Functional and metabolic characterization of endothelial cells in chronic thromboembolic pulmonary hypertension

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CHAPTER 2

ENDOTHELIAL DYSFUNCTION IN PULMONARY HYPERTENSION: CAUSE OR CONSEQUENCE?

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ABSTRACT

Pulmonary arterial hypertension (PAH) is a rare, complex, and progressive disease characterized by abnormal remodelling of the pulmonary arteries that leads to right ventricular failure and death. Although our understanding of the causes for abnormal vascular remodelling in PAH is limited, accumulating evidence indicates that endothelial cell (EC) dysfunction is one of the first triggers initiating this process. EC dysfunction leads to the activation of several cellular signalling pathways in the endothelium, resulting in uncontrolled proliferation of ECs, pulmonary artery smooth muscle cells and fibroblasts, and eventually leads to vascular remodelling and occlusion of the pulmonary blood vessels. Other factors that are related to EC dysfunction in PAH are an increase in endothelial to mesenchymal transition, inflammation, apoptosis, and thrombus formation. In this review, we outline the latest advances on the role of EC dysfunction in PAH and other forms of pulmonary hypertension. We also elaborate on the molecular signals that orchestrate EC dysfunction in PAH. Understanding the role and mechanisms of EC dysfunction will unravel the therapeutic potential of targeting this process in PAH.

Keywords: Pulmonary hypertension – endothelial cell dysfunction – vasoactive factors – TGF-β – EndoMT – epigenetics

INTRODUCTION

Pulmonary hypertension (PH) is a condition defined by a mean pulmonary arterial pressure of more than 20 mmHg at rest and 30 mmHg during exercise. The range of genetic, molecular, and humoral causes that can lead to this increase in pressure is extensive. Therefore, PH is grouped into different classes based on clinical and pathological findings as well as therapeutic interventions [1,2]. The World Health Organization (WHO) classifies PH into five groups, namely: 1. Pulmonary arterial hypertension (PAH), 2. Pulmonary hypertension due to left heart disease (PH-LHD), 3. Pulmonary hypertension due to lung disease (PH-LD), 4. Chronic thromboembolic pulmonary hypertension (CTEPH), 5. Pulmonary hypertension due to unclear and/or multifactorial mechanisms [1,3,4]. PH is becoming more and more a global health issue due to the ageing population. Although PH-LHD and PH-LD are the most prevalent PH groups, research and drug development focuses mainly on PAH and CTEPH, which are rarer diseases that affect mainly younger people [5]. Because of the amount of research conducted in PAH compared to the other four groups, this review will focus mostly on PAH.

PAH is characterized by remodelling of distal pulmonary arteries, causing a progressive increase in vascular resistance. Vascular remodelling is associated with alterations in vasoconstriction, pulmonary artery- endothelial cells (PAECs) and -smooth muscle cells (PASMCs) cell proliferation, inflammation, apoptosis, angiogenesis and thrombosis, which leads to muscularization and occlusion of the lumen of pulmonary arteries by formation of vascular lesions. Plexiform lesions are the most common lesions in PAH, characterized by deregulated endothelial cell (EC) proliferation. Other lesions in PH are thrombotic lesions and neointima formation, which form a layer of myofibroblasts and extracellular matrix between the endothelium and the external elastic lamina [6]. One of the first triggers for development of PAH is EC injury triggering the activation of cellular signalling pathways that are not yet completely understood.

In normal conditions the endothelium is in a quiescent and genetically stable state. However, different types of injury can activate the endothelium. When activated, the endothelium secretes different growth factors and cytokines that affect EC and SMC proliferation, apoptosis, coagulation, attract inflammatory cells or affect vasoactivity to restore homeostasis. EC dysfunction, the loss of cellular functions leading to pathological changes, is crucial in the development of cardiovascular diseases and so too in PAH [7,8]. Many different

factors have been suggested to be involved in the initiation of EC dysfunction in PAH, like shear stress, hypoxia, inflammation, cilia length, and genetic factors (**Figure 1**) [6,9-11]. In PAH the endothelium switches from a quiescent to an overactive state where it starts to secrete vasoconstrictive factors like endothelin-1 (ET-1) [12] and thromboxane [13], and proliferative factors like vascular endothelial growth factor (VEGF) and reduce the secretion of vasodilators like nitric oxide (NO) and prostacyclin, indicating that EC dysfunction might play an central role in the pathogenesis of PAH [7,14].

The purpose of this review is to provide a state-of-the-art overview on EC dysfunction in PAH and to highlight current progress made in understanding this phenomenon. At last, this review discusses several models for studying EC dysfunction in PH and explores possible molecular targets and drugs for restoring EC function in PH.

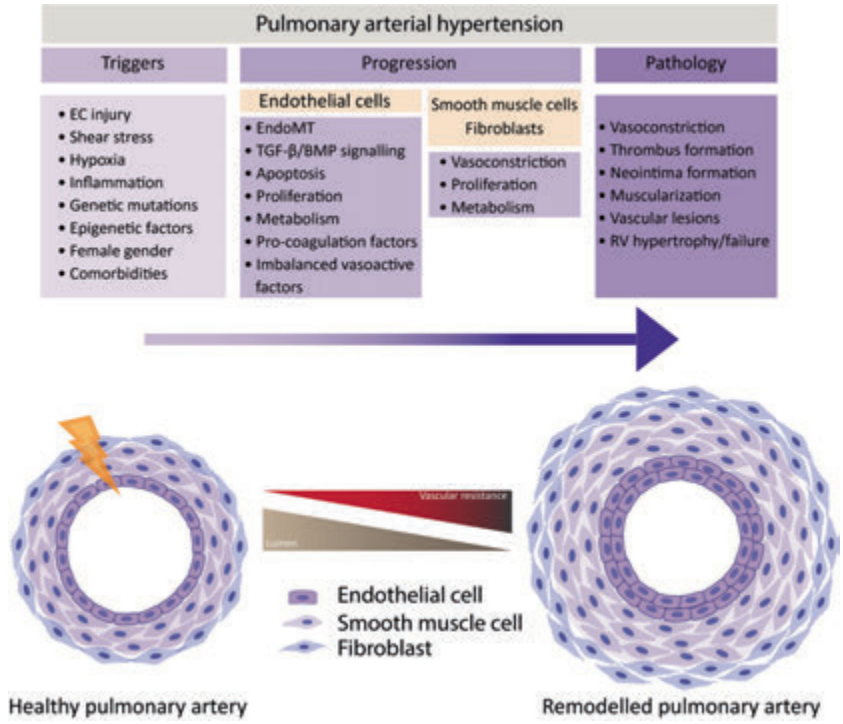

Figure 1. Pulmonary artery remodelling, vascular resistance and pulmonary arterial hypertension (PAH) development. PAH results from a progressive increase in vascular resistance caused by pulmonary vascular remodelling. Molecular mechanisms behind the process of vascular remodelling are still not fully elucidated but endothelial cell (EC) injury is thought to be one of the early triggers. EC injury can be caused by shear stress, hypoxia and inflammation. Host factors such as genetic mutations and gender but also epigenetic factors and comorbidities are thought to play an important role in EC dysfunction. EC dysfunction leads to altered cell signalling that induces cellular processes such as EndoMT, apoptosis and proliferation. In addition, changes are found in cell metabolism and in the secretion of vasoactive, coagulation and thrombotic factors. Also vascular smooth muscle cells and fibroblasts are found to display a diseased cellular phenotype. EC dysfunction eventually promotes vasoconstriction, thrombus formation, neointima formation, muscularization and development of vascular lesions. As lumen size decreases, pulmonary vascular resistance increases and induces right ventricle (RV) hypertrophy with eventually RV failure.

FACTORS CONTRIBUTING TO EC DYSFUNCTION IN PAH

Approximately 80% of familial PAH (hPAH) and 20% of idiopathic cases of PAH (iPAH) are associated with mutations in the bone morphogenic type 2 receptor (BMPR2) but a penetrance of 20-30% suggests secondary stimuli such as endothelial to mesenchymal transition (EndoMT), inflammation, thrombosis, apoptosis and perturbations in vasoactivity as important contributors to EC dysfunction and PAH development [15-17].

Bone morphogenic type 2 receptor

BMPR2 encodes for a transmembrane serine/threonine kinase receptor belonging to the transforming growth factor-β (TGFβ) family of signalling proteins (**Figure 2**) [18]. BMPR2 modulates cellular growth, apoptosis, inflammation and differentiation via binding of bone morphogenetic proteins (BMPs) to a heteromeric complex of a BMP type-I receptor and BMPR2, in a time, concentration and cell type dependent manner [19]. Depending on the localization in the vascular bed, BMPR2 promotes survival of PAECs, while it has an anti-proliferative effect on PASMCs [20-22].

To date over 380 PAH related mutations in *BMPR2* are known, mostly loss of function mutations [23,24]. Low penetrance of disease development associated with *BMPR2* mutations observed in humans has also been confirmed in experimental models of PH, where *BMPR2* deletion alone does not induce PAH in the majority of the cases [25-27]. Interestingly, reduced levels of BMPR2 have also been found in PH patients without *BMPR2* mutations, suggestion additional involvement of genetic modifiers or environmental factors reducing BMPR2 dependent signaling [28-31].

BMPR2 is predominantly present in ECs lining the vascular lumen in the lung and expression is reduced in ECs from PH lung. Therefore mutated *BMPR2* is postulated to play a significant role in EC dysfunction in PAH [24,28]. Association between endothelial BMPR2 and PAH development was further supported by the observation that mice with endothelial specific deletion of *BMPR2* were prone to develop PAH [32,33]. PAECs overexpressing a kinase-inactive BMPR2 mutant show increased susceptibility to apoptosis and conditioned medium from these PAECs stimulated proliferation of PASMCs via increased release of TGFβ1 and fibroblast growth factor (FGF)-2 [34]. BMP9 administration selectively enhanced endothelial *BMPR2* and reversed PH in rats [35]. In line with these findings, several compounds attenuated EC dysfunction via increased BMPR2 signalling and reduced abnormal remodelling in experimental PH[36-38]. Moreover, BMPR2 acts as a gatekeeper to protect ECs from increased TGFβ responses and integrin-mediated mechano-transduction [39].

Loss of endothelial BMPR2 promotes release of pro-inflammatory cytokines in a SOD3-dependent manner, allowing leukocyte transmigration to underlying tissues, causing further vascular remodelling *in vivo* [25,40,41]. Furthermore, loss of BMPR2 signalling in PAECs promotes a pro-inflammatory state during normoxia by enhancing mitochondrial biogenesis, mitochondrial potential and promoting glycolysis [42]. BMPR2 deficiency in iPAH PAECs lacking BMPR2 are associated with loss of DNA damage control via reduced DNA repair related genes such as BRCA1. Increased DNA damage reciprocally leads to further reduction of BMPR2 expression and EC dysfunction [43]. Transcriptome analysis of PAECs from iPAH patients revealed a correlation between reduced BMPR2 levels and downregulation of β-catenin, resulting in reduced Collagen-4 (COL4) and ephrinA1 (EFNA1) expression [44]. Both COL4 and EFNA1 perform intertwining roles in endothelium structure. siRNA mediated silencing of *BMPR2* in PAECs resulted in increased PAEC proliferation, migration, and disruption of cytoskeletal architecture. One of the changes observed was increase in Ras/Raf/ERK signalling, and Ras inhibitors, like nintedanib [45], reversed the enhanced proliferation and hypermotility of BMPR2 silencing in PAECs [46].

Carboxylesterase-1 (CES1) promotes BMP signalling by ensuring proper trafficking of BMPR2 from the endoplasmic reticulum (ER) to the plasma membrane [47]. CES1 is reduced in iPAH patients and impaired ER trafficking will result in decreased BMPR2 availability [47].

Pro-inflammatory cytokines, such as IL-6 and TNFα, have also been found to downregulate BMPR2 expression in PAECs via a STAT3-miR-(Cluster 17/92) and NF-κB-p65 pathway,

respectively [29,48]. Finally, miRNA-21, although primarily induced by BMPR2 signalling, negatively targets BMPR2 expression [49].

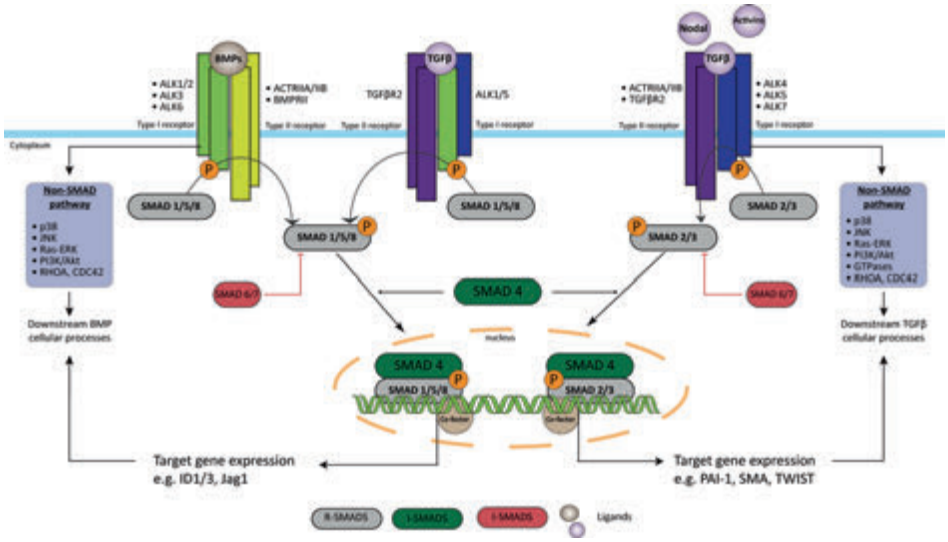

Figure 2. TGF-β superfamily signalling in PAH. The TGF-β superfamily is subdivided into the TGF-β group that include TGFβ, Nodal and activins and the BMP group that includes BMPs. Both groups signal through intracellular mediators, known as Smads. Receptor-regulated Smads (R-Smads) are phosphorylated by type-1 receptors (e.g. ALK1/2/3/6 and ALK4/5/7) and form complexes with a Common mediator Smad (Co-Smad). Subsequently, these complexes translocate into the nucleus where they induce transcriptional responses that alter gene expression of specific targets that influence apoptosis, cell differentiation, inflammation and proliferation. Inhibitory Smads (I-Smads) negatively regulate TGF-β and BMP signalling. Both TGF-β and BMP receptors can also signal independently from Smads and alter downstream cell-specific processes. It is know that TGF-β superfamily signalling plays an important role the initiation of EndoMT by triggering overexpression of genes like TWIST1, αSMA and phospho-vimentin.

Endothelial to mesenchymal transition

EndoMT is a phenomenon where ECs acquire a mesenchymal-like phenotype which is accompanied with loss of endothelial markers and gain of mesenchymal markers. In addition, ECs lose cell-cell contact, change their morphology and adopt a highly migratory and invasive phenotype (**Figure 3A**) [50,51]. In lungs of human PAH patients and monocrotaline (MCT) and Sugen/hypoxia (SuHx) experimental PH rat models, EndoMT was observed whereby cells express high levels of α-SMA and activated phospho-vimentin and VE-cadherin, indicating their endothelial origin [52-54]. Moreover, TWIST1, a key transcription factor in inducing EndoMT, is highly expressed in human PAH lungs compared to healthy lungs [52].

TGFβ treatment of PAECs induces expression of the EndoMT transcription factors TWIST1 and SNAIL1[50,55] and the mesenchymal markers α-SMA and phospho-vimentin[56]. TWIST1 increases

expression of TGFβ, leading to enhanced TGFβ signalling[57]. In addition, reduced BMPR2 signalling promotes EndoMT via upregulation of the High Mobility Group AT-hook 1 and its target gene SLUG, independently of TGFβ signalling [58]. More interestingly, BMP-7, a protein previously described as having anti-inflammatory and anti-tumour effects in several diseases, was attenuated hypoxia-induced EndoMT in PAECs both *in vivo* and *in vitro* by inhibiting the m-TORC1 signalling pathway[59]. BMPR2 favours EndoMT allowing cells of myo-fibroblastic character to create a vicious feed-forward process leading to hyperactivated TGFβ signalling[39]. In summary, alterations in TGFβ/BMP signalling are linked to the process of EndoMT observed in PAH [60].

Hypoxia is also an inducer of EndoMT through hypoxia-inducible transcription factor-1α (HIF-1α) and HIF-2α, and both transcription factors are increased in PAH [61,62]. PAH ECs display increased expression of HIF-2α, leading to SNAIL upregulation [54]. In addition, HIF-1α knockdown alone effectively blocks hypoxia-induced EndoMT but also knockdown of its downstream target gene TWIST1 showed effective blockage of hypoxia-induced EndoMT in microvascular ECs (MVECs), however less pronounced[63]. Nonetheless, microvascular endothelium may differ from arterial endothelial function.

Inflammation

Pulmonary arteries of PAH patients showed infiltration of macrophages, dendritic cells and lymphocytes into the plexiform lesions and an increased migration of monocytes[9,64]. Increased levels of pro-inflammatory cytokines and chemokines, such as IL-1β, TNFα and IL-6, known activators of vascular endothelium, were found (**Figure 3B**) [37,65,66]. IL-1β stimulates endothelial ET-1 production [67]. Administration of IL-6 to experimentally induced PAH in a rat model and overexpression of IL-6 in transgenic mice led to occlusion of pulmonary arteries and RV hypertrophy [68,69]. IL-33 has a dual role as cytokine and a role in the nucleus [70]. Nuclear IL-33 is expressed in nuclei of healthy ECs but is less expressed in nuclei of ECs from iPAH lungs. Nuclear IL-33 modulates gene expression of pro-inflammatory cytokines and IL-33 knock-down in PAECs upregulates expression of IL-6. Therefore, loss of nuclear IL-33 could contribute to EC dysfunction in PAH [70]. Additionally IL-33 may contribute to inflammatory activation of the endothelium by promoting endothelial production of granulocyte macrophage-colony stimulating factor (GM-CSF) and macrophage-CSF [71]. Hypoxia induces

expression of IL-33 and its receptor ST2 on ECs, leading to EC and SMC dysfunction with concomitant PH development [72].

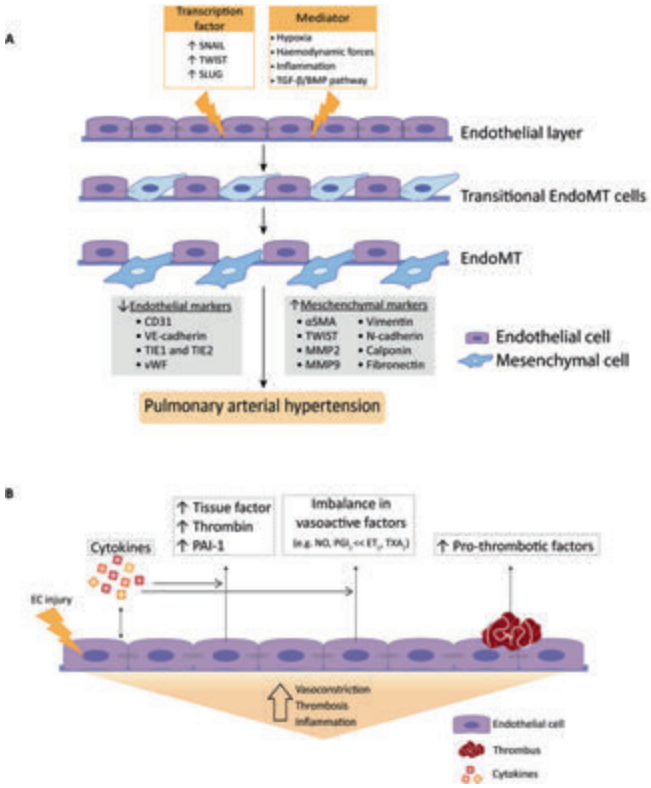

Figure 3. Endothelial to mesenchymal transition (EndoMT) and endothelial dysfunction in PAH. **A)** Upon activation by transcriptional factors, hypoxia, haemodynamic forces, inflammation and TGF-β/BMP pathway signaling pulmonary endothelial cells (PAECs) undergo cellular transition to a mesenchymal phenotype. During transition, PAECs lose endothelial markers and gain mesenchymal markers such as αSMA and TWIST. These mesenchymal-like cells also gain mesenchymal characteristics that trigger vascular remodeling and PAH pathogenesis. **B)** Upon endothelial cell injury, PAECs become dysfunctional and alter their secretion of cytokines and other factors that regulate coagulation, thrombosis and vascular tone. A failure of PAECs in maintaining vessel homeostasis promotes vasoconstriction, thrombosis and inflammation that initiate PAH disease progression.

Thrombosis and coagulation

In situ pulmonary artery thrombosis is regularly found in PAH. However, it remains unclear if thrombosis due to EC dysfunction causes progression of PAH or whether it forms as a result of it. Various factors, e.g. von Willebrand factor, plasminogen activator inhibitor-1 and tissue factor (TF), secreted by EC, have been implicated in coagulation and are found differently expressed in PAH (**Figure 3B**) [73-75].

Recently TF has emerged as an interesting target involved in the pathogenesis of PAH. TF is a glycoprotein expressed on the cell surface of SMCs, macrophages, monocytes and ECs [76]. It plays a role in initiation of coagulation, facilitation of angiogenesis and mediation of arterial injury in the circulation [77,78]. Interestingly, TF is rarely expressed in healthy cells, but is highly expressed in PAECs in PAH, predominantly in plexiform lesions [79]. In PAH animal models , increased TF expression correlates with formation of plexiform-lesions [79]. Furthermore, in PAH patients elevated levels of thrombin, a downstream target of TF and essential for clot formation, are detected [74,80]. Elevated levels of fibrinopeptide-A (FPA) which increases thrombin activity (downstream of TF) are observed in PAH patients [74]. PAH patients have lower thrombomodulin levels, consistent with the hypercoagulable state observed in PAH patients[81]. However, the role of EC dysfunction in this is still unclear.

Apoptosis

EC apoptosis may also play a role in PH development via vascular dropout and selection pressure on ECs, contributing to the apoptosis-resistant phenotype of ECs in vascular lesions [82]. Several attempts were made to elucidate the molecular pathways involved in regulation of PAEC apoptosis. The hypothesis is that disturbed responses to VEGF signalling in combination with hypoxia cause an initial increase in apoptosis in PAECs, leading to the emergence of aggressive apoptosis resistant and hyperproliferative ECs that cause formation of intimal lesions [83-85]. A possible explanation for the initial increase in apoptosis of PAECs is that loss of BMPR2 signalling promotes mitochondrial dysfunction and subsequent PAEC apoptosis [42]. White *et al*., interestingly, proposes a model in which the pro-apoptotic factor programmed cell death-4 (PDCD4) activates cleavage of caspase-3, inducing PAEC apoptosis. Interestingly, they show that reducing PDCD4 levels *in vivo* by overexpressing miRNA-21 prevents PH development in SuHx rats [86]. Besides an initial increase in apoptosis, PAH is also characterized by PAECs that are hyperproliferative and apoptosis resistant [85]. PAECs from iPAH patients showed increased expression of pro-survival factors IL-15, BCL-2 and Mcl-1, together with persistent activation of the pro-survival STAT3 signalling pathway [85]. Furthermore, in lungs from iPAH patients and from SuHx rats Notch1 was elevated. Notch1 contributes to PAH pathogenesis by increasing EC proliferation and inhibiting apoptosis via p21downregulation and regulating BCL-2 and survivin expression. Furthermore, HIF1α expression promotes Notch signalling human PAECs [87].

VASOACTIVE FACTORS CONTRIBUTING TO EC DYSFUNCTION

PAH is characterized by an activated endothelium of which the balance between vasodilation and vasoconstriction, but also growth factor production, is altered, causing perturbations in pulmonary vascular homeostasis that promote vascular remodelling (**Figure 3B**).

Nitric oxide

NO is a fast-reacting endogenous free radical produced by endothelial NO Synthase (eNOS). NO is essential for vasorelaxation via PASMCs but also has antithrombotic effects and controls EC differentiation and growth [88-90]. NO has long been implicated in the pathogenesis of PAH, and lungs of PAH patients have reduced NO expression [91]. However, other studies reported contradictory results and some PH patients even show an increase in eNOS expression [91,92]. Furthermore, $eNOS^{-/-}$ mice show reduced vascular remodelling after chronic hypoxia caused by reduced vascular proliferation [93].

Next to hypoxia as a known regulator of eNOS expression, increasing evidence supports the involvement of epigenetic regulations such as histone acetylation and DNA methylation in expression eNOS. This is independent of the initial hypoxic environment. Experimental models of persistent pulmonary hypertension of the new-born (PPHN) and PPHN PAECs showed that epigenetic modifications can contribute to reduced eNOS expression and subsequent PPHN pathogenesis [94,95]. However, it is unclear whether such mechanisms exist in PAH pathogenesis [96-100].

Reduced NO availability can also be caused by freely circulating endogenous eNOS inhibitors [101], such as asymmetric dimethylarginine (ADMA) [102]. The metabolism of this protein is facilitated by dimethylarginine dimethylaminohydrolase (DDAH) [103]. Increased levels of ADMA are associated with the pathogenesis of PH [101,103,104], and hypoxia-induced increase of miRNA-21 is found to reduce DDAH activity [49,104].

Prostacyclin

Prostacyclin is another important vasodilator produced by EC with additional antithrombotic and antiproliferative properties [7,105-107]. Prostacyclin is synthesized from arachidonic acid, by prostacyclin synthase and cyclo-oxygenase (COX) [108]. Decreased prostacyclin levels are measured in various patients with different forms of PAH, like iPAH and HIV-associated PAH [7,109] explaining in part the increase in pulmonary vasoconstriction, SMC proliferation and

coagulation occurring in these patients. In experimental PAH models, mice overexpressing prostacyclin synthase are protected from developing chronic hypoxia-induced PAH [110].

Endothelin-1

ET-1 is a potent vasoconstrictor, mainly synthesized in EC, but also in smaller amounts in vSMCs, macrophages, fibroblasts, myocytes and epithelial cells [7,111,112]. The lungs show the highest level of ET-1 in the entire body [113]. ET-1 stimulates vSMCs proliferation and platelet aggregation [7,106]. ET-1 exhibits its effects by binding to the ET_A and ET_B receptors, which activate signalling pathways in vSMCs regulating proliferation, vasorelaxation and vasoconstriction [107,113]. ET_A is predominantly expressed on vSMCs and is involved in vasoconstriction and proliferation of these cells, while ET_B is expressed on vSMCs and PAECs, and is involved in stimulating the release of vasodilators, like NO and prostacyclin, and inhibition of apoptosis [67,107,111,113,114]. Expression of ET-1 and its receptors is increased in lungs of PAH patients and experimental PH models [115-118]. Furthermore, a correlation exists between expression of ET-1 and increase in pulmonary resistance in PAH [117]. Multiple PAH associated factors are able to increase ET-1 expression including hypoxia, cytokines, growth factors, TGFβ/BMP signalling and shear stress [119-123]. Increased synthesis of endothelial ET-1, accompanied with an increase in expression of ET_A on PASMCs likely contributes to the increased vasoconstriction and vascular remodelling observed in PAH [106,118,124].

Thromboxane

Thromboxane A_2, produced by ECs and platelets, is a vasoconstrictor, inducer of platelet aggregation and a vSMCs mitogen [7,13]. Its production is increased by hypoxia and oxygen metabolites [125,126]. In PAH thromboxane A_2 is increased, creating an imbalance that might contribute to excessive platelet aggregation and vascular remodelling observed in PAH [13].

Vascular endothelial growth factor

VEGF is an angiogenic factor secreted by ECs. VEGF has multiple roles in maintaining lung structure and homeostasis but also is associated with several vascular disorders [7,127,128]. The pulmonary endothelium does not secrete VEGF during normal homeostasis but iPAH ECs from plexiform lesions show increased expression of VEGF and VEGF receptor 2 [129], and also VEGF plasma levels of PH patients are elevated [130]. The relation between PAH and increased VEGF expression is still poorly understood. It is suggested that VEGF levels in PAECs are elevated in early stages of PAH as a protective response, while during disease progression VEGF keeps

promoting growth of PAECs, causing the formation of plexiform lesions [7]. Rats treated with a VEGF receptor blocker in combination with hypoxia develop angio-obliterative PAH [131]. Furthermore, overexpression of VEGFA slows down the development of hypoxia-induced PAH, and improves endothelial function by increasing eNOS activity among others [132].

EPIGENETICS

In recent years epigenetics has become a growing field of interest in PAH research. Currently the main focus of study for targeting PAH are the following three mechanisms of epigenetic regulation; DNA methylation, histone modifications and RNA interference (**Figure 4**) [14].

DNA methylation profiling of PAECs from iPAH and hPAH patients revealed differences in expression of several genes involved in inflammatory processes, remodelling and lipid metabolism compared to controls [133]. Among those genes ABCA1 was found most differently methylated/ downregulated in the discrimination between PAH and controls. ABCA1 belongs to the family of ATP binding cassette (ABC) transporters that are important for pulmonary homeostasis [133]. Furthermore, ABCA1 is linked to PAH pathophysiology in a MCT animal model of PAH where activation of ABCA1 improved RV hypertrophy and pulmonary haemodynamics [14,133].

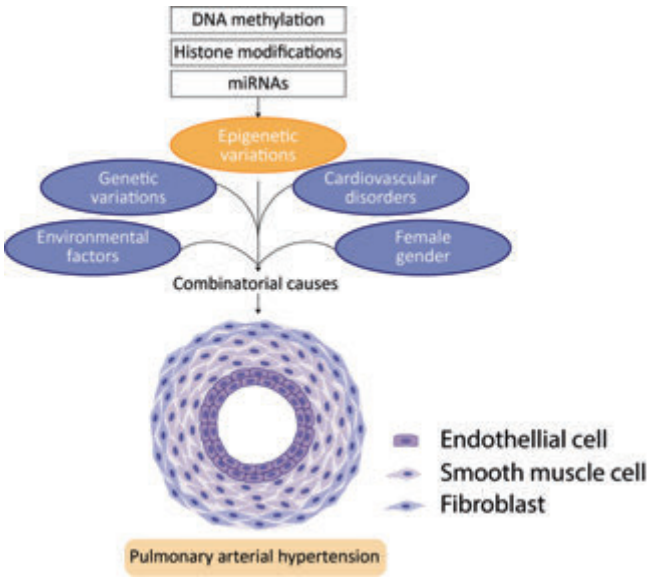

Figure 4. Epigenetics in PAH. In addition to genetic variations and other risk factors such as gender, comorbidities and environmental factors, epigenetic variations in PAH gain interest. Differences in DNA methylation profiles, increased histone acetylation and dysregulated miRNA expression in PAH patients point out a growing field in PAH research that provides better understanding of disease pathology.

Increased histone acetylation through histone-deacetylases (HDAC) is associated with vascular remodelling found in PAH [134,135]. In humans, HDAC enzymes are divided into 4 classes: class-1 HDACs (HDAC-1, -2, -3 and -8), class-2a HDACs (HDAC-4, -5, -7 and -9), class-2b HDACs (HDAC-6 and -10), class-3 HDACs (Sir2-like proteins) and class-4 HDACs (HDAC-11) [136]. HDAC-1 and -5 show increased expression in both lungs of iPAH patients and chronic hypoxic rats whereas HDAC-4 was only increased in human iPAH lungs [135]. More recently HDAC-6 is linked to PAH pathogenesis, possibly through upregulation of HSP90 [137]. HDAC-6 was overexpressed in PAECs and PASMCs of PAH patients and PH experimental models [138]. In the SuHx and MCT rat model pharmacological HDAC-6 inhibition improved PH [138]. Several other studies showed that class-1 HDAC inhibitors attenuate PAH by suppressing arterial remodelling in a chronic hypoxia model and by reducing inflammation in PH-fibroblasts [135,139,140]. In PAECs class-2a HDAC inhibitors restore the levels of myocyte-enhancer-factor-2 and attenuate PAH in both the MCT and SuHx PAH rat models [141].

The epigenetic regulator bromodomain-containing-protein-4 (BRD4) is linked to the pathogenesis of PAH [38]. BRD4 is a member of the Bromodomain and Extra-Terminal (BET) motif family that binds histones to influence gene expression [142]. BRD4 is overexpressed in lungs of PAH patients in a miR-204 dependent manner. It inhibits apoptosis by sending cell survival signals [38,143], and stimulates proliferation of PAEC and PASMC proliferation at these sites [14,143]. Selective inhibition of BRD4 with RVX-208 restored EC function, reversed PAH in the MCT and SuHx rat models, and supported the RV function in pulmonary artery banding model of PAH [38].

EC DYSFUNCTION IN OTHER PH GROUPS

Group 2 PH

Group 2 PH is due to a complication of left heart disease and is most common in patients with heart failure (HF). Therefore research in group 2 PH focuses mostly on left ventricular dysfunction and not so much the lung vasculature. However, EC dysfunction is also associated with PH-LHD [144]. An experimental model of chronic HF showed reduced NO activity and responsiveness to NO in pulmonary arteries [145]. Moreover, ET-1 is elevated in certain PH-LHD phenotypes and ET-1 activity is increased in plasma of patients with chronic HF. Blocking the ET_A receptor caused pulmonary vasodilation in these patients [146,147]. Furthermore, polymorphisms found eNOS also contribute to PH development in patients with LHD [148].

Unfortunately, treating PH-LHD patients with drugs used to treat PAH patients was not beneficial and even harmful [144,149] .

Group 3 PH

Chronic obstructive lung disease (COPD) associated PH is the best described form of PH in group 3. EC dysfunction is one of the causes for these patients to develop PH [150]. Cigarette smoke decreases eNOS and prostacyclin expression in PAECs [151,152]. COPD patients can show overexpression of VEGF and ET-1 in pulmonary arteries [153,154]. These findings have led to the hypothesis that cigarette smoke may be one of the initiating factors for PH in COPD [150]. A role for HIF1α and EndoMT has also been suggested in COPD [155,156]. Although there are similarities in EC dysfunction, drugs used to treat PAH are currently not recommend for group 3 PH, due to lack of evidence how these drugs may influence PH progression in combination with the underlying lung diseases [157].

Group 4 PH

CTEPH develops as a result of a pulmonary embolism (PE) that does not resolve [158]. These organized pulmonary thrombi in the lungs are associated with distal vascular remodelling of non-occluded vessels similar to the remodelling observed in PAH lungs [158]. Activated platelets with a hyper-responsiveness to thrombin are likely to contribute to the CTEPH pathogenesis and progression via enhancing inflammatory responses of pulmonary ECs [159]. EC dysfunction-associated vascular remodelling has been suggested as a common mechanism between CTEPH and PAH [158,160]. Primary cell cultures isolated from endarterectomized tissue co-expressed both EC and SMC markers, suggesting a role for EndoMT in intimal remodelling/lesion development in CTEPH [161]. The existence of endothelial dysfunction in CTEPH pathogenesis is further supported by the fact that conditioned medium from CTEPH derived PAECs, containing high levels of growth factors and inflammatory cytokines, increased PASMC proliferation and monocyte migration [162]. In addition, PAECs from CTEPH patients show an increased proliferation, altered angiogenic potential and metabolism, and apoptosis resistance [163-167]. Increased levels of soluble intracellular adhesion molecule-1 (ICAM1) in PAECs from CTEPH patients and in endarterectomy may contribute to EC proliferation and apoptosis resistance through its effect on cell survival pathways [166]. Also FoxO1, in a PI3K/Akt dependent manner, is a possible contributor to the loss of balance between cell survival and death and was downregulated after PE in a rat model of CTEPH [168].

At last, PAECs isolated from CTEPH patients showed a significant rise in basal calcium levels, which is an important regulatory molecule for EC function [169]. This imbalance in calcium homeostasis is caused by angiostatic factors such as PF4, IP-10 and collagen type 1, that are formed in the microenvironment created by the unresolved clot and eventually lead to EC dysfunction [169]. So far, a soluble guanylate cyclase stimulator (Riociguat) is the only PAH based therapy that has been approved in patients with CTEPH that are not eligible for surgery [170].

CURRENT AND FUTURE PERSPECTIVES

Although much progress has been made to understand EC dysfunction in PAH, to date there is still no definitive cure and patients only have a median survival rate of 2.8 years [171]. Current therapies for PAH, consisting of calcium channel blockers, ET-1 receptor antagonists, phosphodiesterase type 5 inhibitors, prostacyclin-derivatives and more recently also Riociguat, focus on SMC relaxation with limited or no effect on EC dysfunction and subsequent progressive pulmonary vascular remodelling [172-174]. The effects of EC dysfunction are neglected thus far. Therefore, research on EC dysfunction and its stimuli to target structural changes that narrow lumen size in PAH is vital to find a cure.

A first step towards reversing vascular remodelling in PAH is the use of apoptosis-inducing drugs, such as anthracyclines and proteasome inhibitors. They are already used in combination with cardio-protectants such as p53 inhibitors to reduce pulmonary pressure and restore blood flow in experimental models of PAH [175,176]. The combinatorial use is essential to circumvent the lack of cell-type/organ specificity of cell-killing drugs. Cancer patients but also experimental PAH animals treated with only cell-killing drugs show signs of cardiotoxicity which should be prevented in PAH patients that already suffer from reduced right heart function [175,177-179].

Another way to target progressive pulmonary vascular remodelling focuses on restoring signalling pathways and EC function, e.g. using TGFβ inhibitors, like kallistatin, known to inhibit EndoMT in HUVECs, stimulate eNOS expression and prevent TGFβ induced miRNA-21 synthesis [180]. Blocking inflammation to restore normal EC function in PAH, however, was not successful. One explanation might be the complexity of the immune system and by inhibiting the bad side, one also suppresses beneficial inflammatory pathways [181,182].

Modulating BMPR2 has been proposed as therapeutic approach to reverse endothelial dysfunction in PAH too. A recent study comparing human induced pluripotent stem cell-

derived ECs (iPSC-ECs) from unaffected BMPR2-mutation carriers with iPSC-ECs from BMPR2-mutation carriers that present PAH identified several BMPR2 modifiers and differentially expressed genes in unaffected iPSC-ECs. These BMPR2 modifiers exert a protective response against PAH by improving downstream signalling, which compensates against BMPR2 mutation-induced EC dysfunction and offer insights towards new strategies to rescue BMPR2 signalling [183]. A potential therapy for stimulating BMPR2 signalling is through pharmaceuticals [184]. Direct enhancement of endothelial BMPR2 signalling using recombinant BMP9 protein prevents and reverses established experimental PAH [35]. However, in contrast to Long *et al*, Tu *et al* (2019) showed that deletion or inhibition of BMP9, protects against experimental PH via its effect on endothelial production of ET-1, apelin and adrenomedullin [185]. These studies show the BMP receptor family complexity as therapeutics in PAH. More recently, ACTRIIA-Fc, an activin and growth and differentiation factor (GDF) ligand trap, prevented and reversed existing PH in experimental PAH models. ACTRIIA-Fc inhibited SMAD2/3 activation and restored a favourable balance of BMP signalling versus TGFB/activin/GDF signalling. ACTRIIA-Fc is currently tested in a phase-2 clinical trial for efficacy and safety in PAH patients (NCT03496207) [186]. Spiekerkoetter *et al*. uncovered a molecular mechanism where FK506 (tacrolimus) restores defective BMPR2 signalling in PAECs from iPAH patients, and reverses severe PAH in several rat models [184]. Based on improvements in clinical parameters and stabilization of cardiac function of end-stage PAH patients in a phase-2a clinical trial, low dose of FK506 was proposed as potential beneficial in the treatment of end-stage PAH [187].These findings open-up an area in which correcting BMPR2 mutations in combination with other therapies might be more successful in curing PAH. A proposed hypothesis to cure PAH describes collecting iPSCs from PAH patients, restoring the BMPR2 mutation with CRISPR/Cas9 and reinjecting those iPSCs in the patient to normalize EC function and signalling along with administration of drugs that could restore the protective gene expression profile of unaffected BMPR2 mutation carriers [188]. 6-Mercaptopurine (MP), a well-established immunosuppressive drug, inhibits EC dysfunction and reverses development of PH in the SuHx rat model by restoring BMP signalling through upregulation of nuclear receptor Nur77 [189]. A recent proof-of-concept study with MP in a small group of PAH patients showed a significant reduction pulmonary vascular resistance, accompanied by increased BMPR2 mRNA expression in the patients' peripheral blood mononuclear cells. However, unexpected severe side-effects require further dose optimisation and/or use of other thiopurine analogues [36].

Transplantation of mesenchymal cells in rats from the SuHx model improved haemodynamic parameters but more interestingly reduced EndoMT (partially) through modulation of HIF2α expression [190]. Furthermore, mesenchymal stem cells are also suggested to reduce inflammation through secretion of paracrine factors and to attenuate vascular remodelling by lowering collagen deposition [190-192]. However the underlying mechanisms for this observation remain unclear [190].

At last, epigenetic modulation has received growing interests as potential therapeutic intervention. Especially specific HDAC inhibition shows great promise in reversing pulmonary remodelling and pressure [135]. A problem with broad-spectrum HDAC drugs is that they show severe side effects on the right ventricle, which can have fatal consequences in PAH patients with RV failure [139,193,194]. Therefore, searches for more selective HDAC inhibitors that do not show cardiotoxicity are still being done. One example is MGCD0103, a HDAC inhibitor that selectively inhibits class-1 HDACs, which has been tested in a chronic hypoxia rat model. This inhibitor showed improved haemodynamics, reduced wall thickening while RV function was maintained [139]. Also BET inhibitors such as RVX208 seem promising in the treatment of PAH through its beneficial effect on reducing the apoptosis-resistant and pro-inflammatory phenotype in PASMCs and MVECs isolated from PAH patients but also on vascular remodelling and the RV in several experimental models of PH [38]. Finally, miRNA-21 has been associated with multiple pathogenic features, such as TGFB signalling, EndoMT and apoptosis, central to PAH. Therefore, therapeutic modulation of miRNA-21 may be an important issue for future research to restore pathogenic signalling.

CONCLUSION

To date we still do not fully understand what triggers the onset and progression of PAH. We do know that BMPR2 mutations, epigenetics, physiological conditions, and inflammation are important triggers. EC dysfunction plays a central role in all of this, through EC proliferation, EndoMT and a misbalanced production of vasoactive factors resulting in the disorganized growth of PASMCs. However, the question still remains whether EC dysfunction is a cause or consequence of PAH. Despite advancements made in treating this disease, no focus on targeting PAH at its core. A better understanding of the molecular mechanisms involved in EC dysfunction in PAH is of utmost importance for developing successful therapies to save the lung as well as the heart, and maybe cure PAH in the future.

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