Endothelial Autocrine Signaling Contributes to Severe Pulmonary Arterial Hypertension

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Abstract: Endothelial autocrine signaling is essential to maintain vascular hemostasis. There is limited information about the role of endothelial autocrine signaling in regulating severe pulmonary vascular remodeling during the onset of pulmonary arterial hypertension (PAH). In this study, we employed the first severe PAH mouse model, Egln1Tie2Cre (Tie2Cre-mediated disruption of Egln1) mice, to identify the novel autocrine signaling mediating the pulmonary vascular endothelial cells (PVECs) hyperproliferation and the pathogenesis of PAH. PVECs isolated from Egln1Tie2Cre lung expressed upregulation of many growth factors or angiocrine factors such as CXCL12, and exhibited hyperproliferative phenotype in coincident with upregulation of proliferation specific transcriptional factor FoxM1. Treatment of CXCL12 on PVECs increased FoxM1 expression, which was blocked by CXCL12 receptor CXCR4 antagonist AMD3100 in culture human PVECs. Endothelial specific deletion of Cxcl12 (Egln1/Cxcl12Tie2 Cre) or AMD3100 treatment in Egln1Tie2Cre mice downregulated FoxM1 expression in vivo. We then generated and characterized a novel mouse model with endothelial specific FoxM1 deletion in Egln1Tie2Cre mice (Egln1/Foxm1Tie2 Cre), and found that endothelial FoxM1 deletion reduced pulmonary vascular remodeling and right ventricular systolic pressure. Together, our study identified a novel mechanism of endothelial autocrine signaling in regulating PVECs hyperproliferation and pulmonary vascular remodeling in PAH.

Keywords: Hypoxia; Vascular Remodeling; Angiogenesis; Pulmonary hypertension; Endothelium

1. Introduction

Pulmonary arterial hypertension (PAH) is characterized by a progressive increase of vascular resistance and obstructive vascular remodeling affecting pulmonary arterioles, eventually leading to right heart failure and death [1,2]. Endothelial injury and hyperproliferation are hallmarks of PAH [3,4]. Healthy pulmonary vascular endothelial cells (PVECs) maintain vascular hemostasis via preserving vascular integrity, remaining vascular tone, and exhibiting an anti-inflammatory niche. Endothelial injury or dysfunction has been believed to the initial event during the development of PAH [5]. Dysfunctional PVECs produce many kinds of growth factors or angiocrine factors which sustains the pro-proliferative environment. Therapies that attenuate PVECs hyperproliferation may therefore provide benefit to PAH patients.

Both autocrine and paracrine signaling pathways are important to maintain vascular homeostasis and contribute to pathological angiogenesis [5]. PVECs from PAH patients exhibit increased production of growth factors, such as FGF2, IL-6, ET-1, TGF-beta, etc. These factors promote PVECs/smooth muscle cells (SMCs)/fibroblasts proliferation and
survival, stimulate SMCs vasoconstriction, and even recruit leukocytes [5]. For example, our previous studies demonstrated that PVECs from PAH patients or Egln1Tie2Cre mice which develop spontaneous PAH [6], secrete multiple angiocrine factors including CXCL12, PDGF-B, ET-1, and MIF, which induced the expression of proliferation specific transcriptional factor forkhead box M1 (FoxM1) and proliferation of SMCs. These events further lead to pulmonary vascular remodeling and PAH [7]. However, the role of the endothelial autocrine signaling and underline mechanisms in the pathogenesis of PAH remains elusive. FoxM1 is activated after tissue injury and upregulated in the many solid tumors and leukemia [8]. Our previous study also showed that FoxM1 is activated for endothelial regeneration after inflammatory lung injury [9,10], which often, serves as a trigger of PAH development [11]. However, the role of endothelial FoxM1 in the pathogenesis of PAH is not known.

In this study, we employed transcriptome analysis of PVECs isolated from Egln1Tie2Cre mice and identify the intriguing signaling of endothelial autocrine pathway involved CXCL12/CXCR4/FoxM1 mediating endothelial hyperproliferation in the pathogenesis of PAH.

2. Results

Endothelial cells (ECs) hyperproliferation is a hallmark of PAH. Our previous studies demonstrated that Egln1Tie2Cre mice exhibit severe PAH including obliterator pulmonary vascular remodeling, plexiform-like lesions, severe right heart hypertrophy, and failure [6]. To further understand the role of endothelial hyperproliferation in the pathogenesis of PAH, we confirmed that pulmonary vascular endothelial cells (PVECs) were hyperproliferative as evident by increased Ki67+/CD31+ cells in the pulmonary vascular bed in Egln1Tie2Cre mice (Figure 1A). We also performed whole transcriptome RNA sequencing on isolated lung PVECs (CD31+) from WT and Egln1Tie2Cre mice. RNA-seq analysis observed upregulation of many genes related to cell proliferation including Foxm1, E2f1, Cenps, Plk1, Cdk1, Ccnb2, Ccnb1, etc (Figure 1B), and Western Blot demonstrated that FoxM1 protein expression was markedly upregulated in the lung of Egln1Tie2Cre mice (Figure 1C). These data further support the finding that Egln1Tie2Cre lung PVECs is hyperproliferative. Previous studies from multiple groups have demonstrated that FoxM1, a key transcriptional factor for cell cycle progression, is upregulated in PASMCs and contributes to the hyperproliferation of PASMCs and vascular remodeling of PAH [7,12–14]. As pulmonary ECs from PAH patients share similar hyperproliferative features with PAMSCs, therefore we further examined FoxM1 expression in the lung sections from IPAH patients via immunohistochemistry staining. We observed that FoxM1 is markedly increased in pulmonary ECs in IPAH patients compared to that from healthy donors (Figure 1D), suggest that endothelial FoxM1 might be involved in the pathogenesis of PAH.
Vascular system maintains its homeostasis via producing various kinds of factors to act on vasculature and peripheral cells. The RNA-sequencing analysis also suggests that ECs from \textit{Egln1}Tie2Cre mice expressed many genes related to secretive proteins, 123 of 1680 upregulated genes are ligands according to the Secretome database [15] (Figure 2A). Many of these genes are angiocrine factors including Cxcl12, Tgfb1, Edn1, Mif, and Pdgfb (Figure 2B). Cxcl12 is the top list gene (15.6 fold: \textit{Egln1}Tie2Cre vs WT) upregulated in the PVECs from \textit{Egln1}Tie2Cre mice and mediated the development of PAH demonstrated by multiple studies [6,7,16,17]. Cxcl12 receptor Cxcr4 is also increased (3.2 fold: \textit{Egln1}Tie2Cre vs WT) in PVECs from \textit{Egln1}Tie2Cre mice compared to \textit{Egln1}f/f mice (Figure 2B). Thus, we hypothesized that endothelial autocrine signaling through CXCL12/CXCR4 mediates endothelial hyperproliferation and the pathogenesis of PAH. To demonstrate this hypothesis, we incubated healthy human lung microvascular endothelial cells (hLMVECs) with CXCL12 and found that CXCL12 treatment significantly increased FoxM1 expression in hLMVECs, which was blocked by its receptor CXCR4 inhibitor AMD3100 (Figure 2C). To further determine whether CXCL12/CXCR4 signaling regulates FoxM1 expression \textit{in vivo}, we examined the expression of endothelial FoxM1 in \textit{Egln1/Cxcl12}Tie2Cre mice, an endothelial-specific deletion of Cxcl12 in the \textit{Egln1}Tie2Cre mice [6,7]. We found that FoxM1 in the PVECs was reduced in \textit{Egln1/Cxcl12}Tie2Cre mice compared to \textit{Egln1}Tie2Cre mice, accessed by immunofluorescent staining against FoxM1 (Figure 2D). We also observed that FoxM1 expression was attenuated by blocking CXCR4 signaling via AMD3100 treatment in \textit{Egln1}Tie2Cre mice (Figure 2E). Taken together, our studies suggest that endothelial autocrine CXCL12/CXCR4 signaling pathway regulates hyperproliferative FoxM1 expression in PVECs during PAH.
Figure 2. Upregulation of angiocrine factors in the PVECs from Egln1Tie2Cre mice. (A) A diagram showing that 123 of 1803 upregulated genes are ligands. (B) An RNA-seq analysis heatmap showing the representative angiocrine factors in PVECs from Egln1Tie2Cre mice. (C) Immunostaining of anti-FoxM1 showed that endothelial FoxM1 was downregulated in the PVECs from Egln1/Cxcl12Tie2Cre mice (ECx), a mouse model with Cxcl12 deletion in ECs of Egln1Tie2Cre mice (CKO). (D) CXCL12 treatment induced FOXM1 expression in a CXCR4 dependent manner. HLMVECs was treated with CXCL12 (100 ng/ml) and/or CXCR4 inhibitor AMD3100 (AMD, 2μg/ml) for 8 hours. (E) AMD3100 treatment significantly attenuated Foxm1 expression in Egln1Tie2Cre mice. One-way ANOVA with Tukey post hoc analysis for multiple group comparisons. * p<0.05, ** p<0.01. Scale bar =20 μm (D).

Our previous study demonstrated that FoxM1 is required for endothelial regeneration and repair after inflammatory induced acute lung injury and responded to CXCL12 treatment [18]. The role of endothelial FoxM1 in the development of PAH remains elusive. To determine whether endothelial FoxM1 is involved in the development of severe PAH in Egln1Tie2Cre mice, we generated a novel mouse model with Foxm1 deletion in ECs in Egln1Tie2Cre mouse (Egln1/Foxm1Tie2Cre) via breeding Foxm1 floxed mice with Egln1Tie2Cre mice [7,19,20]. RV hemodynamic measurement showed that Egln1/Foxm1Tie2Cre mice exhibit reduction of the right ventricular systolic pressure (RVSP), an indicator of pulmonary arterial pressure, compared to age and gender-matched Egln1Tie2Cre mice (Figure 3A and 3B), suggesting that PAH was attenuated in Egln1/Foxm1Tie2Cre mice. Cardiac dissection showed that RV weight was significantly reduced in Egln1/Foxm1Tie2Cre mice compared to Egln1Tie2Cre mice (Figure 3C). However, we did not observe a significant change of the weight ratio of RV vs Left ventricle plus septum (RV/LV+S) between Egln1/Foxm1Tie2Cre and Egln1Tie2Cre mice (Figure 3D).
Figure 3. Endothelial Foxm1 deletion protected from Egln1 deficiency-induced PAH. (A) RV hemodynamic measurement showed that endothelial Foxm1 deletion (Egln1/Foxm1\textsuperscript{Tie2Cre}, Efoxm1) attenuated RVSP in Egln1\textsuperscript{Tie2Cre} mice (CKO). (B) Cardiac dissection demonstrated that Foxm1 disruption in Egln1\textsuperscript{Tie2Cre} mice reduced RV weight compared with Egln1\textsuperscript{Tie2Cre} mice. (C) The RV/(LV+S) ratio was not changed in Egln1/Foxm1\textsuperscript{Tie2Cre} compared with Egln1\textsuperscript{Tie2Cre} mice. Student t-test, ** p <0.01, *** p <0.001.

We further examine the pulmonary histology via Russel-Movat pentachrome staining and anti-α-SMA staining. We found that Egln1/Foxm1\textsuperscript{Tie2Cre} mice had reduced pulmonary artery (PA) wall thickness when compared to Egln1\textsuperscript{Tie2Cre} mice (Figure 4A and 4B). Muscularization of distal PAs assessed by anti-α-SMA staining was markedly reduced in Egln1/Foxm1\textsuperscript{Tie2Cre} mice compared with Egln1\textsuperscript{Tie2Cre} mice (Figure 4C and 4D). Taken together, our data demonstrate that endothelial FoxM1 contributes to pulmonary vascular remodeling and PAH in mice.

Figure 4. Foxm1 deletion in ECs attenuated pulmonary vascular remodeling in Egln1\textsuperscript{Tie2Cre} mice. (A and B) Representative images of Russel-Movat pentachrome staining showing the reduction of pulmonary wall thickness in Egln1/Foxm1\textsuperscript{Tie2Cre} mice (Efoxm1). (C and
D) Immunostaining against α-SMA demonstrated attenuation of pulmonary vascular remodeling including neointima and muscularization of distal PAs in Egln1/Foxm1\textsuperscript{Tie2Cre} mice. Red arrows indicate the muscularization of distal PAs. V=vessel. Student t-test, * p <0.05, *** p <0.001. Scale bar =50 μm (A and C).

3. Discussion

The present study has demonstrated the autocrine signaling pathway from PVECs through CXCL12/CXCR4 signaling mediates transcriptional factor FoxM1 expression and endothelial hyperproliferation in PAH. For the first time, we found that FoxM1 is upregulated in the lung ECs of IPAH patients and contribute to the hemodynamic increase and vascular remodeling of PAH. Understanding the role of endothelial autocrine signaling and endothelial FoxM1 in PAH pathogenesis will pave the way for FoxM1-targeted new therapy in PAH patients.

Vascular modeling in PAH involves many cell types including EC, SMCs, fibroblasts, and macrophages. Similar to the tumor microenvironment, PVECs in the lung produce many pro-proliferative factors, which sustain vascular cells (including ECs) proliferation. Our transcriptome data demonstrated that upregulation of many growth factors (Cxcl12, Edn1, Mif, Pdgfb), transcription factors (Foxm1, E2F1), and cell cycle-related genes (Cenps, Plk1, Cdk1, Ccnb2, Cdkn2a) in the isolated PVECs from Egln1\textsuperscript{Tie2Cre} mice compared to WT mice, suggest the autocrine signaling pathways mediating PVECs proliferation in Egln1\textsuperscript{Tie2Cre} mice.

CXCL12, also named stromal cell-derived factor 1 (SDF1), is an angiogenic chemokine that acts via its cognate receptor CXCR4. Multiple studies have demonstrated that CXCL12 promotes neovascularization and angiogenesis in several organs including skeletal muscle and cardiac arterial development [21,22]. In pathological conditions, CXCL12 was shown to promote tumor and leukemia progression [23,24], accelerate atherosclerosis [25]. Previous studies also showed that plasma CXCL12 is associated with an unfavorable prognosis in PAH patients[26]. Multiple studies including ours demonstrated that CXCL12/CXCR4 signaling is involved in the development of hypoxia and Egln1 deficiency-induced PH [6,7,27,28], and blocking CXCL12 signaling attenuated PH in mice and rats [17,27,29]. In the present study, we found that Cxcl12 is highly expressed in PVECs and markedly upregulated in the PVECs from Egln1\textsuperscript{Tie2Cre} mice. Treatment of CXCL12 on PVECs induced FoxM1 through CXCR4. Taken together, our data showed that endothelial autocrine signaling through CXCL12/CXCR4/FoxM1 mediated PVECs proliferation in PAH.

Proliferation specific transcription factor FoxM1 has been shown to regulate EC, SMC, fibroblast proliferation in the disease model of acute lung injury [9,20], pulmonary hypertension [7,12–14] and interstitial lung fibrosis [9], respectively. In our previous studies, we first checked the expression of FoxM1 in cultured PAEC from IPAH patients and healthy donors, but we did not observe a significant change of FoxM1 protein expression [7]. This might be because of cultured cells \textit{in vitro} might lose the endogenous feature \textit{in vivo} [30]. In the present studies, we observed that endothelial FoxM1 is upregulated in the PVECs of IPAH patients and severe PAH mouse model Egln1\textsuperscript{Tie2Cre} mice, a mouse model with marked elevation of RVSP, severe RV hypertrophy, and oblitative vascular remodeling, resembling many pathological features of IPAH in patients. Genetic disruption of endothelial FoxM1 in Egln1\textsuperscript{Tie2Cre} mice reduced RVSP, and attenuated pulmonary vascular remodeling evident by reductions of PA wall thickness and muscularization of distal PAs, suggesting FoxM1 in ECs contributes to the severity of vascular remodeling and PAH. This is different from our previous observation that Foxm1\textsuperscript{Tie2Cre} mice did not show protection of hypoxia (10% O\textsubscript{2}, 3 weeks)-induced PAH [7]. This might be due to the fact that hypoxia does not stimulate significant EC proliferation in mice and induces mild pulmonary vascular remodeling and PAH. Another observation in this study is that RV weight but not RV/LV+S ratio was reduced in Egln1/Foxm1\textsuperscript{Tie2Cre\textsubscript{mice} compared with Egln1\textsuperscript{Tie2Cre} mice, which is because LV weight is also reduced in Egln1/Foxm1\textsuperscript{Tie2Cre\textsubscript{mice} compared with Egln1\textsuperscript{Tie2Cre} mice. The data was not shown due to another manuscript on the topic of left ventricular hypertrophy observed in Egln1\textsuperscript{Tie2Cre} mice.
Endothelial FoxM1 has been shown to regulate embryonic development and endothelial proliferation and repair following LPS-induced vascular injury [9,31]. Recent studies also suggest that FoxM1 is a critical driver of TGF-β-induced endothelial-to-mesenchymal transition (EndoMT) [32]. Tang et al. demonstrated that Egln1 deficiency in ECs induced EndoMT in a HIF-2α-dependent manner in vivo and in vitro [33], it is intriguing to determine whether endothelial Foxm1 is also involved in EndoMT in Egln1Tie2Cre mice contributing to severe PAH. Multiple growth factors (PDGF-B, CXCL12, MIF, and ET-1) have been shown to positively regulate FoxM1 expression in SMCs in vitro by our previous studies [7]. We also found that CXCL12 induces FoxM1 expression in lung ECs via p110γ PI3K→FoxO1 signaling and mediating endothelial regeneration in sepsis-induced inflammatory lung injury [20]. BRD4 and FoxO1 have been shown to positively and negatively regulate FoxM1, respectively [34], however, our data showed that both BRD4 and FoxO1 are downregulated in the lung of Egln1Tie2Cre mice (data not shown), suggesting that FoxO1 signaling but not BRD4 might be the direct regulator of FoxM1 in ECs.

In summary, the present study demonstrates that endothelial autocrine signaling through CXCL12/CXCR4 mediated FoxM1 induction in PVECs and contributes to endothelial hyperproliferation and severe pulmonary vascular remodeling in PAH. This study further suggests that FoxM1 inhibition could be a therapeutic approach for PAH patients.

4. Materials and Methods

4.1. Human samples

The human lung tissues and pulmonary type 3 arterial ECs from IPAH patients and healthy donors were provided by the Pulmonary Hypertension Breakthrough Initiative (PHBI) and used as described previously [7,35]. The human lung microvascular ECs (hLMVECs) were obtained from Lonza. The use of human samples was approved by the University of Arizona Institutional Review Board (IRB) # 1907824872.

4.2. Mice

Egln1Tie2Cre, Egln1/Cxcl12Tie2Cre mice were generated as described previously [6]. Foxm1 floxed mice[9] were bred with Egln1Tie2Cre mice to generate Egln1/Foxm1Tie2Cre mice. For CXCR4 inhibitor AMD3100 treatment study, Egln1Tie2Cre mice at the age of 3 weeks were treated with vehicle (PBS) or CXCR4 inhibitor AMD3100 (7.5 mg/kg, daily) for 5 weeks. Right ventricular systolic pressure (RVSP) in mice was measured as described previously [6,7,35]. The experiments were conducted according to National Institutes of Health guidelines on the use of laboratory animals. The animal care and study protocol were approved by the Institutional Animal Care and Use Committee of Northwestern University and the University of Arizona.

4.3. Endothelial Cells Isolation and RNA Sequencing Analysis

Mouse lung ECs were isolated as described previously [7,35]. Purified endothelial cells (EC, CD31+ cells) were lysed for RNA isolation with the RNeasy mini kit including DNase I digestion. Equal amounts of RNA from ECs isolated from three individual WT or Egln1Tie2Cre mice were pooled and sequenced with NovaSeq PE150 at Novogene Corporation Inc. 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 [36].

4.4. Immunofluorescent staining and histological assessment

For immunofluorescent staining of cryosections, human IPAH patients and healthy donors were fixed with 4% paraformaldehyde for 20 mins. The sections were blocked with 5% goat serum and incubated with anti-FoxM1 (Santa Cruz Biotechnology, Cat # sc-376471, 1:25) and anti-CD31 (Abcam, Cat#ab28364, 1:40) were incubated at 4°C overnight, then incubated with Alexa 594 conjugated anti-mouse and Alexa 647 conjugated anti-rab-
bit IgG at room temperature for 1 h. Nuclei were counterstained with DAPI. For immunofluorescent staining of cryosections from mouse lung tissue, mouse lung tissue was well perfused with PBS and inflated with 50% OCT in PBS for sectioning.

For immunofluorescent staining of paraffin sections from mouse lung tissues, mouse lung tissues were perfused with PBS, followed by fixation and inflation with 10% formalin via tracheal instillation for routine tissue processing. The sections were dewaxed and dehydrated, followed by antigen retrieval using Antigen Unmasking Solution (Vector Lab, # H-3300-250) according to the manual. Similarly, after antigen retrieval, mouse lung sections were incubated with anti-α-SMA (Abcam, Cat #ab5694, 1:300) at 4°C overnight and then incubated with Alexa 594 conjugated anti-rabbit IgG at room temperature for 1 h.

For histology assessment, lung sections were dewaxed, dehydrated, and stained with a Russel-Movat pentachrome staining kit (American MasterTech, Cat #KTRMP) according to the manufacturer’s instructions. PA wall thickness was measured using Pentachrome stained sections as described previously [7,35].

4.5. Statistical analysis

Statistical significance was determined by one-way ANOVA with a Tukey post hoc analysis that calculates P values corrected for more than 2 groups comparisons using Prism 9 (Graphpad Software, Inc.). Two-group comparisons were analyzed by the unpaired two-tailed Student’s t test for equal variance or the Welch t test for unequal variance. P less than 0.05 denoted the presence of a statistically significant difference.

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Informed Consent Statement:

Data Availability Statement:

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