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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 *Egln1*^{Tie2Cre} (*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 *Hif2a* but not *Hif1a* in *Egln1*^{Tie2Cre} 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 *Egln1*^{Tie2Cre} 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

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For Sources of Funding and Disclosures, see page 13.

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
PlGF	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.[3,4] Previous studies have demonstrated that angiogenesis stimulated by VEGF (vascular endothelial growth factor)-B or PlGF (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.[5,6] Cardiac vasculature rarefaction is associated with pathological cardiac hypertrophy and heart failure.[7] 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.[8,9] 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 (prolyl-4 hydroxylases 1-3).[10–12] 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-1a and HIF-2α share similar DNA binding site or hypoxia response element.[11,13] 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.[14] HIF-1α is ubiquitously expressed, whereas HIF-2α is more restricted in certain cell types such as ECs and alveolar type 2 epithelial cells,[15] 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* (encoding PHD2) conditional knockout mice, whereas HIF-1α induction and activation is important for endothelial regeneration and vascular repair following inflammatory injury.[16,17]

Egln1-null mutant mice exhibit polycythemia and congestive heart failure.[18] 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,[19] 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.[20] 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.[20] 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 in 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

Egln1^{Tie2Cre} mice*,* *Egln1/Hif1a*^{Tie2Cre}, and *Egln1/Hif2a*^{Tie2Cre} double knockout mice were generated as described previously described.[16] *Egln1*^{EndoCreERT2} mice were generated by crossing *Egln1* 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, *Egln1*^{EndoCreERT2} and *Egln1*$^{f/f}$ mice were treated with 2-mg tamoxifen intraperitoneally for 5 days to induced *Egln1* 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*) clusters were annotated based on the expression of known markers.

Irradiation and Bone Marrow Transplantation

Egln1$^{f/f}$ (Wild-type, WT) or *Egln1*^{Tie2Cre} female mice at 3 weeks old were delivered at a dose of 750 cGy/mouse. At 3 hours following irradiation, mice were transplanted with 1×10^{7} bone marrow cells (in 150 μL of Hanks' Balanced Salt Solution) isolated from male *Egln1*^{Tie2Cre} 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-rabbit 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% H_2O_2 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 costained 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 *Egln1*^{Tie2Cre} mice by

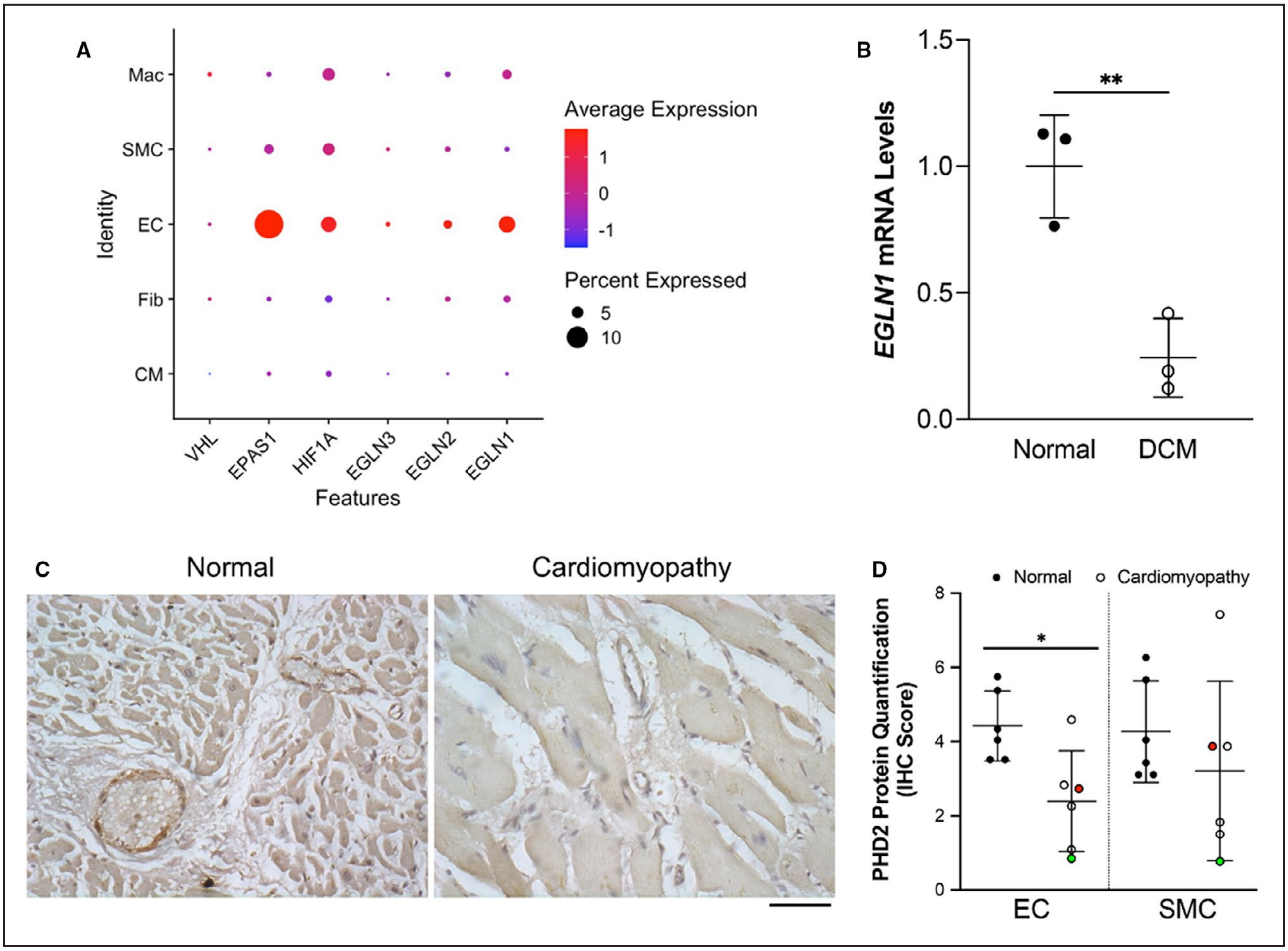


Figure 1. Decreased PHD2 (prolyl-4 hydroxylase 2) expression in heart vascular endothelial cells (ECs) of patients with cardiomyopathy.
A, Single-cell RNA sequencing analysis from human fetal hearts showed that cardiac ECs express higher levels of *EGLN1* and *EPAS1* compared with other cell types. **B**, Quantitative real time-polymerase chain reaction analysis showing that *EGLN1* messenger RNA (mRNA) levels in left ventricle hearts were significantly downregulated in patients with dilated cardiomyopathy (DCM) compared with healthy donors (N=3 in each group). ***P*<0.01 (Student *t* test). **C** and **D**, Immunohistochemistry (IHC) of PHD2 (**C**) and quantification (**D**) demonstrating a significant decrease of PHD2 expression in left ventricle sections, especially in vascular ECs of patients with cardiomyopathy. Immunostaining intensity was graded from 1 to 10, with 10 being the highest. White dots indicate hypertrophic cardiomyopathy, red dots indicate dilated cardiomyopathy, green dots indicate hypertensive cardiomyopathy. Scale bar = 50 μm. **P*<0.05 (linear regression analysis with consideration of age as a covariate). CM indicates cardiomyocyte; Fib, fibroblast; Mac, macrophage; SMC, smooth muscle cell; and VHL, Von Hippel-Lindau.

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 *Egln1*^{Tie2Cre} mice exhibited marked increase of wall thickness of the left ventricle as well as the right ventricle and reduction of the chamber sizes, indicating cardiac hypertrophy (Figure 2F). WGA staining showed that LV cardiomyocytes from *Egln1*^{Tie2Cre} 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 *Egln1*^{Tie2Cre} mice by trichrome staining (Figure 2H). Consistently, *Col1a* expression was also markedly increased in the left ventricle of *Egln1*^{Tie2Cre} 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 *Egln1*^{Tie2Cre} bone marrow cells to lethally irradiated WT or *Egln1*^{Tie2Cre} mice. Three months after transplantation, we did not observe any change of LV/BW ratio in WT mice transplanted with *Egln1*^{Tie2Cre} 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 *Egln1*^{Tie2Cre} mice did not affect LV hypertrophy seen in *Egln1*^{Tie2Cre} mice transplanted with *Egln1*^{Tie2Cre} bone marrow cells (Figure S4A and S4B). These data suggest that loss

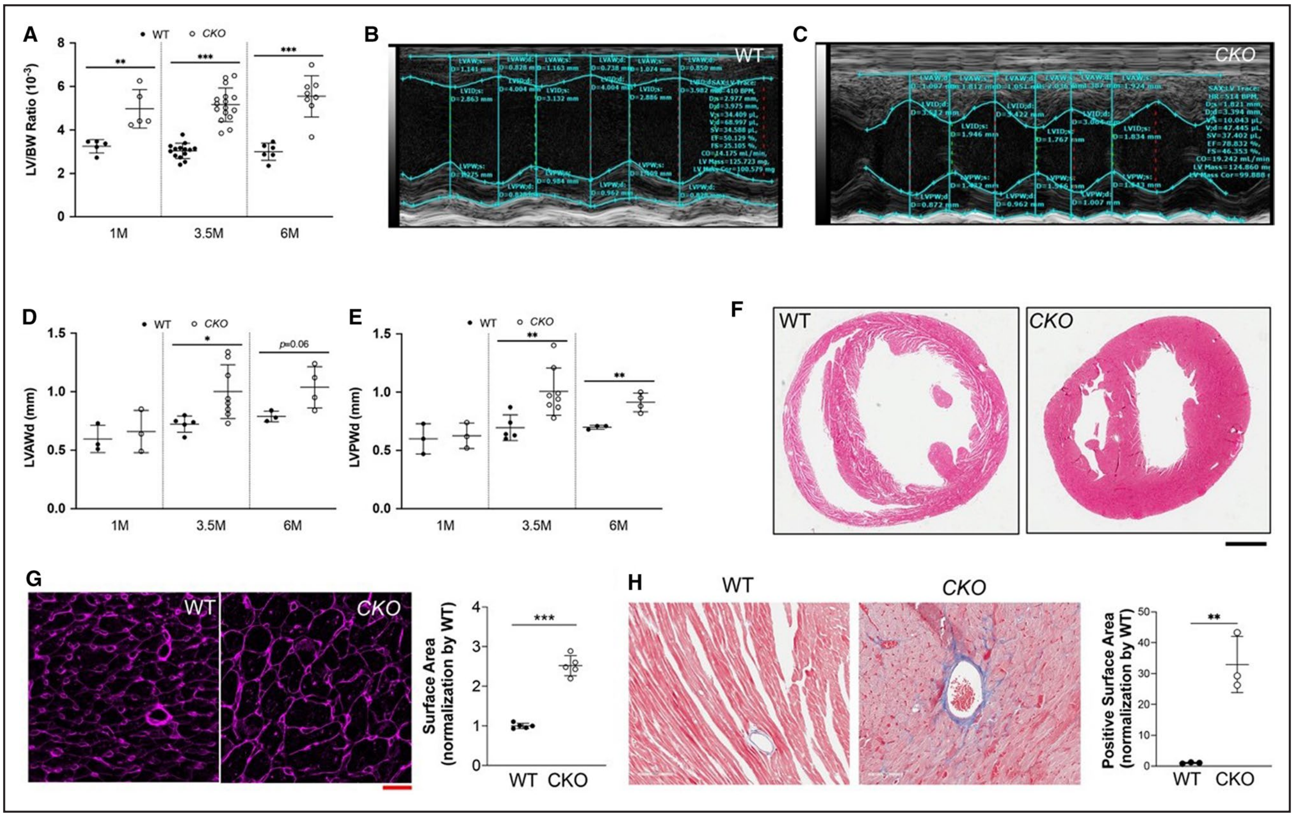


Figure 2. Constitutive loss of endothelial *Egln1* leads to left ventricular hypertrophy and fibrosis.
A, Increased weight ratio of left ventricular/body weight (LV/BW) of *Egln1*^{Tie2Cre} mice (CKO) compared with wild-type (WT) mice at various ages. **B** and **C**, Representative motion model of echocardiography measurement showing that *Egln1*^{Tie2Cre} 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 *Egln1*^{Tie2Cre} mice. **F**, Hematoxylin and eosin staining showing cardiac hypertrophy in *Egln1*^{Tie2Cre} 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 *Egln1*^{Tie2Cre} mice (N=5 per group). Scale bar=20 μm. **H**, Trichrome staining showing prominent cardiac fibrosis in *Egln1*^{Tie2Cre} mice. Scale bar=100 μm. **P*<0.05, ***P*<0.01, ****P*<0.001 (Student *t* test). M indicates month. CKO, *Egln1*^{Tie2Cre} mice; LVAWd, LV anterior wall thicknesses; LVPWd, LV posterior wall thicknesses.

of hematopoietic cell PHD2 is not involved in the development of LV hypertrophy.

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 *Tie2*Cre-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 *Tie2*Cre-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

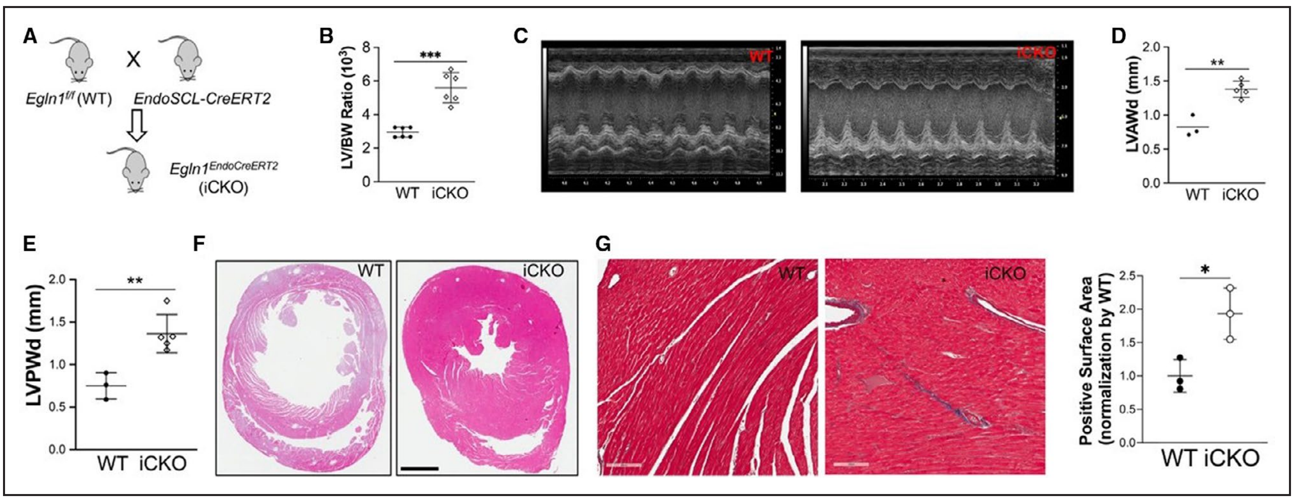


Figure 3. Inducible deletion of endothelial *Egln1* in adult mice leads to left ventricular (LV) hypertrophy and fibrosis.
A, A diagram showing the strategy of generating *Egln1*^{EndoCreERT2} mice (iCKO). **B**, *Egln1*^{EndoCreERT2} mice exhibit increase of left ventricular/body weight (LV/BW) ratio compared with age-matched wild-type (WT) mice at ≈7 months after tamoxifen treatment. **C**, Representative motion model of echocardiography showing LV hypertrophy in *Egln1*^{EndoCreERT2} mice. **D** and **E**, Quantification of LVAWd (LV anterior wall thicknesses) and LVPWd (LV posterior wall thicknesses) showing increased LV wall thicknesses of *Egln1*^{EndoCreERT2} mice. **F**, Hematoxylin and eosin staining demonstrating that *Egln1*^{EndoCreERT2} mice developed LV hypertrophy. Scale bar=1mm. **G**, Trichrome staining showing the deposition of collagen in the left ventricle of *Egln1*^{EndoCreERT2} mice. Scale bar=100 μm. ***P*<0.01, ****P*<0.001 (Students *t* test). CKO, *Egln1*^{Tie2Cre} mice.

mice (Figure 5A) to determine the HIF-α isoform(s) mediating LV hypertrophy. Heart dissection showed that *Egln1/Hif2a*^{Tie2Cre} and *Egln1/Hif1a/Hif2a*^{Tie2Cre} mice exhibited normal LV/BW ratio seen in WT mice, whereas *Egln1/Hif1a*^{Tie2Cre} mice had increased LV/BW ratio compared with *Egln1*^{Tie2Cre} 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*^{Tie2Cre} mice, whereas HIF-1α activation attenuates LV hypertrophy. Echocardiography confirmed that *Egln1/Hif2a*^{Tie2Cre} mice had normal LV wall thickness and LV mass in contrast to *Egln1*^{Tie2Cre} mice (Figure 5C and 5D). Histological assessment demonstrated that *Egln1/Hif2a*^{Tie2Cre} 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*^{Tie2Cre} 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

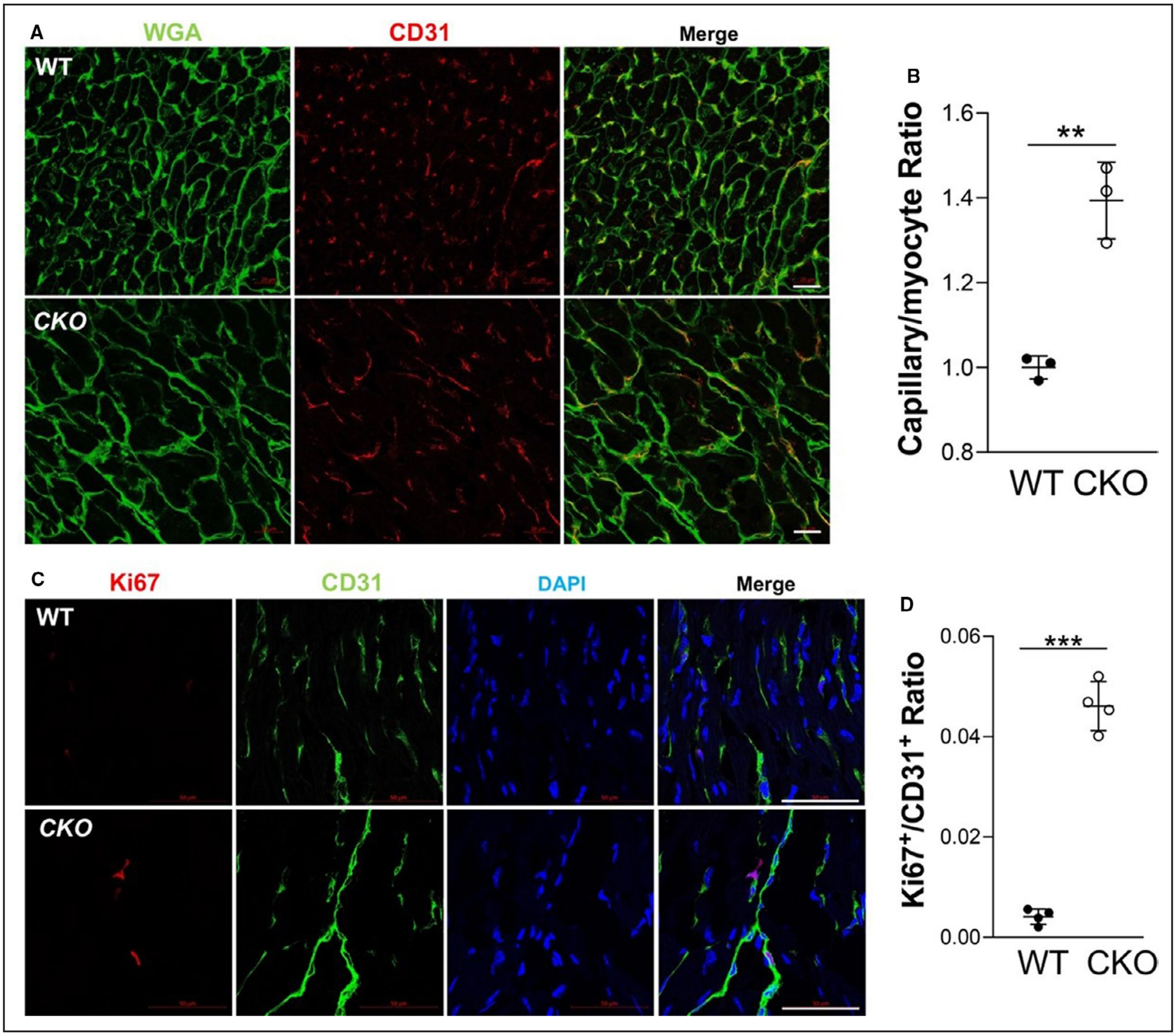


Figure 4. Deletion of endothelial *Egln1* increased angiogenesis in the left ventricles.
A and **B**, Immunostaining of CD31 (cluster of differentiation 31) and WGA (wheat germ agglutinin) and quantification showing increase of capillary endothelial cells (ECs) vs cardiomyocyte number in *Egln1*^{Tie2Cre} 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*^{Tie2Cre} 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, *Egln1*^{Tie2Cre}, and *Egln1/Hif2a*^{Tie2Cre} mice (Figure 6A). First, we found 1454 genes (FPKM>5, fold change >1.5 or <0.66) were changed in *Egln1*^{Tie2Cre} 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 *Egln1*^{Tie2Cre} 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 *Egln1*^{Tie2Cre} mice (Figure 6B). To determine the genes downstream of HIF-2α activation, we did the intersecting analysis of differentially expressed genes from WT_ *Egln1*^{Tie2Cre} and *Egln1*^{Tie2Cre}_ *Egln1/Hif2a*^{Tie2Cre}. There were 864 overlapping genes between WT_ *Egln1*^{Tie2Cre} and *Egln1*^{Tie2Cre}_ *Egln1/Hif2a*^{Tie2Cre}. *Kyoto Encyclopedia of Genes and Genomes* pathway analysis of these genes upregulated in *Egln1*^{Tie2Cre} but normalized in *Egln1/Hif2a*^{Tie2Cre} 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 *Egln1*^{Tie2Cre} mice but normalized in *Egln1/Hif2a*^{Tie2Cre} (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 *Egln1*^{Tie2Cre} mice with the HIF-2α translation inhibitor (C76) Compound 76[31,35] for 11 weeks. C76 treatment inhibited LV hypertrophy of *Egln1*^{Tie2Cre} 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.

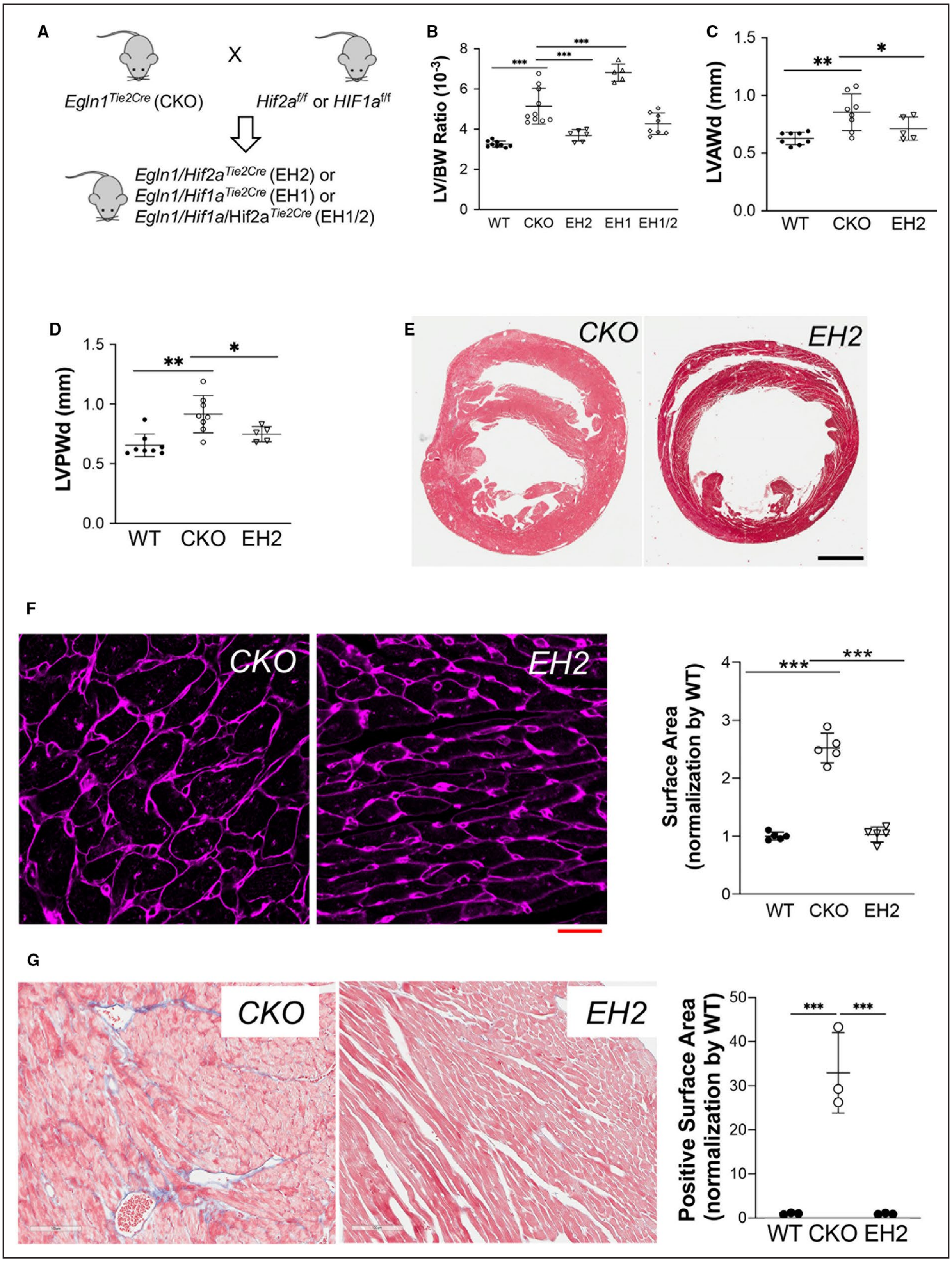
DISCUSSION

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 *Egln1*^{Tie2Cre} 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 *Egln1*^{Tie2Cre} 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 *Tie2*Cre-mediated deletion of *Egln1* develop LV hypertrophy and also RV hypertrophy associated with severe pulmonary hypertension.[16,31] 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.[20] 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.[36] 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 O_2 sensors that use molecular O_2 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.[11,13,37] The cardiac remodeling phenotype in



Egln1^{Tie2Cre} 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 *Egln1*^{Tie2Cre} mice does not show any protection but increases cardiac hypertrophy. Consistently, previous studies show that endothelial *Hif1a*

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/Hif2a*^{Tie2Cre} (EH2), *Egln1/Hif1a*^{Tie2Cre} (EH1), and *Egln1/Hif1a/Hif2a*^{Tie2Cre} (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/Hif2a*^{Tie2Cre} mice. Scale bar=1mm. **F**, Wheat germ agglutinin staining and quantification show a complete normalization of cardiomyocyte hypertrophy in *Egln1/Hif2a*^{Tie2Cre} 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/Hif2a*^{Tie2Cre} 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, *Egln1*^{Tie2Cre} mice.

deletion in transverse aortic constriction–challenged mice induce myocardial hypertrophy and fibrosis, and rapid decompensation.[38] 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 *Egln1*^{Tie2Cre} 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.[39] Moreover, previous studies have shown that induction of myocardial

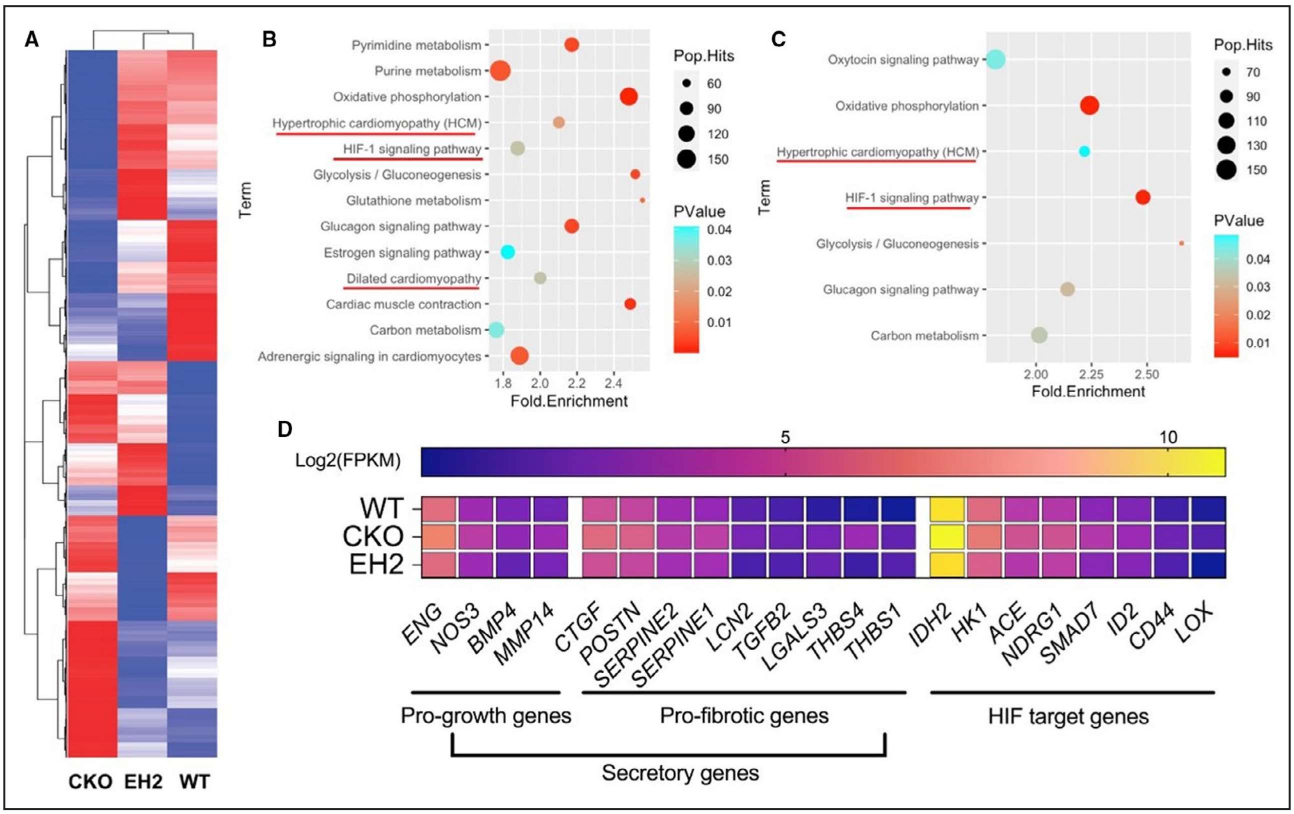


Figure 6. RNA sequencing analysis identifies multiple dysfunctional pathways in regulation of cardiac hypertrophy and fibrosis induced by endothelial *Egln1* deficiency and normalization by hypoxia inducible factor (HIF)-2α disruption.
A, A representative heatmap of RNA sequencing analysis of wild-type (WT), *Egln1*^{Tie2Cre} mice (CKO), and *Egln1/Hif2a*^{Tie2Cre} (EH2) mice. **B**, *Kyoto Encyclopedia of Genes and Genomes* (KEGG) pathway enrichment analysis of upregulated genes in *Egln1*^{Tie2Cre} vs WT mice showing dysregulation of multiple signaling pathways related to cardiac hypertrophy. **C**, Intersecting analysis of differential expression genes between WT_*Egln1*^{Tie2Cre} and *Egln1*^{Tie2Cre}_ *Egln1/Hif2a*^{Tie2Cre} 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.

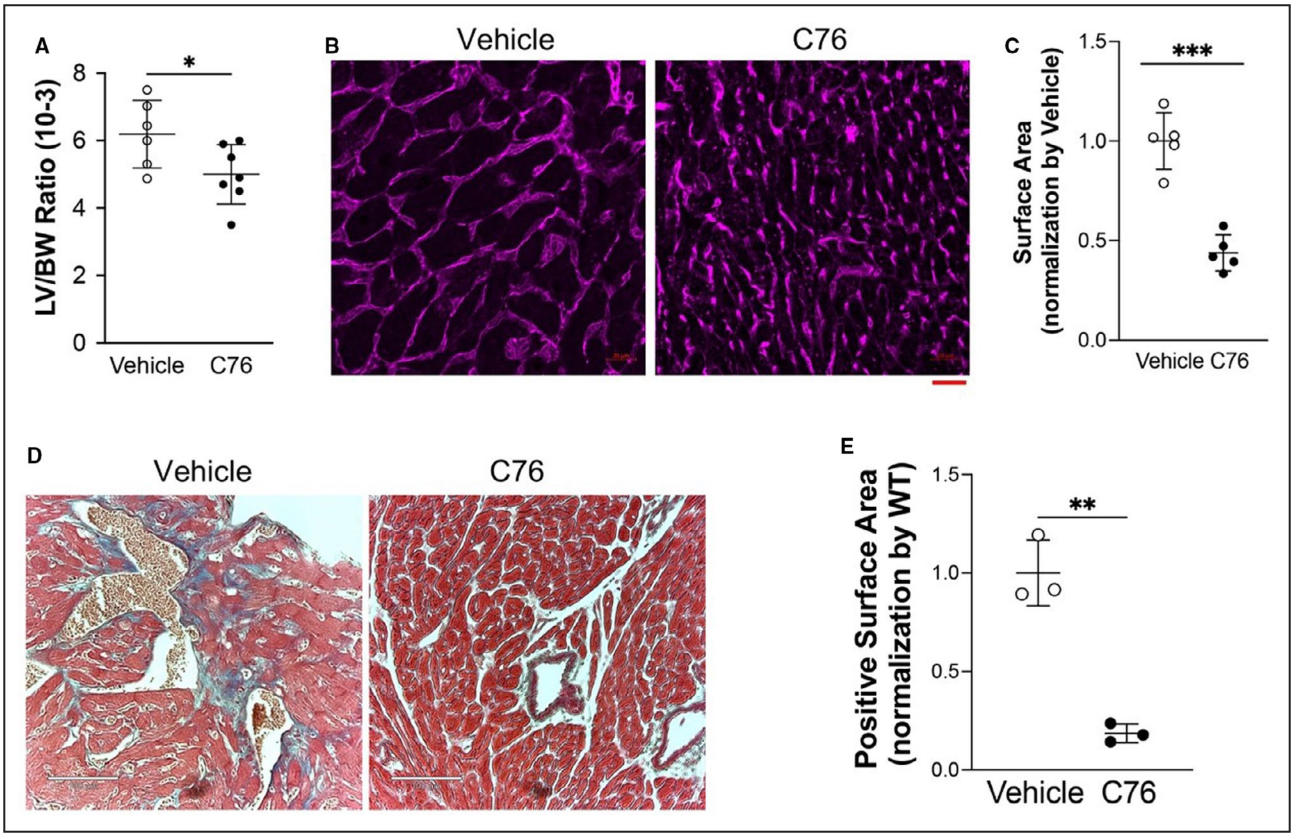


Figure 7. Pharmacological inhibition of hypoxia inducible factor (HIF)-2α attenuated cardiac hypertrophy and fibrosis.
A, Inhibition of HIF-2α reduced weight ratio of left ventricular/body weight (LV/BW) in *Egln1*^{Tie2Cre} mice (CKO). **B** and **C**, Wheat germ agglutinin staining and quantification demonstrates that HIF-2α inhibition reduced cardiomyocyte hypertrophy (N=5 per group). Scale bar=20 μm. **D**, Trichrome staining revealed attenuation of cardiac fibrosis by HIF-2α inhibition. Scale bar=100 μm. **P*<0.05, ***P*<0.01, ****P*<0.001 (Student *t* test). C76, Compound 76.

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.[40] 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.[4,7,41] 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).[8,41] Other endothelium-derived factors, such as endothelin-1, NO, neuregulin, *Bmp4*, *Fgf23*, *Lgals3*, *Lcn2*, *Spp1*, and *Tgfb1*, can directly promote cardiac hypertrophy and fibrosis.[42–46] 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.[31,35] 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.[38] Moreover, HIF-2α–selective antagonists, which target HIF-2α and HIF-β heterodimerization, are under investigation in patients with renal cancer in clinical trials.[47–49] 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

REFERENCES

1. Nakamura M, Sadoshima J. Mechanisms of physiological and pathological cardiac hypertrophy. *Nat Rev Cardiol*. 2018;15:387–407. doi: 10.1038/s41569-018-0007-y
2. Frey N, Olson EN. Cardiac hypertrophy: the good, the bad, and the ugly. *Annu Rev Physiol*. 2003;65:45–79. doi: 10.1146/annurev.physiol.65.092101.142243
3. Gogiraju R, Schroeter MR, Bochenek ML, Hubert A, Münzel T, Hasenfuss G, Schäfer K. Endothelial deletion of protein tyrosine phosphatase-1B protects against pressure overload-induced heart failure in mice. *Cardiovasc Res*. 2016;111:204–216. doi: 10.1093/cvr/cvw101
4. Oka T, Akazawa H, Naito AT, Komuro I. Angiogenesis and cardiac hypertrophy. *Circ Res*. 2014;114:565–571. doi: 10.1161/CIRCRESAHA.114.300507
5. Tirziu D, Chorianopoulos E, Moodie KL, Palac RT, Zhuang ZW, Tjwa M, Roncal C, Eriksson U, Fu Q, Elfenbein A, et al. Myocardial hypertrophy in the absence of external stimuli is induced by angiogenesis in mice. *J Clin Invest*. 2007;117:3188–3197. doi: 10.1172/JCI32024
6. Jaba IM, Zhuang ZW, Li NA, Jiang Y, Martin KA, Sinusas AJ, Papademetris X, Simons M, Sessa WC, Young LH, et al. No triggers RGS4 degradation to coordinate angiogenesis and cardiomyocyte growth. *J Clin Invest*. 2013;123:1718–1731. doi: 10.1172/JCI65112
7. Shiojima I, Sato K, Izumiya Y, Schiekofer S, Ito M, Liao R, Colucci WS, Walsh K. Disruption of coordinated cardiac hypertrophy and angiogenesis contributes to the transition to heart failure. *J Clin Invest*. 2005;115:2108–2118. doi: 10.1172/JCI24682
8. Talman V, Kivelä R. Cardiomyocyte—endothelial cell interactions in cardiac remodeling and regeneration. *Front Cardiovasc Med*. 2018;5:1–8. doi: 10.3389/fcvm.2018.00101
9. Bassat E, Mutlak YE, Genzelinakh A, Shadrin IY, Baruch Umansky K, Yifa O, Kain D, Rajchman D, Leach J, Riabov Bassat D, et al. The extracellular matrix protein agrin promotes heart regeneration in mice. *Nature*. 2017;547:179–184. doi: 10.1038/nature22978
10. Epstein ACR, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, et al. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell*. 2001;107:43–54.
11. Semenza GL. Hypoxia-inducible factors in physiology and medicine. *Cell*. 2012;148:399–408. doi: 10.1016/j.cell.2012.01.021
12. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science*. 2001;292:464–468.
13. Majmundar AJ, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. *Mol Cell*. 2010;40:294–309. doi: 10.1016/j.molcel.2010.09.022
14. Lee KE, Simon MC. SnapShot: hypoxia-inducible factors. *Cell*. 2015;163:1288.e1. doi: 10.1016/j.cell.2015.11.011
15. Wiesener MS, Jürgensen JS, Rosenberger C, Scholze C, Hörstrup JH, Warnecke C, Mandriota S, Bechmann I, Frei UA, Pugh CW, et al. Widespread hypoxia-inducible expression of HIF-2α in distinct cell populations of different organs. *FASEB J*. 2003;17:271–273. doi: 10.1096/fj.02-0445fje
16. Dai Z, Li M, Wharton J, Zhu MM, Zhao YY. Prolyl-4 hydroxylase 2 (PHD2) deficiency in endothelial cells and hematopoietic cells induces obliterative vascular remodeling and severe pulmonary arterial hypertension in mice and humans through hypoxia-inducible factor-2α. *Circulation*. 2016;133:2447–2458. doi: 10.1161/CIRCULATIONAHA.116.021494
17. Huang X, Zhang X, Zhao DX, Yin J, Hu G, Evans CE, Zhao YY. Endothelial hypoxia-inducible factor-1α is required for vascular repair and resolution of inflammatory lung injury through forkhead box protein M1. *Am J Pathol*. 2019;189:1664–1679. doi: 10.1016/j.ajpath.2019.04.014
18. Minamishima YA, Moslehi J, Bardeesy N, Cullen D, Bronson RT, Kaelin WG. Somatic inactivation of the PHD2 prolyl hydroxylase causes polycythemia and congestive heart failure. *Blood*. 2008;111:3236–3244. doi: 10.1182/blood-2007-10-117812
19. Hölscher M, Silter M, Krull S, Von Ahlen M, Hesse A, Schwartz P, Wielockx B, Breier G, Katschinski DM, Zieseniss A. Cardiomyocyte-specific prolyl-4-hydroxylase domain 2 knock out protects from acute myocardial ischemic injury. *J Biol Chem*. 2011;286:11185–11194. doi: 10.1074/jbc.M110.186809
20. Fan Q, Mao H, Angelini A, Coarfa C, Robertson MJ, Lagor WR, Wehrens XHT, Martin JF, Pi X, Xie L. Depletion of endothelial prolyl hydroxylase

domain protein 2 and 3 promotes cardiomyocyte proliferation and prevents ventricular failure induced by myocardial infarction. *Circulation*. 2019;140:440–442. doi: 10.1161/CIRCULATIONAHA.118.039276

21. Gao C, Ren SV, Yu J, Baal U, Thai D, Lu J, Zeng C, Yan H, Wang Y. Glucagon receptor antagonism ameliorates progression of heart failure. *JACC Basic to Transl Sci*. 2019;4:161–172. doi: 10.1016/j.jacbts.2018.11.001
22. Wang Z, Zhang XJ, Ji YX, Zhang P, Deng KQ, Gong J, Ren S, Wang X, Chen I, Wang HE, et al. The long noncoding RNA Chaer defines an epigenetic checkpoint in cardiac hypertrophy. *Nat Med*. 2016;22:1131–1139. doi: 10.1038/nm.4179
23. Tran KA, Zhang X, Predescu D, Huang X, Machado RF, Göthert JR, Malik AB, Valyi-Nagy T, Zhao Y-Y. Endothelial β-catenin signaling is required for maintaining adult blood-brain barrier integrity and central nervous system homeostasis. *Circulation*. 2016;133:177–186. doi: 10.1161/CIRCULATIONAHA.115.015982
24. Han X, Zhou Z, Fei L, Sun H, Wang R, Chen Y, Chen H, Wang J, Tang H, Ge W, et al. Construction of a human cell landscape at single-cell level. *Nature*. 2020;581:303–309. doi: 10.1038/s41586-020-2157-4
25. Stuart T, Butler A, Hoffman P, Hafemeister C, Papalexi E, Mauck WM, Hao Y, Stoeckius M, Smibert P, Satija R. Comprehensive integration of single-cell data. *Cell*. 2019;177:1888–1902.e21. doi: 10.1016/j.cell.2019.05.031
26. Chen J, Sathiyamoorthy K, Zhang X, Schaller S, Perez White BE, Jardetzky TS, Longnecker R. Ephrin receptor A2 is a functional entry receptor for Epstein-Barr virus. *Nat Microbiol*. 2018;3:172–180. doi: 10.1038/s41564-017-0081-7
27. Göthert JR, Gustin SE, Van Eekelen JAM, Schmidt U, Hall MA, Jane SM, Green AR, Göttgens B, Izon DJ, Begley CG. Genetically tagging endothelial cells in vivo: bone marrow-derived cells do not contribute to tumor endothelium. *Blood*. 2004;104:1769–1777. doi: 10.1182/blood-2003-11-3952
28. Nussbaum C, Bannenberg S, Keul P, Gräler MH, Gonçalves-de-Albuquerque CF, Korhonen H, von Wnuck Lipinski K, Heusch G, de Castro Faria Neto HC, Rohwedder I, et al. Sphingosine-1-phosphate receptor 3 promotes leukocyte rolling by mobilizing endothelial P-selectin. *Nat Commun*. 2015;6. doi: 10.1038/ncomms7416
29. Weis SM, Lim ST, Lutu-Fuga KM, Barnes LA, Chen XL, Göthert JR, Shen TL, Guan JL, Schlaepfer DD, Cheresh DA. Compensatory role for Pyk2 during angiogenesis in adult mice lacking endothelial cell FAK. *J Cell Biol*. 2008;181:43–50. doi: 10.1083/jcb.200710038
30. Cheng KT, Xiong S, Ye Z, Hong Z, Di A, Tsang KM, Gao X, An S, Mittal M, Vogel SM, et al. Caspase-11-mediated endothelial pyroptosis underlies endotoxemia-induced lung injury. *J Clin Invest*. 2017;127:4124–4135. doi: 10.1172/JCI94495
31. Dai Z, Zhu MM, Peng Y, Machireddy N, Evans CE, Machado R, Zhang X, Zhao YY. Therapeutic targeting of vascular remodeling and right heart failure in pulmonary arterial hypertension with a HIF-2a inhibitor. *Am J Respir Crit Care Med*. 2018;198:1423–1434. doi: 10.1164/rccm.201710-2079OC
32. Dai Z, Zhu MM, Peng Y, Jin H, Machireddy N, Qian Z, Zhang X, Zhao YY. Endothelial and smooth muscle cell interaction via FoxM1 signaling mediates vascular remodeling and pulmonary hypertension. *Am J Respir Crit Care Med*. 2018;198:788–802. doi: 10.1164/rccm.201709-1835OC
33. Wu S, Nishiyama N, Kano MR, Morishita Y, Miyazono K, Itaka K, Il CU, Kataoka K. Enhancement of angiogenesis through stabilization of hypoxia-inducible factor-1 by silencing prolyl hydroxylase domain-2 gene. *Mol Ther*. 2008;16:1227–1234. doi: 10.1038/mt.2008.90
34. Chan DA, Kawahara TLA, Sutphin PD, Chang HY, Chi JT, Giaccia AJ. Tumor vasculature is regulated by PHD2-mediated angiogenesis and bone marrow-derived cell recruitment. *Cancer Cell*. 2009;15:527–538. doi: 10.1016/j.ccr.2009.04.010
35. Zimmer M, Ebert BL, Neil C, Brenner K, Papaioannou I, Melas A, Tolliday N, Lamb J, Pantopoulos K, Golub T, et al. Small-molecule inhibitors of HIF-2a translation link its 5'UTR iron-responsive element to oxygen sensing. *Mol Cell*. 2008;32:838–848. doi: 10.1016/j.molcel.2008.12.004
36. Frey N, Katus HA, Olson EN, Hill JA. Hypertrophy of the heart: a new therapeutic target? *Circulation*. 2004;109:1580–1589. doi: 10.1161/01.CIR.0000120390.68287.BB
37. Bishop T, Ratcliffe PJ. HIF hydroxylase pathways in cardiovascular physiology and medicine. *Circ Res*. 2015;117:65–79. doi: 10.1161/CIRCRESAHA.117.305109
38. Wei H, Bedja D, Koitabashi N, Xing D, Chen J, Fox-Talbot K, Rouf R, Chen S, Steenbergen C, Harmon JW, et al. Endothelial expression of hypoxia-inducible factor 1 protects the murine heart and aorta from pressure overload by suppression of TGF-β signaling. *Proc Natl Acad Sci USA*. 2012;109:E841–E850.
39. Takeda K, Fong GH. Prolyl hydroxylase domain 2 protein suppresses hypoxia-induced endothelial cell proliferation. *Hypertension*. 2007;49:178–184. doi: 10.1161/01.HYP.0000251360.40838.0f
40. Mohammed SF, Hussain S, Mirzoyev SA, Edwards WD, Maleszewski JJ, Redfield MM. Coronary microvascular rarefaction and myocardial fibrosis in heart failure with preserved ejection fraction. *Circulation*. 2015;131:550–559. doi: 10.1161/CIRCULATIONAHA.114.009625
41. Gogiraju R, Bochenek ML, Schäfer K. Angiogenic endothelial cell signaling in cardiac hypertrophy and heart failure. *Front Cardiovasc Med*. 2019;6. doi: 10.3389/fcvm.2019.00020
42. Grabner A, Schramm K, Silswal N, Hendrix M, Yanucil C, Czaya B, Singh S, Wolf M, Hermann S, Stypmann J, et al. FGF23/FGFR4-mediated left ventricular hypertrophy is reversible. *Sci Rep*. 2017;7:1–12. doi: 10.1038/s41598-017-02068-6
43. Sun BO, Huo R, Sheng Y, Li Y, Xie X, Chen C, Liu H-B, Li NA, Li CB, Guo WT, et al. Bone morphogenetic protein-4 mediates cardiac hypertrophy, apoptosis, and fibrosis in experimentally pathological cardiac hypertrophy. *Hypertension*. 2013;61:352–360. doi: 10.1161/HYPERTENSIONAHA.111.00562
44. de Boer RA, Edelmann F, Cohen-Solal A, Mamas MA, Maisel A, Pieske B. Galectin-3 in heart failure with preserved ejection fraction. *Eur J Heart Fail*. 2013;15:1095–1101. doi: 10.1093/eurjhf/hft077
45. Khalil H, Kanisicak O, Prasad V, Correll RN, Fu X, Schips T, Vagnozzi RJ, Liu R, Huynh T, Lee S-J, et al. Fibroblast-specific TGF-β–Smad2/3 signaling underlies cardiac fibrosis. *J Clin Invest*. 2017;127:3770–3783. doi: 10.1172/JCI94753
46. López B, González A, Lindner D, Westermann D, Ravassa S, Beaumont J, Gallego I, Zudaire A, Brugnolaro C, Querejeta R, et al. Osteopontin-mediated myocardial fibrosis in heart failure: a role for lysyl oxidase? *Cardiovasc Res*. 2013;99:111–120. doi: 10.1093/cvr/cvt100
47. Cho H, Du X, Rizzi JP, Liberzon E, Chakraborty AA, Gao W, Carvo I, Signoretti S, Bruick RK, Josey JA, et al. On-target efficacy of a HIF-2α antagonist in preclinical kidney cancer models. *Nature*. 2016;539:107–111. doi: 10.1038/nature19795
48. Chen W, Hill H, Christie A, Kim MS, Holloman E, Pavia-Jimenez A, Homayoun F, Ma Y, Patel N, Yell P, et al. Targeting renal cell carcinoma with a HIF-2 antagonist. *Nature*. 2016;539:112–117. doi: 10.1038/nature19796
49. Courtney KD, Infante JR, Lam ET, Figlin RA, Rini BI, Brugarolas J, Zojwalla NJ, Lowe AM, Wang K, Wallace EM, et al. Phase I dose-escalation trial of PT2385, a first-in-class hypoxia-inducible factor-2α antagonist in patients with previously treated advanced clear cell renal cell carcinoma. *J Clin Oncol*. 2018;36:867–874. doi: 10.1200/JCO.2017.74.2627

Supplemental Material

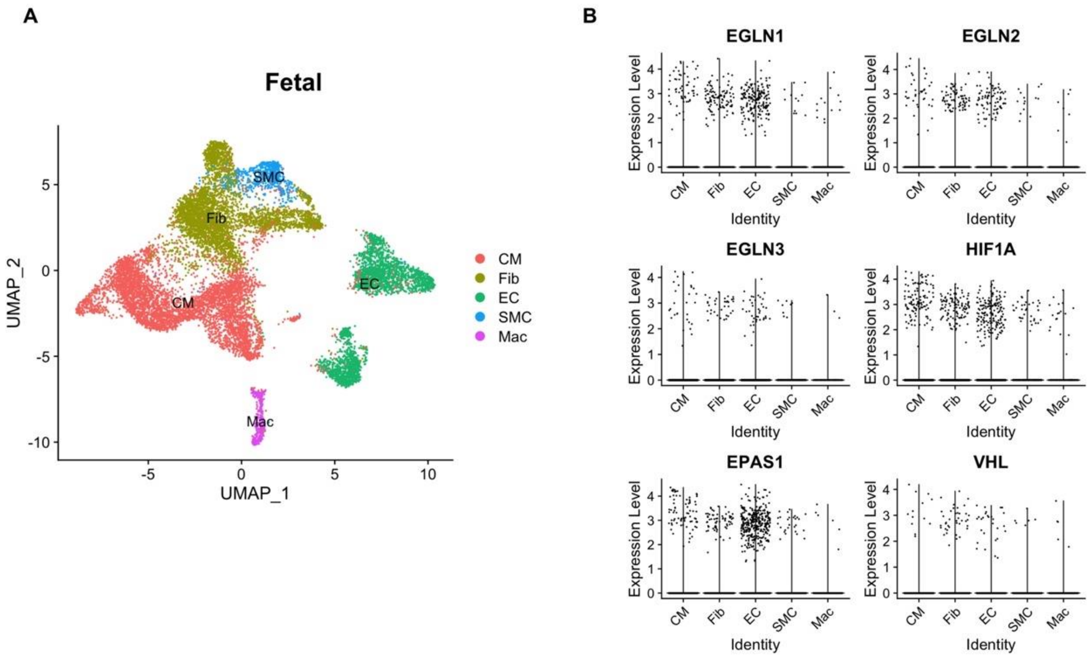
Table S1. Patients' information.

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.

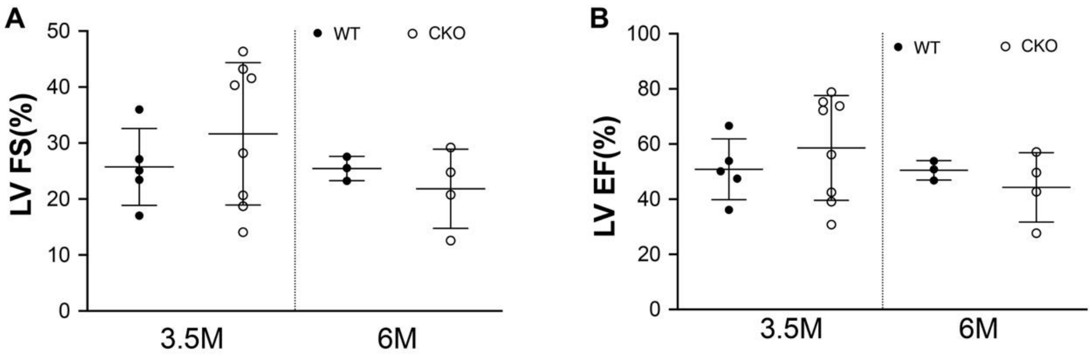
Gene name	Forward primer	Reverse primer
hEGLN1	*AAAGACTGGGATGCCAAGGT*	*CTCGTGCTCTCTCATCTGCA*
hGAPDH	*GTCTCCTCTGACTTCAACAGCG*	*ACCACCCTGTTGCTGTAGCCAA*
mAnp	*GATAGATGAAGGCAGGAAGCCGC*	*AGGATTGGAGCCCAGAGTGGACTAGG*
mBnp	*TGTTTCTGCTTTTCCTTTATCTGTC*	*CTCCGACTTTTCTCTTATCAGCTC*
mMyh7	*TGCAAAGGCTCCAGGTCTGAGGGC*	*GCCAACACCAACCTGTCCAAGTTC*
mCola1	*TCACCAAACTCAGAAGATGTAGGA*	*GACCAGGAGGACCAGGAAG*

Figure S1. Single-cell RNA sequencing analysis of human fetal hearts.



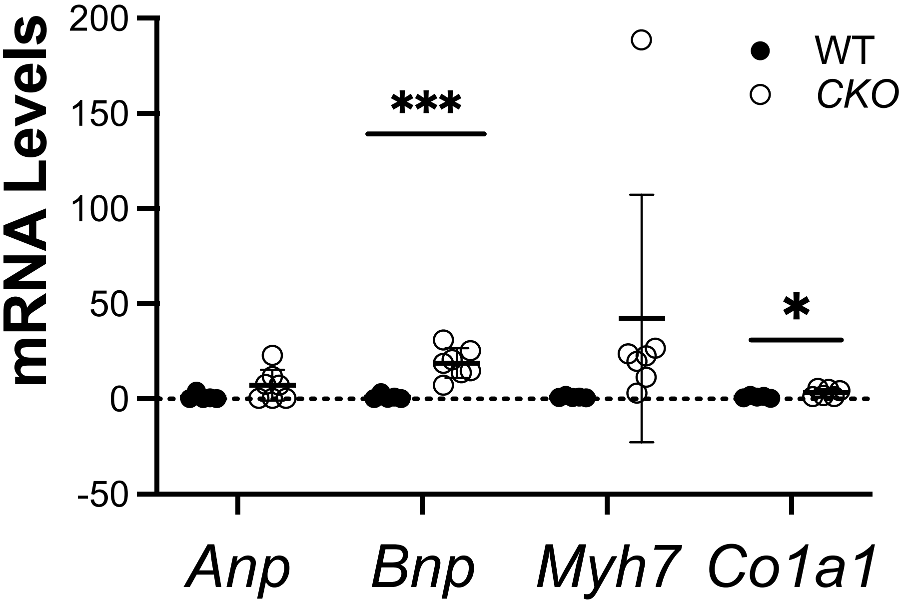
(**A**) A UMAP plot showing the major cardiac cell types including ECs, cardiomyocytes (CM), fibroblasts (Fib), smooth muscle cells (SMC) and macrophages (Mac). (**B**) A Violin plot showing the expression levels of PHD2/HIF signaling genes in cardiac cells.

Figure S2. Constitutive loss of endothelial *Egln1* in mice does not affect cardiac function.



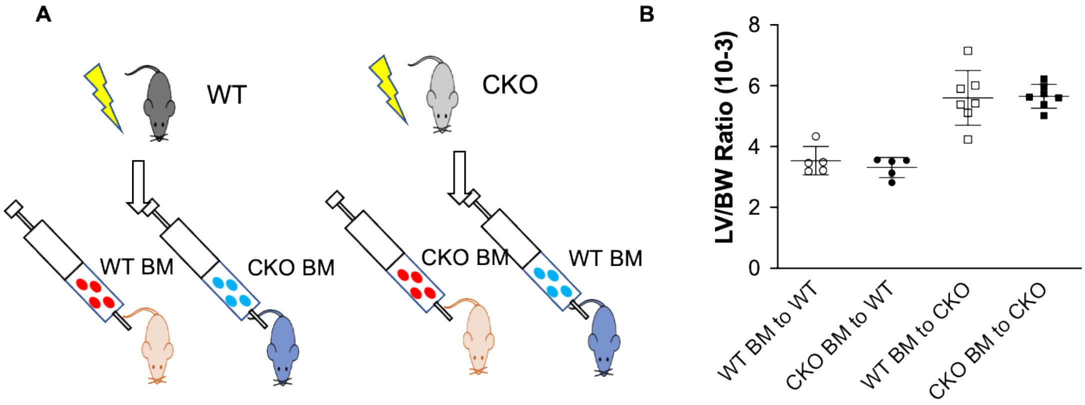
Echocardiography measurement showing similar LV fraction shorting (FS%) and ejection fraction (EF%) in adult *Egln1*^{Tie2Cre} mice compared to WT mice.

Figure S3. Upregulation of fetal gene including *Bnp* and fibrotic gene *Col1a* in the LV of *Egln1*^{Tie2Cre} 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*^{Tie2Cre} (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*^{Tie2Cre} (CKO) mice.