Endothelial dysfunction in pulmonary arterial hypertension: an evolving landscape (2017 Grover Conference Series)

Benoît Ranchoux[1], Lloyd D. Harvey[2], Ramon J. Ayon[3], Aleksandra Babicheva[3], Sebastien Bonnet[1], Stephen Y. Chan[2], Jason X.-J. Yuan[3] and Vinicio de Jesus Perez[4,5,6]

[1]Centre de Recherche de l'IUCPQ, Quebec Canada; [2]University of Pittsburgh Vascular Medicine Institute Division of Cardiology, Pittsburgh, PA, USA; [3]Division of Translational and Regenerative Medicine, The University of Arizona College of Medicine, Tucson, AZ, USA; [4]Division of Pulmonary and Critical Care Medicine, Stanford University Medical Center, Stanford, CA, USA; [5]The Vera Moulton Wall Center for Pulmonary Vascular Medicine, Stanford University Medical Center, Stanford, CA, USA; [6]Stanford Cardiovascular Institute, Stanford University Medical Center, Stanford, CA, USA

Abstract

Endothelial dysfunction is a major player in the development and progression of vascular pathology in pulmonary arterial hypertension (PAH), a disease associated with small vessel loss and obstructive vasculopathy that leads to increased pulmonary vascular resistance, subsequent right heart failure, and premature death. Over the past ten years, there has been tremendous progress in our understanding of pulmonary endothelial biology as it pertains to the genetic and molecular mechanisms that orchestrate the endothelial response to direct or indirect injury, and how their dysregulation can contribute to the pathogenesis of PAH. As one of the major topics included in the 2017 Grover Conference Series, discussion centered on recent developments in four areas of pulmonary endothelial biology: (1) angiogenesis; (2) endothelial-mesenchymal transition (EndMT); (3) epigenetics; and (4) biology of voltage-gated ion channels. The present review will summarize the content of these discussions and provide a perspective on the most promising aspects of endothelial dysfunction that may be amenable for therapeutic development.

Introduction

Pulmonary arterial hypertension (PAH) is a life-threatening disorder characterized by progressive elevation of pulmonary pressures that lead to right heart failure and death.[1] With an estimated prevalence of ~12–15 per million, PAH is considered a rare disease that predominantly targets women and has a poor prognosis with a three-year survival rate of 58%.[2] Although vasodilation therapies can provide temporary symptomatic relief, PAH progresses until lung transplantation is the only option.[2] Thus, there is an unmet need for novel therapeutic approaches that can effectively target the underlying pathological mechanisms driving disease progression and alter the natural history of this devastating disease.

The abnormal rise in pulmonary pressures seen in PAH is a consequence of increased pulmonary vascular resistance (PVR) due to progressive loss and obliteration of small pulmonary arteries. The initial trigger is thought to be a combination of genetic and environmental factors that lead to endothelial cell (EC) injury and impaired vascular regeneration, resulting in aberrant vascular remodeling and loss of small pulmonary arteries.[1–4] The abnormal remodeling ranges from marked hypertrophy and hyperplasia of the medial layer to cell-rich (i.e. plexogenic) lesions that cause luminal obstruction and reduced pulmonary blood flow.

At present, none of the available therapeutic modalities have shown the potential to prevent small vessel loss, induce vascular regeneration, and/or reverse plexogenic

Corresponding author:
Vinicio de Jesus Perez, Stanford University Medical Center, 300 Pasteur Drive, Grant S140B, Stanford, CA 94301, USA.
Email: vdejesus@stanford.edu

arteriopathy. A major barrier to the development of effective treatments for PAH is an incomplete understanding of the signaling pathways that drive the endothelium to produce and respond to vasoactive factors that orchestrate angiogenesis, the process by which damaged vessels are regenerated. These new blood vessels, comprising arteries, capillaries, and veins, are necessary for normal tissue growth and response to injury.[3,4] When the dynamic regulation of angiogenesis is disrupted, these processes can rapidly become contributing factors to pathogenic processes.[5] For example, multiple cancers exploit angiogenic and vasculogenic pathways to supply oxygen and nutrients to tumors, driving their growth and fueling inflammatory responses.[6–8] In addition, angiogenesis and vasculogenesis are relevant to many non-cancerous conditions. Specifically, impaired and/or inappropriate angiogenesis and vasculogenesis have been linked to multiple cardiovascular diseases, such as peripheral vascular disease,[9] coronary artery disease,[10] and diabetic retinopathy,[11,12] and may also occur in PAH pathogenesis.[13]

Angiogenesis—driven by the coordinated action of ECs—involves remodeling of the extracellular matrix and recruitment of mural cells, such as pericytes, fibroblasts, and smooth muscle cells (SMCs). There are two significant forms of angiogenesis: (1) intussusceptive angiogenesis, where new vessels are formed through pillars that result from the fusing of the plasma membrane of pre-existing vessels; and (2) sprouting angiogenesis, which is driven by coordinated movements of ECs in response to cytokine gradients and tissue hypoxia (Fig. 1). In sprouting angiogenesis, a highly motile, filopodia-enriched "tip cell" acts as the "pathfinder" that creates a path for its adjacent neighbors (the highly proliferative "stalk" cells) through the extracellular matrix. As a result of the coordinated action of tip and stalk cells, a new vessel branch arises that reaches through zones of hypoxia to merge with neighboring vessels for the re-establishment of blood flow and oxygen delivery to these areas. Through a stochastic process, any EC is intrinsically capable of becoming a tip or stalk cell, or to remain as a quiescent EC, or phalanx cell, in the stable vessel.[14] Interestingly, individual ECs actively rotate between each phenotype by the rapid induction and repression of a complex genetic program driven by the integration of extracellular pro- and anti-angiogenic chemotactic cues from surrounding tissues and neighboring vascular ECs.[15–17] Furthermore, the ratio of tip cells to stalk cells is crucial for the proper formation of new vessels and is tightly regulated by cross-talk among several signaling pathways, such as ephrin, notch, bone morphogenetic protein (BMP), and Wnt signaling (Fig. 1).[5,17–21]

Any effort to understand the basis of the vascular pathology seen in PAH must take into consideration the contribution of each of the major signaling pathways that orchestrates angiogenesis. One of the most important is the vascular endothelial growth factor (VEGF) pathway, a crucial regulator of vascular development.[22,23] In the setting of hypoxia, there is an increase in the expression of the ligand VEGF-A, which upon its release interacts with the VEGF receptor (VEGFR) 2 and triggers a complex signaling cascade that results in endothelial proliferation, survival, motility, and ultimately angiogenesis.[22] While the action and regulation of the VEGF pathway are well characterized in
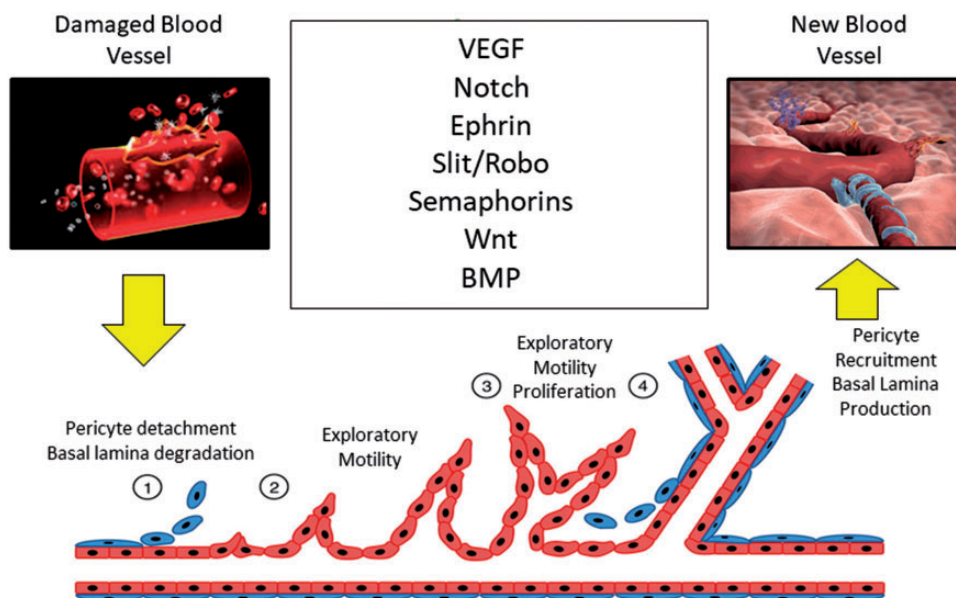
Fig. 1. Mechanism of sprouting angiogenesis. When blood vessel is damaged or under proangiogenic factors activation, the pulmonary artery smooth muscle cells (PASMC) and pericytes (blue) leave the arterial wall and endothelial cells (PAECs) (red) start to degrade the basal lamina (1). PAECs then migrate in response to cytokine gradients and tissue hypoxia and create sprouts (2). The sprouts split and growth as the PAECs proliferate (3) until perivascular cells are recruited and a new basal lamina is created to stabilize the new microvessels.

the systemic circulation, little is known about these functions in the pulmonary circulation. Immunohistochemical studies performed in lung sections of PAH patients have revealed that plexogenic lesions contain high levels of VEGF-A and VEGFR2, supporting the argument that these cells may be of endothelial origin and that the plexogenic lesions may arise from a process of "misguided" or inappropriate angiogenesis.[24] While these findings argue for pathological "hyperactivation" of the VEGF pathway, studies in pulmonary microvascular ECs (PMVECs) isolated from PAH patients suggest that, despite VEGF stimulation, these cells have a limited capacity to tolerate injury and assemble smaller vascular tube networks in vitro compared to healthy PMVECs.[25–27] These findings suggest that, despite increased expression of VEGF ligands and receptors, PMVECs in PAH may have intrinsic defects that prevent them from appropriately responding to VEGF-A stimulation.

Besides being a master regulator of angiogenesis, the VEGF pathway is also known to cross-talk with an extensive network of signaling also implicated in proper vessel assembly. Research efforts should, therefore, prioritize dissection of these critical interactions along with the epigenetic and genetic alterations responsible for unleashing the angiogenic response required to regenerate lost or injured vessels. This review will summarize some of the most recent discoveries in the evolving landscape of angiogenesis research in PAH and how therapeutic interventions can be tailored to address the dysregulation associated with vascular remodeling in patient lungs.

Endothelial-to-mesenchymal transition in pulmonary hypertension

Aberrant vascular remodeling implicated in the increased PVR include concentric arterial wall thickening, occlusive intimal lesions, and neomuscularization of precapillary arterioles. Concentric pulmonary vascular wall thickening is characterized by significant intimal and medial hypertrophy due to dysfunction of pulmonary artery ECs (PAECs) and elevated proliferation/attenuated apoptosis of pulmonary arterial SMCs (PASMCs). While an imbalanced ratio of proliferation/apoptosis in PASMCs mainly contributes to the medial thickening, altered endothelial function contributes to neointimal formation, intimal thickening, and distal pulmonary arteries obliteration.[28]

Pathological studies indicate that cell types found in the intraluminal occlusions express mesenchymal markers and SMC markers.[29] Increased expression of smooth muscle α-smooth muscle actin (α-SMA or Acta2) is a nearly universal finding in the remodeled pulmonary arteries and the occlusive lesions in patients with PAH. Until recently, resident PASMCs, via concentric migration, were considered the predominant source of the newly appearing α-SMA-expressing cells in the obliterative lesions. However, recent studies provided evidence that other sources might play an important pathogenic role, such as dedifferentiated α-SMA-positive cells through the KLF signaling pathway[30] and myofibroblasts derived from ECs via endothelial-to-mesenchymal transition (EndMT).

EndMT is a process by which ECs acquire a mesenchymal phenotype in association with the downregulation of EC-specific genes, such as platelet EC adhesion molecule 1 (PECAM1 or CD31) and vascular endothelial cadherin (VE-cadherin or CD144), and with the upregulation of SMC-specific genes, such as α-SMA and fibroblast-specific genes like vimentin. TWIST1, SNAIL (or SNAI1), and SLUG (or SNAI2) are transcription factors that initiate EndMT (and epithelial-to-mesenchymal transition). The obtained mesenchymal cells can then differentiate into other phenotypes such as fibroblasts, chondrocytes, or osteoblasts.[31–36] As such, EndMT is considered to be a major contributor to tissue regeneration.

During EndMT, ECs lose their junctions to the endothelium and gain migratory and proliferative capacities as they gradually switch from endothelial to a mesenchymal phenotype. Recently, EndMT has emerged as a critical player not only in the pathogenesis of tissue fibrosis but also in the pulmonary vascular remodeling processes seen in PAH.[37–39] In patients with PAH, the presence of EndMT was confirmed in remodeled arteries as typified by cells expressing both endothelial (CD31, CD34, VE-cadherin) and mesenchymal markers (αSMA, fibronectin 1). A signature of EndMT, including decreased expression of endothelial junction protein p120-catenin, increased expression of mesenchymal markers phospho-vimentine and over expression of transcription factors TWIST1, SNAIL, and SLUG was found in lungs from PAH patients and in an experimental pulmonary hypertension (PH) model using monocrotaline (MCT).[37,39] It has been reported that 5% of ECs from the experimental PAH Sugen 5416/hypoxia mouse and systemic sclerosis-associated PAH also express both endothelial and mesenchymal phenotypes.[38]

The molecular mechanisms inducing EndMT in PAH remain elusive with several pathways likely implicated. For example, the transforming growth factor β (TGF-β) signaling pathway is critical to PAH development and is also an important player in EndMT. Indeed, TGF-β induces α-SMA and type I collagen expression in detriment to VE-cadherin expression in PAECs.[40] Similarly, activation of the Wnt/β-catenin axis by TGF-β can potentiate EndMT by repressing VE-cadherin messenger RNA (mRNA) expression and by promoting vimentin and SLUG mRNA expression.[41] EndMT may also occur after exposure of PAEC to inflammatory molecules, such as IL-1β and TNF-α, or in response to vasoactive molecules like ET-1, all of which are implicated in PAH etiology.[42,43] Another hallmark of PAH is the disruption of bone morphogenetic protein receptor type 2 (BMPR2) signaling. BMPR2 expression is reduced in idiopathic PAH and a mutation in *BMPR2* are found in 70% of familial PAH and 25% of idiopathic PAH.[44–46] Interestingly, Hopper

et al. recently demonstrated that dysfunctional BMPR2 signaling promotes EndMT via activation of High Hobility Group AT-hook 1 (HMGA1) and SLUG pathways.[47]

EndMT can also be controlled epigenetically by several microRNAs (miRNAs), including miR-21 which is critical to PAH development.[48,49] TGF-β can significantly increase miR-21 expression in ECs and induce EndMT through an AKT-dependent mechanism.[50] Finally, HIF-2α, a key regulator of the molecular response to hypoxia, was also linked to EndMT. Besides its endothelial effect on vasoconstriction, through arginase[51] and endotheline-1,[52] and on PASMC proliferation, through endothelial CXCL12 overexpression,[53] it has been recently reported that HIF2-α also upregulates SNAIL and SLUG.[39] Under normoxic conditions, HIF2-α is mainly under control of prolyl hydroxylase domain protein 2 (PHD2) that enables its proteasomal degradation.[54,55] Interestingly PHD2 expression was found decreased in PAECs from PAH patients which leads to abnormal HIF2-α stabilization under normoxic conditions and SNAIL/SLUG upregulation.[39] The endothelial depletion of PHD2 gene (egln1) in mice leads to spontaneous severe PAH,[39,52,53] which was associated with overexpression of EndMT markers in remodeled vessels.[39]

Epigenetic regulation of endothelial cell phenotypes in pulmonary hypertension

Genetic information is stored in a linear sequence of complementary DNA base pairings, which is further organized into a hierarchical biological infrastructure upon which additional regulation may be exerted to influence gene expression. This concept is known as epigenetics. Classically, epigenetics is defined as the study of changes in phenotype and gene expression not due to alterations in the nucleotide sequence but rather through the chemical modifications of DNA and its associated organizing histone proteins.[56] Epigenetic modifications are remarkably stable—thus conferring heritability—yet also highly dynamic reorganizations of chromatin that maintain specific cellular phenotypes and alter activity states via the selective expression or repression of various genetic programs.

Our understanding of PAH as a disease with multifactorial etiologies suggests that its manifestation may be due to a series of independent mechanisms converging into a single clinical phenotype. Moreover, instances of unexplained biological phenomena in PAH indicate that a role for epigenetic influence may exist. For example, a genetic predisposition to heritable PAH has long been established with known causative mutations, such as the autosomal dominant mutation in the BMPR2; however, the mutation in BMPR2 demonstrates markedly reduced penetrance in its carriers and, when coupled with the high rates of "sporadic" PAH, further confirms a requirement for other inciting stimuli in disease initiation.[56] Consequently, the inability of a singular

sequence alteration to explain PAH pathophysiology strengthens arguments in favor for a role of epigenetic influence in disease manifestation.

ECs demonstrate an initial predisposition to apoptosis followed by an apparent resistance to apoptosis and a secondary hyperproliferative phenotype.[57,58] When PAECs are isolated from the lung vessels of patients with long-standing PAH and cultured ex vivo, they maintain this resistance to apoptosis and hyperproliferative state, which is suggestive of a heritable and stable phenotype.[59] Accordingly, the observations of both endothelial apoptosis and proliferation in PAECs provides the foundation for a spatiotemporal model of EC reprogramming in PAH that may be, in part, mediated via epigenetic mechanisms. In fact, EC dysfunction in PAH models has been shown to act through DNA methylation, histone protein modifications, and non-coding RNAs, which, when taken together, may help reconcile the observed biological phenomena into a more unifying and cohesive epigenetic theory of PAH (Fig. 2).
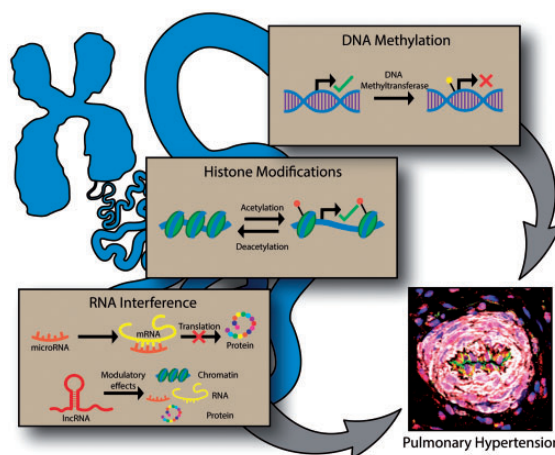

Fig. 2. Mechanisms of endothelial epigenetic regulation. in PH. The transcription of DNA is repressed via the covalent addition of a methyl group at the C5 position on cytosine bases by DNA methyltransferase. Methylation sterically hinders the binding of molecules necessary for messenger RNA synthesis, thereby preventing gene expression. Histones can be modified with the addition or removal of various molecules. The acetylation of histones alters chromatin structure to allow for enhanced gene expression. Likewise, the deacetylation of histones compacts chromatin and reduces the availability of genes for expression. RNA interference occurs through non-coding RNA. miRNAs negatively regulate gene expression by binding to a mRNA and targeting it for degradation or preventing its translation. lncRNAs are more widespread in their effects with the ability to bind to chromatin, RNA, or protein to modify function at various points both before and after the transcriptional and translational processing of genetic information. These various mechanisms of epigenetic regulation have been shown to have roles in the pathogenesis of PH. (Confocal image is reproduced from the *Journal of Clinical Investigation* with permission from the American Society of Clinical Investigation.)

Mechanisms of epigenetic regulation: DNA methylation, histone post-translational modifications, and RNA interference

One of the most well-studied mechanisms of epigenetic regulation is the repression of DNA transcription via the methylation of DNA at cytosine residues in CpG dinucleotide sequences, where a methyl group is covalently attached at the C5 position of the nucleotide base by DNA methyltransferases.[60] These CpG islands are often found in the promoter or enhancer regions of genes, thereby conferring a mechanism through which DNA methylation can "shut off" or reduce gene expression through steric hindrance. Likewise, hypomethylation of CpG islands within promoter regions can lead to overexpression of genes. Within normal biology, DNA methylation is a stable yet highly dynamic mechanism in developmental and cellular differentiation processes, such as through the selective expression of tissue-specific gene programs.[61] The relevance of possessing both characteristics allows for cellular phenotypes to exist within a spatiotemporal framework, where cellular activity states can adapt to microenvironmental stimuli and maintain such activity states as cells divide. On the other hand, this mechanism of maintaining cellular identity in the face of external stress can also serve as a substrate for pathologic manifestations, which is increasingly demonstrated in various human diseases.[62] In the case of PAH, external stimuli may result in specific methylation patterns within vascular cells that alter cellular activity states to resist apoptosis initially and then subsequently become hyperproliferative.

The next level of organization of genetic material is within a functional unit known as the nucleosome. The nucleosome is an octameric complex of histone proteins with two copies of each H2A, H2B, H3, and H4 which DNA wraps tightly around. The nucleosome confers an added layer of epigenetic modification in which its chemical modification can restructure chromatin to control the accessibility and thus transcriptional activity of a given gene. Post-translational modifications of histones can occur in many ways, but the most common modification is acetylation. Histone acetylation often occurs at H3 and H4, where increased acetylation enhances gene expression, and decreased acetylation represses gene expression. Unlike DNA methylation, however, histone post-translational modifications are not stable, allowing for fluid and dynamic alterations in the structure of chromatin.

Lastly, RNA interference is mediated through non-coding RNA—transcripts from genes that do not code for proteins. Non-coding RNA is arbitrarily divided by nucleotide length as either small non-coding RNA (<200 nt) or long non-coding RNA (>200 nt).[63] miRNAs represent a type of small non-coding RNA that are evolutionarily conserved and negatively regulate gene expression at a post-transcriptional level by either targeting the mRNA transcript for degradation or through inhibition of its translation. Long non-coding RNAs (lncRNAs), on the other hand, can bind to chromatin, RNA, or protein to modify cellular function at multiple points before and after transcriptional and translational processes of genetic information. Within recent years, the functions of miRNAs have expanded to include direct regulation of other epigenetic mechanisms, such as DNA methylation and histone modifications.[64] This suggests that certain miRNAs may serve as master regulators in pathogenic, epigenetically mediated pathways, and that the selective targeting of regulatory miRNAs may serve as a viable therapeutic strategy.

DNA methylation in pulmonary hypertension

A recently published study examined differential gene expression through DNA methylation status in PAECs from PAH patients versus controls. DNA methylation profiles from both idiopathic and heritable PAH patients demonstrated only three promoter sites of differential methylation, thereby presenting a remarkably similar methylation profile despite the difference in etiologic origin.[65] Moreover, there was significant differential methylation in genes related to cholesterol and lipid transport pathways between PAH and control patients, such as ABCA1 (ATP-binding cassette 1), ADIPOQ (adiponectin), and APOA4 (apolipoprotein A4).[65] Interestingly, ABCA1 belongs to the super-family ATP binding cassette (ABC) transporters, which has demonstrated significance to pulmonary hemostasis in cases of persistent PH of the newborn secondary to loss-of-function mutations in *ABCA3*.[66–68] The relevance of *ABCA1* was further demonstrated using monocrotaline (MCT)-induced PH rat models. In this case, the administration of a positive regulator of ABCA1 expression—the liver X receptor-activating ligand T0901317—improved MCT-induced PH at both hemodynamic and histologic measures.[65] The significance of this work demonstrates how, unlike genetic mutations, epigenetic markings such as DNA methylation are pharmacologically reversible, making them an attractive target for the treatment of PAH.

In persistent PH of the newborn, there is a failure of the pulmonary vasculature to relax at birth, resulting in high PVR, and the development of PH and severe hypoxemia.[69] In this patient population, decreased gene expression of endothelial nitric oxide synthase (eNOS) is observed in vitro in umbilical vein ECs cultured immediately postpartum;[70] however, as hypoxemia worsens, there is a compensatory increase in the expression of eNOS within the pulmonary vasculature.[71] The highly restricted expression of eNOS to the endothelium via epigenetic mechanisms suggested that similar mechanisms may also be implicated in PAH pathophysiology.[72–76] Increased eNOS expression was also reported in a neonatal rodent model of persistent PH of the newborn induced by hypoxia and indomethacin when compared to controls. In this model, the increased expression of eNOS was associated with decreases in DNA

methylation and increases in H3 and H4 histone acetylation within its promoter region, suggesting a regulation of eNOS via epigenetic mechanisms.[77]

Histone post-translational modifications in pulmonary hypertension

The relevance of histone post-translational modifications in PAH has also been related to PAH pathogenesis. Recent studies reported that the use of histone deacetylase (HDACs) inhibitors had improved various animal models of PAH.[78–81] In one such study, PAECs isolated from PAH patients demonstrated a reduced expression of myocyte enhancer factor 2 (MEF2) mediated by a concomitant nuclear increase in HDAC4 and HDAC5.[82] Selective pharmacological inhibition of these class IIa HDACs restored MEF2 activity in PAECs and rescued MCT-induced and Sugen-5416/hypoxia models of PH. Furthermore, this study established that MEF2 controls the expression of miR-424/503, two miRNAs previously determined to be involved in pulmonary vascular homeostasis. HDAC6 was also found increased in PAEC and PASMC and its inhibition by Tubastatin A improved PH in Sugen 5416/hypoxia and MCT rats.[81] Those results highlight a complex interplay of epigenetic mechanisms, where chemical modifications of DNA or histone proteins can unleash or repress other epigenetic mechanisms. Understanding how these mechanisms exist within a network is crucial to minimize non-specific and unintended alterations of therapeutic strategies.

More recently, there has been an increased appreciation for epigenetic readers, such as bromodomain (BRD)-containing proteins, in gene regulation. BRD-containing proteins recognize and bind to histones, where they serve as molecular scaffolds for the recruitment of transcriptional regulators to influence gene expression.[83] The role for one such member of this protein family, BRD4, was demonstrated to be overexpressed in lung tissue from PAH patients—serving as the first observation for the role of an epigenetic reader in PAH.[84] Moreover, this same study demonstrated that BRD4 influences over at least three oncogenes involved in the hyperproliferative, anti-apoptotic cellular phenotype observed in PAH, suggesting a broad epigenetic role in disease pathogenesis.

Endothelial-specific RNA interference in pulmonary hypertension

microRNAs in PAH

Although numerous miRNAs have been implicated in the pathogenesis of PAH, only a subset of those have been linked directly to an EC, cell-type specific dysfunction in this disease.[49,85–87] As previously discussed, miR-424/503 have been established as critically vital components of EC homeostasis within the pulmonary vasculature.

More specifically, disruption of apelin (APLN) signaling within PAECs results in increased fibroblast growth factor 2 (FGF2) and FGF receptor 1 (FGFR1) expression.[88] The downregulation of APLN—a positive regulator of miR-424/503—results in miR-424/503 downregulation and thus prevents their ability to negatively attenuate FGF2 and FGFR1 expression, thereby leading to a hyperproliferative phenotype observed in PAECs. This observed phenotype, however, can be reversed in vivo with the restoration of miR-424/503 in experimental models of PH.[88] Interestingly, it was later demonstrated that the APLN-miR-424/503-FGF2 axis and ultimately EC proliferation is under the control of the miR-130/301 family—yet another example of the complex interplay among various epigenetic mechanisms.[89]

Similarly, the miR-17/92 cluster has shown relevance in EC biology and angiogenesis. In human umbilical vein ECs (HUVECs), overexpression of miR-17 demonstrated an inhibition of endothelial angiogenic cell sprouting in vitro and in vivo.[90] Moreover, in HUVECs overexpressing miR-92a, there was downregulation of integrin α5, which directly attenuated expression of eNOS.[91]

In order to sustain the hyperproliferative state observed in PAECs derived from PAH patients, miR-210 has shown regulatory roles in the facilitation of the metabolic shift toward glycolysis under hypoxic conditions by targeting hypoxia-inducible factor.[92] miR-210 downregulated expression of iron-sulfur cluster assembly proteins 1 and 2 (ISCU1/2)—prosthetic groups necessary for the proper functioning of the citric acid cycle and electron transport chain.[93–95] Specifically, downregulation of ISCU1/2 by hypoxic miR-210 attenuated Fe-S-dependent mitochondrial respiration in favor of glycolysis, thereby promoting a metabolic shift to sustain a hyperproliferative phenotype in PAECs.[95] Although this metabolic shift is acutely protective, its chronic repression promotes PH in rodent models.[96]

Analysis of miRNA expression within ECs reveals that miR-21 is highly expressed.[97] In one study, hypoxia and BMPR2 signaling—two stimuli involved in the pathogenesis of PH—independently upregulated miR-21 in PAECs in vitro. Furthermore, miR-21 directly repressed RhoB expression and Rho-kinase activity, thereby resulting in decreased angiogenesis and vasodilation. This finding was corroborated in rodent models of PH and in human PH, where miR-21 expression is upregulated in pulmonary tissue demonstrating a protective role of miR-21. As would be expected, miR-21^{-/-} mice demonstrated severe PH characteristics.[48] Another study demonstrated the role of miR-21 in PAEC apoptosis. When subjected to apoptotic stimuli, PAECs induced a programmed cell death 4 (PDCD4)/caspase-3 apoptotic pathway, which could be indirectly repressed by delivery of a miR-21 mimetic. In miR-21^{-/-} mice, there was activation of the PDCD4/caspase-3 axis and the development of PH. Similarly, miR-21 overexpressing mice demonstrated reduced PDCD4/caspase-3 axis activation and were partially resistant to

PH-inciting stimuli.[98] On the other hand, however, studies have also demonstrated that inhibition of miR-21 may have therapeutic relevance. In a chronic hypoxia PH mouse model, the inhibition of miR-21 reversed pulmonary vascular remodeling and decreased the proliferation of PASMCs in vitro.[99] In another study, increased levels of the endogenous eNOS inhibitor asymmetric dimethylarginine (ADMA) is contributory in PH pathogenesis, and reduced levels of its metabolizing enzymes—dimethylarginine dimethylaminohydrolases (DDAH) 1 and 2—which are under the regulation of miR-21. Hypoxic exposure of human PAECs in vitro upregulated miR-21 and downregulated DDAH1 to induce EC dysfunction, which could be prevented by DDAH1 overexpression or miR-21 blockade.[100] In addition to influencing over other epigenetic mechanisms, miRNAs have demonstrated the ability to act as paracrine signals in cell–cell communication. Using multiple stimuli in the expression of the miR-143/145 cluster, it was demonstrated that upregulation of miR-143 in PASMCs is excreted via exosomes and taken up by neighboring PAECs to induce angiogenesis and cell migration.[101]

Other miRNAs have shown EC-specific activities in the diseased pulmonary vasculature. For instance, the miRNA let-7b was found to have differential expression among various organ-specific ECs, suggestive of a potential role for miRNA signatures in the diversity of EC phenotypes.[102] Additionally, circulating let-7b is decreased in a patient population of chronic thromboembolic PH. In this same study, let-7b was shown to directly regulate the expression of endothelin-1 and TGF-β receptor 1 in PAECs.[103] In PAECs under conditions of hypoxia, there is an observed upregulation of miR-27a that directly reduces peroxisome proliferator-activated receptor gamma (PPARγ) and increases endothelin-1 to stimulate PAEC proliferation in vitro.[104] In another study, increased miR-138 was found to downregulate expression of the calcium-binding protein S100A1 in ECs in a hypoxia-dependent manner, which is a critical activator of eNOS, thereby contributing to EC dysfunction.[105]

Long non-coding RNAs in PAH

More recently, the role of lncRNAs in PAH has begun to be elucidated over the past few years. As early as 2015, comprehensive lncRNA and mRNA profiling of endothelial tissues from the pulmonary arteries of chronic thromboembolic PH patients was conducted with microarray analysis. Significant alterations were observed in the lncRNAs NR_036693, NR_027783, NR_033766, and NR_001284, which demonstrated through gene ontology and pathway analysis possible regulatory roles in disease pathogenesis either through regulation of mRNAs or vice versa.[106]

Another study published in 2016 utilized next-generation sequencing technologies to examine transcriptomic changes occurring in HUVECs under hypoxic stress, demonstrating differential expression of lncRNAs H19, MIR210HG, MEG9, MALAT1, and MIR22HG.[107] Knockdown of H19 in vitro decreased HUVEC growth and promoted cellular accumulation in G1 of the cell cycle, while also decreasing HUVEC ability to form capillary-like structures.[107] Moreover, knockdown of MALAT1 in ECs increased cell migration in vitro, while reducing capillary density and blood flow in a model of hind limb ischemia.[108] Interestingly, a paper published this year described a functional polymorphism in MALAT that contributes to PAH in a Chinese cohort.[109] Lastly, an epigenetically controlled lncRNA—MANTIS—is controlled by the histone demethylase JARID1B. In MCT-induced PH rat models and PAH patients, MANTIS is downregulated, and when modeled in vitro through small interfering RNA silencing or CRISPR/Cas9 deletion of MANTIS inhibited angiogenic sprouting of ECs.[110]

Ion channels and EC dysfunction. Ion channels are key regulator of vasoconstriction and proliferative/apoptotic phenotypes, and are therefore involved in PAH pathogenesis.[111] Their role is complex and may differ among vascular cell types. In this section, we will focus on the most studied endothelial ion channels that can contribute to altered functions of the PAECs.

Transient receptor potential channels. Transient receptor potential (TRP) channels are a group of voltage-independent cation channels located mostly in the cytoplasm of numerous vascular ECs, including PAECs. More than 15 TRP channels from different subfamilies, including canonical TRP (TRPC), melastatin TRP (TRPM), and vanilloid TRP (TRPV), are expressed in human PAECs.[112] Numerous studies demonstrated that certain TRP channels regulate vascular function. For example, TRPC1/4/6 are involved in the control of vascular remodeling and vascular permeability,[113,114] TRPV1/4 play a role in the regulation of vascular tone and vascular remodeling, TRPM6/7 contribute to vascular remodeling, and TRPM2/7 mediate the response to oxidative stress.

Canonical TRP channels

Accumulating evidence suggests that PAEC and endothelial cytosolic Ca^{2+} play a major role in the initiation of pulmonary vascular remodeling during PAH. The endothelium or PAEC is believed to be a source of proteins and molecules that stimulate PASMC proliferation in a paracrine manner in patients with PAH. PASMC isolated from healthy subjects are quiescent within the medial layer of the pulmonary artery wall. During vascular remodeling or when the endothelium is injured, expression of endothelium-derived growth factors (e.g. platelet-derived growth factor [PDGF] and VEGF) in PAECs is upregulated. This change contributes to the subsequent alterations in the growth, migration, and differentiation of PASMCs in patients with PAH. PDGF released by PAECs upregulates TRPC6, Orai2, and STIM2 in PASMCs, essential components of

receptor-operated Ca^{2+} channels (ROC) and store-operated Ca^{2+} channels (SOC).[115–117] Genetic deletion or pharmacological inhibition of TRPC6 significantly attenuates both chronic hypoxia-mediated pulmonary vascular remodeling and acute hypoxia-induced pulmonary vasoconstriction.[118] Hypoxia-induced expression of TRPC6 is mediated by Notch signaling,[118] which in turn plays a major role in EndMT, alterations of cell phenotype from a cobblestone-like shape to a spindle-shaped fibroblast-like morphology, and increased endothelial proliferation in patients with idiopathic PAH.[119–121] Accumulating data indicate that TRPC1/6 also regulate endothelial permeability by enhancing intracellular Ca^{2+} concentration,[122,123] while TRPC1/4 interact with STIM1 and Orai1 to form SOC and disrupt endothelial barrier function.[124] Recently Orai1 and Stim1 have been established as key players in VEGF-mediated Ca^{2+} entry.[125,126] Interestingly, knockdown of Stim1/2 and Orai1 inhibited EC proliferation but Orai1 silencing had more effect than that of Stim proteins.[125] Orai1 and TRPC1 contribute to cytosolic Ca^{2+} increase following store depletion but have different cellular functions. TRPC1-mediated Ca^{2+} entry is the primary regulator of K_{Ca} channel and partially activates NF-κB pathway while Orai1-mediated Ca^{2+} entry contributes to NFAT signaling.[127] This evidence suggests that activation of SOC is important stimuli for EC morphological change[128] and that EndMT could be a Ca^{2+}-dependent event in PAH.

We demonstrated that TRPC4 might also participate in hypoxia-induced vascular remodeling.[129] Increased expression of TRPC4 in human PAECs exposed to hypoxia was associated with capacitative Ca^{2+} entry via SOC, which is considered an important mechanism for raising cytosolic Ca^{2+} concentration in PAECs. Interestingly, it results in the increased binding of activating protein-1 (AP-1) transcription factors involved in cell proliferation and migration by promoting genes such as VEGF and PDGF. These data allow us to suggest a link among endothelial TRPC4/6 activation, Ca^{2+} entry, and pulmonary vascular remodeling during PAH.

Recent studies reported that TRPC channels could also be involved in the resistance of ECs to apoptosis. For example, Ca^{2+} entry through TRPC1 channels is required to protect HUVECs from apoptosis via NF-kB signaling.[130,131] Inhibition of NF-kB pathway reverses EndMT in animals with MCT-induced PH and human PMVECs treated with TGF-β1.[132] Interestingly, that 15% cyclic stretch for 48 h induces EndMT and changes cell morphology in HUVECs.[133] Mechanical stretch upregulates another TRPC3 that leads to Ca^{2+} influx and activation of the NF-kB pathway; however, the role of TRPC channels in the development of EndMT remains unknown.[134,135]

Melastatin TRP channels

At least three endothelial melastatin TRP channels, TRPM2, 4, and 7, are known to be activated by oxidative stress. Numerous studies showed that these channels modulate oxidative stress-induced cell death[136–138] and endothelial proliferation.[139] However, different splice variants of TRPM2 (long and short isoforms) and their roles in tumor growth have been identified in neuroblastoma cells.[140] Cells expressing the short-length isoform of TRPM2 had reduced Ca^{2+} entry which led to reduced HIF-1/2α activation due to oxidative stress, increased mitochondrial dysfunction, and decreased cell viability. On the other hand, cells expressing the full-length isoform of TRPM2 had higher Ca^{2+} entry which led to increased HIF-1/2α activation and higher cell proliferation and survival. As previously described, elevated HIF-2α in PAEC might play a key role in PAH pathogenesis through arginase and endothelin-1 overexpression which promote vasoconstriction,[51,52] and through CXCL12 and SNAIL/SLUG upregulations which contribute to vascular remodeling in PAH.[39,53] Furthermore, the report by Ding et al. postulated that upregulation of endothelial TRPM4 channel in response to oxidative stress is connected with endothelial lesions.[141] The authors suggest that oxidative stress is one of the mechanisms underlying aldosterone-induced endothelial injury in hypertensive patients. Downregulation of TRPM7 prevents endotoxin-induced endothelial fibrosis, suggesting that the involvement of the subunit in the EndMT mechanism.[142] The direct role of TRPM channels in EndMT needs to be fully understood, but recent reports provided evidence that these calcium-permeable ion channels are involved in calcium-dependent epithelial-to-mesenchymal transition (EMT) induction in cancer cells.[143,144] Moreover, TRPM7 may play a role in the cellular phenotype changes of PASMC by phosphorylating annexin A1, a Ca^{2+}-dependent membrane-binding protein.[145] Lately, Yi et al. confirmed that upregulation of annexin A1 mediates hypoxia-induced pulmonary arterial remodeling in the development of PH.[146]

Vanilloid TRP channels

Published data on the role of vanilloid TRP channels indicate that these channels are involved in the regulation of endothelial function. Thus, Ca^{2+}-mediated activation of TRPV1 channels ameliorates EC inflammation, and vascular oxidative stress improves endothelium-dependent vasorelaxation and protects animals from systemic hypertension through the eNOS/NO pathway.[147–150] Simvastatin activates TRPV1 and leads to the formation of the Ca^{2+}/calmodulin-dependent protein kinase II- eNOS (CaMKII-eNOS) complex as well as inhibits TGF-β1–induced EndMT in vitro.[151,152] Interestingly, we recently revealed that upregulated TRPV1 had the opposite effect in PASMCs and could contribute to idiopathic PAH due to augmented Ca^{2+} influx and excessive PASMC proliferation.[153] Genetic ablation of TRPV1 markedly enhanced TGF-β1 and VEGF expression, resulting in post-myocardial infarction fibrosis.[154] Geng et al. speculate that the long-term consumption of chili-pepper-rich diets can decrease the risk of cancer development because it promotes TRPV1

level and inactivates NF-κB signaling, thereby reducing lung epithelial proliferation and EMT.[155] Surprisingly, capsaicin (an ingredient of chili pepper) induces apoptosis in human small cell lung cancer cells via TRPV6 and but not TRPV1.[156] The authors reported a robust expression of the TRPV6 receptor in patients but no expression in normal lung tissues. Also, capsaicin induced very little apoptosis in normal lung epithelial cells. TRPV5 and 6 are the only known Ca^{2+}-selective channels within TRP family. They are involved in a higher proliferation rate, increasing cell survival and apoptosis resistance in human cancer cells.[157,158] Little is known about TRPV2 and 4 channels, but recent publications suggest a role in cell survival by regulating ERK- and Akt-pathways, respectively.[159,160] It was also shown that increased lung endothelial permeability in response to 14,15-epoxyeicosatrienoic acid (14,15-EET) in rat lung requires Ca^{2+} entry via TRPV4 channels.[161–163] More recently, it was shown that Ca^{2+} entry via TRPV4 channels selectively activates IK and SK, but not BK, channels; and that inhibition of IK and SK3 channel activity attenuates TRPV4-induced lung permeability.[164] Thus, these data suggest a functional link between TRPV4/IK/SK channels and Ca^{2+}-dependent endothelial lung injury. Also in ECs TRPV4 acts as a mechanosensor. Both shear stress and agonist activation of TRPV4 leads to enhancement of EC proliferation and triggers collateral growth after arterial occlusion.[165]

K⁺ channels. The membrane potential is an important regulator of cellular excitability. Therefore, it comes as no surprise that K⁺ channel activity plays a critical role in regulating pulmonary vascular tone. The resting membrane potential of PAECs typically falls in the range of −55 to −30 mV, which is close to the predicted equilibrium potential for K⁺ channels (–85 mV). Increased efflux or outward movement of K⁺ ions from the cytoplasm to the extracellular space leads to membrane hyperpolarization. A small change in K⁺ channel activity can have a significant impact on membrane potential. Downregulated or dysfunctional K⁺ channels can result in membrane depolarization which enhances Ca^{2+} entry via voltage-dependent calcium channels (VDCCs). Increased Ca^{2+} signaling in the pulmonary vasculature inevitably promotes excessive vascular remodeling and sustained vasoconstriction, two characteristic hallmarks of PAH. The main families of K⁺ channels identified in the pulmonary vasculature are: (1) voltage-gated K⁺ channels (Kv); (2) large-, intermediate-, and small-conductance Ca^{2+}-activated K⁺ channels (BK_{Ca}, IK_{Ca}, and SK_{Ca}, respectively); (3) inward rectifier K⁺ channels (K_{ir}); (4) ATP-sensitive K⁺ channels (K_{ATP}); and (5) two-pore-domain K⁺ channels (K_{2P}).[166]

Voltage-gated K⁺ channels

Another field of investigation in PH is focused on K⁺ channels. Kv channels are the most diverse family of K⁺ channel ubiquitously expressed in the pulmonary vasculature.[167–169] There are currently more than 20 distinct Kv channels known to be expressed in the pulmonary vasculature. Although several Kv channels are ubiquitously expressed in ECs, very little is known about their function in the endothelium. A recent study suggests that Kv1.5 may play a major role in oxidative vascular endothelial injury.[170] It was demonstrated that pharmacological inhibition of Kv1.5 was capable of attenuating H_2O_2-induced EC apoptosis in rat carotid artery. Furthermore, targeted knockdown of Kv1.5 could mitigate EC injury, and decrease intracellular and mitochondrial ROS levels, whereas overexpression of Kv1.5 had the opposite effect.

Ca^{2+}-activated K⁺ channels

K_{Ca} channels are activated by an increase in $[Ca^{2+}]_{cyt}$ and membrane depolarization. These channels are subcategorized by their conductance: large (BK), intermediate (IK), and small (SK). Although all three groups are activated by intracellular $[Ca^{2+}]_i$, SK and IK display greater sensitivity to $[Ca^{2+}]_i$ than BK channels. Furthermore, BK channels are highly voltage-sensitive, whereas SK and IK are predominately voltage-independent. Although all three subgroups of K_{Ca} channels are expressed in PASMCs and PAECs, BK channels are primarily expressed in PASMCs, while IK and SK channels are almost exclusively expressed in the PAECs. Increased expression of BK channels in PH associated with chronic obstructive pulmonary disease is linked to pulmonary vascular remodeling.[171] It was previously demonstrated that oxidized low-density lipoprotein (oxLDL), which plays an integral role in endothelial dysfunction, activates BK. Additionally, acetylcholine-induced NO synthesis is modulated by BK.[172] SK channels are preferentially localized to homocellular endothelial gap junctions and caveolin-rich domains and are linked with various connexins (Cx), while IK are preferentially localized at the sites of endothelial projections often related to myoendothelial gap junctions.[173–176] Interestingly in PAECs, activation of K_{Ca} channels does not reduce calcium influx. Quite the opposite occurs as membrane potential hyperpolarization promotes calcium entry. As PAECs do not express VDCC, it is very likely that activation of TRP channels (TRPC1/3/4/6 and TRPV1/4) leads to an elevation of the electrochemical driving force for Ca^{2+}.[177,178] Endothelial IK and SK channels play a fundamental role in controlling vascular tone as they have been shown to contribute to PAEC membrane hyperpolarization, which in turn evokes NO synthesis to mediate NO-dependent vasorelaxation.[179,180] Kefaloyianni et al. found that shear-stress can induce expression of several ion channel subunits, including $K_{Ca}2.2$, $K_{Ca}2.3$ (SK), and $K_{Ca}3.1$ (IK), in human coronary endothelial cells. Thus they suggested that altered expression of these ion channels may contribute to vascular pathologies associated with shear stress.[181] Another study focused on the relationship

between ER-stress and IK/SK activity. It was indicated that IK/SK currents inhibited by the known ER stressor homocysteine, which ultimately leads to endothelial dysfunction. It is well established that coronary artery endothelial dysfunction is closely associated with diabetes. As such, Liu et al. demonstrated that diabetes significantly decreases endothelial SK/IK currents and endothelial-dependent relaxation compared with that of non-diabetics, suggesting that inactivation of endothelial SK/IK channels may contribute to endothelial dysfunction in diabetic patients.[182] In humans, diabetes during pregnancy is associated with an abnormal regulation of peripheral vascular tone in part due to vascular endothelial dysfunction.[183,184] A recent study reported that IK channels play a prevalent role over SK channels in the initiation of endothelial K^+ currents and vasodilation of uteroplacental arteries and that impaired function of IK channels contributes to diabetes-induced uterine endothelial dysfunction.[138] Because K^+ channels are implicated in these endothelium-dependent responses, pharmacological activation of endothelial and/or smooth muscle K_{Ca} could prevent or reduce the occurrence of endothelial dysfunction.[185]

Inward rectifier K^+ channels

Inward rectifier K^+ (K_{IR}) channels conduct inward K^+ current, however, little outward current; they are blocked by intracellular Mg^{2+} and Cs^+ and extracellular Ba^{2+}. K_{IR} channels in PASMCs and PAECs are involved in setting the resting Em, preventing membrane hyperpolarization

by the electrogenic Na^+, K^+-ATPase, mediating K^+-induced vasodilation, and minimizing loss of intracellular K^+. It was previously shown that pharmacologically increasing cGMP levels in bovine pulmonary artery and microvascular ECs causes membrane depolarization which is due, in part, to PKG-independent inhibition of K_{IR} currents. It was suggested that this ensuing depolarization could be a protective mechanism that prevents EC barrier dysfunction by reducing the driving force for Ca^{2+} entry.[186] Additionally, suppression of K_{IR} currents is one of the critical factors associated with hypercholesterolemia-induced endothelial dysfunction.[187]

ATP-sensitive K^+ channels

Native K_{ATP} channels are voltage-dependent channels that have low open probability under basal conditions. K_{ATP} channels are inhibited by intracellular ATP and glibenclamide and activated by intracellular adenosine diphosphate (ADP) and cromakalim. K_{ATP} agonist can reduce cardiac remodeling and improves endothelial dysfunction.[188] It has also been demonstrated that activation of K_{ATP} channels induces ischemic preconditioning (IPC) which affords endothelial protection in guinea pig hearts.[189] Another study found that glibenclamide (a K_{ATP} channel antagonist) abolishes, while diazoxide (a K^+ channel activator) mimics endothelial IPC in humans.[190] Thus, activation agents that activate or modulate K_{ATP} channel function may have future therapeutic implications in vascular diseases associated with endothelial dysfunction.
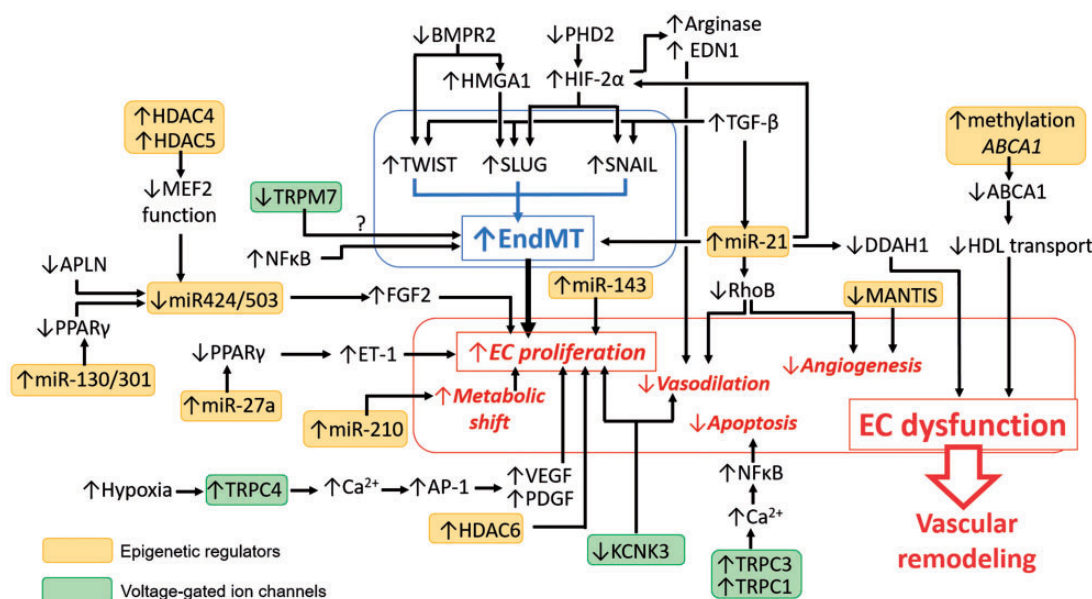

Fig. 3. Molecular pathways implicated in the endothelial dysfunction implicated in PAH pathogenesis. PAH is a complex and multifactorial disease where endothelial cell (EC) dysfunction appears to play a key role through impaired vasoconstriction, unbalanced EC proliferation and apoptosis, aberrant EndMT, and altered production of endothelial vasoactive mediators. The present figure summarized how the presented epigenetic factors (yellow) and ion channels (green) can interact with the molecular pathways implicated EndMT and EC dysfunction that lead to vascular remodeling in PAH.

Two-pore-domain K$^+$ channels

K$_{2P}$ channels are key in stabilizing the negative membrane potential at rest and counterbalance depolarization by mediating leak K$^+$ conductance.[191,192] K$_{2P}$ channels are classified in six subfamilies (TWIK, TREK, TASK, TALK, THIK, and TRESK) based on functional similarity and sequence homology. K$_{2P}$ channels are also related to PAH pathogenesis as whole-exome sequencing revealed that mutation on *KCNK3* might predispose individuals to PAH. The gene codes for K$^+$ channel subfamily K member 3 (KCNK3), a K$_{2P}$ channel also called TWIK-related acid-sensitive K$^+$channel (TASK1).[193] Antigny et al. recently discovered that KCNK3 expression and function are significantly reduced in ECs and PASMCs from idiopathic and heritable PAH but also in the MCT-induced rat model, where it correlates to PH development. Additionally, the inhibition of KCNK3 modulates pulmonary arterial tone and contributes to vasoconstriction. They also reported that KCNK3 inhibition *in vivo* increased PAEC and PASMC proliferation and distal artery remodeling, which lead to early signs of PH.[194]

Conclusion

PAH involves a multifactorial and complex disease due to aberrant remodeling of distal pulmonary arteries and impaired angiogenesis. Vascular remodeling involves pre-capillary micro-vessels neomuscularization, the rise of neo-intima and media thickening that leads to increased PVR. EC dysfunction appears to play a key role in such pathological process through impaired vasoconstriction, unbalanced EC proliferation, aberrant EndMT, and altered production of endothelial vasoactive mediators. Although tremendous progress were made in the last decade, a lot of work remains to understand PAH complex mechanisms completely. In the present review, we summarized the current knowledge on how EC actively participate in PAH pathogenesis via aberrant mesenchymal transition, epigenetic dysregulation, altered ions homeostasis, and dysfunctional ion channels (Fig. 3). The identification of these molecular mechanisms and their role in the global PAH pathogenesis are crucial to the development of promising targeted therapies.

2017 Grover Conference Series

This review article is part of the 2017 Grover Conference Series. The American Thoracic Society and the conference organizing

References

1. Rubin LJ. Pulmonary arterial hypertension. *Proc Am Thorac Soc* 2006; 3: 111–115.
2. McLaughlin VV, Archer SL, Badesch DB, et al. ACCF/AHA 2009 expert consensus document on pulmonary hypertension a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents and the American Heart Association developed in collaboration with the American College of Chest Physicians; American Thoracic Society, Inc.; and the Pulmonary Hypertension Association. *J Am Coll Cardiol* 2009; 53: 1573–1619.
3. Patan S. Vasculogenesis and angiogenesis as mechanisms of vascular network formation, growth and remodeling. *J Neurooncol* 2000; 50: 1–15.
4. Risau W. Mechanisms of angiogenesis. *Nature* 1997; 386: 671–674.
5. Carmeliet P and Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature* 2011; 473: 298–307.
6. Carmeliet P. Angiogenesis in health and disease. *Nat Med* 2003; 9: 653–660.
7. Hanahan D and Weinberg RA. The hallmarks of cancer. *Cell* 2000; 100: 57–70.
8. Moschetta M, Mishima Y, Sahin I, et al. Role of endothelial progenitor cells in cancer progression. *Biochim Biophys Acta* 2014; 1846: 26–39.
9. Collinson DJ and Donnelly R. Therapeutic angiogenesis in peripheral arterial disease: can biotechnology produce an effective collateral circulation? *Eur J Vasc Endovasc Surg Off J Eur Soc Vasc Surg* 2004; 28: 9–23.
10. Vasa M, Fichtlscherer S, Aicher A, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 2001; 89: E1–7.
11. Adamis AP, Miller JW, Bernal MT, et al. Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. *Am J Ophthalmol* 1994; 118: 445–450.
12. Crawford TN, Alfaro DV, Kerrison JB, et al. Diabetic retinopathy and angiogenesis. *Curr Diabetes Rev* 2009; 5: 8–13.
13. Voelkel NF and Gomez-Arroyo J. The role of vascular endothelial growth factor in pulmonary arterial hypertension. The angiogenesis paradox. *Am J Respir Cell Mol Biol* 2014; 51: 474–484.
14. De Bock K, Georgiadou M and Carmeliet P. Role of Endothelial Cell Metabolism in Vessel Sprouting. *Cell Metab* 2013; 18: 634–647.
15. del Toro R, Prahst C, Mathivet T, et al. Identification and functional analysis of endothelial tip cell-enriched genes. *Blood* 2010; 116: 4025–4033.
16. Strasser GA, Kaminker JS and Tessier-Lavigne M. Microarray analysis of retinal endothelial tip cells identifies CXCR4 as a mediator of tip cell morphology and branching. *Blood* 2010; 115: 5102–5110.

17. Beets K, Huylebroeck D, Moya IM, et al. Robustness in angiogenesis: notch and BMP shaping waves. *Trends Genet TIG* 2013; 29: 140–149.

18. Jin Y, Kaluza D and Jakobsson L. VEGF, Notch and TGFβ/BMPs in regulation of sprouting angiogenesis and vascular patterning. *Biochem Soc Trans* 2014; 42: 1576–1583.

19. Blanco R and Gerhardt H. VEGF and Notch in tip and stalk cell selection. *Cold Spring Harb Perspect Med* 2013; 3: a006569.

20. Aspalter IM, Gordon E, Dubrac A, et al. Alk1 and Alk5 inhibition by Nrp1 controls vascular sprouting downstream of Notch. *Nat Commun* 2015; 6: 7264.

21. Oka T, Akazawa H, Naito AT, et al. Angiogenesis and cardiac hypertrophy: maintenance of cardiac function and causative roles in heart failure. *Circ Res* 2014; 114: 565–571.

22. Adams RH and Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Biol* 2007; 8: 464–478.

23. Olsson A-K, Dimberg A, Kreuger J, et al. VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol* 2006; 7: 359–371.

24. Tuder RM, Chacon M, Alger L, et al. Expression of angiogenesis-related molecules in plexiform lesions in severe pulmonary hypertension: evidence for a process of disordered angiogenesis. *J Pathol* 2001; 195: 367–374.

25. Masri FA, Anand-Apte B, Vasanji A, et al. Definitive evidence of fundamental and inherent alteration in the phenotype of primary pulmonary hypertension endothelial cells in angiogenesis. *Chest* 2005; 128: 571S.

26. de Jesus Perez VA, Yuan K, Lyuksyutova MA, et al. Whole-exome sequencing reveals TopBP1 as a novel gene in idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2014; 189: 1260–1272.

27. de Jesus Perez VA, Yuan K, Orcholski ME, et al. Loss of adenomatous poliposis coli-α3 integrin interaction promotes endothelial apoptosis in mice and humans. *Circ Res* 2012; 111: 1551–1564.

28. Humbert M, Montani D, Perros F, et al. Endothelial cell dysfunction and cross talk between endothelium and smooth muscle cells in pulmonary arterial hypertension. *Vascul Pharmacol* 2008; 49: 113–118.

29. Yi ES, Kim H, Ahn H, et al. Distribution of obstructive intimal lesions and their cellular phenotypes in chronic pulmonary hypertension. A morphometric and immunohistochemical study. *Am J Respir Crit Care Med* 2000; 162: 1577–1586.

30. Courboulin A, Tremblay VL, Barrier M, et al. Krüppel-like factor 5 contributes to pulmonary artery smooth muscle proliferation and resistance to apoptosis in human pulmonary arterial hypertension. *Respir Res* 2011; 12: 128.

31. Medici D, Shore EM, Lounev VY, et al. Conversion of vascular endothelial cells into multipotent stem-like cells. *Nat Med* 2010; 16: 1400–1406.

32. Medici D and Kalluri R. Endothelial-mesenchymal transition and its contribution to the emergence of stem cell phenotype. *Semin Cancer Biol* 2012; 22: 379–384.

33. Yu W, Liu Z, An S, et al. The endothelial-mesenchymal transition (EndMT) and tissue regeneration. *Curr Stem Cell Res Ther* 2014; 9: 196–204.

34. Lamouille S, Xu J and Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* 2014; 15: 178–196.

35. Mani SA, Guo W, Liao M-J, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 2008; 133: 704–715.

36. Shoshani O and Zipori D. Transition of endothelium to cartilage and bone. *Cell Stem Cell* 2011; 8: 10–11.

37. Ranchoux B, Antigny F, Rucker-Martin C, et al. Endothelial-to-mesenchymal transition in pulmonary hypertension. *Circulation* 2015; 131: 1006–1018.

38. Good RB, Gilbane AJ, Trinder SL, et al. Endothelial to mesenchymal transition contributes to endothelial dysfunction in pulmonary arterial hypertension. *Am J Pathol* 2015; 185: 1850–1858.

39. Tang H, Babicheva A, McDermott KM, et al. Endothelial HIF-2α contributes to severe pulmonary hypertension by inducing endothelial-to-mesenchymal transition. *Am J Physiol Lung Cell Mol Physiol* 2017; doi:10.1152/ajplung.00096.2017.

40. Li Z and Jimenez SA. Protein kinase Cδ and c-Abl kinase are required for transforming growth factor β induction of endothelial-mesenchymal transition in vitro. *Arthritis Rheum* 2011; 63: 2473–2483.

41. Lee WJ, Park JH, Shin JU, et al. Endothelial-to-mesenchymal transition induced by Wnt 3a in keloid pathogenesis. *Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc* 2015; 23: 435–442.

42. Rieder F, Kessler SP, West GA, et al. Inflammation-induced endothelial-to-mesenchymal transition: a novel mechanism of intestinal fibrosis. *Am J Pathol* 2011; 179: 2660–2673.

43. Groth A, Vrugt B, Brock M, et al. Inflammatory cytokines in pulmonary hypertension. *Respir Res* 2014; 15: 47.

44. Atkinson C, Stewart S, Upton PD, et al. Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. *Circulation* 2002; 105: 1672–1678.

45. Fessel JP, Loyd JE and Austin ED. The genetics of pulmonary arterial hypertension in the post-BMPR2 era. *Pulm Circ* 2011; 1: 305–319.

46. Thomson J, Machado R, Pauciulo M, et al. Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding BMPR-II, a receptor member of the TGF-β family. *J Med Genet* 2000; 37: 741–745.

47. Hopper RK, Moonen J-RAJ, Diebold I, et al. In pulmonary arterial hypertension, reduced BMPR2 promotes endothelial-to-mesenchymal transition via HMGA1 and its target slug. *Circulation* 2016; 133: 1783–1794.

48. Parikh VN, Jin RC, Rabello S, et al. MicroRNA-21 integrates pathogenic signaling to control pulmonary hypertension: results of a network bioinformatics approach. *Circulation* 2012; 125: 1520–1532.

49. Babicheva A, McDermott KM, Williams SC, et al. Pathogenic and Therapeutic Role of MicroRNA in Pulmonary Arterial Hypertension. In: *Diagnosis and Treatment of Pulmonary Hypertension.* Springer, Singapore, pp. 31–54.

50. Guo Y, Li P, Bledsoe G, et al. Kallistatin inhibits TGF-β-induced endothelial-mesenchymal transition by differential regulation of microRNA-21 and eNOS expression. *Exp Cell Res* 2015; 337: 103–110.

51. Cowburn AS, Crosby A, Macias D, et al. HIF2α–arginase axis is essential for the development of pulmonary hypertension. *Proc Natl Acad Sci* 2016; 113: 8801–8806.

52. Kapitsinou PP, Rajendran G, Astleford L, et al. The endothelial PHD2/HIF-2 axis regulates pulmonary artery pressure in mice. *Mol Cell Biol* 2016; 36: 1584–1594.

53. Dai Z, Li M, Wharton J, et al. PHD2 Deficiency in endothelial cells and hematopoietic cells induces obliterative vascular remodeling and severe pulmonary arterial hypertension in mice and humans through HIF-2α. *Circulation* 2016; 133: 2447–2458.

54. Loenarz C and Schofield CJ. Expanding chemical biology of 2-oxoglutarate oxygenases. *Nat Chem Biol* 2008; 4: 152–156.

55. Appelhoff RJ, Tian Y-M, Raval RR, et al. Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. *J Biol Chem* 2004; 279: 38458–38465.

56. Lane KB, Machado RD, et al. International PPH Consortium. Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. *Nat Genet* 2000; 26: 81–84.

57. Michelakis ED. Spatio-temporal diversity of apoptosis within the vascular wall in pulmonary arterial hypertension: heterogeneous BMP signaling may have therapeutic implications. *Circ Res* 2006; 98: 172–175.

58. Sakao S, Taraseviciene-Stewart L, Lee JD, et al. Initial apoptosis is followed by increased proliferation of apoptosis-resistant endothelial cells. *FASEB J Off Publ Fed Am Soc Exp Biol* 2005; 19: 1178–1180.

59. Masri FA, Xu W, Comhair SAA, et al. Hyperproliferative apoptosis-resistant endothelial cells in idiopathic pulmonary arterial hypertension. *Am J Physiol Lung Cell Mol Physiol* 2007; 293: L548–554.

60. Bestor TH. The DNA methyltransferases of mammals. *Hum Mol Genet* 2000; 9: 2395–2402.

61. Feng S, Jacobsen SE and Reik W. Epigenetic reprogramming in plant and animal development. *Science* 2010; 330: 622–627.

62. Kanwal R and Gupta S. Epigenetic modifications in cancer. *Clin Genet* 2012; 81: 303–311.

63. Nagano T and Fraser P. No-nonsense functions for long noncoding RNAs. *Cell* 2011; 145: 178–181.

64. Chuang JC and Jones PA. Epigenetics and microRNAs. *Pediatr Res* 2007; 61: 24R–29R.

65. Hautefort A, Chesné J, Preussner J, et al. Pulmonary endothelial cell DNA methylation signature in pulmonary arterial hypertension. *Oncotarget* 2017; 8: 52995–53016.

66. Danhaive O, Peca D and Boldrini R. ABCA3 mutation and pulmonary hypertension: a link with alveolar capillary dysplasia? *J Pediatr* 2008; 152: 891–892.

67. Kunig AM, Parker TA, Nogee LM, et al. ABCA3 deficiency presenting as persistent pulmonary hypertension of the newborn. *J Pediatr* 2007; 151: 322–324.

68. Ota C, Kimura M and Kure S. ABCA3 mutations led to pulmonary fibrosis and emphysema with pulmonary hypertension in an 8-year-old girl. *Pediatr Pulmonol* 2016; 51: E21–23.

69. Thérèse P . Persistent pulmonary hypertension of the newborn. *Paediatr Respir Rev* 2006; 7(Suppl 1): S175–176.

70. Villanueva ME, Zaher FM, Svinarich DM, et al. Decreased gene expression of endothelial nitric oxide synthase in newborns with persistent pulmonary hypertension. *Pediatr Res* 1998; 44: 338–343.

71. Hoehn T, Preston AA, McPhaden AR, et al. Endothelial nitric oxide synthase (NOS) is upregulated in rapid progressive pulmonary hypertension of the newborn. *Intensive Care Med* 2003; 29: 1757–1762.

72. Chan Y, Fish JE, D'Abreo C, et al. The cell-specific expression of endothelial nitric-oxide synthase: a role for DNA methylation. *J Biol Chem* 2004; 279: 35087–35100.

73. Fish JE and Marsden PA. Endothelial nitric oxide synthase: insight into cell-specific gene regulation in the vascular endothelium. *Cell Mol Life Sci CMLS* 2006; 63: 144–162.

74. Fish JE, Matouk CC, Rachlis A, et al. The expression of endothelial nitric-oxide synthase is controlled by a cell-specific histone code. *J Biol Chem* 2005; 280: 24824–24838.

75. Zhang M-X, Zhang C, Shen YH, et al. Effect of 27nt small RNA on endothelial nitric-oxide synthase expression. *Mol Biol Cell* 2008; 19: 3997–4005.

76. Gan Y, Shen YH, Wang J, et al. Role of histone deacetylation in cell-specific expression of endothelial nitric-oxide synthase. *J Biol Chem* 2005; 280: 16467–16475.

77. Xu X-F, Ma X-L, Shen Z, et al. Epigenetic regulation of the endothelial nitric oxide synthase gene in persistent pulmonary hypertension of the newborn rat. *J Hypertens* 2010; 28: 2227–2235.

78. Cavasin MA, Demos-Davies K, Horn TR, et al. Selective class I histone deacetylase inhibition suppresses hypoxia-induced cardiopulmonary remodeling through an antiproliferative mechanism. *Circ Res* 2012; 110: 739–748.

79. Li M, Riddle SR, Frid MG, et al. Emergence of fibroblasts with a proinflammatory epigenetically altered phenotype in severe hypoxic pulmonary hypertension. *J Immunol Baltim Md 1950* 2011; 187: 2711–2722.

80. Zhao L, Chen C-N, Hajji N, et al. Histone deacetylation inhibition in pulmonary hypertension: therapeutic potential of valproic acid and suberoylanilide hydroxamic acid. *Circulation* 2012; 126: 455–467.

81. Boucherat O, Chabot S, Paulin R, et al. HDAC6: a novel histone deacetylase implicated in pulmonary arterial hypertension. *Sci Rep* 2017; 7: 4546.

82. Kim J, Hwangbo C, Hu X, et al. Restoration of impaired endothelial myocyte enhancer factor 2 function rescues pulmonary arterial hypertension. *Circulation* 2015; 131: 190–199.

83. Fujisawa T and Filippakopoulos P. Functions of bromodomain-containing proteins and their roles in homeostasis and cancer. *Nat Rev Mol Cell Biol* 2017; 18: 246–262.

84. Meloche J, Potus F, Vaillancourt M, et al. Bromodomain-containing protein 4: the epigenetic origin of pulmonary arterial hypertension. *Circ Res* 2015; 117: 525–535.

85. Courboulin A, Ranchoux B, Cohen-Kaminsky S, et al. MicroRNA networks in pulmonary arterial hypertension: share mechanisms with cancer? *Curr Opin Oncol* 2016; 28: 72–82.

86. Boucherat O, Potus F and Bonnet S. microRNA and pulmonary hypertension. *Adv Exp Med Biol* 2015; 888: 237–252.

87. Chun HJ, Bonnet S and Chan SY. Translational advances in the field of pulmonary hypertension. Translating MicroRNA biology in pulmonary hypertension. It will take more than 'miR' words. *Am J Respir Crit Care Med* 2017; 195: 167–178.

88. Kim J, Kang Y, Kojima Y, et al. An endothelial apelin-FGF link mediated by miR-424 and miR-503 is disrupted in pulmonary arterial hypertension. *Nat Med* 2013; 19: 74–82.

89. Bertero T, Lu Y, Annis S, et al. Systems-level regulation of microRNA networks by miR-130/301 promotes pulmonary hypertension. *J Clin Invest* 2014; 124: 3514–3528.

90. Doebele C, Bonauer A, Fischer A, et al. Members of the microRNA-17-92 cluster exhibit a cell-intrinsic antiangiogenic function in endothelial cells. *Blood* 2010; 115: 4944–4950.

91. Bonauer A, Carmona G, Iwasaki M, et al. MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. *Science* 2009; 324: 1710–1713.

92. Chan SY and Loscalzo J. MicroRNA-210: a unique and pleiotropic hypoxamir. *Cell Cycle Georget Tex* 2010; 9: 1072–1083.

93. Beinert H, Holm RH and Münck E. Iron-sulfur clusters: nature's modular, multipurpose structures. *Science* 1997; 277: 653–659.

94. Rouault TA and Tong WH. Iron-sulfur cluster biogenesis and human disease. *Trends Genet TIG* 2008; 24: 398–407.

95. Chan SY, Zhang Y-Y, Hemann C, et al. MicroRNA-210 controls mitochondrial metabolism during hypoxia by repressing the iron-sulfur cluster assembly proteins ISCU1/2. *Cell Metab* 2009; 10: 273–284.

96. White K, Lu Y, Annis S, et al. Genetic and hypoxic alterations of the microRNA-210-ISCU1/2 axis promote iron-sulfur deficiency and pulmonary hypertension. *EMBO Mol Med* 2015; 7: 695–713.

97. Kuehbacher A, Urbich C, Zeiher AM, et al. Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis. *Circ Res* 2007; 101: 59–68.

98. White K, Dempsie Y, Caruso P, et al. Endothelial apoptosis in pulmonary hypertension is controlled by a microRNA/programmed cell death 4/caspase-3 axis. *Hypertens Dallas Tex 1979* 2014; 64: 185–194.

99. Yang S, Banerjee S, Freitas A de, et al. miR-21 regulates chronic hypoxia-induced pulmonary vascular remodeling. *Am J Physiol Lung Cell Mol Physiol* 2012; 302: L521–529.

100. Iannone L, Zhao L, Dubois O, et al. miR-21/DDAH1 pathway regulates pulmonary vascular responses to hypoxia. *Biochem J* 2014; 462: 103–112.

101. Deng L, Blanco FJ, Stevens H, et al. MicroRNA-143 activation regulates smooth muscle and endothelial cell crosstalk in pulmonary arterial hypertension. *Circ Res* 2015; 117: 870–883.

102. McCall MN, Kent OA, Yu J, et al. MicroRNA profiling of diverse endothelial cell types. *BMC Med Genomics* 2011; 4: 78.

103. Guo L, Yang Y, Liu J, et al. Differentially expressed plasma microRNAs and the potential regulatory function of Let-7b in chronic thromboembolic pulmonary hypertension. *PLoS One* 2014; 9: e101055.

104. Kang B-Y, Park KK, Green DE, et al. Hypoxia mediates mutual repression between microRNA-27a and PPARγ in the pulmonary vasculature. *PLoS One* 2013; 8: e79503.

105. Sen A, Ren S, Lerchenmüller C, et al. MicroRNA-138 regulates hypoxia-induced endothelial cell dysfunction by targeting S100A1. *PLoS One* 2013; 8: e78684.

106. Gu S, Li G, Zhang X, et al. Aberrant expression of long noncoding RNAs in chronic thromboembolic pulmonary hypertension. *Mol Med Rep* 2015; 11: 2631–2643.

107. Voellenkle C, Garcia-Manteiga JM, Pedrotti S, et al. Implication of long noncoding RNAs in the endothelial cell response to hypoxia revealed by RNA-sequencing. *Sci Rep* 2016; 6: 24141.

108. Michalik KM, You X, Manavski Y, et al. Long noncoding RNA MALAT1 regulates endothelial cell function and vessel growth. *Circ Res* 2014; 114: 1389–1397.

109. Zhuo Y, Zeng Q, Zhang P, et al. Functional polymorphism of lncRNA MALAT1 contributes to pulmonary arterial hypertension susceptibility in Chinese people. *Clin Chem Lab Med* 2017; 55: 38–46.

110. Leisegang MS, Fork C, Josipovic I, et al. Long noncoding RNA MANTIS facilitates endothelial angiogenic function. *Circulation* 2017; 136: 65–79.

111. Guibert C, Marthan R and Savineau J-P. Modulation of ion channels in pulmonary arterial hypertension. *Curr Pharm Des* 2007; 13: 2443–2455.

112. Firth AL, Remillard CV and Yuan JX-J. TRP channels in hypertension. *Biochim Biophys Acta* 2007; 1772: 895–906.

113. Sukriti S, Tauseef M, Yazbeck P, et al. Mechanisms regulating endothelial permeability. *Pulm Circ* 2014; 4: 535–551.

114. Villalta PC and Townsley MI. Transient receptor potential channels and regulation of lung endothelial permeability. *Pulm Circ* 2013; 3: 802–815.

115. Yu Y, Fantozzi I, Remillard CV, et al. Enhanced expression of transient receptor potential channels in idiopathic pulmonary arterial hypertension. *Proc Natl Acad Sci U S A* 2004; 101: 13861–13866.

116. Yu Y, Keller SH, Remillard CV, et al. A functional single-nucleotide polymorphism in the TRPC6 gene promoter associated with idiopathic pulmonary arterial hypertension. *Circulation* 2009; 119: 2313–2322.

117. Yu Y, Sweeney M, Zhang S, et al. PDGF stimulates pulmonary vascular smooth muscle cell proliferation by upregulating TRPC6 expression. *Am J Physiol Cell Physiol* 2003; 284: C316–330.

118. Smith KA, Voiriot G, Tang H, et al. Notch Activation of Ca(2+) Signaling in the development of hypoxic pulmonary vasoconstriction and pulmonary hypertension. *Am J Respir Cell Mol Biol* 2015; 53: 355–367.

119. Noseda M, McLean G, Niessen K, et al. Notch activation results in phenotypic and functional changes consistent with endothelial-to-mesenchymal transformation. *Circ Res* 2004; 94: 910–917.

120. Dabral S, Tian X, Kojonazarov B, et al. Notch1 signalling regulates endothelial proliferation and apoptosis in pulmonary arterial hypertension. *Eur Respir J* 2016; 48: 1137–1149.

121. Geng H and Guan J. MiR-18a-5p inhibits endothelial-mesenchymal transition and cardiac fibrosis through the Notch2 pathway. *Biochem Biophys Res Commun* 2017; 491: 329–336.

122. Pocock TM, Foster RR and Bates DO. Evidence of a role for TRPC channels in VEGF-mediated increased vascular permeability in vivo. *Am J Physiol Heart Circ Physiol* 2004; 286: H1015–1026.

123. Paria BC, Vogel SM, Ahmmed GU, et al. Tumor necrosis factor-alpha-induced TRPC1 expression amplifies store-operated Ca2+ influx and endothelial permeability. *Am J Physiol Lung Cell Mol Physiol* 2004; 287: L1303–1313.

124. Sundivakkam PC, Freichel M, Singh V, et al. The Ca(2+) sensor stromal interaction molecule 1 (STIM1) is necessary and sufficient for the store-operated Ca(2+) entry function of transient receptor potential canonical (TRPC) 1 and 4 channels in endothelial cells. *Mol Pharmacol* 2012; 81: 510–526.

125. Abdullaev IF, Bisaillon JM, Potier M, et al. Stim1 and Orai1 mediate CRAC currents and store-operated calcium entry important for endothelial cell proliferation. *Circ Res* 2008; 103: 1289–1299.

126. Li J, Cubbon RM, Wilson LA, et al. Orai1 and CRAC channel dependence of VEGF-activated Ca2+ entry and endothelial tube formation. *Circ Res* 2011; 108: 1190–1198.

127. Cheng KT, Liu X, Ong HL, et al. Local Ca2+ entry via orai1 regulates plasma membrane recruitment of TRPC1 and controls cytosolic Ca2+ signals required for specific cell functions. *PLoS Biol* 2011; 9: e1001025.

128. Moore TM, Brough GH, Babal P, et al. Store-operated calcium entry promotes shape change in pulmonary endothelial cells expressing Trp1. *Am J Physiol* 1998; 275: L574–582.

129. Fantozzi I, Zhang S, Platoshyn O, et al. Hypoxia increases AP-1 binding activity by enhancing capacitative Ca2+ entry in human pulmonary artery endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 2003; 285: L1233–1245.

130. Paria BC, Bair AM, Xue J, et al. Ca2+ influx induced by protease-activated receptor-1 activates a feed-forward mechanism of TRPC1 expression via nuclear factor-kappaB activation in endothelial cells. *J Biol Chem* 2006; 281: 20715–20727.

131. Thippegowda PB, Singh V, Sundivakkam PC, et al. Ca2+ influx via TRPC channels induces NF-kappaB-dependent A20 expression to prevent thrombin-induced apoptosis in endothelial cells. *Am J Physiol Cell Physiol* 2010; 298: C656–664.

132. Li L, Wei C, Kim I-K, et al. Inhibition of nuclear factor-κB in the lungs prevents monocrotaline-induced pulmonary hypertension in mice. *Hypertens Dallas Tex 1979* 2014; 63: 1260–1269.

133. Mai J, Hu Q, Xie Y, et al. Dyssynchronous pacing triggers endothelial-mesenchymal transition through heterogeneity of mechanical stretch in a canine model. *Circ J Off J Jpn Circ Soc* 2015; 79: 201–209.

134. Yeon S-I, Kim JY, Yeon D-S, et al. Transient receptor potential canonical type 3 channels control the vascular contractility of mouse mesenteric arteries. *PLoS One* 2014; 9: e110413.

135. Smedlund K, Tano J-Y and Vazquez G. The constitutive function of native TRPC3 channels modulates vascular cell adhesion molecule-1 expression in coronary endothelial cells through nuclear factor kappaB signaling. *Circ Res* 2010; 106: 1479–1488.

136. Hara Y, Wakamori M, Ishii M, et al. LTRPC2 Ca2+-permeable channel activated by changes in redox status confers susceptibility to cell death. *Mol Cell* 2002; 9: 163–173.

137. Chen H-C, Su L-T, González-Pagán O, et al. A key role for Mg(2+) in TRPM7's control of ROS levels during cell stress. *Biochem J* 2012; 445: 441–448.

138. Simon F, Varela D and Cabello-Verrugio C. Oxidative stress-modulated TRPM ion channels in cell dysfunction and pathological conditions in humans. *Cell Signal* 2013; 25: 1614–1624.

139. Baldoli E, Castiglioni S and Maier JAM. Regulation and function of TRPM7 in human endothelial cells: TRPM7 as a potential novel regulator of endothelial function. *PLoS One* 2013; 8: e59891.

140. Chen S, Hoffman NE, Shanmughapriya S, et al. A splice variant of the human ion channel TRPM2 modulates neuroblastoma tumor growth through hypoxia-inducible factor (HIF)-1/2α. *J Biol Chem* 2014; 289: 36284–36302.

141. Ding X-Q, Ban T, Liu Z-Y, et al. Transient receptor potential melastatin 4 (TRPM4) contributes to high salt diet-mediated early-stage endothelial injury. *Cell Physiol Biochem Int J Exp Cell Physiol Biochem Pharmacol* 2017; 41: 835–848.

142. Echeverría C, Montorfano I, Hermosilla T, et al. Endotoxin induces fibrosis in vascular endothelial cells through a mechanism dependent on transient receptor protein melastatin 7 activity. *PLoS One* 2014; 9: e94146.

143. Davis FM, Azimi I, Faville RA, et al. Induction of epithelial-mesenchymal transition (EMT) in breast cancer cells is calcium signal dependent. *Oncogene* 2014; 33: 2307–2316.

144. Liu J, Chen Y, Shuai S, et al. TRPM8 promotes aggressiveness of breast cancer cells by regulating EMT via activating AKT/GSK-3β pathway. *Tumour Biol J Int Soc Oncodevelopmental Biol Med* 2014; 35: 8969–8977.

145. Dorovkov MV, Kostyukova AS and Ryazanov AG. Phosphorylation of annexin A1 by TRPM7 kinase: a switch regulating the induction of an α-helix. *Biochemistry (Mosc)* 2011; 50: 2187–2193.

146. Yi B, Cui J, Ning J, et al. cGMP-dependent protein kinase Iα transfection inhibits hypoxia-induced migration, phenotype modulation and annexins A1 expression in human pulmonary artery smooth muscle cells. *Biochem Biophys Res Commun* 2012; 418: 598–602.

147. Wang Y, Cui L, Xu H, et al. TRPV1 agonism inhibits endothelial cell inflammation via activation of eNOS/NO pathway. *Atherosclerosis* 2017; 260: 13–19.

148. Yang D, Luo Z, Ma S, et al. Activation of TRPV1 by dietary capsaicin improves endothelium-dependent vasorelaxation and prevents hypertension. *Cell Metab* 2010; 12: 130–141.

149. Hao X, Chen J, Luo Z, et al. TRPV1 activation prevents high-salt diet-induced nocturnal hypertension in mice. *Pflugers Arch* 2011; 461: 345–353.

150. Sun J, Pu Y, Wang P, et al. TRPV1-mediated UCP2 upregulation ameliorates hyperglycemia-induced endothelial dysfunction. *Cardiovasc Diabetol* 2013; 12: 69.

151. Su K-H, Lin S-J, Wei J, et al. The essential role of transient receptor potential vanilloid 1 in simvastatin-induced activation of endothelial nitric oxide synthase and angiogenesis. *Acta Physiol Oxf Engl* 2014; 212: 191–204.

152. Tuuminen R, Syrjälä S, Krebs R, et al. Donor simvastatin treatment abolishes rat cardiac allograft ischemia/reperfusion injury and chronic rejection through microvascular protection. *Circulation* 2011; 124: 1138–1150.

153. Song S, Ayon RJ, Yamamura A, et al. Capsaicin-induced Ca(2+) signaling is enhanced via upregulated TRPV1 channels in pulmonary artery smooth muscle cells from patients with idiopathic PAH. *Am J Physiol Lung Cell Mol Physiol* 2017; 312: L309–L325.

154. Huang W, Rubinstein J, Prieto AR, et al. Enhanced postmyocardial infarction fibrosis via stimulation of the transforming growth factor-beta-Smad2 signaling pathway: role of transient receptor potential vanilloid type 1 channels. *J Hypertens* 2010; 28: 367–376.

155. Geng S, Zheng Y, Meng M, et al. Gingerol reverses the cancer-promoting effect of capsaicin by increased TRPV1 level in a urethane-induced lung carcinogenic model. *J Agric Food Chem* 2016; 64: 6203–6211.

156. Lau JK, Brown KC, Dom AM, et al. Capsaicin induces apoptosis in human small cell lung cancer via the TRPV6 receptor and the calpain pathway. *Apoptosis Int J Program Cell Death* 2014; 19: 1190–1201.

157. Lehen'kyi V, Flourakis M, Skryma R, et al. TRPV6 channel controls prostate cancer cell proliferation via Ca(2+)/NFAT-dependent pathways. *Oncogene* 2007; 26: 7380–7385.

158. Dai W, Bai Y, Hebda L, et al. Calcium deficiency-induced and TRP channel-regulated IGF1R-PI3K-Akt signaling regulates abnormal epithelial cell proliferation. *Cell Death Differ* 2014; 21: 568–581.

159. Nabissi M, Morelli MB, Amantini C, et al. TRPV2 channel negatively controls glioma cell proliferation and resistance to Fas-induced apoptosis in ERK-dependent manner. *Carcinogenesis* 2010; 31: 794–803.

160. Zhan L, Yang Y, Ma T-T, et al. Transient receptor potential vanilloid 4 inhibits rat HSC-T6 apoptosis through induction of autophagy. *Mol Cell Biochem* 2015; 402: 9–22.

161. Alvarez DF, King JA, Weber D, et al. Transient receptor potential vanilloid 4-mediated disruption of the alveolar septal barrier: a novel mechanism of acute lung injury. *Circ Res* 2006; 99: 988–995.

162. Jian M-Y, King JA, Al-Mehdi A-B, et al. High vascular pressure-induced lung injury requires P450 epoxygenase-dependent activation of TRPV4. *Am J Respir Cell Mol Biol* 2008; 38: 386–392.

163. Wu S, Jian M-Y, Xu Y-C, et al. Ca2+ entry via alpha1G and TRPV4 channels differentially regulates surface expression of P-selectin and barrier integrity in pulmonary capillary endothelium. *Am J Physiol Lung Cell Mol Physiol* 2009; 297: L650–657.

164. Lin MT, Jian M-Y, Taylor MS, et al. Functional coupling of TRPV4, IK, and SK channels contributes to Ca(2+)-dependent endothelial injury in rodent lung. *Pulm Circ* 2015; 5: 279–290.

165. Troidl C, Troidl K, Schierling W, et al. Trpv4 induces collateral vessel growth during regeneration of the arterial circulation. *J Cell Mol Med* 2009; 13: 2613–2621.

166. Boucherat O, Chabot S, Antigny F, et al. Potassium channels in pulmonary arterial hypertension. *Eur Respir J* 2015; 46: 1167–1177.

167. Chen TT, Luykenaar KD, Walsh EJ, et al. Key role of Kv1 channels in vasoregulation. *Circ Res* 2006; 99: 53–60.

168. Plane F, Johnson R, Kerr P, et al. Heteromultimeric Kv1 channels contribute to myogenic control of arterial diameter. *Circ Res* 2005; 96: 216–224.

169. Firth AL, Remillard CV, Platoshyn O, et al. Functional ion channels in human pulmonary artery smooth muscle cells: Voltage-dependent cation channels. *Pulm Circ* 2011; 1: 48–71.

170. Chen W-L, Huang X-Q, Zhao L-Y, et al. Involvement of Kv1.5 protein in oxidative vascular endothelial cell injury. *PLoS One* 2012; 7: e49758.

171. Peinado VI, París R, Ramírez J, et al. Expression of BK(Ca) channels in human pulmonary arteries: relationship with remodeling and hypoxic pulmonary vasoconstriction. *Vascul Pharmacol* 2008; 49: 178–184.

172. Kuhlmann CRW, Schäfer M, Li F, et al. Modulation of endothelial Ca(2+)-activated K(+) channels by oxidized LDL and its contribution to endothelial proliferation. *Cardiovasc Res* 2003; 60: 626–634.

173. Sandow SL, Neylon CB, Chen MX, et al. Spatial separation of endothelial small- and intermediate-conductance calcium-activated potassium channels (K(Ca)) and connexins: possible relationship to vasodilator function? *J Anat* 2006; 209: 689–698.

174. Absi M, Burnham MP, Weston AH, et al. Effects of methyl beta-cyclodextrin on EDHF responses in pig and rat arteries; association between SK(Ca) channels and caveolin-rich domains. *Br J Pharmacol* 2007; 151: 332–340.

175. Dora KA, Gallagher NT, McNeish A, et al. Modulation of endothelial cell KCa3.1 channels during endothelium-derived hyperpolarizing factor signaling in mesenteric resistance arteries. *Circ Res* 2008; 102: 1247–1255.

176. Ledoux J, Taylor MS, Bonev AD, et al. Functional architecture of inositol 1,4,5-trisphosphate signaling in restricted spaces of myoendothelial projections. *Proc Natl Acad Sci U S A* 2008; 105: 9627–9632.

177. Ahmmed GU and Malik AB. Functional role of TRPC channels in the regulation of endothelial permeability. *Pflugers Arch* 2005; 451: 131–142.

178. Watanabe H, Vriens J, Prenen J, et al. Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. *Nature* 2003; 424: 434–438.

179. Sheng J-Z and Braun AP. Small- and intermediate-conductance Ca2+-activated K+ channels directly control agonist-evoked nitric oxide synthesis in human vascular endothelial cells. *Am J Physiol Cell Physiol* 2007; 293: C458–467.

180. Taylor MS, Bonev AD, Gross TP, et al. Altered expression of small-conductance Ca2+-activated K+ (SK3) channels modulates arterial tone and blood pressure. *Circ Res* 2003; 93: 124–131.

181. Kefaloyianni E and Coetzee WA. Transcriptional remodeling of ion channel subunits by flow adaptation in human coronary artery endothelial cells. *J Vasc Res* 2011; 48: 357–367.

182. Liu Y, Xie A, Singh AK, et al. Inactivation of endothelial small/intermediate conductance of calcium-activated potassium channels contributes to coronary arteriolar dysfunction in diabetic patients. *J Am Heart Assoc* 2015; 4: e002062.

183. Gokina NI, Bonev AD, Phillips J, et al. Impairment of IKCa channels contributes to uteroplacental endothelial dysfunction in rat diabetic pregnancy. *Am J Physiol Heart Circ Physiol* 2015; 309: H592–604.

184. Paradisi G, Biaggi A, Ferrazzani S, et al. Abnormal carbohydrate metabolism during pregnancy: association with endothelial dysfunction. *Diabetes Care* 2002; 25: 560–564.

185. Félétou M. Calcium-activated potassium channels and endothelial dysfunction: therapeutic options? *Br J Pharmacol* 2009; 156: 545–562.

186. Shimoda LA, Welsh LE and Pearse DB. Inhibition of inwardly rectifying K(+) channels by cGMP in pulmonary vascular endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 2002; 283: L297–304.

187. Fang Y, Mohler ER, Hsieh E, et al. Hypercholesterolemia suppresses inwardly rectifying K+ channels in aortic endothelium in vitro and in vivo. *Circ Res* 2006; 98: 1064–1071.

188. Tang Y, Long C-L, Wang R-H, et al. Activation of SUR2B/Kir6.1 subtype of adenosine triphosphate-sensitive potassium channel improves pressure overload-induced cardiac remodeling via protecting endothelial function. *J Cardiovasc Pharmacol* 2010; 56: 345–353.

189. Maczewski M and Beresewicz A. The role of adenosine and ATP-sensitive potassium channels in the protection afforded by ischemic preconditioning against the post-ischemic endothelial dysfunction in guinea-pig hearts. *J Mol Cell Cardiol* 1998; 30: 1735–1747.

190. Broadhead MW, Kharbanda RK, Peters MJ, et al. KATP channel activation induces ischemic preconditioning of the endothelium in humans in vivo. *Circulation* 2004; 110: 2077–2082.

191. Enyedi P and Czirják G. Molecular background of leak K+ currents: two-pore domain potassium channels. *Physiol Rev* 2010; 90: 559–605.

192. Goldstein SA, Bockenhauer D, O'Kelly I, et al. Potassium leak channels and the KCNK family of two-P-domain subunits. *Nat Rev Neurosci* 2001; 2: 175–184.

193. Ma L, Roman-Campos D, Austin ED, et al. A novel channelopathy in pulmonary arterial hypertension. *N Engl J Med* 2013; 369: 351–361.

194. Antigny F, Hautefort A, Meloche J, et al. Potassium channel subfamily K member 3 (KCNK3) contributes to the development of pulmonary arterial hypertension. *Circulation* 2016; 133: 1371–1385.