Endothelial Krüppel-Like Factor 4 Modulates Pulmonary Arterial Hypertension

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Abstract

Krüppel-like factor 4 (KLF4) is a transcription factor expressed in the vascular endothelium, where it promotes anti-inflammatory and anticoagulant states, and increases endothelial nitric oxide synthase expression. We examined the role of endothelial KLF4 in pulmonary arterial (PA) hypertension (PAH). Mice with endothelial KLF4 knockdown were exposed to hypoxia for 3 weeks, followed by measurement of right ventricular and PA pressures, pulmonary vascular muscularization, and right ventricular hypertrophy. The effect of KLF4 on target gene expression was assessed in lungs from these mice, verified *in vitro* by small interfering RNA (siRNA) knockdown of KLF4, and further studied at the promoter level with cotransfection experiments. KLF4 expression was measured in lung tissue from patients with PAH and normal control subjects. We found that, after hypoxia, right ventricular and PA pressures were significantly higher in

KLF4 knockdown animals than controls. Knockdown animals also had more severe pulmonary vascular muscularization and right ventricular hypertrophy. KLF4 knockdown resulted in increased pulmonary expression of endothelin-1 and decreased expression of endothelial nitric oxide synthase, endothelin receptor subtype B, and prostacyclin synthase. Concordant findings were observed *in vitro*, both with siRNA knockdown of KLF4 and promoter activity assays. Finally, KLF4 expression was reduced in lungs from patients with PAH. In conclusion, endothelial KLF4 regulates the transcription of genes involved in key pathways implicated in PAH, and its loss exacerbates pulmonary hypertension in response to chronic hypoxia in mice. These results introduce a novel transcriptional modulator of PAH, with the potential of becoming a new therapeutic target.

Keywords: Krüppel-like factor 4; pulmonary arterial hypertension; pulmonary endothelium

Pulmonary arterial (PA) hypertension (PAH) is characterized by a sustained elevation in PA pressure (PAP), due to increased resistance in small PAs (1). The disease has a complex and multifactorial pathobiology, which remains incompletely understood. Some of the key disturbances in the pulmonary vasculature that result in

PAH are vascular wall remodeling, with muscularization of pulmonary arteriole, vasoconstriction, thrombosis, and probably inflammation (2, 3). These homeostatic imbalances are largely thought to be consequences of endothelial dysfunction (4).

The Krüppel-like family is a group of transcription factors characterized by the

presence of a three-zinc-finger DNA binding domain. A total of 18 mammalian Krüppel-like factors (KLFs) have been described to date, three of which have been identified as being expressed in endothelial cells: KLF2, -4, and -6 (5). Our group has previously shown that KLF4 is expressed in arterial and venous endothelial cells of the

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pulmonary circulation, and that KLF4 up-regulates the expression of endothelial nitric oxide (NO) synthase (eNOS), leading to increased endothelial eNOS activity (6). We have also shown that endothelial KLF4 has anti-inflammatory and antithrombotic effects (7). Thus, there is evidence that endothelial KLF4 modulates certain critical processes (vascular tone, inflammation, and thrombosis) involved in the pathogenesis of PAH. We therefore hypothesized that loss of endothelial KLF4 would exacerbate PAH.

Materials and Methods

Animals

Mice with endothelial KLF4 knockdown were generated by crossing floxed KLF4 mice with vascular endothelial cadherin (VE-cadherin)-driven Cre mice from a C57BL/6 background, as previously described (7). This achieved 80% or greater loss of endothelial KLF4 expression, but partial loss of KLF4 expression was also observed in macrophages and lymphocytes (7). All animal protocols were approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

Hypoxia-Induced PAH Model

Male mice, 8–12 weeks of age, were housed for 3 weeks at an oxygen concentration of 10%, maintained by aProOx 110 oxygen controller (BioSpherix, Lacona, NY). Control mice were simultaneously housed at 21% oxygen under identical conditions. At the end of the 3 weeks, pressure measurements were obtained using the technique reported by Skuli and colleagues (8), with slight modification. Briefly, animals were anesthetized, intubated, and mechanically ventilated. A parasternal incision was made, and a Mikro-Tip Catheter (Millar Instruments, Houston, TX) was inserted through a small puncture into the right ventricle (RV), then advanced to the PA. Pressures were recorded using PowerLab Data Acquisition System and analyzed using LabChart 7 Software (ADInstruments, Colorado Springs, CO).

Immune Staining

Mouse lung tissue was formalin fixed and paraffin embedded. For pulmonary vessel staining, 7- μ m sections were stained with antibodies to α -smooth muscle actin

(α -SMA; Sigma-Aldrich, St. Louis, MO) and von Willebrand factor (Vector Laboratories, Burlingame, CA). Inflammatory cell staining was performed with antibody to CD 45 (BD Biosciences, San Jose, CA). Additional mouse lung tissue samples were fresh frozen in optimal cutting temperature compound (OCT), and 10- μ m sections stained with antibodies to KLF4 (R&D Systems, Minneapolis, MN) and platelet endothelial cell adhesion molecule (PECAM) (BD Biosciences). RV tissue was fresh frozen in OCT, and 8- μ m sections were stained with rhodamine-labeled wheat germ agglutinin (Vector Laboratories) to outline cardiomyocytes. Images were analyzed using ImageJ software (National Institute of Health, Bethesda, MD).

KLF4 Knockdown

Human umbilical vein endothelial cells (HUVECs) at passages 3–6 were transfected with nonspecific or KLF4-specific small interfering RNA (siRNA) (50 nM; Dharmacon, Waltham, MA) using LipofectamineRNAiMAX (Invitrogen, Grand Island, NY) following the manufacturer's instructions. RNA was isolated 48 hours after transfection.

Human Lung Tissue

Use of human tissue was approved by the University of Pittsburgh Institutional Review Board. Explanted lung tissue from patients with idiopathic PAH (IPAH) was obtained at the time of transplantation. Normal lung tissue was obtained either from donor lungs that were not suitable for transplantation or from deceased patients without known lung disease through a rapid autopsy program. The tissue was snap frozen in liquid nitrogen and stored at -80°C until the time of use.

RNA Isolation, RT-Quantitative PCR, Western Blot, and Promoter Assays

RNA isolation, reverse transcription, quantitative PCR (QPCR), Western blot, and promoter activity assays were performed as described in the online supplement.

Statistical Analysis

All data are presented as means ($\pm$ SEM). Statistical analyses were done using two-tailed Student's *t* test for two-group comparisons and ANOVA with *post hoc* Tukey's test for multiple group comparisons. A *P* value of 0.05 or less was considered significant.

Results

Mice Deficient in Endothelial KLF4 Develop More Severe Pulmonary Hypertension in Response to Chronic Hypoxia

Under normoxic conditions, there was no significant difference in right ventricular systolic pressure (RVSP) between VE-Cadherin Cre/KLF4 wild-type (WT) mice, referred to as Cre/WT, and VE-cadherin Cre/KLF4 floxed mice, referred to as Cre/Flox. Exposure to hypoxia for 3 weeks resulted in elevation of RVSP in both groups, but it was significantly more elevated in the Cre/Flox than in the Cre/WT group (Figure 1A).

Similar results were found for the pulmonary artery pressure (PAP), which was not significantly different between the two mouse groups under normoxic conditions, but more elevated after hypoxia in the Cre/Flox group than in the Cre/WT group (Figure 1B).

Loss of Endothelial KLF4 Exacerbates Pulmonary Vessel Muscularization

Sections of lung tissue were examined after staining for von Willebrand factor and α -SMA. Small vessels ($< 100 \mu\text{m}$) were classified into three categories (9): nonmuscularized (no α -SMA staining), partially muscularized, or fully muscularized (unbroken wall of smooth muscle) (Figure 1C).

The percentage of nonmuscularized pulmonary vessels was greater in Cre/WT animals than in Cre/Flox animals. Conversely, the percentage of fully muscularized vessels was greater in Cre/Flox animals. There was no significant difference in the percentage of partially muscularized vessels (Figure 1D).

Because the Cre/Flox animals have partial knockdown of KLF4 in a subset of hematopoietic cells, this could have potentially contributed to the observed difference between the two mouse groups by modifying inflammatory cells in the lung. To explore this possibility, we examined lung tissue after hypoxia for inflammatory cell infiltration by staining for CD45, a marker for cells of hematopoietic origin. We found no significant difference in CD45 staining between lungs from Cre/WT and Cre/Flox animals (see Figure E1 in the online supplement).

Loss of Endothelial KLF4 Exacerbates RV Hypertrophy

Cross-sections of hearts from mice exposed to hypoxia demonstrated greater thickening

Figure 1. Pressure measurements and pulmonary vascular muscularization after 3 weeks of hypoxia. (A) Right ventricular (RV) systolic pressure (RVSP) in VE-cadherin Cre mice with either Krüppel-like factor (KLF) 4 wild-type (Cre/WT) or KLF4 floxed (Cre/Flox) with and without hypoxia (five animals per group). (B) Mean pulmonary arterial (PA) pressure (mPAP) in the same mice. (C) Lung immunofluorescent staining for von Willebrand factor (vWF; green) and α -smooth muscle actin (α -SMA; red), showing examples of vessels that are fully muscularized (yellow arrow), nonmuscularized (blue arrow), or partially muscularized (white arrow). (D) Percentages of vessels with different degrees of muscularization in Cre/WT and Cre/Flox animals after hypoxia exposure. Vessels were counted from five animals per group, with at least three sections per animal. Error bars represent SEM. * $P \leq 0.05$, ** $P \leq 0.01$.

of the RV wall in Cre/Flox mice than in Cre/WT mice (Figure 2A). To quantify RV hypertrophy, we used the percent ratio of the weight of the RV to the weight of the left ventricle with septum, RV/(left ventricle + septum). This ratio was higher in the Cre/Flox group than in the Cre/WT group (40.4 ± 1.4 and 32.2 ± 1.3 , respectively; $P < 0.001$; Figure 2B).

To further characterize the RV hypertrophy, sections of the RV were examined with measurement of the cross-sectional area of RV cardiomyocytes. This area was significantly greater after hypoxia in Cre/Flox mice compared with Cre/WT mice (Figures 2C and 2D).

Endothelial KLF4 Knockdown Alters the Expression of Genes Involved in the Pathogenesis of PAH

To investigate potential mechanisms by which endothelial deletion of KLF4 exacerbates hypoxia-induced pulmonary hypertension, we assessed the expression of a number of genes that are expressed in the endothelium, and which are known to play a role in the pathogenesis of PAH. Lung tissue from hypoxic animals was homogenized, RNA was extracted for RT-QPCR, and protein was extracted and subjected to SDS-PAGE.

Cre/Flox animals had decreased expression of eNOS, increased expression of endothelin (EDN) 1, decreased expression of EDN receptor subtype B (EDNRB), and decreased expression of prostacyclin synthase (PTGIS; Figures 3A and 3B).

To further confirm the effect of endothelial loss of KLF4 on the genes identified in total lung tissue, we performed siRNA knockdown experiments for KLF4 in cultured HUVECs. Results were concordant with the murine lung findings, as KLF4 knockdown in HUVECs resulted in decreased mRNA levels for eNOS, increased mRNA for EDN1, and decreased mRNA for EDNRB and PTGIS (Figures 3C and 3D).

KLF4 Expression Alters Promoter Activity of Target Genes

Because KLF4 is a transcription factor, we anticipated that its effects on the target genes identified previously here were, at least in part, mediated by modulating transcription. To explore this issue, transient transfection experiments were performed wherein PA endothelial cells were cotransfected with reporter plasmids for eNOS, EDN1, EDNRB, or PTGIS, together with a control vector, a full-length KLF4 expression plasmid, or an expression plasmid of KLF4 lacking the DNA-binding zinc finger domain.

We found that KLF4 induces eNOS promoter activity, represses EDN1 promoter activity, and induces EDNRB and PTGIS promoter activities. This effect seems to be, at least in part, dependent on DNA binding. When using the DNA-binding zinc finger domain expression plasmid, the ability to induce eNOS and PTGIS was lost, EDN1 was induced instead of repressed, and the induction of EDNRB was reduced (Figures 4A–4D).

Lung Tissue from Patients with IPAH Has Lower Expression of KLF4 Compared with Normal Control Subjects

Based on the findings presented previously here, we predicted that patients with PAH may have altered expression of KLF4 in the lung. To test this, we measured KLF4 expression by QPCR and Western blot in lung tissue from patients with IPAH and from normal control subjects. We found that IPAH lungs had significantly lower levels of KLF4 mRNA and protein compared with normal lungs (Figure 5A).

Discussion

Despite recent advances in our understanding of the pathogenesis of PAH, there remain

Figure 2. RV hypertrophy after hypoxia exposure. (A) Heart cross-sections from Cre/WT and Cre/Flox mice after hypoxia, showing greater thickening of the RV wall in Cre/Flox mice. (B) Weight ratio of the RV to left ventricle plus septum (RV/(LV + S)) in the two mouse groups with and without hypoxia. (C) Fluorescent staining with rhodamine-labeled wheat germ agglutinin for RV myocytes in sections of the RV from a Cre/WT mouse and a Cre/Flox mouse after hypoxia, showing larger cross-sectional areas of RV myocytes from the Cre/Flox mouse. (D) Average cross-sectional area of RV myocytes from Cre/WT and Cre/Flox animals with and without hypoxia (three to four animals per group, with at least three sections per animal). Error bars represent SEM. ** $P \leq 0.01$.

large gaps in our knowledge base. Currently available therapies are of limited efficacy (10), and the disease prognosis remains poor, with 1-year mortality of approximately 15% on modern therapy (11).

PAH has a multifactorial pathobiology, with multiple cell types likely involved (2). Although proliferation of smooth muscle cells in the pulmonary arterioles seems to be a key feature of the disease, there is accumulating evidence of cross-talk between endothelial cells and smooth muscle cells (2, 4, 12). In fact, endothelial cell dysfunction seems to play a critical role in the development of the disease (13), and endothelial cell alterations precede muscularization of distal PAs (14).

In this study, we examined the role of KLF4 in PAH. KLF4 is a transcription factor that we have previously shown to be expressed in endothelial cells of both the pulmonary and systemic circulations (6), and the transcriptional repertoire of which favors anti-inflammatory and antithrombotic states (6, 7). Other researchers have also shown that conditional global knockout of KLF4 results in accelerated neointimal formation after vascular injury (15).

We demonstrate here that endothelial deficiency of KLF4 exacerbates hypoxia-induced pulmonary hypertension in mice, resulting in higher PAPs, more pulmonary arteriolar muscularization, and more pronounced RV hypertrophy. This suggests a protective role played by endothelial KLF4 against the development of PAH. We suspect that this role is complex and involves transcriptional regulation of multiple target genes.

Consistent with this view, we show that KLF4 regulates the transcription of genes that are part of the three key pathways involved in the pathogenesis of PAH (16)—namely, the NO pathway, EDN pathway, and prostacyclin pathway.

NO is one of the most important endothelium-derived vasodilators, and a key regulator of vascular tone (17). This role applies to the pulmonary as well as the systemic circulation (18). Lungs from patients with PAH have reduced expression of eNOS (19). Our findings indicate that endothelial KLF4 deficiency results in reduced pulmonary eNOS expression in mice exposed to chronic hypoxia. We also show that the expression of KLF4 induces eNOS promoter activity, and this is largely

dependent on an intact zinc finger domain of KLF4, suggesting that DNA binding is required for KLF4 to exert this effect.

EDN1 is a potent vasoconstrictor (20) and an inducer of pulmonary vascular smooth muscle proliferation (21). Pulmonary hypertension is associated with increased expression of EDN1 in pulmonary vascular endothelial cells (22), and EDN receptor antagonists are an important class of therapy available for treatment of PAH (23). EDN has complex actions in vascular biology. Its vasoconstrictor action is thought to be predominantly mediated by binding to EDN receptors of the subtype A, present in vascular smooth muscle cells. In contrast, EDNRB is expressed in other cell types, including endothelial cells (24), and mediates a vasodilator action, at least in part through inducing NO synthesis (25). Our results suggest that endothelial KLF4 modulates EDN signaling at two different points: by repressing the transcription of the EDN1 gene and inducing the transcription of the gene for EDNRB.

Prostacyclin is a potent vasodilator and inhibitor of platelet aggregation (26). It also inhibits the proliferation of PA smooth

Figure 3. Expression of genes altered with KLF4 knockdown. (A) Relative mRNA expression in lung tissue homogenates from Cre/WT and Cre/Flox animals after hypoxia for the genes of KLF4, endothelial nitric oxide synthase (eNOS), endothelin (EDN) 1, EDN receptor subtype B (EDNRB), and prostacyclin synthase (PTGIS) ($n = 15$ animals per group). (B) Western blot analysis for protein levels of eNOS and EDN1 (ET-1) in lung homogenates. (C) small interfering RNA (siRNA) knockdown was performed in cultured human umbilical vein endothelial cells (HUVECs) and the same targets as in (A) were assessed. mRNA levels are expressed for cells treated with KLF4-specific siRNA (siKLF4) relative to those treated with nonspecific siRNA (siNS; $n = 10$ experiments). (D) Representative Western blot analysis for selected gene targets in HUVECs treated similar to those in (C) ($n = 3$ experiments). Error bars represent SEM. * $P \leq 0.05$, ** $P \leq 0.01$.

muscle cells (27). Patients with PAH have decreased expression of PTGIS in pulmonary vasculature (28) and reduced levels of prostacyclin metabolites excreted in the urine (29). Prostacyclin analogs are considered the

first line therapy in patients with severe PAH and severe functional impairment (World Health Organization functional class IV) (30). Our study shows that endothelial KLF4 induces the expression of PTGIS.

These findings led us to hypothesize that KLF4 expression may be altered in human PAH. A study that used microarray analysis to examine lung gene expression patterns in patients with PAH found

Figure 4. Promoter activity assays. Transient transfection was performed on bovine PA endothelial cells (BPAECs) with reporter plasmids of respective targets together with expression plasmids for either full-length KLF4 (KLF4), KLF4 with deleted zinc finger DNA-binding domain (KLF4ΔZnF), or empty plasmid vector (Vector). (A) Full-length KLF4, but not KLF4ΔZnF, induces eNOS promoter activity. (B) Full-length KLF4 represses EDN1 promoter activity, whereas KLF4ΔZnF induces it. (C) Full-length KLF4 induces EDNRB promoter activity, but KLF4ΔZnF causes less induction. (D) Full-length KLF4, but not KLF4ΔZnF, induces PTGIS promoter activity ($n = 3$ –5 experiments for each promoter, done in triplicates). Error bars represent SEM. ** $P \leq 0.01$ in comparison to empty vector cotransfection.

Figure 5. (A) KLF4 expression is decreased at both the mRNA and protein levels in lungs from patients with idiopathic PA hypertension (IPAH) compared with control subjects ($n = 4$ per group). Error bars represent SEM. $*P \leq 0.05$. (B) A proposed mechanism by which KLF4 in the endothelial cell (EC) modulates PAH by regulating the expression of the genes for eNOS, EDN1, EDNRB, and PTGIS.

decreased expression of KLF4, which was labeled in the study as zinc finger transcription factor (human erythroid zinc finger transcription factor, hEZF) (31). Here, we found a similar pattern using QPCR to quantify gene expression, together with Western blot to confirm the results at the protein level. Although these findings clearly do not distinguish whether the low KLF4 expression is a risk factor for or a consequence of PAH, they are consistent with a role for KLF4 in modulating PAH.

Our study introduces endothelial KLF4 as a novel factor that plays a role in protection from PAH, and as a regulator of key molecular pathways involved in the pathogenesis of the disease. These results enhance our understanding of the regulation of a complex signaling network, the perturbation of which leads to the development of PAH. The apparent central role of KLF4 in impacting multiple pathways makes it an attractive potential therapeutic target to be explored. Transcription factors have traditionally been considered too

difficult to target by drugs, but this view is being increasingly challenged by new technological advances (32, 33). In fact, small-molecule compounds have been identified that can modulate the expression of KLF5, a closely related transcription factor to KLF4 (34).

Limitations to our study include the use of the hypoxia mouse model for PAH, which, like most other mouse models, does not entirely recapitulate the morphologic findings of human PAH. Another limitation is the use of VE-cadherin-driven Cre for endothelial knockdown of KLF4, which is also expressed in a subset of hematopoietic cells (35). However, our animals display only partial knockdown in hematopoietic cells (7), and we demonstrate here the regulatory effect of KLF4 on endothelial targets implicated in PAH, without a significant impact on inflammatory cell infiltration of the lung. Finally, our analysis of KLF4 expression in lungs of patients with IPAH was performed in homogenized lung tissue, so we were unable to confirm altered expression specifically in endothelial cells.

Our findings are yet another demonstration of the important role that KLF4 plays in protecting the integrity of endothelial function, and they further broaden this role to include the pulmonary circulation. The ability of KLF4 to regulate the transcription of a wide range of genes places it in a central position in promoting vascular health.

Additional studies will be needed to further characterize the mechanisms by which KLF4 seems to regulate the interaction of endothelial cells with other cell types, impacting vascular smooth muscle cells and RV cardiomyocytes, thereby extending the influence of endothelial KLF4 beyond the endothelial compartment. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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