ORIGINAL RESEARCH

Loss of Endothelial Hypoxia Inducible Factor-Prolyl Hydroxylase 2 Induces Cardiac Hypertrophy and Fibrosis

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BACKGROUND: Cardiac hypertrophy and fibrosis are common adaptive responses to injury and stress, eventually leading to heart failure. Hypoxia signaling is important to the (patho)physiological process of cardiac remodeling. However, the role of endothelial PHD2 (prolyl-4 hydroxylase 2)/hypoxia inducible factor (HIF) signaling in the pathogenesis of cardiac hypertrophy and heart failure remains elusive.

METHODS AND RESULTS: Mice with Egln1Tie2Cre (Tie2-Cre-mediated deletion of Egln1 [encoding PHD2]) exhibited left ventricular hypertrophy evident by increased thickness of anterior and posterior wall and left ventricular mass, as well as cardiac fibrosis. Tamoxifen-induced endothelial Egln1 deletion in adult mice also induced left ventricular hypertrophy and fibrosis. Additionally, we observed a marked decrease of PHD2 expression in heart tissues and cardiovascular endothelial cells from patients with cardiomyopathy. Moreover, genetic ablation of Hif2α but not Hif1α in Egln1Tie2Cre mice normalized cardiac size and function. RNA sequencing analysis also demonstrated HIF-2α as a critical mediator of signaling related to cardiac hypertrophy and fibrosis. Pharmacological inhibition of HIF-2α attenuated cardiac hypertrophy and fibrosis in Egln1Tie2Cre mice.

CONCLUSIONS: The present study defines for the first time an unexpected role of endothelial PHD2 deficiency in inducing cardiac hypertrophy and fibrosis in an HIF-2α-dependent manner. PHD2 was markedly decreased in cardiovascular endothelial cells in patients with cardiomyopathy. Thus, targeting PHD2/HIF-2α signaling may represent a novel therapeutic approach for the treatment of pathological cardiac hypertrophy and failure.

Key Words: angiogenesis ■ cardiac hypertrophy ■ endothelial cells ■ heart failure ■ hypoxia-inducible factor

During development, the heart grows by cardiomyocyte proliferation and hypertrophy, whereas after birth, the cardiomyocytes lose proliferative potential, and heart growth is mainly via cardiomyocyte hypertrophy. Cardiac hypertrophy can happen in physiological and pathophysiological conditions. Pathological hypertrophy induced by hypertension, myocardial infarction, and/or cardiomyopathy results in ventricular remodeling, which is associated with systolic and diastolic dysfunction and interstitial fibrosis, and finally leads to deleterious outcomes such as heart failure.1,2 Understanding the mechanistic molecular signaling in the event of physiological and pathological cardiac hypertrophy will lead to identifying novel therapeutic approaches for patients with heart failure. There are multiple cell types including cardiomyocytes, endothelial cells (ECs), fibroblasts, and smooth muscle cells in the heart. ECs lining the inner layer of the blood
CLINICAL PERSPECTIVE

What Is New?
• Our studies show that HIF-2α (hypoxia inducible factor 2α) activation in the endothelial cells secondary to PHD2 (prolyl-4 hydroxylase 2) deficiency leads to the development of cardiac hypertrophy and fibrosis.
• The therapeutic approach against HIF-2α represents a promising strategy for the prevention and treatment of pathological cardiac hypertrophy, fibrosis, and failure.

What Are the Clinical Implications?
• Chronic activation of HIF-2α in the cardiac endothelial cells is detrimental to patients with cardiac hypertrophy and fibrosis.
• Inhibiting HIF-2α via HIF-2α antagonist or HIF PHD2 agonist may improve clinical outcomes in patients with cardiac hypertrophy, fibrosis, and failure.

Nonstandard Abbreviations and Acronyms

| BW  | body weight |
| ECs | endothelial cells |
| FGF2 | basic fibroblast growth factor |
| HIF | hypoxia inducible factor |
| LDHA | lactate dehydrogenase A |
| OCT4 | octamer-binding transcription factor 4 |
| PDGF | platelet-derived growth factor |
| PHD2 | prolyl-4 hydroxylase 2 |
| PIGF | placental growth factor |
| WGA | wheat germ agglutinin |
| WT | wild-type |

vessels account for the greatest number of cells in the heart. One of the major functions of cardiac vascular ECs is to supply oxygen and nutrients to support cardiomyocytes. Previous studies have demonstrated that angiogenesis stimulated by VEGF (vascular endothelial growth factor)-B or PIGF (placental growth factor) induces a marked increase of cardiac mass in rodents, whereas inhibition of angiogenesis results in decreased capillary density, contractile dysfunction, and impaired cardiac growth. Cardiac vasculature rarefaction is associated with pathological cardiac hypertrophy and heart failure. Recent studies have also demonstrated the important role of EC-derived paracrine factors such as endothelin-1, apelin, neuregulin, and agrin in regulating cardiac hypertrophy, regeneration, and repair. However, how ECs crosstalk with cardiomyocytes in the pathogenesis of cardiac hypertrophy and dysfunction is not fully understood.

HIFs (hypoxia-inducible factors) are key transcriptional factors mediating cellular response to oxygen levels. The α subunit of HIF is delicately controlled by HIF–PHD1-3 (proply-4 hydroxylases 1-3). Under normoxia conditions, PHDs (prolyl-4 hydroxylases) hydroxylate HIF-α (mainly HIF-1α and HIF-2α), then E3 ligase Von Hippel-Lindau promotes degradation of hydroxylated HIF-α through a proteasome-degradation pathway. Under hypoxia conditions, PHD activities are inhibited, leading to stabilization of HIF-α proteins that activate downstream target gene expression. Both HIF-1α and HIF-2α share similar DNA binding site or hypoxia response element. Thus, some genes are coregulated by HIF-1α and HIF-2α, such as CXCL12 (C-X-C motif chemokine ligand 12). However, HIF-1α and HIF-2α also control some sets of unique downstream targets; for example, LDHA (lactate dehydrogenase A) is a HIF-1α target, whereas OCT4 (octamer-binding transcription factor 4) is an HIF-2α target. HIF-1α is ubiquitously expressed, whereas HIF-2α is more restricted in certain cell types such as ECs and alveolar type 2 epithelial cells, suggesting that HIF-α has a distinct function in different cell types under different (patho) physiological conditions. Our previous studies have shown that HIF-2α but not HIF-1α activation causes severe pulmonary hypertension in Egln1 knockout mice, whereas HIF-1α induction and activation is important for endothelial regeneration and vascular repair following inflammatory injury. Egln1-null mutant mice exhibit polycythemia and congestive heart failure. However, cardiomyocyte-specific disruption of Egln1 causes only mild abnormality with the presence of occasional myocytes with increased hypereosinophilia and blurring of the cross-striations, indicating that loss of PHD2 cells other than cardiomyocytes causes congestive heart failure. Fan et al show that EC deletion of both PHD2 and 3 induces spontaneous cardiomegaly because of enhanced cardiomyocyte proliferation and also prevents heart failure induced by myocardial infarction. However, it is unknown whether these phenotypes are mediated by endothelial loss of either PHD2 or PHD3 alone. Studies have shown loss of both PHD2 and 3 but not PHD2 alone in cardiomyocytes induces ischemic cardiomyopathy. Thus, it is important to determine whether loss of PHD2 alone in ECs affects cardiomyocyte and heart function in adult mice. To our surprise, we observed spontaneous left ventricular (LV) hypertrophy and cardiac fibrosis in tamoxifen-inducible EC-specific Egln1 knockout adult mice. Using the mice with endothelial deletion of Egln1 alone, Egln1 and Hif1a, or Egln1 and Hif2a, we
found that loss of endothelial PHD2-induced cardiac hypertrophy and fibrosis was mediated by HIF-2α activation, and pharmacological inhibition of HIF-2α inhibited LV hypertrophy and cardiac fibrosis. Analysis of single-cell RNA sequencing data sets revealed that EGLN1 was mainly expressed in vascular ECs of the human heart under normal conditions. In patients with hypertrophic cardiomyopathy, EGLN1 expression in LV heart tissue (messenger RNA [mRNA]) and cardiac vascular ECs (protein) was markedly deceased, which validates the clinical relevance of our findings that endothelial PHD2 deficiency induces LV hypertrophy and cardiac fibrosis. Thus, targeting PHD2/HIF-2α signaling could be a therapeutic approach for pathological cardiac hypertrophy leading to cardiomyopathy and heart failure.

**METHODS**

**Data Availability**

Scripts used for single-cell RNA sequencing analysis and analyzed data in R objects are available at Figshare (https://doi.org/10.6084/m9.figshare.14068268.v1). The RNA sequencing raw data and analyzed data are available at the National Center for Biotechnology Information Gene Expression Omnibus database (GSE182863).

**Human Samples and Data**

Archived human heart sections from the National Center for Medico-Legal Expertise of Sun Yat-sen University were used. The patient heart samples were collected from 6 patients with cardiomyopathy including 4 patients with hypertrophic cardiomyopathy, 1 with dilated cardiomyopathy, and 1 with hypertensive cardiomyopathy. Control samples were collected from similar age-range individuals (5 men, 1 woman; 27–53 years old) without heart disease (Table S1). The cause of death for controls was mechanical injuries. All data and sample collection were approved by the ethics committee (institutional review board) of Sun Yat-sen University. Informed consent was obtained from the legal representatives of the patients. EGLN1 mRNA expression in heart tissues from patients with dilated cardiomyopathy were provided by Drs Chen Gao and Yibin Wang. 21,22 Failing heart samples were obtained from the LV anterior wall during heart transplantation or implantation of an LV assistant device. The nonfailing heart samples were obtained from the LV free wall and procured from the National Disease Research Interchange and University of Pennsylvania. Nonfailing heart donors showed no laboratory signs of cardiac disease. Tissue collection was approved by the University of California, Los Angeles Institutional Review Board numbers 11-001053 and 12-000207.

**Animals**

Egl1\textsubscript{Tie2Cre} mice, Egl1/Hif1a\textsubscript{Tie2Cre}, and Egl1/Hif2a\textsubscript{Tie2Cre} double knockout mice were generated as described previously.18 Egl1\textsubscript{EndoCreERT2} mice were generated by crossing Egl1 floxed mice with EndoSCL-CreERT2 transgenic mice expressing the tamoxifen-inducible Cre recombinase under the control of the 5′ endothelial enhancer of the stem cell leukemia locus.23 At 8 weeks old, Egl1\textsubscript{EndoCreERT2} and Egl1\textsubscript{f/f} mice were treated with 2-mg tamoxifen intraperitoneally for 5 days to induced Egl1 deletion only in ECs. Mice were euthanized at the age of ≈9 months. Both male and female mice were used in these studies. The use of animals was in compliance with the guidelines of the Animal Care and Use Committee of the Northwestern University and of the University of Arizona.

**Echocardiography**

Echocardiography was performed on a FUJIFILM VisualSonics (Bothell, WA) Vevo 2100 using an MS550D (40 MHz) transducer as described previously. Briefly, mice were anesthetized in an induction chamber filled with 1% isoflurane. The left ventricle anterior/posterior wall thickness during diastole, the left ventricle internal dimension during diastole, the LV fractional shortening, and the cardiac output were obtained from the parasternal short axis view using M-mode. Results were calculated using VisualSonics Vevo 2100 analysis software (version 1.6) with a cardiac measurements package, and were based on the average of at least 3 cardiac cycles.

**Reanalysis of Public Single-Cell RNA Sequencing Data Sets**

We used the publicly available metadata from 2 human fetal hearts (GSM4008686 and GSM4008687).24 The metadata were processed in R (version 4.0.2; The R Foundation for Statistical Computing, Vienna, Austria) via the Seurat package version 3.2.3.25 Briefly, cells that expressed <100 genes and cells that expressed >4000 genes, and cells with unique molecular identifiers >10% from the mitochondrial genome were filtered out. The data were normalized and integrated in Seurat, followed by Scaled, and summarized by principal component analysis, and then visualized using uniform manifold approximation and projection plot. FindClusters function (resolution=0.5) in Seurat was used to cluster cells based on the gene expression profile. Cardiomyocyte (NPPA, TNNT2), endothelial cells (CDH5), fibroblasts (FN1, VIM), smooth muscle cells (ACTA2), and macrophages (CD68, CD14)
Irradiation and Bone Marrow Transplantation

Egln1WT (Wild-type, WT) or Egln1Tie2Cre female mice at 3 weeks old were delivered at a dose of 750 cGy/mouse. At 3 hours following irradiation, mice were transplanted with 1×107 bone marrow cells (in 150 μL of Hanks’ Balanced Salt Solution) isolated from male Egln1Tie2Cre or WT mice through tail vein injection. Mice were used for heart dissection at 3.5 months old as described previously.16

Immunofluorescent, Immunohistochemical, and Histological Staining

Following PBS perfusion, heart tissue was embedded in optimal cutting temperature compound for cryosectioning for immunofluorescent staining. Heart sections (5 μm) were fixed with 4% paraformaldehyde, followed by blocking with 0.1% Triton X-100 and 5% normal goat serum at room temperature for 1 hour. After 3 washes, they were incubated with anti-Ki67 (1:25, cat no. ab1667; Abcam), anti-CD31 antibody (1:25, cat no. 550274; BD Biosciences) at 4 °C overnight and were then incubated with either Alexa 647 conjugated anti-rabbit immunoglobulin G (Life Technologies) or Alexa 594 conjugated anti-rat immunoglobulin G, or Alexa 488 conjugated anti-rat immunoglobulin G at room temperature for 1 hour after 3 washes. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole contained in Prolong Gold mounting media (Life Technologies).

For wheat germ agglutinin (WGA) staining, WGA conjugated with fluorescein isothiocyanate or WGA conjugated with Alexa 647 were stained with cryosectioned slides at room temperature for 10 minutes.

For immunohistochemistry staining on paraffin sections, the tissues were cut into 5-μm-thick sections after paraffin processing. Heart sections were then dewaxed and dehydrated. Antigen retrieval was performed by boiling the slides in 10 mmol/L sodium citrate (pH 6.0) for 10 minutes. After blocking, slides were incubated with anti-PHD2 antibody (1:200, cat no. 4835; Cell Signaling Technology) at 4 °C overnight. Slides were incubated with 6% H2O2 for 30 minutes after primary antibody incubation and were then biotinylated with a rabbit immunoglobulin G and VECTASTAIN ABC kit (Vector Labs) for immunohistochemistry. The nucleus was stained with hematoxylin (Sigma-Aldrich).

For histological assessment, hearts were harvested and washed with PBS, followed by fixation in 4% formalin and dehydrated in 70% ethanol. After paraffin processing, the tissues were cut into semithin 5μm-thick sections. Sections were stained with hematoxylin and eosin staining or Masson’s trichrome kit as a service of charge at the core facility.

Quantitative Real Time-Polymerase Chain Reaction

Total RNA was isolated from frozen left ventricle tissues with Trizol reagents (Invitrogen) followed by purification with the RNeasy Mini kit including DNase I digestion (Qiagen). One microgram of RNA was transcribed into complementary DNA using the high-capacity complementary DNA reverse transcription kits (Applied Biosystems) according to the manufacturer’s protocol. Quantitative real time-polymerase chain reaction analysis was performed on an ABI ViiA 7 Real-Time polymerase chain reaction system (Applied Biosystems) with the FastStart SYBR Green Master kit (Roche Applied Science). Target mRNA was determined using the comparative cycle threshold method of relative quantitation. Cyclophilin was used as an internal control for analysis of expression of mouse genes. The primer sequences are provided in Table S2.

RNA Sequencing

Total RNA was isolated from left ventricle tissues with Trizol reagents (Invitrogen) followed by purification with the RNeasy Mini kit including DNase I digestion (Qiagen). RNA sequencing was performed by Novogene Corporation on the Illumina Hiseq platform. The original sequencing data were trimmed using FASTX and aligned to the reference genome using TopHat2. The differential expression analysis was performed using Cuffdiff software.26

Statistical Analysis

Statistical analysis of data was done with Prism 7 (GraphPad Software). Statistical significance for multiple-group comparisons was determined by 1-way ANOVA with Tukey post hoc analysis that calculates corrected P values. Two-group comparisons were analyzed by the unpaired 2-tailed Student t test. Because the covariates may affect the response, 2-group comparisons of data from samples of patients with cardiopathy and controls were examined by linear regression models, where the covariates including age were included/adjusted. All bar graphs represent mean±SD.

RESULTS

Decreased PHD2 Expression in Patients With Cardiomyopathy

PHD, Von Hippel-Lindau, and HIF signaling have been implicated in many physiological and pathological conditions of heart development and heart diseases.
Leveraging the public single-cell RNA sequencing data set, we first analyzed the mRNA expression of the key molecules of PHD, Von Hippel-Lindau, and HIF signaling from fetal and adult hearts. Our data demonstrated that EGLN1 and EPAS1 (encoding HIF-2α) are highly expressed in cardiac ECs in both fetal and adult hearts (Figure 1A and Figure S1). We then examined the expression levels of PHD2 in heart tissues of patients with cardiomyopathy and normal donors by quantitative real-time polymerase chain reaction. EGLN1 mRNA levels were drastically decreased in the LV cardiac tissue from patients with cardiomyopathy compared with normal donors (Figure 1B). To further determine the cell-specific loss of PHD2 expression in the heart, we performed immunohistochemistry on left ventricle heart sections. PHD2 was highly expressed in ECs as well as smooth muscle cells but only mildly in cardiomyocytes in normal donor hearts. However, its levels were dramatically reduced in cardiovascular ECs but not in smooth muscle cells of patients with cardiomyopathy (Figure 1C and 1D). These data demonstrate a marked loss of endothelial PHD2 expression in patients with cardiomyopathy, suggesting a crucial role of endothelial PHD2 in cardiac function.

Constitutive Loss of Endothelial PHD2 Induces LV Hypertrophy and Fibrosis
To investigate the role of endothelial PHD2 in the heart in vivo, we generated Egln1Tie2Cre mice by
breeding Egln1 floxed mice with Tie2Cre transgenic mice. Dissection of cardiac tissue showed marked increase of the LV versus body weight (LV/BW) ratio, indicative of LV hypertrophy (Figure 2A). Echocardiography revealed marked increases of LV anterior and posterior wall thicknesses (Figure 2B through 2E). There was no significant change of LV systolic function by evaluation of fractional shortening and ejection fraction (Figure S2A and S2B). Histological examination demonstrated that Egln1Tie2Cre mice exhibited marked increase of wall thickness of the left ventricle as well as the right ventricle and reduction of the chamber sizes, indicative of cardiac hypertrophy (Figure 2F). WGA staining showed that LV cardiomyocytes from Egln1Tie2Cre mice had marked increase of cellular surface, indicative of cardiomyocyte hypertrophy (Figure 2G). Quantitative real time-polymerase chain reaction analysis revealed a marked increase of expression of Anp, Bnp, and Myh7, further supporting cardiac hypertrophy (Figure S3). We also observed significant perivascular and intercardiomyocyte fibrosis in the left ventricle of Egln1Tie2Cre mice by trichrome staining (Figure 2H). Consistently, Col1a expression was also markedly increased in the left ventricle of Egln1Tie2Cre mice (Figure S3).

Because Tie2Cre also expresses in hematopoietic cells in addition to ECs, we next determined whether hematopoietic cell-expressed PHD2 plays a role in inducing LV hypertrophy. We performed bone marrow transplantation via transplanting WT or Egln1Tie2Cre bone marrow cells to lethally irradiated WT or Egln1Tie2Cre mice. Three months after transplantation, we did not observe any change of LV/BW ratio in WT mice transplanted with Egln1Tie2Cre bone marrow cells compared with WT mice with WT bone marrow cells, indicating loss of PHD2 in bone marrow cells, per se, did not induce LV hypertrophy. Similarly, WT bone marrow cell transplantation to Egln1Tie2Cre mice did not affect LV hypertrophy seen in Egln1Tie2Cre mice transplanted with Egln1Tie2Cre bone marrow cells (Figure S4A and S4B). These data suggest that loss

Figure 2. Constitutive loss of endothelial Egln1 leads to left ventricular hypertrophy and fibrosis.
A, Increased weight ratio of left ventricular/body weight (LV/BW) of Egln1Tie2Cre mice (CKO) compared with wild-type (WT) mice at various ages. B and C, Representative motion model of echocardiography measurement showing that Egln1Tie2Cre mice exhibit increase of LV anterior and posterior wall thicknesses, and reduction of LV internal dimension (LVID) compared with WT mice at 3.5 months old. D and E, Quantification of LV anterior and posterior wall thicknesses via echocardiography measurement of WT and Egln1Tie2Cre mice. F, Hematoxylin and eosin staining showing cardiac hypertrophy in Egln1Tie2Cre mice at 3.5 months old. Scale bar=1 mm. G, Wheat germ agglutinin staining of cardiac sections and quantification demonstrating enlargement of cardiomyocytes in Egln1Tie2Cre mice (N=5 per group). Scale bar=20 μm. H, Trichrome staining showing prominent cardiac fibrosis in Egln1Tie2Cre mice. Scale bar=100 μm. *P<0.05, **P<0.01, ***P<0.001 (Student t test). M indicates month. CKO, Egln1Tie2Cre mice; LVAWd, LV anterior wall thicknesses; LVPWd, LV posterior wall thicknesses.
Inducible Deletion of Endothelial Egln1 in Adult Mice Leads to Development of LV Hypertrophy and Fibrosis

To determine if the cardiac hypertrophy in the Egln1 Tie2Cre mice is ascribed to potential developmental defects in mice with Tie2Cre-mediated deletion of Egln1, we generated mice with tamoxifen-inducible endothelial Egln1 deletion via breeding Egln1 floxed mice with EndoSCL-CreERT2 mice\(^27\) (Figure 3A), which induces EC-restricted gene disruption.\(^{23,28–30}\) Tamoxifen was administrated to Egln1 EndoCreERT2 mice at 8 weeks old. Seven months after tamoxifen treatment, echocardiography, cardiac dissection, and histological analysis were performed to evaluate the cardiac phenotype. Egln1 EndoCreERT2 mice exhibited a marked increase of LV/BW ratio (Figure 3B), LV wall thicknesses, and LV mass (Figure 3C through 3E). Histologic analysis also demonstrated marked increase of LV wall thickness, reduction of LV chamber size, and perivascular fibrosis by trichrome staining (Figure 3F and 3G). However, the right ventricular (RV) wall thickness and chamber size were not affected. Different from the Egln1 Tie2Cre mice by Tie2Cre-mediated deletion, which also exhibits severe pulmonary hypertension,\(^{16,31,32}\) the Egln1 EndoCreERT2 mice had only a mild increase of RV systolic pressure (data not shown), indicative of mild pulmonary hypertension consistent with minimal changes in RV wall thickness and chamber size. These data support the idea that loss of endothelial PHD2 in adult mice selectively induces LV hypertrophy.

Increased Endothelial Proliferation and Angiogenesis in the Left Ventricle of Endothelial PHD2-Deficient Mice

Previous studies demonstrated that stimulation of angiogenesis in the absence of other insults can drive myocardial hypertrophy in mice via overexpression of a secreted angiogenic growth factor PR39 or VEGF-B (vascular endothelial growth factor B).\(^5\) PHD2 deficiency has been shown to induce EC proliferation and angiogenesis in vitro and in vivo.\(^{33,34}\) To determine if vascular mass is increased in the LV of the Egln1 Tie2Cre mice, we performed immunostaining with endothelial marker CD31 (cluster of differentiation 31) and WGA. The capillary/myocyte ratio was drastically increased in the left ventricle of Egln1 Tie2Cre mice compared with WT mice (Figure 4A and 4B). Anti-Ki67 immunostaining demonstrated a marked increase of Ki67+/CD31+ cells in the left ventricle of Egln1 Tie2Cre mice (Figure 4C and 4D), suggesting that PHD2 deficiency induces EC proliferation in the left ventricle, which explains the marked increase of capillary/myocyte ratio.

Distinct Role of Endothelial HIF-1α Versus HIF-2α in LV Hypertrophy

Because Egln1 deletion stabilizes both HIF-1α and HIF-2α, we generated Egln1/Hif1a Tie2Cre, Egln1/Hif2a Tie2Cre double knockout and Egln1/Hif1a/Hif2a Tie2Cre triple knockout
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mice (Figure 5A) to determine the HIF-α isoform(s) mediating LV hypertrophy. Heart dissection showed that Egln1/Hif2a\(^{\text{Cre}}\) and Egln1/Hif1α/Hif2a\(^{\text{Cre}}\) mice exhibited normal LV/BW ratio seen in WT mice, whereas Egln1/Hif1α\(^{\text{Cre}}\) mice had increased LV/BW ratio compared with Egln1\(^{\text{Cre}}\) mice (Figure 5B). These data provide in vivo evidence that endothelial HIF-2α activation secondary to loss of endothelial PHD2 is responsible for LV hypertrophy seen in Egln1\(^{\text{Cre}}\) mice, whereas HIF-1α activation attenuates LV hypertrophy. Echocardiography confirmed that Egln1/Hif2a\(^{\text{Cre}}\) mice had normal LV wall thickness and LV mass in contrast to Egln1\(^{\text{Cre}}\) mice (Figure 5C and 5D). Histological assessment demonstrated that Egln1/

Hif2a\(^{\text{Cre}}\) mice had no cardiac hypertrophy and fibrosis, as well as normal cardiomyocyte surface area (Figure 5E through 5G). These data demonstrate the causal role of endothelial HIF-2α activation in mediating LV hypertrophy and fibrosis seen in Egln1\(^{\text{Cre}}\) mice.

RNA Sequencing Analysis Identifies HIF-2α-Mediated Signaling Pathways Regulating Cardiac Hypertrophy and Fibrosis

To understand the downstream mechanisms of endothelial HIF-2α in regulating cardiac function, we

![Image of Figure 4](image-url)

**Figure 4.** Deletion of endothelial Egln1 increased angiogenesis in the left ventricles. A and B, Immunostaining of CD31 (cluster of differentiation 31) and WGA (wheat germ agglutinin) and quantification showing increase of capillary endothelial cells (ECs) vs cardiomyocyte number in Egln1\(^{\text{Cre}}\) mice. Left heart sections were stained with anti-CD31 (red, marker for ECs) and WGA (green) (N=4 per group). Scale bar=20 μm. C and D, Anti-Ki67 staining and quantification demonstrates that cardiac ECs were hyperproliferative in the ventricles of Egln1\(^{\text{Cre}}\) mice (CKO). Left heart sections were immunostained with anti-Ki67 (red, cell proliferation marker) and anti-CD31 (green). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (N=4 per group). Scale bar=50 μm. **P<0.01, ***P<0.001 (Student t test). WT indicates wild-type.
performed whole transcriptome RNA sequencing of LV tissue dissected from WT, Egln1Tie2Cre, and Egln1/Hif2aTie2Cre mice (Figure 6A). First, we found 1454 genes (FPKM>5, fold change >1.5 or <0.66) were changed in Egln1Tie2Cre left ventricle compared with WT left ventricle. Then, we performed the enriched Kyoto Encyclopedia of Genes and Genomes pathways analysis on the upregulated genes in Egln1Tie2Cre versus WT mice. The analysis revealed that there were alterations of upregulated pathways related to adrenergic signaling in cardiomyocytes, hypertrophic cardiomyopathy, dilated cardiomyopathy, and cardiac muscle contraction, which are consistent with the hypertrophic phenotype we observed in Egln1Tie2Cre mice (Figure 6B). To determine the genes downstream of HIF-2α activation, we did the intersecting analysis of differentially expressed genes from WT_ Egln1Tie2Cre and Egln1Tie2Cre_ Egln1/Hif2aTie2Cre. There were 864 overlapping genes between WT_ Egln1Tie2Cre and Egln1Tie2Cre_ Egln1/Hif2aTie2Cre. Kyoto Encyclopedia of Genes and Genomes pathway analysis of these genes upregulated in Egln1Tie2Cre but normalized in Egln1/Hif2aTie2Cre hearts also showed enrichment of hypertrophic cardiomyopathy and dilated cardiomyopathy (Figure 6C), suggesting that these pathway abnormalities are downstream of HIF-2α activation. We also observed that many genes related to endothelium and EC-derived factors-mediated cell growth and genes related to cardiac fibrosis were altered in Egln1Tie2Cre mice but normalized in Egln1/Hif2aTie2Cre (Figure 6D). These data provide mechanistic understanding of the distinct roles of endothelial HIF-1α and HIF-2α in regulating LV hypertrophy and fibrosis.

Pharmacological Inhibition of HIF-2α Inhibits LV Hypertrophy

To assess the therapeutic potential of HIF-2α inhibition for cardiomyopathy, we treated 3- week-old Egln1Tie2Cre mice with the HIF-2α translation inhibitor (C76) Compound 76 for 11 weeks. C76 treatment inhibited LV hypertrophy of Egln1Tie2Cre mice evident by decreased LV/BW ratio compared with vehicle treatment (Figure 7A). C76 treatment also significantly attenuated the cardiomyocyte surface area assessed by WGA staining (Figure 7B and 7C) and perivascular fibrosis by trichrome staining (Figure 7D). Taken together, these data demonstrated that inhibition of HIF-2α via a pharmacologic approach suppressed endothelial PHD2 deficiency-induced LV hypertrophy and cardiac fibrosis.

DISCUSSION

In this study we demonstrate that endothelial PHD2 is markedly decreased in the hearts of patients with cardiomyopathy. Genetic deletion of endothelial Egln1 in mice induces spontaneously severe cardiac hypertrophy and fibrosis. Through genetic deletion of Hif2a or Hif1a in Egln1Tie2Cre mice, we also demonstrate that endothelial HIF-2α but not HIF-1α activation is responsible for PHD2 deficiency-induced LV hypertrophy and fibrosis. Moreover, pharmacological inhibition of HIF-2α reduces cardiac hypertrophy in Egln1Tie2Cre mice. Thus, our data provide strong evidence that endothelial homeostasis is crucial to maintain normal cardiac function.

We demonstrate that PHD2 is mainly expressed in the vascular endothelium in human heart under normal condition, and its expression is markedly reduced in cardiac tissue and ECs of patients with cardiomyopathy. As far as we know, our study for the first time indicates the clinical importance of PHD2 deficiency in the pathogenesis of cardiomyopathy and heart failure.

Mice with Tie2Cre-mediated deletion of Egln1 develop LV hypertrophy and also RV hypertrophy associated with severe pulmonary hypertension. The RV hypertrophy is secondary to marked increase of pulmonary artery pressure. Furthermore, we used the tamoxifen-inducible Egln1 knockout mice to determine the role of endothelial PHD2 deficiency in heart function. Seven months after tamoxifen treatment of 8-week-old adult mice, we observed only LV hypertrophy in the mutant mice with normal RV size. These data provide clear evidence that endothelial PHD2 deficiency in adult mice selectively induces LV hypertrophy. Published studies have shown that loss of both endothelial PHD2 and PHD3 leads to enhanced ejection fraction and LV cardiomegaly because of increased cardiomyocyte proliferation. Our study demonstrates that loss of endothelial PHD2 alone is sufficient to induce LV hypertrophy without marked changes in cardiomyocyte proliferation and LV contractility and ejection fraction. We also observed marked LV cardiac fibrosis predominantly in the perivascular regions and less in the intercardiomyocytes area, which may lead to heart dysfunction. Cardiac hypertrophy associated with fibrosis indicates maladaptive deleterious remodeling. However, our data did not suggest that fibrosis is associated with heart failure, which might be because of the variability among animals. For example, some mice still are in the compensation stage phase, whereas other mice already show an early sign of heart failure.

PHDs are O2 sensors that use molecular O2 as a substrate to hydroxylate proline residues of HIF-α. Deficiency of PHD2 results in stabilization and accumulation of HIF-α, and formation of HIF-α/HIF-β heterodimer, which consequently activates expression of many HIF target genes that regulate angiogenesis, inflammation, and metabolism. The cardiac remodeling phenotype in
Egln1Tie2Cre mice is ascribed to activation of HIF-2α but not HIF-1α, because Hif2a deletion in EC protects from Egln1 deficiency-induced cardiac hypertrophy and fibrosis, whereas Hif1a deletion in Egln1Tie2Cre mice does not show any protection but increases cardiac hypertrophy. Consistently, previous studies show that endothelial Hif1a
deletion in transverse aortic constriction–challenged mice induce myocardial hypertrophy and fibrosis, and rapid decompensation. Although both HIF-1α and HIF-2α express in ECs and share similar DNA binding site, endothelial HIF-1α and HIF-2α play distinct roles in cardiac homeostasis. We speculate that it might be because of the distinct sets of genes activated by HIF-1α and HIF-2α in ECs.

Increased vascular mass and EC proliferation are evident in the left ventricle of Egln1Tie2Cre mice, demonstrating that PHD2 deficiency in cardiac vascular ECs induces EC proliferation and angiogenesis in mice. Our finding is consistent with previous studies that report silencing of endogenous PHD2 in ECs enhances hypoxia-induced EC proliferation. Moreover, previous studies have shown that induction of myocardial

Figure 5. Distinct role of endothelial hypoxia inducible factor (HIF)-1α and HIF-2α in Egln1 deficiency-induced left heart hypertrophy and fibrosis.

A, A diagram demonstrating generation of Egln1/Hif2aTae2Cre (EH2), Egln1/Hif1aTae2Cre (EH1), and Egln1/Hif1a/Hif2aTae2Cre (EH1/2) mice. B, Cardiac dissection showing that endothelial HIF-2α deletion protected from endothelial Egln1 deficiency-induced left ventricular (LV) hypertrophy, whereas HIF-1α deletion augmented LV hypertrophy. C and D, Echocardiography demonstrate that HIF-2α deletion in endothelial cells protected from LV wall thickening induced by Egln1 deficiency. E, Hematoxylin and eosin staining show normalization of LV hypertrophy in Egln1/Hif2aTae2Cre mice. Scale bar=1mm. F, Wheat germ agglutinin staining and quantification show a complete normalization of cardiomyocyte hypertrophy in Egln1/Hif2aTae2Cre mice. The same surface area data of wild-type (WT) and KO in Figure 2H were used (N=5 per group). Scale bar=20 μm. G, Trichrome staining demonstrates absence of collagen deposition in the left ventricle of Egln1/Hif2aTae2Cre mice. Scale bar=100 μm. *P<0.05, **P<0.01, ***P<0.001 (1-way ANOVA with Tukey post hoc analysis for multiple group comparisons). LV/BW indicates left ventricular/body weight. LVAWd, LV anterior wall thicknesses; LVPWd, LV posterior wall thicknesses. CKO, Egln1Tie2Cre mice.

Figure 6. RNA sequencing analysis identifies multiple dysfunctional pathways in regulation of cardiac hypertrophy and fibrosis induced by endothelial Egln1 deficiency and normalization by hypoxia inducible factor (HIF)-2α disruption.

A, A representative heatmap of RNA sequencing analysis of wild-type (WT), Egln1Tae2Cre mice (CKO), and Egln1/Hif2aTae2Cre (EH2) mice. B, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of upregulated genes in Egln1Tae2Cre vs WT mice showing dysregulation of multiple signaling pathways related to cardiac hypertrophy. C, Intersecting analysis of differential expression genes between WT, Egln1Tae2Cre and Egln1/Hif2aTae2Cre, Egln1/Hif1aTae2Cre mice. D, KEGG pathway enrichment analysis indicates that HIF-2α–activated downstream genes are related to hypertrophic cardiomyopathy (HCM). E, A heatmap showing the expression of genes related to endothelium and endothelial cell-derived factors-mediated cell growth, genes related to cardiac fibrosis, and HIF target genes. FPKM, fragments per kilobase of transcript per million mapped reads; the “pop hits” is the number of all genes enriched in a certain term.
angiogenesis promotes cardiomyocyte growth and cardiac hypertrophy.\(^5\) Thus, it is possible that PHD2 deficiency in ECs induces angiogenesis, which promotes cardiomyocyte hypertrophy leading to LV hypertrophy. Rarefaction of cardiac microvasculature is associated with pathological hypertrophy.\(^4\) We observed a marked increase of capillary density in \(Egln1^{Tie2Cre}\) hearts, which may help to explain normal cardiac function including fractional shortening and ejection fraction in \(Egln1^{Tie2Cre}\) hearts.

The heart is highly organized and consists of multiple cell types including cardiomyocytes, ECs, and fibroblasts. Cell–cell communication in the heart is important for cardiac development and adaptation to stress such as pressure overload. It is well documented that soluble factors secreted by ECs maintain tissue homeostasis in different physiological and pathological microenvironments including cancer and bone marrow niche.\(^4\) To date, a growing number of cardioactive factors derived from ECs have been shown to regulate cardiac angiogenesis contributing to cardiac hypertrophy and/or dysfunction, including NO, neuregulin-1, basic FGF2 (fibroblast growth factor 2), PDGF (platelet-derived growth factor), and VEGF (vascular endothelial growth factor).\(^8\) Other endothelium-derived factors, such as endothelin-1, NO, neuregulin, Bmp4, Fgf23, Lgals3, Lcn2, Spp1, and Tgfβ1, can directly promote cardiac hypertrophy and fibrosis.\(^4\) Our data suggest that endothelial-derived factors mediate EC–myocyte crosstalk to induce cardiac hypertrophy and fibrosis through excessive angiogenesis and/or paracrine effects.

Pathological cardiac hypertrophy worsens clinical outcomes and progresses to heart failure and death. Modulation of abnormal cardiac growth is becoming a potential approach for preventing and treating heart failure in patients.\(^2\) Our study demonstrates that pharmacological inhibition of HIF-2α reduces cardiac hypertrophy in \(Egln1^{Tie2Cre}\) mice, which provides strong evidence that targeting PHD2/HIF-2α signaling is a promising strategy for patients with pathological cardiac hypertrophy. The HIF-2α translation inhibitor C76 used in this study selectively inhibits HIF-2α translation by enhancing the binding of iron-regulatory protein 1 to the 5'-untranslated region of HIF2A mRNA without affecting HIF-1α expression.\(^3\) Inhibition of HIF-1α might be detrimental based on previous findings and our findings.
that endothelial HIF-1α is protective in terms of cardiac hypertrophy. Moreover, HIF-2α-selective antagonists, which target HIF-2α and HIF-β heterodimerization, are under investigation in patients with renal cancer in clinical trials. Further studies will be warranted to study this class of HIF-2α inhibitors for the treatment of pathological cardiac hypertrophy and heart failure.

In conclusion, our findings demonstrate for the first time that endothelial PHD2 deficiency in mice induces spontaneous cardiac hypertrophy and fibrosis via HIF-2α activation but not HIF-1α. PHD2 expression was markedly decreased in cardiovascular ECs of patients with cardiomyopathy, validating the clinical relevance of our findings in mice. Thus, selective targeting the abnormality of PHD2/HIF-2α signaling is a potential therapeutic strategy to treat patients with pathological cardiac hypertrophy and fibrosis.

ARTICLE INFORMATION

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Disclosures

None.

Supplementary Material

Tables S1–S2

Figures S1–S4

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Supplemental Material
### Table S1. Patients’ information.

| ID     | Group                        | Age | Sex  |
|--------|------------------------------|-----|------|
| 9374   | Control                      | 27  | male |
| 8795   | Control                      | 53  | male |
| 9499   | Control                      | 35  | female |
| 8978   | Control                      | 43  | male |
| 5069   | Control                      | 32  | male |
| 9546   | control                      | 40  | male |
| 9370   | Hypertrophic cardiomyopathy | 39  | male |
| 8670   | Hypertrophic cardiomyopathy | 43  | male |
| 9312   | Dilated cardiomyopathy      | 27  | male |
| 9822   | Hypertensive cardiomyopathy | 53  | male |
| 9458   | Hypertrophic cardiomyopathy | 35  | female |
| 6758   | Hypertrophic cardiomyopathy | 32  | male |
**Table S2. QRT-PCR primers.**

| Gene name | Forward primer       | Reverse primer       |
|-----------|----------------------|----------------------|
| hEGLN1    | AAAGACTGGGATGCCAAGGT | CTCGTGCTCTTCTCATCTGCA |
| hGAPDH    | GTCTCCTCTGACTTCAACAGC | ACCACCCTGTGTGCTTAGCCAA |
| mAnp      | GATAGATGAAGGCAGGAGCCGC | AGGATGGGAGCCCAGAGTGGAATAGG |
| mBnp      | TGTTTCTGCTTTCTTTATCTGTC | CTCCGACTTTTTCTTTATCAGGTC |
| mMyh7     | TGCAAAGGCTCCAGGTCTGAGGGC | GCCAACACCAACTGTCCAGTTTC |
| mCola1    | TCACCAAACTCAGAAGATGTAGGA | GACCAGGGGACCAGGAAG |
Figure S1. Single-cell RNA sequencing analysis of human fetal hearts.

(A) A UMAP plot showing the major cardiac cell types including ECs, cardiomyocytes (CM), fibroblasts (Fib), smooth muscle cells (SMC) and macrophages (Mac). (B) A Violin plot showing the expression levels of PHD2/HIF signaling genes in cardiac cells.
Figure S2. Constitutive loss of endothelial *Egln1* in mice does not affect cardiac function.

Echocardiography measurement showing similar LV fraction shorting (FS%) and ejection fraction (EF%) in adult *Egln1*Tie2Cre mice compared to WT mice.
Figure S3. Upregulation of fetal gene including *Bnp* and fibrotic gene *Col1a* in the LV of *Egln1<sup>Tie2Cre</sup>* mice.

* p <0.05, ** p <0.01 (Student’s t test).
Figure S4. *Egln1* deficiency in bone marrow cells does not contribute to cardiac hypertrophy.

(A) A diagram showing the strategy of bone marrow cell transplantation. Lethally gamma-irradiated WT were transplanted with bone marrow (BM) cells freshly isolated from WT or *Egln1* 	ext{Tie2}Cre (CKO) mice. Similarly, irradiated CKO mice were reconstituted with WT or CKO bone marrow cells. (B) Cardiac dissection showed that *Egln1*-deficient bone marrow cells did not contribute to cardiac hypertrophy seen in *Egln1* 	ext{Tie2}Cre (CKO) mice.