KLF6 in Pulmonary Hypertension: The Dual Role of Friend and Foe

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ABSTRACT

Background: Pulmonary arterial hypertension (PAH) is a severe lung condition with an unmet clinical need. Endothelial damage is followed by excessive repair and narrowing of lung arteries, but the mechanisms are unclear.

Methods: We investigated the role of Krüppel-like transcription factor 6 (KLF6), known for its involvement in tissue injury response and cancer onset, in PAH through functional and expression analyses of human pulmonary artery endothelial cells (HPAECs) and PAH lung tissues. KLF6 activation patterns and transcriptional programs were compared with those regulated by KLF2 and KLF4, previously linked to PAH.

Results: KLF6 expression increased due to hypoxic and inflammatory triggers in early rodent PAH. KLF6 overexpression improved endothelial survival and induced angiogenesis in cultured HPAECs and human pulmonary arterial explants. Transcriptomic analysis of KLF6-overexpressing HPAECs revealed its role in regulating endothelial homeostasis and expression of arterial identity genes, such as *SOX17, ERG, BMPR2, KDR, TEK, ENG, ACVRL1* and *CDH5*. DisGeNET analysis showed a significant association of KLF6-regulated genes with PAH. Spatial transcriptomic analysis of PAH lung vascular tissues (n=6/group) revealed significant enrichment of KLF6-regulated genes in angiogenesis, cell motility, VEGFR signalling, and extracellular matrix organization. $KLF6^+$ Erg^+ vWF^+ cells accumulated in vascular channels of remodelled PAH lungs and. KLF6 expression was elevated in PAH blood-derived endothelial progenitor cells (n=5) and pulmonary arterial cells in PAH associated with Alveolar Capillary Dysplasia.

Conclusions: KLF6 uniquely orchestrates endothelial repair but its sustained activation promotes development of the apoptosis-resistant, angio-proliferative vascular phenotype in human PAH. Dysregulation of KLF6 signalling may have broader implications for pulmonary vascular disease.

Key words: endothelial, angiogenesis, pulmonary arterial hypertension, KLF, vascular

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a progressive and fatal disease, despite targeted treatment with multiple therapies. Pathologically, PAH is characterized by sustained pulmonary vasoconstriction and progressive obliteration of resistance pulmonary arteries and arterioles due to medial thickening, intimal fibrosis, and formation of angioproliferative (plexiform) lesions[1]. These changes result in the loss of small distal vessels, often described as "vascular pruning"[2].

PAH is a multifactorial condition where a combination of genetic predisposition, hypoxia, and inflammation play contributory roles. The most commonly recognized molecular mechanism underlying PAH is the loss of function of bone morphogenetic protein receptor 2 (BMPR2), a TGF-β receptor, either due to mutations or increased protein degradation and reduced membrane recycling [3]. In recent years, mutations in other genes, mostly from the TGF-β superfamily, have also been implicated in PAH[4].

In the process of PAH development, endothelial damage caused by genetic, epigenetic or environmental factors triggers exaggerated repair, during which clonal expansion of apoptosis-resistant endothelial cells gives rise to complex angio-proliferative (plexiform) lesions[5,6]. Plexiform lesions are a hallmark of human disease but are poorly replicated in experimental PAH[7]. In addition, loss of endothelial barrier function, pro-inflammatory and pro-thrombotic activation, endothelial-to-mesenchymal transition as well as the proliferation and migration of pulmonary arterial smooth muscle cells (PASMCs) and adventitial fibroblasts drive pulmonary vascular remodelling[1]. The mechanisms that initiate the formation of plexiform lesions are not known, but a contribution of circulating endothelial progenitor cells has been suggested[8]. None of the existing therapies can prevent small vessel loss and/or reverse plexogenic arteriopathy, partly because mechanisms that drive endothelial repair are incompletely understood[6 9]

Endothelial dysfunction in PAH has been attributed to reduced activity of two closely related, flow-activated Krüppel-like transcription factors, KLF2 and KLF4[10,11]. Both of these transcription factors maintain endothelial homeostasis by controlling expression of anti-proliferative, anti-inflammatory, anti-angiogenic and anti-thrombotic genes[12,13]. Previous work from our group and others has also suggested that expression levels of KLF6, another KLF family member, are dysregulated in pulmonary disease[14,15].

KLF6 plays a critical role in vascular development and repair[12], evidenced by embryonic lethality of KLF6$^{-/-}$ mice due to defects in yolk sac vascularization[16]. Changes in the expression of KLF6 have been linked to cancer progression as well as inflammatory and fibrotic diseases[17]. Of potential relevance to PAH, KLF6 activates endoglin and activin receptor-like kinase 1 (ALK1)[18], two TGF-β receptors involved in the regulation of pulmonary vascular remodelling[4]. However, the role of KLF6 in PAH or its relationship with the structurally and functionally related transcription factors KLF2 and KLF4 has not been investigated.

This study is the first to provide evidence of KLF6 dysregulation in PAH. We show that KLF6 is activated in response to endothelial injury in the early stages of the disease to enhance endothelial resilience and orchestrate endothelial repair. Its continued activation, evident in human PAH, leads to exaggerated repair and resistance to apoptosis, augmenting the formation of occlusive plexiform lesions. Additionally, we uncover similarities and differences in the activation patterns and transcriptional programs regulated by KLF2, KLF4, and KLF6 in lung endothelial cells, highlighting the specific role of KLF6 in promoting endothelial dysfunction in PAH.

MATERIALS AND METHODS

Detailed materials and methods information has been provided in Supplement.

RESULTS

KLF6 expression is increased under PAH-related vascular stress conditions

We studied the impact of hypoxia and inflammation - two important triggers of PAH[19] - on KLF6 expression in rodent models of PAH, specifically in chronically hypoxic mice and monocrotaline (MCT) rats, as well as in cultured human pulmonary artery endothelial cells (HPAECs). In mice, development of PAH was confirmed by elevated RVSP, right ventricular hypertrophy (RVH), and increased pulmonary arterial muscularization (Supplementary Figure S1). PAH MCT rats were characterised in our previous study[20].

Mice exhibited a transient 3-fold increase in lung KLF6 expression after 24 hours of hypoxic exposure ($p<0.0001$), which returned to control levels 2 weeks later, once the disease was

established (Figure 1B). An early rise in KLF6 expression was also observed in monocrotaline (MCT) rats, which typically display a more severe phenotype and greater inflammatory response, compared to chronically hypoxic animals[7] (Figure 1C).

To mimic the PAH-associated vascular stress conditions *in vitro*, human pulmonary artery endothelial cells (HPAECs) were exposed to hypoxia (2% O_2) and TNF-α, either separately or in combination. The combined treatment was performed to create the "double hit" conditions shown to induce a more severe vascular phenotype in experimental PAH models[21,22]. When combined, hypoxia and TNF-α induced a more pronounced increase in KLF6 expression in HPAECs than either factor alone (Figure 1D). Notably, under the same experimental conditions, HPAECs showed reduced expression of KLF2 and KLF4 (Supplementary Figure S2A and B).

KLF2- and KLF4- dependent endothelial adaptation to flow[23] is compromised in PAH[24]. Acute exposure to flow increased KLF6 expression in HPAECs, but its expression pattern differed from those of KLF2 and KLF4. The peak response for KLF6 occurred at later time points following the onset of flow (Supplementary Figure S2C and D), likely indicating distinct roles for these transcription factors in regulating endothelial responses to mechanosensory stimulation.

Altogether these analyses reveal that KLF6 is over-expressed in both *in vitro* and *in vivo* models of PAH and follows a distinct pattern from the previously implicated in PAH KLF2 and KLF4.

KLF6 improves endothelial survival and stimulates angiogenesis.

To investigate the role of stress induced KLF6 activation, KLF6 was overexpressed in HPAECs via adenoviral gene transfer. KLF6 exhibited nuclear localization, both when exogenously expressed and when induced by the onset of flow (Figure 1F and Supplemental Figures S2E, F).

The overexpression of KLF6 inhibited the activation of the pro-inflammatory transcription factor NFκB in HPAECs exposed to hypoxia and TNF-α (Figure 1G and H), while silencing of KLF6 had an opposite effect (Supplemental Figure S3A-C).

KLF6 overexpression enhanced endothelial resilience and improved endothelial survival. KLF6 attenuated hypoxia-, TNF-α- and thrombin-induced breakdown in endothelial barrier function (Figure 1I, J) and inhibited HPAEC apoptosis induced by serum starvation and treatment with staurosporine (STS)[25] (Figure 1K). Conversely, KLF6 silencing markedly enhanced inflammatory activation and apoptosis in HPAECs (Supplementary Figure S3D).

In PAH, endothelial damage is thought to trigger exaggerated repair, which leads to the emergence of highly proliferative, angiogenic, cancer-like cells, contributing to intimal hyperplasia and the formation of plexiform lesions [26].

In addition to its pro-survival effects, KLF6 markedly enhanced endothelial angiogenesis, as evidenced by increased capillary network formation in KLF6-overexpressing HPAECs (Figure 2A-D). Increased angiogenic sprouting was also observed in human pulmonary artery explants transduced with AdKLF6 (Figure 2E, F). Adenoviral transduction efficiency in the arterial endothelium exceeded 80%, similar to the transduction efficiency observed in cultured HPAECs (Supplementary Figure S4).

Angiogenesis is a complex process involving endothelial cell proliferation, migration, and junctional remodelling. To gain further insights into the mechanisms involved, we studied endothelial proliferation and migration following manipulation of KLF6 expression in HPAECs. KLF6 inhibited HPAEC proliferation (~ 2-fold decrease, $p < 0.001$) (Figure 2G), but markedly increased endothelial migration, resulting in accelerated endothelial wound closure *in vitro*. Enhanced endothelial wound repair was observed under both optimal and serum- and growth factor-reduced culture conditions (Figure 2H-I and Supplementary Figure S5 A, B). Conversely, KLF6 silencing markedly reduced endothelial wound repair (Supplementary Figure S5 C, D).

In summary, functional studies suggest that KLF6 is induced under vascular stress conditions to reduce endothelial damage and stimulate endothelial repair and angiogenesis.

KLF6-regulated endothelial transcriptome HPAECs under healthy and disease conditions.

KLFs can act as either transcriptional activators or repressors[27], and the functional assays described above suggest a key role for KLF6 in regulating endothelial gene programs. Thus, we conducted RNA-seq analysis on HPAECs infected with AdCTRL or AdKLF6 (n=4

biological replicates/condition), to characterise the set of endothelial genes regulated by KLF6 (Figure 3A). The viral transduction was optimised to induce a ~6-fold increase in KLF6 protein, comparable with changes seen during placental differentiation[28] and endothelial injury[29]. We detected a total of 6,621 differentially expressed genes (DEGs) in HPAECs overexpressing KLF6 (FDR < 0.05, $\log_2$ |FC| > 0.25), including 3,225 downregulated and 3,396 upregulated genes. This included 624 downregulated and 232 upregulated genes passing a more stringent criteria of FDR <0.01 and $\log_2$ |FC|>2 (Supplementary Figure S6 and Supplementary File S1).

Metascape [30] gene annotation analysis showed that the genes upregulated upon KLF6 overexpression were significantly enriched in annotation terms associated with endothelial homeostasis and function, including angiogenesis, cell migration, wound repair, arterial development and tissue homeostasis. Notably, KLF6 enhanced expression of several key regulators of arterial identity, repair and angiogenesis, including *ERG, BMPR2, SOX17, KDR, TIE1, TIE2, HES1*, *CDH5, PECAM1* and *FLT1*. In contrast, the downregulated genes were mainly enriched in generic terms, including cell cycle progression, DNA replication, and DNA metabolic process (Figure 3B and C). Changes in the expression of selected genes involved in endothelial homeostasis and function were replicated by qPCR in an additional set of HPAECs (Supplementary Figure S7).

Our initial functional analyses suggested that KLF6 modulates the response of HPAECs to hypoxia and TNF-α (Figure 1G, H, Supplemental Figure S3A-C). We therefore set out to investigate at the transcriptional level the impact of KLF6 on the endothelial response to these two key PAH contributory factors.

RNA-seq analysis showed that HPAEC exposure to either hypoxia or TNF-α (10 ng/mL, 24 hours) alone resulted in detectable changes in gene expression. Two hundred genes were modulated by hypoxia (FDR <0.05, $\log_2$|FC|>0.25; comparison AdCTRL vs AdCTRL+ hypoxia), enriched, as expected, in HIF-1 and TGF-β signalling pathways (Supplementary Figure S8A). The response to TNF-α exposure showed 751 DEGs (FDR <0.05, $\log_2$|FC|>0.25; comparison AdCTRL vs AdCTRL+ TNF-α), significantly enriched for TNF-α and NF-κB signalling pathways (Supplementary Figure S9A).

We then compared the transcriptional signatures of HPAECs exposed to hypoxia or TNF-α-induced inflammation with, or without of KLF6 overexpression. KLF6 overexpression under hypoxic conditions revealed 3,405 DEGs (FDR <0.05, log2|FC|>0.25, Supplementary Files

S2-S4) which showed significant association with inflammatory responses, autophagy, pathways controlling cell proliferation, growth factor and oestrogen signalling (Supplementary Figure S8B).

Upon overexpression of KLF6, TNF-α-treated HPAECs showed differential expression of 4,361 genes (FDR < 0.05, $\log_2|FC|> 0.25$, Supplementary Files S5 and S6). The upregulated genes were enriched in FOXO, p53, PI3K-AKT, JAK-STAT, TNF-α and NF-κB signalling pathways. The inhibitory effect of KLF6 on TNF-α-induced NF-κB activity in cultured HPAECs is consistent with previously reported anti-inflammatory actions of KLF6[31-33] and may result from increased expression of NF-κB Inhibitor Alpha (*NFKBIA*). However, KLF6-induced expression of IL-6, typically seen during acute response to endothelial injury, may augment immune responses under sustained vascular stress conditions *in vivo*.[34]

In both treatment groups, KLF6-downregulated genes were enriched in cell cycle and DNA replication, consistent with anti-proliferative effects of KLF6 overexpression seen in cultured HPAECs (Supplementary Figures S8B and S9B).

In summary, transcriptomic profiling and pathway analysis of KLF6-overexpressing HPAECs reaffirms the role of KLF6 as a regulator of arterial identity, endothelial survival, repair and angiogenesis and an upstream regulator of numerous critical genes and pathways implicated in the development of PAH.

Comparative analysis of KLF2-, KLF4 -and KLF6-induced transcriptomic changes in HPAECs reveals unique effects of KLF6

KLF2, 4 and 6 are expressed in the pulmonary endothelium and share numerous structural and functional similarities but their individual roles have not been well delineated [13,17]. To identify shared as well as unique genes regulated by these three transcription factors, RNA-seq was conducted on control HPAECs, as well as HPAECs overexpressing either one of these KLF factors. Adenoviral infection was optimised to ensure a comparable ~6-fold increase in protein expression in all KLFs (Supplementary Figure S10 A-D).

KLFs are major regulators of broad gene expression programs. Indeed, the extent of perturbation of the endothelial transcriptome by overexpression of either KLF2 or KLF4 was similar to that observed in KLF6-overexpressing cells (6,621 DEGs), with 6,655 and 6,703

DEGs for KLF2- and KLF4-overexpressing HPAECs, respectively (FDR <0.05, $\log_2|FC|>0.25$) (Supplementary Figure S10 E, F and Supplementary Files S7 and S8).

We compared all three transcriptomic datasets, focussing on the set of genes showing the strongest differential expression (FDR<0.01, $\log_2|FC|>2$). The analysis revealed individual and shared contributions of KLFs to regulating endothelial transcription. Namely, 214 DEGs unique to KLF6 were enriched in key functions orchestrating endothelial repair such as wound healing, vasculature development, angiogenesis, actin filament bundle assembly, extracellular matrix organization and regulation of glycolytic process (Figure 4A, B, Supplementary Figure S11 A, B and Supplementary Table S1). The 248 DEGs shared among KLF2, KLF4 and KLF6 (Figure 4B, Supplementary Figure S11A, B and Supplementary Table S2) showed enrichment in processes controlling cell proliferation such as DNA metabolism, DNA replication and cell cycle regulation (Figure 4C, D).

Transcription factors are often part of complex regulatory networks whereby they can regulate each other via feedback loops and/or work within a specific hierarchy. We therefore harnessed the transcriptomic datasets corresponding to overexpression of different KLFs to investigate their relationships in HPAECs. The analysis of mutual regulatory interactions revealed that KLF6 increased expression of KLF2, but KLF2 inhibited expression of KLF6 (Suppl. Fig S11 D, F). This result suggests that KLF2, which promotes endothelial quiescence and homeostasis, may act to restrict the actions of KLF6. No significant regulatory relationship was observed between the expression of KLF6 and KLF4 even though KLF4-overexpressing cells appeared to express more KLF6 (p=0.056, Supplementary Figure S11 E, G).

Collectively, these analyses suggest that KLF6 plays a unique role in orchestrating pulmonary endothelial response to injury. KLF6 can function in conjunction with KLF2 and KLF4 to inhibit endothelial proliferation, apoptosis, and inflammatory activation. The net effect of KLF6 activation, supported by functional and transcriptomic analyses, is twofold: minimizing injury and promoting endothelial regeneration.

KLF6-regulated genes in PAH

The transcriptomic analyses described above demonstrate a core role for KLF6 in lung endothelial homeostasis. This prompted us to investigate the relationship between KLF6 and

PAH-relevant genes. DisGeNET disease enrichment analysis[35] revealed significant associations of KLF6-upregulated genes with vascular diseases, including PAH, IPAH and pulmonary emphysema, whereas the down-regulated genes showed links with abnormal arterial morphology (Figure 5A and B). Interestingly, KLF6 altered the expression of numerous genes with known PAH-linked mutations, including *BMPR2, ENG, THBS1, SOX17, KDR, CAV1, ACVRL1, SMAD1, SMAD4, EDN1, ATP13A3, KLK1, NOTCH3, TOPBP1* and *BMPR1B* (Figure 5C). KLF6 DEGs also showed significant overlaps with publicly available sets of differentially expressed genes in PAH and IPAH, including PAH HPAECs and ECFCs from microfluidic models of PAH [14], IPAH PAECs[36] and a previously curated list of PAH-associated genes (named herein as "known PAH genes")[37] (Supplementary Figure S12 and Supplementary Table S3).

Considering that KLF6-induced increase in expression of endothelin-1[38] and TGF-β 1family genes[39] in pulmonary arterial endothelial cells may potentially affect the behaviour of underlaying arterial smooth muscle cells, we studied HPASMC proliferation in co-culture with AdCTRL- and KLF6-infected HPAECs. KLF6 overexpression in HPAECs had a stimulatory effect on HPASMC proliferation only when the cells remained in direct contact with each other through a porous membrane (Supplementary Figure S13), whilst co-culture of spatially separated HPAECs and HPASMCs had no effect (Supplementary Figure S13).

To further investigate the contribution of KLF6 to vascular remodelling in PAH and to analyse site-specific changes in gene expression, spatial transcriptomic analysis of remodelled human PAH vasculature was carried out, with two separate categories, one for the concentric intimal lesions with different degrees of medial and adventitial thickening and the other one for vessels located within the complex plexiform lesions (Figure 6A) [30 ROIs/lung in sections from patients with (n=6)] and equivalent sixed pulmonary arterioles from age- and sex-matched healthy controls (n=5).

A total of 541 genes were detected as differentially expressed in plexiform lesions by comparing the expression profiles of remodelled vessels with those from plexiform lesions (FDR<0.05, $\log_2|FC|>0.25$) (Figure 6B and Supplementary File S9). Remarkably, more than half of plexiform lesion DEGs (433/541 genes) were identified as KLF6-regulated in HPAECs (p= 3.3E-18) (Figure 6B and Supplementary Table S4). Comparison of our data with a recently published spatial transcriptomic analysis of IPAH vasculature[40] showed that

KLF6-regulated genes constituted 65% of top plexiform-specific DEGs, consistent with our findings described in this study (Supplementary Figure S14).

These KLF6-regulated plexiform lesion DEGs displayed significant enrichment in several pathways and functions related to angiogenesis, growth, VEGF signalling, extracellular matrix organization, positive regulation of cell motility, Rho GTPase signalling, cell junction organization, apoptosis, and response to mechanical stimuli (Figure 6B). Furthermore, these DEGs were involved in various processes driving vascular remodelling in pulmonary arterial hypertension, including actin cytoskeleton reorganization, cell proliferation, migration, and ROS metabolic processes (Supplementary Figure S15).

Immunostaining of human PAH lung showed that KLF6 expression was increased predominantly in cells lining the vascular channels in plexiform lesions and displaying endothelial identity markers ERG and vWF. These cells were also noted, albeit less frequently, in and around the remodelled vessels (Figure 6C-G and Supplementary Figures S16 and S17). A marked upregulation of KLF6 expression was also found in PAH ECFCs, compared with the corresponding healthy controls (Figure 6H). PAH blood-derived endothelial colony forming cells (ECFCs) display numerous characteristics of PAH endothelium, including increased proliferative and angiogenic responses [41,42] and are thought to play a contributory role in the formation of angioproliferative lesions in human PAH.

In the healthy human lung, single $KLF6^+$ cells were sparsely dispersed in the lung parenchyma (Supplementary Figure S16 C) but did not exhibit any particular pattern of localization. Human placental tissue, known to express high levels of KLF6[43], served as a positive control (Supplementary Figure S16 E). Lung tissue from a patient with tuberculosis, taken as disease control, showed increased numbers of $KLF6^+$ cells compared with healthy controls (Supplementary Figure S18 C), however, in contrast to PAH lung tissue, these cells did not show a collective pattern of localization. Lung tissues from pulmonary hypertensive MCT rats and hypoxic mice did not show an accumulation of $KLF6^+$ cells (Supplementary Figure S18 A, B), whilst Sugen/hypoxia rats exhibited increased number of $KLF6^+$ cells in the lung (Supplementary Figure S18 A). These cells however did not display the collective organization observed in human PAH lung tissues.

Alveolar capillary dysplasia (ACD) is a rare lung developmental disorder leading to persistent pulmonary arterial hypertension and fatal outcomes in newborns[44]. Recently, Guo et al. reported an integrated analysis of single nucleus RNA-seq (snRNA-seq) of ACDMPV

(n=5) and control lungs (three 3-year-old and three preterm neonate lungs) and identified six EC subtypes, including capillary 1 (CAP1) cells, capillary 2 (CAP2) cells, systemic vascular ECs (SVECs; also known as bronchial vessels), venous ECs (VECs), arterial ECs (AECs), and lymphatic ECs (LECs), in the integrated data [45]. Given the pervasive role of KLF6 in regulating transcriptional programs essential for endothelial homeostasis, and its involvement in PAH-associated vascular remodelling, we performed a single cell comparative analysis of *KLF6* expression in ACDMPV vs. control lungs within each EC subtype using this integrated snRNA-seq dataset. The analysis revealed a disease-related increase in KLF6 expression in arterial ECs, and to a lesser extent in venous ECs, when compared to age-matched controls and only in arterial ECs when compared with preterm neonate controls (n=3/group) (Supplementary Figure S19 and Supplementary Table S5).

These results collectively demonstrate that the accumulation and structural reorganization of KLF6+ endothelial cells are characteristic of human PAH. The elevated expression of KLF6 in arterial endothelial cells in ACDMPV lungs implies a wider relevance of KLF6 upregulation in pulmonary vascular disease.

DISCUSSION

This study identifies KLF6 as a key driver of the endothelial transcriptional response to injury in early pulmonary arterial hypertension (PAH) and a novel regulator of pulmonary vascular remodelling. The proposed role of KLF6 in PAH is illustrated in Figure 7.

Much as its target gene *VEGF*, KLF6 is likely to play a dual role in PAH, with an early protection and a late pathogenic effect[46]. The endothelium-protective and pro-angiogenic effects of KLF6 can be linked with increased expression of arterial identity and blood vessel development genes[29,47], such as SRY-box 17 (*SOX17*), ETS-related gene (*ERG*), VEGF receptors *FLT1* and *KDR*, angiopoietin receptor TIE2 (*TEK*), endoglin (*ENG*), activin receptor-like kinase 1 (ACVRL1), and bone morphogenetic protein receptor 2 (*BMPR2*). In addition, increased expression of endothelial motility inducers, matrix metalloproteinases (*MMP2* and *MMP14*) and junctional proteins VE-cadherin (*CDH5*) and PECAM-1 (*PECAM1*) can facilitate re-establishment of endothelial junctions during endothelial healing and promote maturation of newly formed blood vessels.

A significant association between KLF6 and PAH was confirmed by DisGeNET disease enrichment analysis[30], with KLF6-regulated genes enriched in critical PAH pathways such as FOXO, p53, TNF-α, NF-kB, PI3K-Akt, and Ras signalling [48-52]. Early protective effects of KLF6 may result from a compensatory increase in the expression of PAH-associated genes such as *BMPR2, SOX17, KDR, ENG, CAV1, ACVRL1, SMAD1, SMAD4 and CDH5*[26] as well as increased expression of eNOS, prostacyclin and suppression of glycolysis[53]. Likewise, KLF6 activation may potentially benefit patients with hereditary haemorrhagic telangiectasia (HHT) where loss-of-function mutations in KLF6-upregulated genes, such as *ENG, ACVRL1*, or *SMAD4*, plays a contributory role[54].

Though KLF6 may attenuate endothelial injury in early PAH, its sustained activation is likely to promote vascular remodelling by dysregulation of angiogenesis, increased expression of endothelin-1 and activation of pro-fibrotic and pro-proliferative TGF-β signalling[55]. In support of this scenario, KLF6 was shown to promote neointima formation in denuded mouse femoral arteries[29]. Our data also highlight potential stimulatory effect of KLF6 on medial cell hyperplasia, most likely involving altered gap junctional communication and changes in the extracellular matrix composition, in addition to paracrine signalling[29]. Further, KLF6-induced sustained elevation of IL-6 expression may augment chronic inflammatory responses in PAH [56]. IL-6 expression is transiently increased in response to environmental stress which activates host defense mechanisms to counteract tissue damage. However, chronically elevated IL-6 serum levels in PAH are a predictor of poor outcome, associated with increased inflammation, angiogenesis, vascular remodelling[34] increased expression hepcidin and reduced serum iron levels[56,57].

Spatial transcriptomic analysis of PAH lung vascular tissues has uncovered a significant enrichment of KLF6-regulated genes in molecular pathways and biological processes that drive angiogenesis, including cell motility, VEGFR signalling and extracellular matrix organization. Comparison of our data with a recently published spatial transcriptomic analysis of IPAH vasculature[40] shows that in both studies, KLF6-regulated genes account for more than half of all DEGs found in plexiform lesions.

PAH lung sections showed accumulation of $KLF6^+$ cells within vascular channels in plexiform lesions and, to a lesser extent, in other parts of the remodelled lung. These $KLF6^+$ vWF^+ ERG^+ cells may potentially represent a subpopulation of endothelial progenitor cells, known to accumulate in lung tissues in the end-stage PAH [41,58,59]. Consistent with this premise, we observed a significantly elevated expression of KLF6 in PAH blood-derived endothelial

progenitor cells (also called the late outgrowth colony forming cells; ECFCs). ECFCs are often used as endothelial surrogates in PAH and other cardiovascular diseases [60]. High expression levels of KLF6 in these cells is likely to account for their pro-migratory, pro-angiogenic behaviour[61 62], contributing to the formation of angio-proliferative vascular lesions in PAH lung. Selective overexpression of KLF6 in PAH endothelium is corroborated by recent data showing that only 2 out of 8 endothelial cell clusters in PAH lung display a pro-angiogenic phenotype[63].

By employing an alternative analytical approach, we also analysed single nucleus RNA sequencing data of samples from a distinct condition - alveolar capillary dysplasia characterized by severe pulmonary hypertension[64]. Through this analysis, we verified an elevation in KLF6 expression within pulmonary arterial endothelial cells. The KLF6-induced activation of VEGF/KDR signalling, prominent in both PAH and ACD[45] lungs, may contribute to the disruption of the alveolar microvasculature, observed in both conditions.

KLF6 expression was induced concurrently with the inhibition of KLF2 and KLF4 in PAH lungs and cultured cells. Functional analyses and transcriptional profiling revealed that KLF2, KLF4, and KLF6 share anti-proliferative effects, but KLF6 uniquely stimulates endothelial repair and angiogenesis. Our results suggest that the regulatory relationship between KLF2 and KLF6 is crucial for the timely execution of endothelial repair. In the proposed scenario, the KLF6-induced increase in KLF2 expression suppresses KLF6 once endothelial repair is complete, thereby restoring endothelial homeostasis. This regulatory feedback response is unlikely to operate in PAH, where the expression and activity of KLF2 are chronically suppressed. Though we did not see a direct relationship between KLF4 and KLF6 expression, these two transcription factors are likely to interact via their transcriptional targets. The N-terminal domain of KLF6 is responsible for recruiting and interacting with different transcription factors and cofactors, including Sp1, KLF4, p53, hypoxia-inducible factor 1 alpha (HIF1α), runt related transcription factor 1 (RUNX1), E2F1, GTF3C1 and histone deacetylase 3 (HDAC3), to regulate the transcription process in a context-dependent manner[17]. Characterizing the complex cross-talk between KLF2, KLF4, and KLF6 under healthy and disease conditions will require further investigation.

Whilst selective pharmacological inhibitors or activators of KLF6 have not been identified, in cancer cells KLF6 expression was regulated by a number of factors, including large HIF2α-activated and H3 acetylation-dependent super enhancer[65], miR-191-5p, miR-200c-3p, miRNA-543 [66-68], long non-coding RNA CR749391 [69], transcription factor SP2 and HIF-1[70]

[71,72]. Pharmacologically, KLF6 is upregulated by Azilsartan, an angiotensin II receptor antagonist [73] as well as non-steroidal anti-inflammatory drug Naproxen and phosphodiesterase type-4 (PDE4) inhibitor Apremilast [74,75].

In summary, we are first to identify KLF6 as a regulator of key processes orchestrating vascular remodelling in PAH. KLF6 activation is a prominent feature of apoptosis-resistant, angio-proliferative pulmonary vascular phenotype in human PAH. Dysregulation of KLF6 signalling may have broader implications for pulmonary vascular disease.

NOVELTY AND SIGNIFICANCE

What is known.

- Pulmonary arterial hypertension (PAH) is a severe and currently incurable lung condition characterized by progressive pulmonary vascular remodelling.
- Endothelial damage followed by excessive repair contributes to the formation of occlusive angioproliferative lesions, but the mechanisms are unclear.
- Krüppel-like transcription factors 2 and 4 (KLF2 and KLF4) have been implicated in PAH, but the role of KLF6, involved in tissue injury response and cancer, has not been investigated.

What new information does this article contribute.

- KLF6 is activated by hypoxic and inflammatory triggers in early PAH.
- KLF6 uniquely orchestrates endothelial repair, but its sustained activation promotes development of apoptosis-resistant, angio-proliferative vascular phenotype in human PAH.
- Dysregulation of KLF6 signalling may have broader implications for pulmonary vascular disease

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AUTHOR CONTRIBUTIONS

RA designed and performed experiments, analysed data, and wrote the manuscript; NF, HM analysed RNA-seq data, IC analysed RNA-seq data and critically evaluated manuscript; NM, NL, AL and MK analysed spatial transcriptomics data and critically evaluated manuscript, LZ and CNC provided lung tissues from pre-clinical PAH and critically evaluated manuscript, MAS provided surgical samples of pulmonary arteries, JAW and MG provided and analysed ACD snRNA-seq data patient data and critically analysed evaluated manuscript; B.W.S. conceived the study, secured funding, performed experiments and wrote the manuscript.

DATA AVAILABILITY

The RNA-sequencing data generated and analysed during this study are currently being uploaded to European Genome-phenome Archive (EGA). All data generated or analysed during this study are included in this published article (and its supplementary information files) or are available from the corresponding author upon request.

COMPETING INTERESTS

The authors declare no competing interests.

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MAIN FIGURES

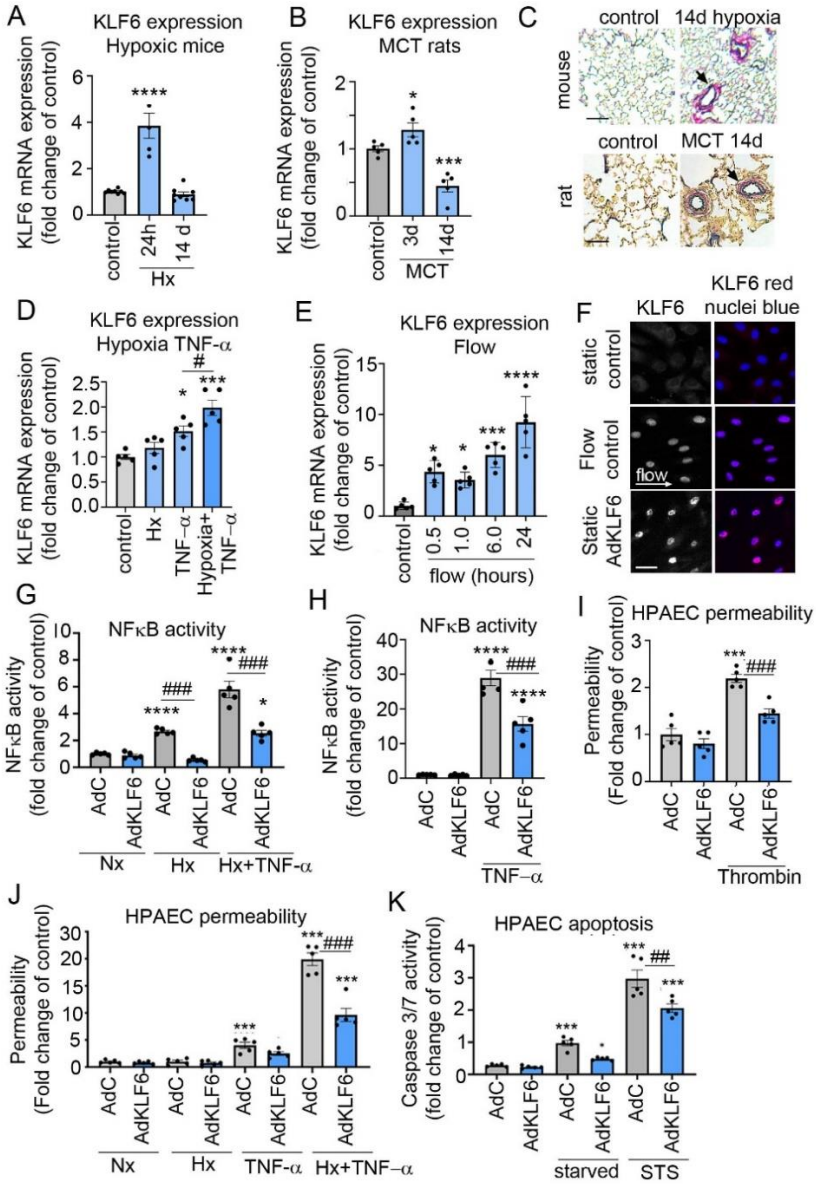

Figure 1. KLF6 in endothelial responses to PAH-associated stress conditions. **(A)** KLF6 mRNA expression changes in hypoxic mice (10% O_2) and **(B)** MCT rats at different experimental time points, as indicated. **(C)** Corresponding representative images of healthy and PAH mouse and rat lung tissues; arrows point to muscularised vessels (EVG); Bar=25 µm. **(D)** KLF6 mRNA expression in HPAECs exposed to hypoxia (2% O_2, 24h) with, or without TNF-α (10 ng/ml, 24h) or **(E)** in HPAECs exposed to flow (4 dynes/cm^2 0-24h). n=5. **(F)** Localization of KLF6 in HPAECs under flow or HPAECs infected with AdKLF6. In merged images, nuclei are blue and KLF6 is red, confocal microscopy. Scale bar = 10 µm. **(G)**, (**H**) and **(I)** show effects of KLF6 overexpression on NF-κB activity in HPAECs treated with TNF-α (10ng/ml, 24h), hypoxia (2% O2, 24h) or thrombin (1U/mL, 1h), as indicated. AdCTRL: adenoviral control; AdKLF6: adenoviral KLF6. **(J)** Effect of KLF6 overexpression on endothelial permeability in hypoxia (Hx) and TNF-a-treated HPAECs, as indicated. **(K)** Effect of KLF6 on starvation- and staurosporine (STS)-induced endothelial apoptosis. In all graphs, bars show mean fold-changes of control±SEM. KLF6 expression was normalised to B2M. *P<0.05, ***P<0.001, ****P<0.0001, comparisons with untreated or viral controls, as appropriate. #P<0.05, ##P<0.01, ###P<0.001, comparison with corresponding treatment controls, as indicated. One-way ANOVA with Tukey's post-hoc test; (A) n=4-7, (B-K) n=5.

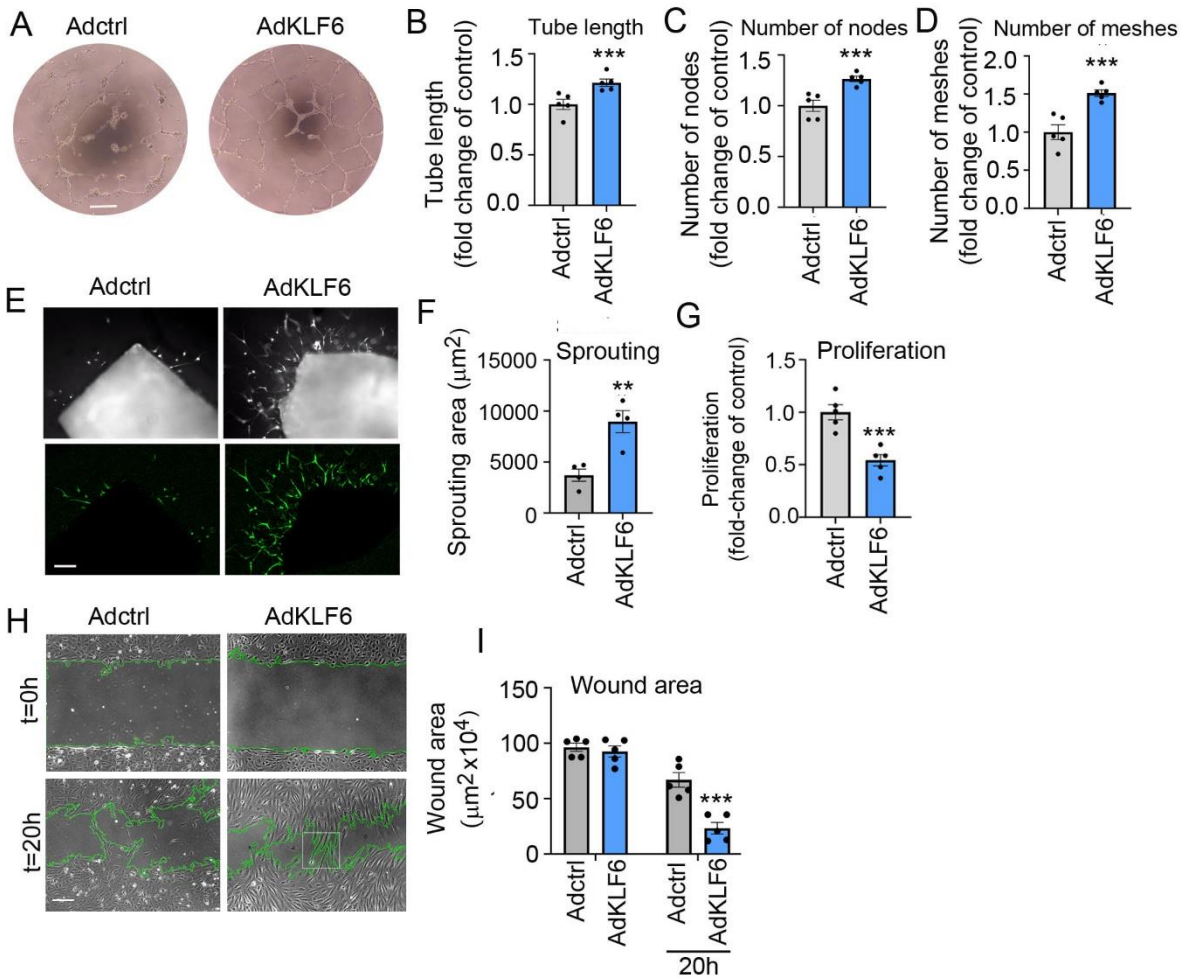

Figure 2. Effects of KLF6 on endothelial angiogenesis, proliferation and migration. (A) Images of tube formation in AdCTRLand AdKLF6-treated HPAECs; phase contrast microscopy. Scale bar = 50μm. **(B)** Total tube length, **(C)** number of nodes and **(D)** meshes in HPAECs treated, as indicated. ImageJ with Angiogenesis Analyzer plugin. **(E)** Sprouting angiogenesis in human pulmonary artery explants treated with AdCTRLor AdKLF6. Explants were labelled with Vybrant CFDA SE cell tracker green, fluorescent microscopy. Scale bar=50μm. **(F)** Sprouting area in explants treated, as indicated. n=4 donors; 8 explants/donor were analysed. **(G)** HPAEC proliferation; EdU incorporation assay. Images in **(H)** and corresponding graph in **(I)** show endothelial wound healing in control and KLF6-overexpressing HPAECs. Boxed area shows well polarised AdKLF6-overexpressing cells migrating into the wound area. Wound edges are traced in green. In (B, D, F, G) ***P<0.001, comparison with adenoviral controls, unpaired t-test; In (I) **P <0.01, ***P<0.001, comparison with adenoviral controls; two-way ANOVA with Tukey's post-hoc test; n=4-5. Bars show mean ±SEM.

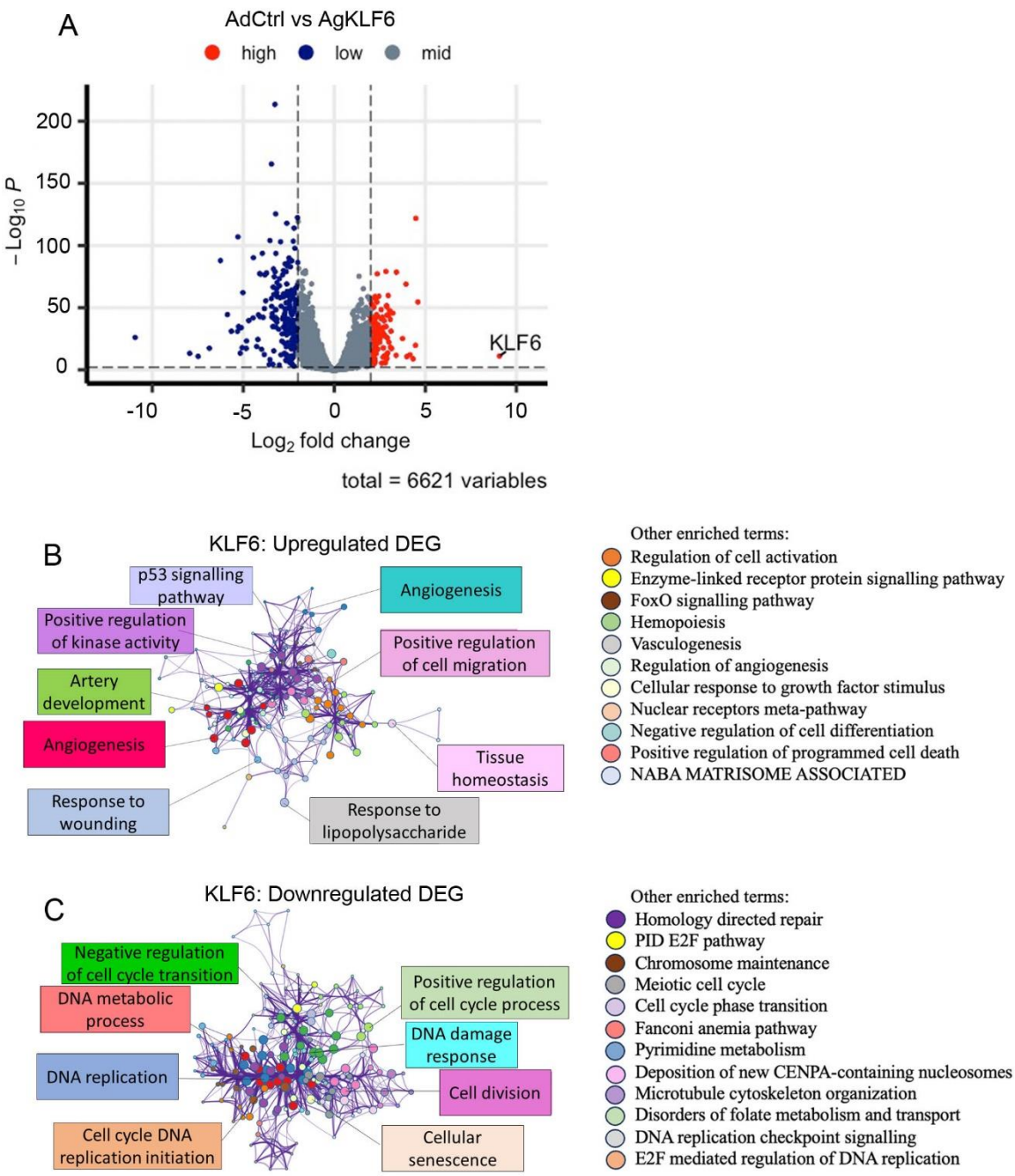

Figure 3. Enrichment analysis of KLF6 DEGs. **(A)** Volcano plot showing differentially expressed genes (DEGs) in AdKLF6 and AdCTRL treated HPAECs. Downregulated genes are blue and upregulated genes are red. $p<0.05$ and Log_2 fold change <-0.25 or >0.25. Metascape network of enriched terms in **(B)** upregulated and **(C)** downregulated DEGs coloured by cluster ID.

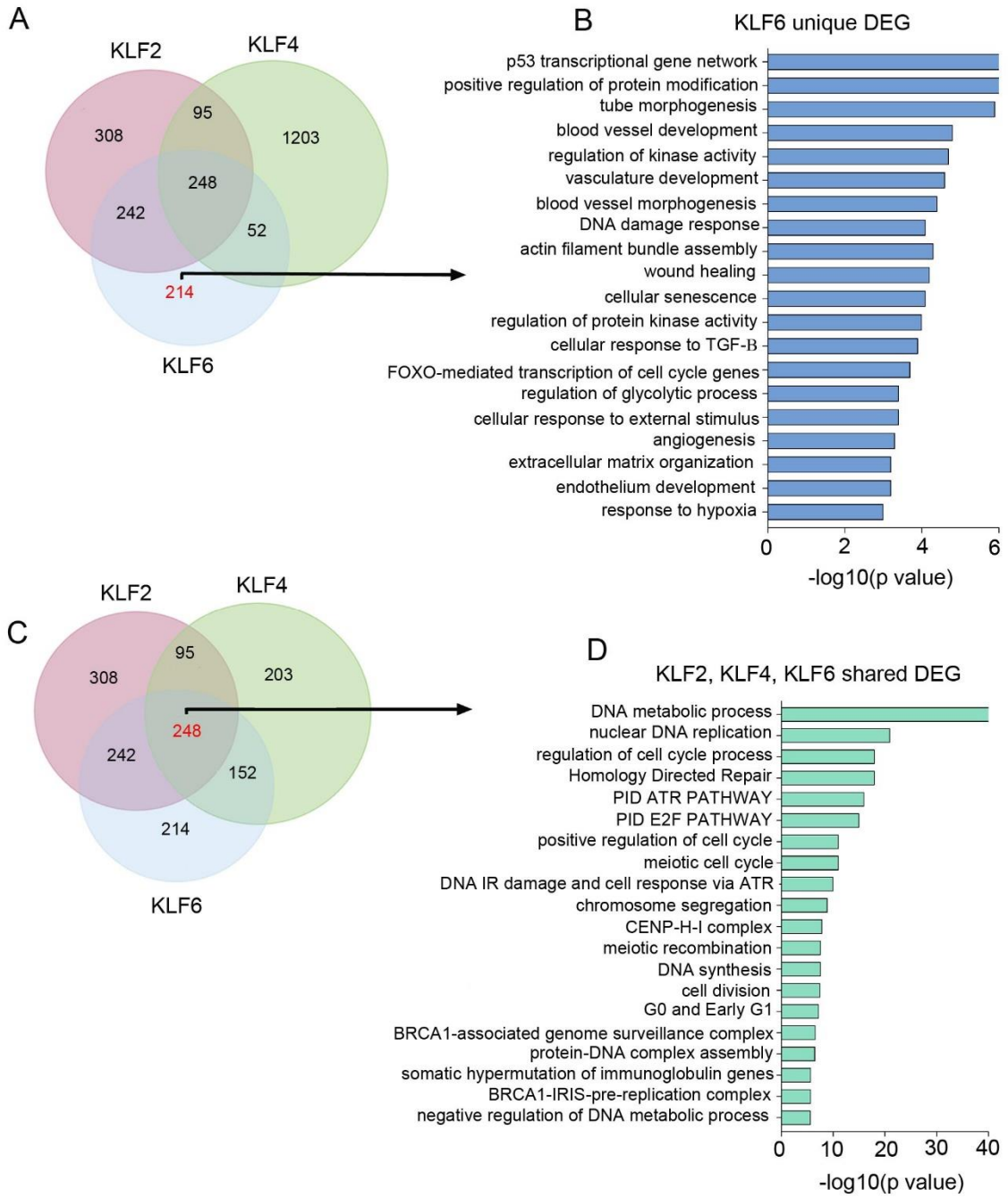

Figure 4: Metascape pathway and functional enrichment analysis of shared and unique KLF6-regulated DEGs. (A) Venn diagram shows the number of KLF2-, KLF4- and KLF6-regulated DEGs, with DEGs unique to KLF6 marked in red. **(B)** A corresponding bar graph shows Metascape pathway and process enrichment analysis of unique KLF6-regulated DEGs. **(C)** Venn diagram shows the number of shared KLF2-, KLF4- and KLF6-regulated DEGs in HPAECs, where shared DEGs are marked in red. **(D)** Metascape pathway and process enrichment analysis of shared DEGs. DEG selection criteria were FDR<0.01, log2|FC|>2

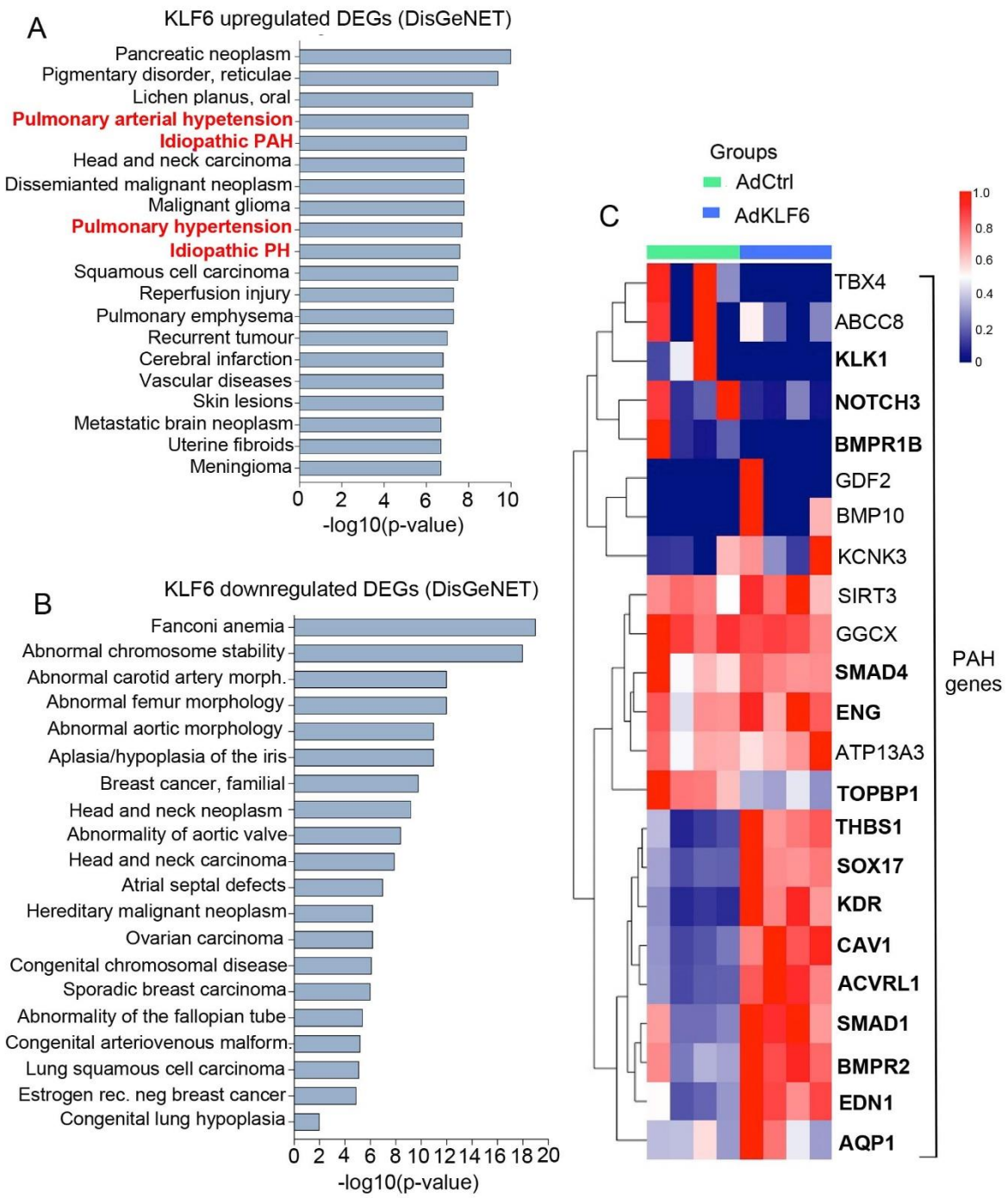

Figure 5. DisGeNET enrichment analysis of KLF6-regulated DEGs. Disease gene enrichment analysis of **(A)** upregulated and **(B)** downregulated KLF6 DEGs. Pulmonary hypertension terms are highlighted in purple **(C)** Heatmap shows colour-coded changes in expression of PAH-associated genes, with hierarchical clustering in control and KLF6-overexpressing HPAECs. DEGs are marked in bold. Each column represents one experimental repeat; n= 4/group.

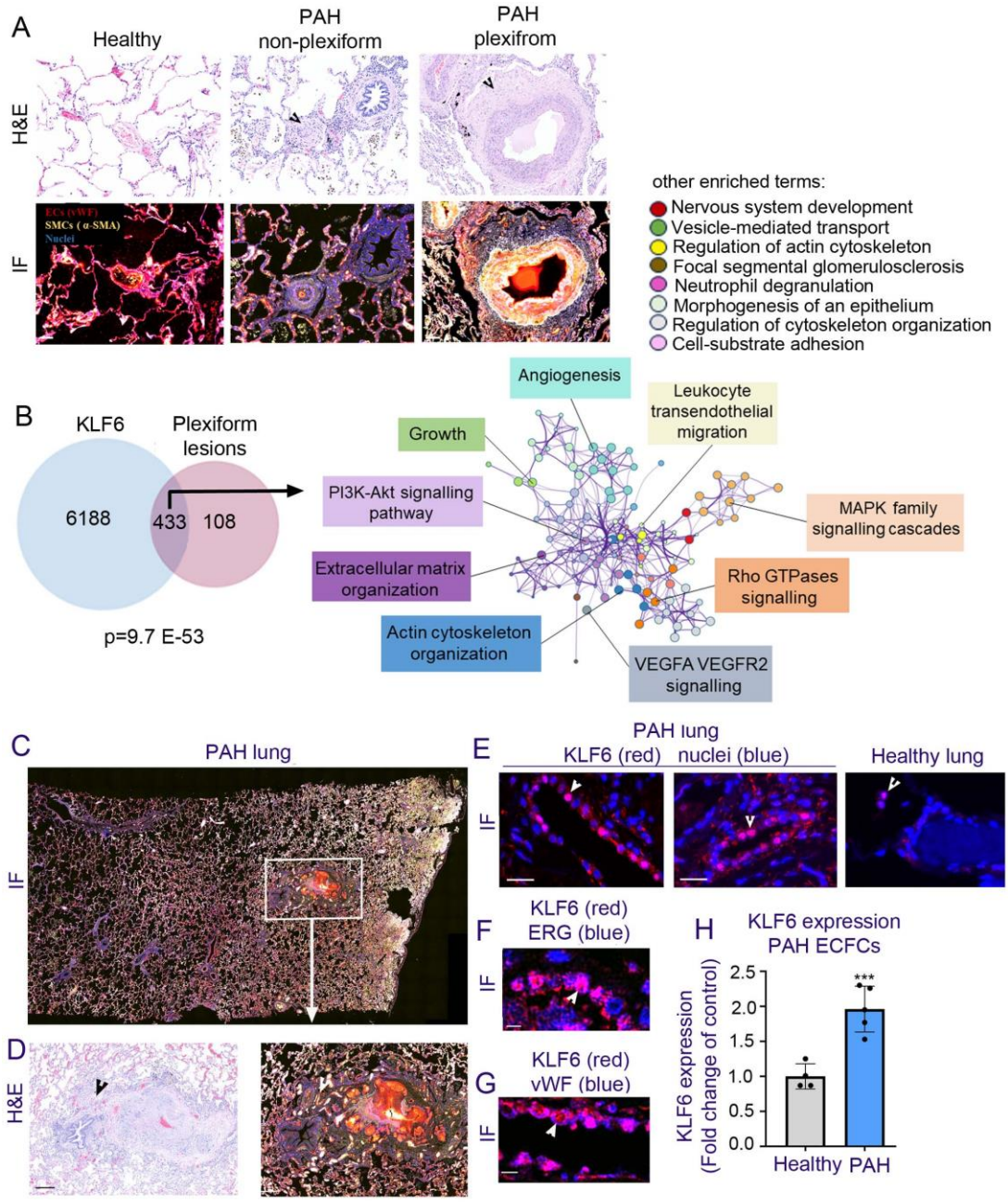

Figure 6. KLF6 in PAH endothelium. (A) Representative examples of healthy lung tissues, non-plexiform remodelled vessel and plexiform lesion in PAH lung used for spatial transcriptomics. H&E and IF staining was used to guide the selection of ROIs in GeoMx Digital Spatial Profiler. Von Willebrand factor (vWF; red), α-smooth muscle actin (α-SMA; yellow) and (SYTO 13, blue). 30-45 ROIs were selected in lung sections from healthy (n=5) and PAH (n=6) individuals, with separate annotation for non-plexiform plexiform lesions. **(B)** Venn diagram showing an overlap between KLF6-regulated DEGs in HPAECs and plexiform lesions DEGs identified by spatial transcriptomics; P= 9.7E-53, Fisher's exact test in the GeneOverlap package in R. The associated Metascape network of enriched terms in the shared pool of DEGs coloured by cluster ID, is shown on the right. FDR<0.05, log2|FC|>0.25 **(C)** PAH lung section (Immunofluorescence) with a plexiform lesion within the boxed area. **(D)** Enlarged images of the plexiform lesion (H&E and IF staining), with arrowheads pointing to the area enlarged in (E). **(E)** Localization of KLF6 in in PAH and healthy lung, as indicated. KLF6 is red and nuclei are blue; Arrowheads point to nuclear localization of KLF6 (pink). **(F, G)** show KLF6, vWF and ERG staining, as indicated. Scale bars in (D) =200 µm, in (E)= 50µm and in (F, G)=10 µm. **(H)** KLF6 mRNA expression in healthy and PAH ECFCs. Bars are means ±SEM, ***P<0.001, n=4-5, Student t-test.

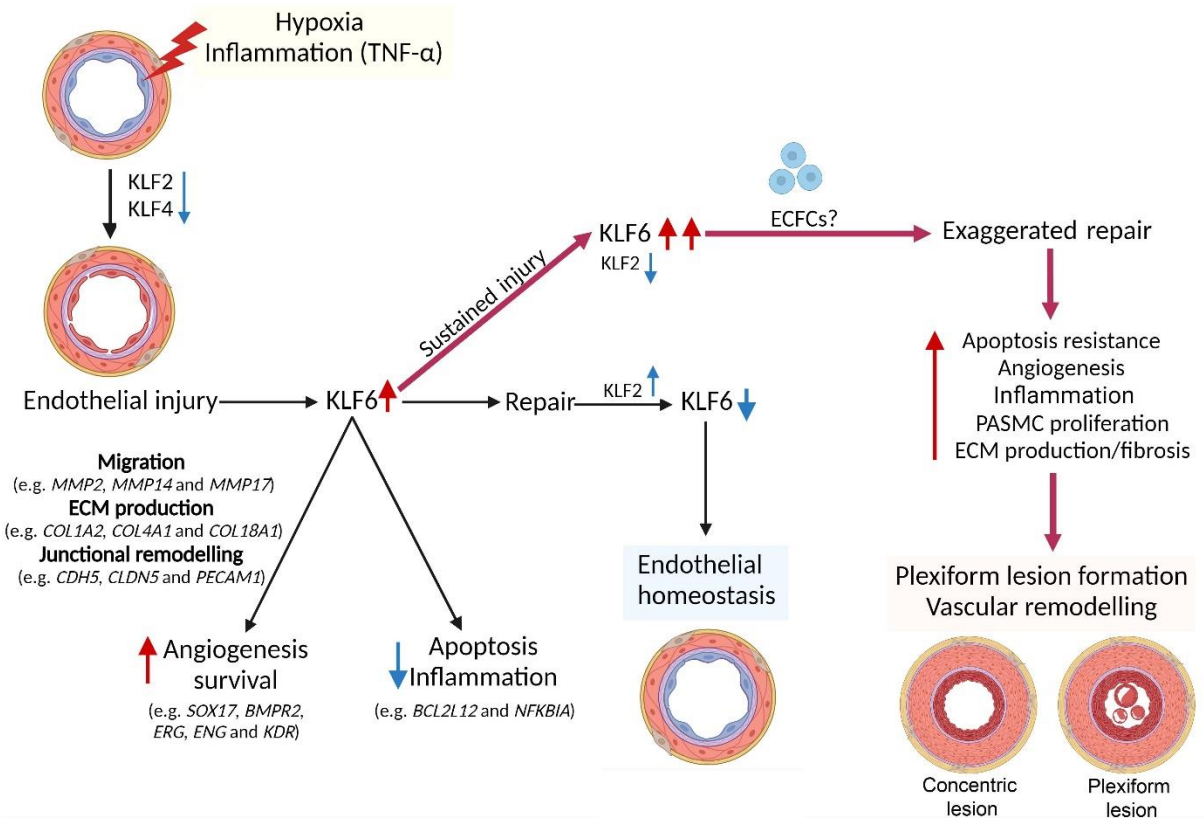

Figure 7. Schematic diagram of the proposed role of KLF6 in PAH. KLF6 is activated in response to endothelial injury to orchestrate the process of endothelial repair. This process is characterized by increased endothelial cell survival, angiogenesis, reduced apoptosis, and reduced inflammatory response. Under normal, healthy conditions, KLF6 activity is downregulated once the process of endothelial repair is complete. However, its continued activation under chronic hypoxic and inflammatory conditions induces endothelial apoptosis resistance, angiogenesis, fibrosis, inflammation and PASMC proliferation, augmenting vascular remodelling. Examples of KLF6-regulated genes are shown in italics. Further detail can be found in Discussion