, rather than HIF-1α, in pulmonary endothelial cells. Sowter et al. (27) found that in a panel of hypoxia-inducible genes, responses were critically dependent on HIF-2α in renal carcinoma cells. Therefore, we investigated the potential relationship between HIF-2α and transgelin in the present study.

MATERIALS AND METHODS

Cells and culture. Human PASMCs were purchased from Cascade Biologics (Portland, OR). The cells were maintained in smooth muscle growth medium (medium 231; Cascade Biologics) and were supplemented with Smooth Muscle Growth Supplement (SMGS; Cascade Biologics). The cells were incubated in a Heraeus CO2 incubator set at 37°C in an atmosphere of 5% CO2, and 4- to 10-passage cells were used in all experiments. Cells were also cultured in a hypoxia incubator (Thermo Electron, Forma) under hypoxic conditions (1% O2, 94% N2, and 5% CO2) at 37°C.

Reverse transcription-polymerase chain reaction. Fluorescence real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed according to the previous report (37). The following specific oligonucleotide primers were used for transgelin: sense, 5’-GGA GTG GAT CAT AGT GCA GTG T-3’; antisense, 5’-GCT TGG AGC CAT CAG GTA-3’.

Western blot analysis. Western blot was performed according to the previous report (37). The transgelin protein was detected by using a rabbit anti-transgelin polyclonal antibody (Proteintech Group). HIF-1α was detected by using a mouse anti-HIF-1α monoclonal antibody (BD Biosciences, San Diego, CA). HIF-2α was detected by

---

![Graph](https://example.com/graph1.png)

**Fig. 1. Hypoxia-inducible factor 2α (HIF-2α)-dependent transgelin expression. Human pulmonary arterial smooth muscle cells (PASMCs) were transiently transfected with siRNA targeting HIF-1α (100 nM), HIF-2α (100 nM), or HIF-1α + HIF-2α (50 nM each). SiRandom was used as the control siRNA. After transfection the cells were incubated under normoxic and hypoxic conditions for 72 h. A: the mRNA level of transgelin was assessed by real-time RT-PCR with β-actin as an internal control. Values (means ± SE) are expressed as fold increases, with the control (under normoxia) taken as 1. All results were repeated with 3 independent experiments. **P < 0.01 compared with the control under normoxia; #P < 0.05 compared with the control under hypoxia; ###P < 0.01 compared with the control under hypoxia. B: transgelin, HIF-1α, and HIF-2α proteins were detected by Western blotting with β-actin as the loading control. Values (means ± SE) are expressed as fold increases, with the control (under normoxia) taken as 1. All results were repeated with 5 independent experiments. **P < 0.01 compared with the control under normoxia; #P < 0.05 compared with the control under hypoxia.**
using a mouse anti-HIF-2α monoclonal antibody (Novus Biologicals, Littleton, CO).

siRNA knockdown of HIF-1α, HIF-2α, and transgelin expression. siRNAs were designed and transfected into cells as described elsewhere (37, 38), with some modifications. Human PASMCs were treated with siRNA targeting HIF-1α (100 nM), HIF-2α (100 nM), transgelin (100 nM), or HIF-1α + HIF-2α (50 nM each); after transfection the cells were incubated under normoxic and hypoxic conditions for 72 h.

Expression constructs and luciferase assay. The procedure was conducted according to the previous report (37). Human transgelin promoter luciferase constructs included the following sequences: -2,000 nt of the 5′-flanking region from the transcription start site. Human PASMCs were treated with varying concentrations of TGF-β1, HIF-2α (100 nM), or HIF-1α (100 nM), and were incubated overnight. The cells were cotransfected with 50 ng of pGL3-transgelin and 1 ng of pSV40-Renilla and then treated with the pEF-BOS empty vector. Promoter activity was measured as the proportion of luciferase activity 36 h after transfection. The promoter activity in the presence of different concentrations of pEF-BOS-HIF-2α expression vectors was defined relative to that value. The value in the panel represents the fold increase ± SE of 3 independent samples with the same treatment. •, HRE; ○ with arrow, transcription start site. Human PASMCs were treated under 1% O2 and normoxia for different durations, and the concentration of TGF-β1 in cell culture supernatants was measured by ELISA. The value in the panel represents means ± SE of 3 independent samples with the same treatment. *P < 0.05 compared with normoxia controls (C). Next, human PASMCs were treated with different doses of TGF-β1, D: the mRNA level of transgelin was assessed by real-time RT-PCR with GAPDH as an internal control. E: transgelin protein was detected by Western blotting with GAPDH as the loading control. Values (means ± SE) are expressed as fold increases, with a zero concentration of TGF-β1 taken as a fold increase of 1. All results were repeated with 3 independent experiments. *P < 0.05 compared with samples without TGF-β1 treatment; **P < 0.01 compared with samples without TGF-β1 treatment.

Chromatin immunoprecipitation assay. As described in a previous report (35), cells were treated with TGF-β1 (2 ng/ml) for 60 min, 1% formaldehyde for 10 min, and 0.125 M glycine for 5 min and were analyzed with the Chromatin Immunoprecipitation Assay (Upstate) with anti-Smad3 and control IgG (Upstate/Millipore). PCR was performed with primers to amplify the transgelin gene. The following PCR primer pairs were used to amplify the transgelin promoter region (–17 to +106): sense, 5′-AGG GAA GGC TGA CAT-3′; antisense, 5′-TCG CAG GAA GGA TGT-3′ (35).

TGF-β1 concentration detection in culture medium. Human PASMCs were plated at 1×10⁵ cells/well in 12-well plates. When confluent, the cells were incubated with 1 ml of fresh medium and then cultured under hypoxia or normoxia. After the specified intervals, the cell culture supernatants were aspirated and frozen at −80°C until assayed. TGF-β1 concentrations in the culture medium were detected by using ELISA kits according to the manufacturer’s instructions.

Cell growth and migration assay. As described in a previous report (39), cell growth was evaluated by WST-8 [2-methoxy-4-nitrophenyl-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] cleavage assays (Cell Counting Kit-8; Dojindo, Kumamoto, Japan). Cell count was also analyzed. The migration assay was conducted according to the previous report (38).
Production of the transgelin shRNA lentiviral vector. A pair of miRNA oligosequences, including the transgelin interference target, were designed (Transgelin-MIR-1-F, 5'-TGCTGCAATCTCTTCAATTTGTTATGGTTTGGGCCACCTGACCAACAAAGATTTGAAAGGATTATG-3; Transgelin-MIR-1-R, 5'-CTCTGGCTTTCTTCAATCTTCTGTGTCAGTCAGTGCGCCACCAAGATTTGAAAGGATTATG-3'; the sequences in bold type represent the target sequences) and were cloned into pcDNA6.2-EGFP-miR (Invitrogen, Carlsbad, CA) by use of Bsal. The newly formed vector was named pcDNA6.2-EGFP-Transgelin-miR. The Transgelin-miR target sequence, combined with green fluorescent protein (EGFP-Transgelin-miR), was amplified from pcDNA6.2-EGFP-Transgelin-miR by PCR (Lenti-Asc1-F, 5'-TACTG-CCGCAGCCGCCCCACATGGTGAGCAAGGGCGAGGA-3'; Lenti-Pme1-R, 5'-ACTAGTTTAAACTGCGGCCAGATCTGGGC-3'). Next, the EGFP-Transgelin-miR fragment was cloned into pLenti6.3-MCS5/Dest (Invitrogen) by use of AscI and PmeI. Finally, the newly formed vector was named pLenti6.3-EGFP-Transgelin-miR. Viral medium, containing Lenti6.3-EGFP-Transgelin-miR, was collected, concentrated, and titered. The concentration of viral particles was determined by using the HIV-1 p24 antigen ELISA assay (Beckman Coulter) following the manufacturer’s instructions.

Inhibiting transgelin expression in vitro. Cultured rat PASMCs (donated by Dr. Chuanxin Liu, Medical School of Shanghai Jiaotong University, Shanghai, China) were infected with either Lenti6.3-EGFP-Transgelin-miR or Lenti6.3-EGFP for 72 h, and the cells were then harvested. Transgelin was examined by Western blotting.

Determining lentiviral transduction efficiency of the lung. After anesthesia with intraperitoneal ketamine (7 mg/100 g) and xylazine (1 mg/100 g), rat lungs were perfused for 2 min with 100 ml of cold phosphate-buffered saline (PBS) at a pressure of 100 mm Hg and were then harvested. Transgelin was examined by Western blotting.

Fig. 3. TGF-β1 transactivated the transgelin promoter. A: scheme of the promoter region of human transgelin. B: the pGL3-transgelin reporter plasmid was transiently cotransfected with the pSV40-Renilla plasmid into Cos-7 cells. The transfected cells were treated with different doses of TGF-β1, and then the promoter activity was measured as the proportion of luciferase activity. The promoter activity without TGF-β1 treatment was defined as 1, and the promoter activity in the presence of different concentrations of TGF-β1 was defined relative to that value. The value in the panel represents the fold increase ± SE of 3 independent samples with the same treatment. *P < 0.05 compared with samples without TGF-β1 treatment. C: chromatin immunoprecipitation assay of Smad3 binding in TGF-β1-exposed human PASMCs. Chromatin from cells exposed to different doses of TGF-β1 was immunoprecipitated with IgG or anti-Smad3 and was analyzed by PCR using primers spanning the candidate Smad3 binding site. Genomic DNA (gDNA) was used as a positive control in which PCR was performed with use of total human gDNA rather than immunoprecipitated chromatin. F, forward; R, reverse; •, Smad3 binding site; # with arrow, transcription start site. Values (means ± SE) are the densitometric proportions of PCR products, with IgG (0 ng of TGF-β1) taken as 1. All results were repeated with 3 independent experiments. **P < 0.01. D: human PASMCs were transiently transfected with siRNA targeting HIF-1α or HIF-2α (100 nM). SiRandom was used as the control siRNA. After transfection the cells were incubated under normoxic and hypoxic conditions. The TGF-β1 concentration in cell culture supernatants was estimated by ELISA. Values in the panel represent means ± SE of 3 independent samples with the same treatment. **P < 0.01 compared with the control under normoxia; #P < 0.05 compared with the control under hypoxia.

AJP-Lung Cell Mol Physiol • doi:10.1152/ajplung.00327.2013 • www.ajplung.org
mg/100 g), the rats received intratracheal instillation of Lent6.3-EGFP (1.5×10⁶ transducing units in 300 µl PBS) or 300 µl PBS. One week following the gene transfer, the animals were euthanized, and their lungs were perfused with PBS. Next, the lung tissues were frozen, and 5-µm-thick lung frozen sections were cut. The intensity of EGFP in the lung tissue sections was observed under a fluorescence microscope.

Animals and delivery of lentiviral vectors to the lungs. Male Sprague-Dawley rats (12 wk old) were used in this study; the animals were obtained from the Animal Experimental Center of Zhejiang University, China. After anesthesia with intraperitoneal ketamine (7 mg/100 g) and xylazine (1 mg/100 g), the rats received intratracheal instillation of Lent6.3-EGFP-Transgelin-miR or Lent6.3-EGFP as the control. The amount of gene delivery of Lent6.3-EGFP-Transgelin-miR or Lent6.3-EGFP for one rat was 1.5×10⁶ transducing units (in total 300 µl PBS, 50 µl was instilled every day for 6 days). After 7 days of lentivirus delivery, the rats were exposed to normobaric hypoxia (10% O₂ environment) or normoxia (21% O₂ environment) for 3 wk. All of in vivo studies were approved by the Zhejiang University Institutional Animal Care and Use Committee.

Isolation of PASMCS. Primary PASMCS from the rats (received Lent6.3-EGFP-Transgelin-miR or Lent6.3-EGFP and exposed to normobaric hypoxia or normoxia for 3 wk) were prepared as previously described (31). Cells from passages 4–10 were used to detect transgelin by RT-PCR and Western blot.

Right ventricular systolic pressure and right ventricular hypertrophy measurements. Right ventricular systolic pressure (RVSP) was measured after 3 wk of hypoxia or normoxia treatment. The procedure was conducted as the previous report (31). The right ventricle, left ventricular wall, and ventricular septum were also weighed. The ratio of the right ventricular wall weight to the left ventricular wall plus septum weight [RV/(LV + S)] was used as an index of right ventricular hypertrophy (n = 6 in each group).

Immunohistochemical analysis. Immunohistochemical staining for α-smooth muscle actin and transgelin protein was performed as the previous reports (31, 32). The sections were observed via light microscopy. At 40× magnification, 10–20 muscular arteries per rat were photographed, and 60–80 intra-acinar vessels per rat accompanying either alveolar ducts or alveoli were analyzed by two observers blinded to the experiment. Only vessels with 30- to 90-μm diameters were analyzed. The external diameter and internal diameter of these muscular arteries were measured by use of ImageJ software (National Institutes of Health, Bethesda, MD), version 1.45s. The arterial wall thickness was calculated as follows: percentage wall thickness = [(external diameter − internal diameter)/external diameter]×100 (n = 6 rats per group) (32). Intra-acinar vessels were categorized as fully

---

**Fig. 4. RNA interference-mediated reduction in transgelin expression resulted in impaired cell migration and proliferation ability. Human PASMCs were transiently transfected with siRNA targeting transgelin. Random siRNA was used as the control siRNA. Transgelin protein was detected by Western blotting with β-actin as the loading control (A). B: effect of transgelin knockdown on migration. Transfected human PASMCs were trypsinized and plated onto a Millicell insert at 2×10⁴ cells per 24-well plate. Then the cells were incubated under normoxia and hypoxia. The number of cells that migrated to the lower membrane surface was counted after 6 h. All results are presented as means ± SE of 3 independent tests. **P < 0.01 compared with the control under hypoxia. Effects of transgelin knockdown on proliferation was analyzed. Cells transfected with targeting or nontransgelin siRNA were cultured under hypoxia and normoxia for different durations. C: WST-8 cleavage in each group was measured (absorbance OD450). All results are presented as means ± SE of 3 independent tests. *P < 0.05 compared with the control under normoxia; #P < 0.05 compared with the control under hypoxia. D: cell counting assay was also done. All results are presented as means ± SE of 3 independent tests. *P < 0.05 compared with the control under hypoxia; **P < 0.05 compared with the control under normoxia; #P < 0.05 compared with the control under hypoxia.
Transgelin was upregulated by HIF-2α. Transgelin was upregulated under hypoxia. RNA interference for HIF-1α, HIF-2α, or the combination of HIF-1α and HIF-2α was employed by transfecting specific siRNA motifs to human PASMCs. The effects were analyzed and compared with those of control-siRNA-transfected cells. We found that transgelin expression was diminished in HIF-2α-siRNA-treated and the transgelin expression was unchanged at both the mRNA and protein levels under hypoxia. However, there were no further decreases in transgelin expression with the combination of HIF-1α and HIF-2α siRNA. Transgelin expression was unchanged at both the mRNA and protein levels in HIF-1α-siRNA-treated cells compared with the controls (Fig. 1).

HIF-2α did not enhance the transcriptional activity of transgelin. Because both HIF-1α- and HIF-2α-dependent target genes are controlled by the HREs of their respective promoters, bioinformatics analysis was performed by using an ~2-kb region (2,000 bp upstream to 100 bp downstream of the transcription start site) of human transgelin gene. The results showed two typical HREs (A/GCGTG) in the putative promoter region of transgelin, located at −1,481 bp and 194 bp of the gene (Fig. 2A). A fragment of the human transgelin promoter (+2,000 to −100 bp) was subcloned into the luciferase reporter plasmid pGL3-basic to form pGL3-transgelin).

We measured the promoter activities using a dual luciferase reporter assay, as described in our previous study (37). Different amounts of the HIF-2α expression vector pEF-BOS-HIF-2α and HIF-1β expression vector pEF-BOS-HIF-1β were

![Image](https://example.com/image.png)
cotransfected, along with the pGL3-transgelin plasmid into cells. The results demonstrated that the transgelin promoter activity of luciferase was unchanged compared with that of the control (Fig. 2B).

**Accumulated TGF-β1 is a mediator in the upregulation of transgelin by HIF-2α.** Previous studies have shown that TGF-β1 can induce the synthesis of transgelin at both the mRNA and protein levels (26, 35). TGF-β1 has also been demonstrated to induce transgelin expression under the control of SBEs (Smad-binding elements) in the promoter region (3, 35). In the present study, we found that TGF-β1 concentration in human PASMC culture medium was higher under hypoxia than normoxia at the same time point (Fig. 2C). Subsequently, cultured human PASMCs were incubated with recombinant human TGF-β1. Our results indicated that TGF-β1 upregulated transgelin expression at the mRNA and protein levels, and this effect was dose dependent (Fig. 2, D and E).

Yu and colleagues (35) found that transgelin was a direct target of TGF-β1/Smad3-dependent gene expression in ATII cells. Bioinformatics analysis showed that there were 16 SBEs (-CAGA-) in the promoter region of human transgelin (Fig. 3A). The promoter activities were measured again after the cells were treated with TGF-β1, and the results demonstrated that the transgelin promoter activity of luciferase was significantly increased when TGF-β1 was used (Fig. 3B). To confirm the enhanced binding of TGF-β1/Smad3 to the transgelin promoter in live human PASMCs, we performed a chromatin immunoprecipitation assay using primers covering the suggested SBE binding sites (35). Consistent with the previous report (35), TGF-β1 augmented the binding of Smad3 to the transgelin promoter in human PASMCs (Fig. 3C).

To investigate whether accumulated TGF-β1 under hypoxia was regulated by HIF-2α, RNA interference for HIF-1α and HIF-2α was employed, and the concentration of TGF-β1 in the culture medium was measured. The results showed that the concentration of TGF-β1 was lower in HIF-2α-siRNA-treated cells under hypoxia than in the controls (Fig. 3D). These results indicated that accumulated TGF-β1 was a mediator of the upregulation of transgelin by HIF-2α under hypoxia.

RNA interference-mediated reductions in transgelin expression resulted in impaired abilities of proliferation and migration. Transgelin protein expression was decreased in transgelin-siRNA-treated cells compared with controls (Fig. 4A). For the migration assay, the number of cells that translocated across the membranes during hypoxia was increased compared with that during normoxia. However, the number of cells decreased when transgelin expression was inhibited under hypoxia (Fig. 4B). In the cell proliferation assay, human PASMCs had higher WST-8 cleavage levels under hypoxia. We found that cells transfected with transgelin-targeted siRNA exhibited significantly lower WST-8 cleavage levels than controls under hypoxia (Fig. 4C). Cell counting assay showed the similar results (Fig. 4D). This study demonstrated that upregulated transgelin could enhance the proliferation and migration of PASMCs under hypoxia.

**Inhibiting transgelin expression in vitro and in vivo.** Lentiv6.3-EGFP-Transgelin-miR could inhibit transgelin expression in vitro (Fig. 5A). Rat pulmonary tissues were observed 7 days after intratracheal gene transfer with Lentiv6.3-EGFP. The transfection efficiency was indicated by green fluorescence in the lung tissues. The intratracheal administration of Lentiv6.3-EGFP resulted in stronger green fluorescence in the pulmonary arterioles than in those of PBS-treated rats (Fig. 5B). Transgelin mRNA and protein levels were increased in PASMCs isolated from rats exposed to normobaric hypoxia for 3 wk. This effect was attenuated by Lentiv6.3-EGFP-Transgelin-miR transfer (Fig. 5, C and D). Immunohistochemistry revealed that the immunoactive intensity of transgelin in the pulmonary arterioles was markedly enhanced by 3 wk of hypoxia treatment. This effect was attenuated by Lentiv6.3-EGFP-Transgelin-miR transfer (Fig. 5E).

**Attenuated of HPH and associated cardiac and pulmonary artery remodeling by Lentiv6.3-EGFP-transgelin-miR.** Three weeks of hypoxia treatment resulted in an increase in RVSP (35.6 ± 2.2 mmHg in the hypoxia group vs. 27.0 ± 2.4 mmHg in the control group; P < 0.05; Fig. 6, A and B) and an ∼40% increase in the RV/(LV+S) (0.34 ± 0.03 in the hypoxia group vs. 0.24 ± 0.02 in the control group; Fig. 6C). Lentiv6.3-EGFP-Transgelin-miR administration in hypoxic rats resulted in significant attenuation of RVSP (30.5 ± 2.8 mmHg; Fig. 6, A and B) and RV/(LV+S) (0.29 ± 0.03; Fig. 6C). Immunostaining with an antibody directed against α-smooth muscle actin showed that the medial walls of pulmonary arterioles were

![Fig. 6](http://ajplung.physiology.org/)
markedly thickened by 3 wk of hypoxia treatment and were also significantly attenuated by Lentiv6.3-EGFP-Transgelin-miR (Fig. 7, A and B). Furthermore, in normal lungs, 70.0 ± 3.3% of the arterioles were nonmuscularized, and 9.0 ± 1.4% were fully muscularized. In contrast, the hypoxia-treated animals showed a substantially greater proportion of small vessels with partial (30.0 ± 3.7%) and full muscularization (44.0 ± 6.5%), as well as a smaller proportion of small vessels that were nonmuscularized (26.0 ± 5.4%; Fig. 7C). Lentiv6.3-EGFP-Transgelin-miR gene transfer, before exposure to hypoxia, significantly reduced the percentage of small vessels exhibiting full muscularization (25.0 ± 3.2 vs. 44.0 ± 6.5%; \( P < 0.05 \); Fig. 7C) and increased the percentage of nonmuscularized vessels (42.0 ± 5.2 vs. 26.0 ± 5.4%; \( P < 0.05 \); Fig. 7C).

**DISCUSSION**

In the present study, our results indicated that hypoxia stimulated transgelin expression via HIF-2α-dependent pathways. However, HIF-2α did not transactivate the transgelin promoter directly. We also investigated the role of transgelin in HPH pathogenesis in vivo. Our results indicated that intratracheal introduction of Lentiv6.3-EGFP-Transgelin-miR into rats could attenuate HPH and associated cardiac and pulmonary vessel remodeling by inhibiting transgelin expression.

Our results showed that hypoxia induced TGF-β1 and transgelin. Consistent with the previous report (20), we identified the transgelin gene as an immediate target of TGF-β1/Smad3-dependent gene expression in human PASMCs using a Smad3 chromatin immunoprecipitation assay. We next sought to determine whether the accumulated TGF-β1 was a key mediator in the upregulation of transgelin by HIF-2α. Although the present results showed that the concentration of TGF-β1 was lower in HIF-2α-siRNA-treated cells under hypoxia than in control cells, the effects of si-HIF-2α on the inhibition of TGF-β1 are only mild. These results indicated that the accumulated TGF-β1 may be only one mediator in the upregulation of transgelin. Consistent with the previous report (20), we identified transgelin as a target of TGF-β1 and transgelin. Our results showed that hypoxia induced TGF-β1 and transgelin. Consistent with the previous report (20), we identified the transgelin gene as an immediate target of TGF-β1/Smad3-dependent gene expression in human PASMCs using a Smad3 chromatin immunoprecipitation assay. We next sought to determine whether the accumulated TGF-β1 was a key mediator in the upregulation of transgelin by HIF-2α. Although the present results showed that the concentration of TGF-β1 was lower in HIF-2α-siRNA-treated cells under hypoxia than in control cells, the effects of si-HIF-2α on the inhibition of TGF-β1 are only mild. These results indicated that the accumulated TGF-β1 may be only one mediator in the upregulation of transgelin by HIF-2α under hypoxic conditions. However, other factors may also take part in the regulation of transgelin by HIF-2α. Our results demonstrated an extended role for TGF-β1 in the regulation of hypoxic lung vascular remodeling.

PASMC migration and proliferation into the arterial wall play pivotal roles in the development of HPH. We found that reduced transgelin expression resulted in the reduced ability of cells to migrate during hypoxia. Previous studies have indicated that transgelin contributes to the motility of many types of cells (2, 11, 19, 29). This effect might be due to cytoskeletal alterations and rearrangements induced by transgelin (7). In the present study, we also found that reduced transgelin expression resulted in the reduced ability of cells to proliferate during hypoxia. Different studies have produced divergent results regarding the proliferation function of transgelin. Daniel and colleagues (5) found that transgelin could promote mesangial cell proliferation after injury, and Yu et al. (35) found that transgelin contributed to the proliferation of A549 epithelial cells and primary alveolar epithelial type II cells. In contrast, Lv and his colleagues (21) found that transgelin can also be phosphorylated and induce effects via phosphorylation. Both downregulation and phosphorylation of transgelin are associated with hypertrophy and hyperplasia of vessel smooth muscle cells in vitro and in vivo. Dong and colleagues (6) found that the high expression of transgelin in vivo inhibited balloon injury-induced neointimal hyperplasia by suppressing vascular smooth muscle cell proliferation. Their results also indicated that overexpression of transgelin markedly inhibited the Ras-Raf-MEK-ERK1/2 signaling cascade and resulted in cell cycle arrest in G0–G1, thereby blocking the mitogen-stimulated proliferation of vascular smooth muscle cells and preventing injury-induced neointimal hyperplasia. Similarly, Kim and colleagues (17) demonstrated that transgelin overexpression inhibited the activation of IGF-1R/Akt and Erk, consequently suppressing cell proliferation. They also found that transgelin overexpressing cells became resistant to apoptotic cell death caused by cytotoxic agents. These results suggest that transgelin-
lin serves various proliferation functions under different conditions. Despite our increasing knowledge concerning the function of transgelin, the molecular mechanisms that govern cellular proliferation remain largely elusive.

Our in vivo study showed that lentiviral vector treatment, before the induction of PH, resulted in prevention of increases in RVSP and right ventricular hypertrophy, as well as attenuation of the thickening of pulmonary vessels. These effects were associated with a significant inhibition of the muscularization of arterioles. Notably, our data provide evidence that transgelin represents a novel target for interventions aimed at attenuating lung vascular remodeling and subsequent right heart hypertrophy. However, further evidence is necessary to clarify these issues.

A major limitation of this study is that the potential role of transgelin in the sustained pulmonary vasoconstriction of chronically hypoxic rats is not considered. There is now considerable evidence that the increased pulmonary vascular resistance in chronically hypoxic rats is due largely to Rho kinase-mediated vasoconstriction, rather than to the vascular remodeling (4, 10, 22, 23). Transgelin is important in vascular resistance in chronically hypoxic rats is not considered. There is now

GRANTS
This study was supported by research grants 81000019 and 81001103 from the National Natural Science Foundation of China and by research grant 2013KYB158 from the Ministry of Health of Zhejiang Province in China.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


