Cellular and Molecular Basis of Pulmonary Arterial Hypertension

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Pulmonary arterial hypertension (PAH) is caused by functional and structural changes in the pulmonary vasculature, leading to increased pulmonary vascular resistance. The process of pulmonary vascular remodeling is accompanied by endothelial dysfunction, activation of fibroblasts and smooth muscle cells, crosstalk between cells within the vascular wall, and recruitment of circulating progenitor cells. Recent findings have reestablished the role of chronic vasoconstriction in the remodeling process. Although the pathology of PAH in the lung is well known, this article is concerned with the cellular and molecular processes involved. In particular, we focus on the role of the Rho family guanosine triphosphatases in endothelial function and vasoconstriction. The crosstalk between endothelium and vascular smooth muscle is explored in the context of mutations in the bone morphogenetic protein type II receptor, alterations in angiopoietin-1/TIE2 signaling, and the serotonin pathway. We also review the role of voltage-gated K^+ channels and transient receptor potential channels in the regulation of cytosolic $[Ca^{2+}]$ and $[K^+]$, vasoconstriction, proliferation, and cell survival. We highlight the importance of the extracellular matrix as an active regulator of cell behavior and phenotype and evaluate the contribution of the glycoprotein tenascin-c as a key mediator of smooth muscle cell growth and survival. Finally, we discuss the origins of a cell type critical to the process of pulmonary vascular remodeling, the myofibroblast, and review the evidence supporting a contribution for the involvement of endothelial-mesenchymal transition and recruitment of circulating mesenchymal progenitor cells.   (J Am Coll Cardiol 2009;54:S20–31) © 2009 by the American College of Cardiology Foundation

Despite the recognized success of existing drug interventions in the relief of symptoms of pulmonary arterial hypertension (PAH), and possibly improvement in survival, most patients eventually become resistant to therapy and succumb to the disease. The past few years have

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seen a remarkable increase in our knowledge of the cellular and molecular mechanisms responsible for the pathobiology of PAH. This summary aims to present the current state of our understanding of some of the key mechanisms (Fig. 1). We also indicate further areas and directions of research and suggest novel approaches to therapy.

Endothelial Dysfunction in PAH

Endothelial cells (ECs) are recognized as major regulators of vascular function, and endothelial dysfunction has come to mean a multifaceted imbalance in EC production of vasoconstrictors versus vasodilators, activators versus inhibitors of smooth muscle cell (SMC) growth and migration, prothrombotic versus antithrombotic mediators, and proinflammatory versus anti-inflammatory signals.

Rho guanosine triphosphatases (GTPases) in endothelial dysfunction. Rho (*Ras ho*mologous) GTP-binding proteins regulate many cellular processes, including gene transcription,

differentiation, proliferation, hypertrophy, apoptosis, phagocytosis, adhesion, migration, and contraction (1). In the prototypical mechanism of RhoA GTPase signaling, environmental cues, acting through G-protein−coupled receptors or receptor-dependent and receptor-independent tyrosine kinases, activate guanine nucleotide exchange factors, which induce exchange of guanosine diphosphate for GTP binding and translocation of GTP-RhoA to the plasma membrane. The membrane translocation requires post-translational prenylation. Upon translocation to the plasma membrane, GTP-RhoA activates its effectors, including the 2 isoforms of Rho kinase (ROCK), ROCK I (ROKβ) and ROCK II (ROKα). Negative regulators of RhoA activation include guanine nucleotide disassociation inhibitors, which oppose the exchange of GTP for guanosine diphosphate; GTPase activating proteins, which catalyze dephosphorylation and inactivation of membrane-bound GTP-RhoA; statins, which inhibit isoprenylation of RhoA and thereby prevent translocation of GTP-RhoA to the cell membrane (2); and protein kinases A and G, which, by phosphorylating RhoA, also prevent membrane translocation of the GTP-bound protein (3).

Rho GTPases and EC permeability. An increase in EC permeability may be an important component of the pathogenesis of PAH. The GTPases RhoA and Rac1 play opposing roles in the regulation of EC barrier function. While stimuli such as thrombin activate RhoA/ROCK, which increases formation of F-actin stress fibers, cell contraction, and permeability, barrier-enhancing mediators such as sphingosine-1-phosphate and prostacyclin (PGI$_2$) stimulate Rac1/p21-activated kinase (PAK), which counteracts the effects of RhoA/ROCK and promotes cortical F-actin ring formation and barrier integrity (4). Pulmonary artery ECs cultured from chronically hypoxic piglets demonstrate low Rac1 and high RhoA activities, which correlate with increased stress fiber formation and permeability (5). Activation of Rac1/PAK-1 and inhibition of RhoA reverse these changes.

Rho GTPases and EC proliferation, migration, and apoptosis. Rho GTPases participate in EC proliferation and apoptosis. Interestingly, the hyperproliferative, apoptosis-resistant phenotype of PAH ECs may be due to persistent activation of signal transducer and activator of transcription 3 (6), a downstream target of Rho GTPases. Signal transducer and activator of transcription 3 mediates RhoA-induced nuclear factor-κB and cyclin D1 transcription and is involved in nuclear factor-κB nuclear translocation (7).

Role of rho GTPases in thrombosis. In situ thrombosis of small peripheral pulmonary arteries contributes to PAH. The ECs are directly involved in the fibrinolytic process through synthesis and release of the profibrinolytic tissue plasminogen activator and the antifibrinolytic/prothrombotic plasminogen activator inhibitor (PAI)-1. The stimulation of systemic artery EC PAI-1 expression by angiotensin II, C-reactive protein, high glucose, and monocyte adhesion is dependent on activation of RhoA/ROCK signaling. Similarly, EC expression of tissue factor, another prothrombotic mediator, increased in the pulmonary arteries (PAs) of PAH lungs, is upregulated by RhoA/ROCK signaling (8). The RhoA/ROCK and Rac/PAK signaling pathways are implicated in thrombin- and thromboxane A$_2$-induced platelet activation and aggregation (9).

Nitric oxide (NO) and PGI$_2$. Endothelial dysfunction in PAH is reflected by reduced production of the vasodilators/growth inhibitors NO and PGI$_2$ and increased production of the vasoconstrictor/co-mitogens, for example, endothelin-1 and thromboxane A2. Nitric oxide signaling is mediated mainly by the guanylate cyclase/cyclic guanosine monophosphate (cGMP) pathway. Degradation of the second messenger of NO, cGMP, by phosphodiesterases is mainly accomplished by phosphodiesterase-5.

Reduced NO bioavailability in PAH can be due to decreased expression of endothelial NO synthase (eNOS), inhibition of eNOS enzymatic activity, and inactivation of NO by superoxide anion. Activation of endothelial RhoA/ROCK signaling can be involved in at least the first 2 processes. For example, RhoA/ROCK activation mediates hypoxia- and thrombin-induced inhibition of both eNOS expression and its activity in cultured ECs (10). The activity of arginase II, which reduces NO synthesis by competing with eNOS for the substrate L-arginine, is increased in PAH ECs (11), and RhoA/ROCK signaling mediates thrombin- and tumor necrosis factor-α/lipopolysaccharide-induced activation of eNOS (12). Patients with idiopathic PAH (IPAH) have increased plasma levels of the endogenous inhibitor of eNOS, asymmetric dimethylarginine (13), and the levels of asymmetric dimethylarginine and the enzyme that degrades it, dimethylarginine

Abbreviations and Acronyms

ALK = activin-receptorlike kinase

Ang = angiopoeitin

BMP = bone morphogenetic protein

BMPR = bone morphogenetic protein receptor

cGMP = cyclic guanosine monophosphate

EC = endothelial cell

ECM = extracellular matrix

enMT = endothelial mesenchymal transition

eNOS = endothelial nitric oxide synthase

GTPase = guanosine triphosphatase

5-HT = hydroxytryptamine (serotonin)

5-HTT = hydroxytryptamine (serotonin) transporter

IPAH = idiopathic pulmonary arterial hypertension

MLC = myosin light chain

MLCK = myosin light chain kinase

MLCP = myosin light chain phosphatase

NO = nitric oxide

PA = pulmonary artery

PAEC = pulmonary artery endothelial cell

PAH = pulmonary arterial hypertension

PAI = plasminogen-activator inhibitor

PAK = p21-activated kinase

PASMC = pulmonary artery smooth muscle cell

PGI$_2$ = prostacyclin

PH = pulmonary hypertension

PK = protein kinase

ROCK = Rho kinase

SMC = smooth muscle cell

TGF = transforming growth factor

TRPC = canonical transient receptor potential
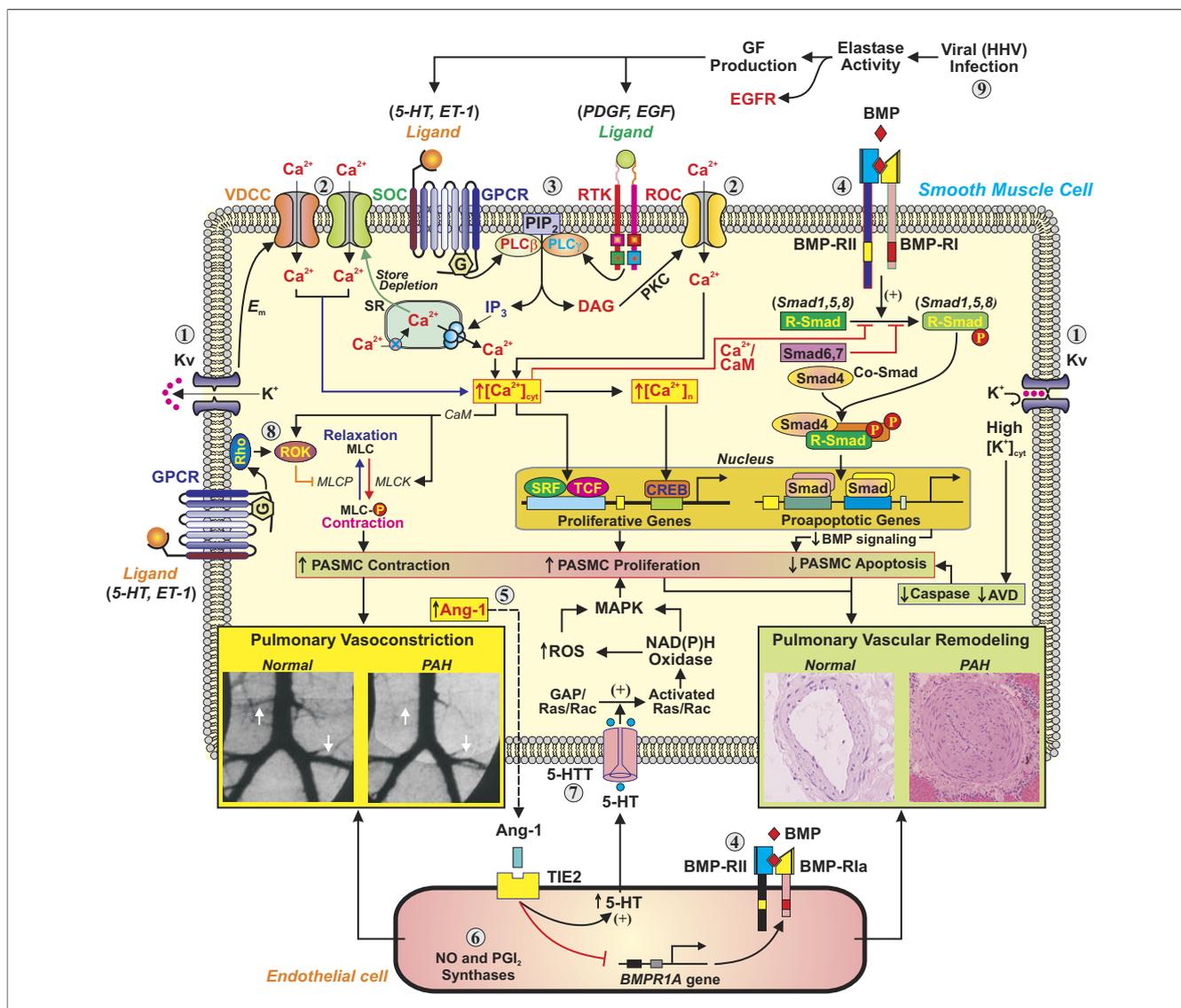
Figure 1 **Potential Mechanisms Involved in the Development of PAH**

Schematic diagram depicting potential mechanisms involved in the development of pulmonary arterial hypertension (PAH). Ang = angiopoeitin; AVD = apoptotic volume decrease; BMP = bone morphogenetic protein; BMPR = bone morphogenetic protein receptor; CaM = calmodulin; CREB = cAMP-response element binding protein; DAG = diacylglycerol; E_m = membrane potential; EGF = epidermal growth factor; EGFR = epidermal growth factor receptor; ET = endothelin; GAP = GTPase activating protein; GPCR = G protein-coupled receptor; HHV = human herpes virus; HT = hydroxytryptamine (serotonin); HTT = hydroxytryptamine (serotonin) transporter; IP_3 = inositol 1,4,5-trisphosphate; Kv = voltage-gated K^+; MAPK = mitogen-activated protein kinase; MLC = myosin light chain; MLCK = myosin light chain kinase; NA(D)PH = nicotinamide adenine dinucleotide phosphate; NO = nitric oxide; PASMC = pulmonary artery smooth muscle cell; PDGF = platelet-derived growth factor; PGI_2 = prostacyclin; PKC = protein kinase C; PLC = phospholipase C; ROC = receptor-operated Ca^{2+} channels; ROS = reactive oxygen species; RTK = receptor tyrosine kinase; SR = sarcoplasmic reticulum; SRF = serum response factor; TCF = T cell factor; TIE = endothelial-specific tyrosine kinase; VDCC = voltage-dependent calcium channel.

dimethylaminohydrolase, are, respectively, increased and decreased in the PA endothelium of IPAH patients (13).

Prostacyclin stimulates the formation of cyclic adenosine monophosphate, which also inhibits the proliferation of SMCs and decreases platelet aggregation. A deficiency of PGI_2 and PGI_2 synthase and an excess of thromboxane are found in PAH (14). Moreover, PGI_2-receptor knockout mice develop more severe hypoxia-induced pulmonary hypertension (PH) (15). Conversely, PGI_2 overex-

pressing mice are protected against hypoxia-induced PH (16).

Angiopoietin and TIE2. Angiopoietin (Ang)-1 is an oligomeric-secreted glycoprotein, which, along with angiopoietin-2 and angiopoietin-3/4, comprises a family of growth factors. The angiopoietin ligands exert their effects through the endothelial-specific tyrosine kinase, TIE2 (17). During lung development, both Ang-1 and TIE2 are expressed in growing blood vessels: Ang-1 is made and

secreted by vascular SMCs and pericytes, whereas TIE2 is a transmembrane receptor expressed on endothelial cells (18). In the adult, Ang-1 expression in the lung is minimal, whereas TIE2 expression remains constitutive (19).

Several lines of evidence suggest that Ang-1 regulates pathologic SMC hyperplasia in PAH. Ang-1 is overexpressed in most forms of nonfamilial PAH (18,20). In PAH, Ang-1 causes activation of the TIE2 receptor by tyrosine autophosphorylation in the pulmonary vascular endothelium (20,21). Enhanced TIE2 levels and a 4-fold increase in TIE2 phosphorylation are found in human PAH lung tissue, compared with control subjects (20,22).

Virally mediated overexpression of Ang-1 in the rat lung results in PH (21,23). Ang-1 transgenic animals show increased pulmonary vascular endothelial TIE2 phosphorylation and SMC hyperplasia in small pulmonary arterioles. Further, overexpression of a soluble TIE2 ectodomain, which sequesters Ang-1, suppresses the PH phenotype in monocrotaline- and Ang-1–induced models of this disease (24).

There is a reciprocal relationship between bone morphogenetic protein receptor (BMPR) 1A and Ang-1 expression in the lungs of patients with nonfamilial PAH (20). Ang-1 downregulates BMPR1A expression through a TIE2 pathway in human pulmonary artery endothelial cells (PAECs). Stimulation of human PAECs with Ang-1 induces release of 5-hydroxytryptamine (HT [serotonin]), a potent stimulator of SMC proliferation (21,22). There is controversy in this field. In contrast to a causative role, Ang-1 has been reported to protect against the development of PAH in the rat monocrotoline and hypoxia models of disease (25).

The SMC in PAH

Serotonin, serotonin transporter, and receptors. Patients with IPAH have increased circulating 5-HT levels, even after heart-lung transplantation (26). In contrast to the constricting action of 5-HT on SMCs, which is mainly mediated by 5-HT receptors 1B/D, 2A, and 2B (27), the mitogenic and co-mitogenic effects of 5-HT require internalization through the serotonin transporter, 5-HTT (28). That may require co-stimulation of the 5-HT$_{1B}$ receptor (29). Drugs that competitively inhibit 5-HTT block the mitogenic effects of 5-HT on SMCs (30). The appetite suppressants fenfluramine, d-fenfluramine, and aminorex differ from selective 5-HTT inhibitors in that they not only inhibit 5-HT reuptake but also trigger indoleamine release and interact with 5-HTT and -HT receptors in a specific manner (30).

SEROTONIN TRANSPORTER. 5-HTT is abundantly expressed on pulmonary artery smooth muscle cells (PASMCs) (31). Mice with targeted 5-HTT gene disruption develop less severe hypoxic PH than do wild-type controls (32,33). Conversely, increased 5-HTT expression is associated with increased severity of hypoxic PH (34,35). Indeed, specific overexpression of 5-HTT in PASMCs is sufficient to produce spontaneous PH (33).

5-HT receptors in PH. Of the 14 distinct 5-HT receptors, the 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{1B}$ receptors are particularly relevant to PAH.

5-HT$_{2A}$ RECEPTOR. In most nonhuman mammals, the 5-HT$_{2A}$ receptor mediates vasoconstriction in both the systemic and pulmonary circulations (36). However, the 5-HT$_{2A}$ receptor antagonist ketanserin is not specific for the pulmonary circulation, and systemic effects have limited its use in PAH, where it fails to improve pulmonary hemodynamics significantly (37).

5-HT$_{2B}$ RECEPTOR. The development of hypoxia-induced PH in mice is ablated in 5-HT$_{2B}$ receptor knockout mice (38), and this receptor may control 5-HT plasma levels in mice. However, the 5-HT$_{2B}$ receptor may also mediate vasodilation of the PA (39), and loss of the 5-HT$_{2B}$ receptor function may predispose to fenfluramine-associated PH in humans (40).

5-HT$_{1B}$ RECEPTOR. The 5-HT$_{1B}$ receptor mediates constriction in human PAs (41) and plays a role in the development of PAH (36,42), because inhibition, either by genetic knockout or pharmacologic antagonism, reduces hypoxia-induced pulmonary vascular remodeling (36). There is cooperation between the 5-HT$_{1B}$ receptor and the 5-HTT in mediating pulmonary vascular contraction (43). In addition, 5-HT$_{1B}$ receptor expression is increased in mice overexpressing the human 5-HTT and in the fawn-hooded rat, which also demonstrates increased 5-HTT expression (43). Both these models are predisposed to hypoxia-induced pulmonary vascular remodeling. Remodeled PAs from patients with PAH overexpress the 5-HT$_{1B}$ receptor. 5-HT$_{1B}$ receptor-mediated changes are specific to the pulmonary circulation, making this receptor an attractive therapeutic target for PH.

5-HT SYNTHESIS IN PH. The rate-limiting step in 5-HT biosynthesis is catalyzed by the enzyme tryptophan hydroxylase. Although peripheral 5-HT is synthesized chiefly by the enterochromaffin cells in the gut, human PAECs produce 5-HT and express the tryptophan hydroxylase-1 isoform. Both 5-HT synthesis and tryptophan hydroxylase-1 expression are increased in cells from patients with IPAH compared with controls (44). Mice lacking tryptophan hydroxylase-1 are resistant to hypoxia- and dexfenfluramine-induced PH (45,46).

K$^+$ and Ca$^+$ channels in PAH. In PASMCs, the free Ca^{2+} concentration in the cytosol ([Ca^{2+}]$_{cyt}$) is an important determinant of contraction, migration, and proliferation. The [Ca^{2+}]$_{cyt}$ in PASMCs can be increased by: 1) Ca^{2+} influx through voltage-dependent Ca^{2+} channels, receptor-operated Ca^{2+} channels, and store-operated Ca^{2+} channels; and 2) Ca^{2+} release from intracellular stores (e.g., sarcoplasmic reticulum) through Ca^{2+} release channels (e.g., inositol 1,4,5-trisphosphate receptors and ryanodine receptors). Inward transport of Ca^{2+} through Ca^{2+} transporters in the plasma membrane, such as the reverse mode

of Na^+/Ca^{2+} exchanger, is also an important pathway for increasing $[Ca^{2+}]_{cyt}$. In contrast, $[Ca^{2+}]_{cyt}$ in PASMCs can be decreased by: 1) Ca^{2+} extrusion by the Ca^{2+}-Mg^{2+} adenosine triphosphatase (Ca^{2+} pump) and by the forward mode of Na^+/Ca^{2+} exchanger in the plasma membrane; and 2) Ca^{2+} sequestration by the Ca^{2+}-Mg^{2+} adenosine triphosphatase in the sarcoplasmic reticulum.

INHIBITION OF K^+ CHANNEL ACTIVITY. Decreased expression and/or function of K^+ channels leads to membrane depolarization and contributes to sustained elevation of $[Ca^{2+}]_{cyt}$ by: 1) activating voltage-dependent calcium channel (VDCC); 2) facilitating the production of inositol 1,4,5-trisphosphate, which stimulates the release of sarcoplasmic reticulum Ca^{2+} into the cytoplasm; and 3) promoting Ca^{2+} entry through the reverse mode of Na^+/Ca^{2+} exchange.

ROLE OF RECEPTOR-OPERATED AND STORE-OPERATED CA^{2+} CHANNELS IN REGULATING $[CA^{2+}]_{CYT}$. The influx of Ca^{2+} through store-operated calcium channels, referred to as capacitative Ca^{2+} entry, is critical for refilling the empty sarcoplasmic reticulum with Ca^{2+}. Store-operated calcium channels in vascular SMC include the transient receptor potential channels. Some canonical transient receptor potential (TRPC) channel genes are expressed in human PASMCs and PAECs.

Proliferation of PASMC is associated with a significant increase in messenger ribonucleic acid and protein expression of TRPC channels such as TRPC1, TRPC3, and TRPC6 (47,48). Inhibition of TRPC expression with antisense oligonucleotides markedly decreases the amplitude of capacitative calcium entry and significantly inhibits PASMC proliferation. Thus, upregulation of TRPC channels may be a significant mechanism in the induction of PASMC proliferation.

PATHOGENIC ROLE OF DOWNREGULATED KV CHANNELS AND UPREGULATED TRP CHANNELS. In PASMCs from IPAH patients, the amplitude of whole-cell $I_{K(V)}$ and mRNA/protein expression levels of Kv channel subunits (e.g., Kv1.2 and Kv1.5) are both significantly decreased in comparison with cells from controls or patients with secondary PH (49). The downregulated Kv channels and decreased $I_{K(V)}$ are associated with a more depolarized E_m in IPAH PASMCs, and the resting $[Ca^{2+}]_{cyt}$ is much higher than in PASMCs from controls. The magnitude of capacitative calcium entry, evoked by passive store depletion with cyclopiazonic acid, is significantly greater in PASMCs from IPAH patients than in cells from secondary PH patients. Enhanced capacitative calcium entry, possibly by upregulation of TRPC channels, may represent a critical mechanism involved in the development of severe PAH.

KV CHANNELS, MITOCHONDRIAL METABOLISM, AND PAH. Warburg (50) proposed that a metabolic shift from oxidative phosphorylation to glycolysis, occurring despite adequate oxygen availability, was a characteristic of cancers. Recent data suggest that PAH and cancer share this "Warburg phenotype" (51,52). Both are characterized by mitochondrial hyperpolarization, depressed pyruvate dehydrogenase complex activity, and depressed H_2O_2 production (53). In both, there is also an O_2-independent perpetuation of the rapid, reversible metabolic/redox shifts that normally occur in response to hypoxia and initiate hypoxic pulmonary vasoconstriction (54,55). This metabolic shift creates a "pseudohypoxic environment" with glycolytic predominance and normoxic hypoxia-inducible factor-1α activation. The metabolic shift suppresses Kv1.5 expression, leading to membrane depolarization and elevation of cytosolic K^+ and Ca^{2+}. In both PAH PASMCs and cancer cell lines, this creates a proliferative, apoptosis-resistant phenotype.

As in familial PAH, PAH in the fawn-hooded rat is heritable. The fawn-hooded rat's PASMC mitochondrial reticulum is fragmented even before PAH develops. The observed hyperpolarization of $\Delta\Psi m$ and reduction in production of reactive oxygen species also occurs in PASMCs from IPAH patients (51). In PAH, mitochondrial abnormalities that shift metabolism away from oxidative phosphorylation toward glycolysis lead to a decreased electron flux and reduced reactive oxygen species production, which falsely signifies hypoxia, resulting in normoxic hypoxia-inducible factor-1α activation. Both the hypoxia-inducible factor-1α activation and the related decrease in Kv1.5 expression are reversed by low doses of exogenous H_2O_2, consistent with the redox theory for their etiology. A hypoxia-inducible factor-1α dominant-negative construct also restores Kv1.5 expression in fawn-hooded rat PASMC (51). Decreased Kv expression is an emerging hallmark of the PAH PASMC, occurring in human PAH (49,51) and all known experimental models (56–58). Interestingly, both Kv channels involved in hypoxic pulmonary vasoconstriction (Kv1.5 and Kv2.1) are inhibited by the anorexigens (59) and by 5-HT (60). In addition, endothelin-1 reversibly reduces the Kv1.5 currents (61). Restoring Kv1.5 expression reduces chronic hypoxic PH and restores hypoxic pulmonary vasoconstriction (62).

Mitochondrial therapy, for example, inhibition of pyruvate dehydrogenase kinase by dichloroacetate or Kv1.5 gene therapy partially regresses both PAH and cancer (51,52,62), consistent with the concept that PAH and cancer share a mitochondrial basis. Dichloroacetate restores oxidative metabolism in fawn-hooded rat PASMCs, shifting them away from the proliferative/apoptosis resistant glycolytic state. Dichloroacetate also causes regression of PAH induced by chronic hypoxia or monocrotaline (51,56,57).

RhoA/ROCK-mediated vasoconstriction. It is now clear that activation of RhoA/ROCK signaling is a major regulator of vascular tone (63). Smooth muscle cell tension is determined primarily by phosphorylation (contraction) and dephosphorylation (relaxation) of the regulatory myosin light chain (MLC), as described in the preceding text. At a given level of cytosolic Ca^{2+}, second messenger-mediated pathways can modulate the activity of myosin light chain kinase (MLCK) and myosin light chain phosphatases

(MLCPs) (e.g., MYPT1) to modify MLC phosphorylation and force, namely, to modify the Ca^{2+} sensitivity of contraction. Two major pathways in vascular smooth muscle (SM) are inhibition of MLCP action by ROCK-mediated phosphorylation of MYPT1, and protein kinase C-mediated phosphorylation and activation of the MLCP-inhibitor protein CPI-17.

Desensitization of Ca^{2+} is also a mechanism of vasodilation. Besides inducing SMC relaxation by desensitizing receptors and decreasing cytosolic $[Ca^{2+}]$ and MLCK activity, the NO/soluble guanylate cyclase/cGMP/PKG pathway also decreases Ca^{2+} sensitivity by phosphorylating and inactivating RhoA protein, or by directly phosphorylating MLCP, which increases MLCP activity (3). Similarly, vasodilation by stimuli that activate the adenylate kinase/cyclic adenosine monophosphate/PKA pathway is also attributable partly to inhibition of RhoA/ROCK signaling (3).

RhoA/ROCK in acute pulmonary vasoconstriction. ROCK-mediated Ca^{2+} sensitization is necessary for the sustained phase of acute hypoxic pulmonary vasoconstriction (64). Similarly, hypoxia directly activates RhoA in cultured PASMCs (65). Many studies have demonstrated the participation of ROCK in acute pulmonary vasoconstriction due to a variety of stimuli.

RhoA/ROCK in human PAH. Studies of RhoA/ROCK signaling in human PAH are limited. Low intravenous doses of fasudil acutely cause modest decreases in pulmonary vascular resistance in patients with PAH (66). Clinical trials examining the inhibition of RhoA/ROCK are under way.

Crosstalk Between Vascular Cells

Whether SM hyperplasia results from inherent characteristics of PASMCs or from dysregulation of molecular events that govern PASMC growth, such as signals originating from PAECs, remains an open question (67). In addition, there is evidence of crosstalk between adventitial cells and medial SMCs.

Endothelial dysfunction in PAH may follow excessive release of paracrine factors that act either as growth factors to induce PASMC proliferation or as chemokines to recruit circulating inflammatory cells (44,68). Thus, exposure of PASMCs to culture medium from PAECs induces PASMC proliferation, and this effect is exaggerated when PAECs from patients with PAH are used (44).

The role of ECs in angiogenesis and remodeling is now better understood (69,70). In maturation, ECs no longer proliferate or migrate but promote vessel stabilization by recruiting periendothelial support cells, which differentiate into SM-like cells (71). Failure of interactions between the 2 cell types, as seen in numerous genetic mouse models, results in severe and often lethal cardiovascular defects. Deficiencies in this process may lead to abnormal dilation of resistance pulmonary vessels, such as that seen in hereditary hemorrhagic telangectasia. Several studies suggest that the

crosstalk between PAECs and PASMCs may be under the control of diverse pathways including the angiopoietin-1/TIE2, transforming growth factor (TGF)-β/activin-receptorlike kinase (ALK)-1, and bone morphogenetic protein (BMP)/BMPR-II pathways (21,22,72). PAECs constitutively produce and release excessive amounts of soluble factors that act on PASMCs and inflammatory circulating cells to initiate or enhance pulmonary vascular remodeling and inflammation.

Cellular and Molecular Consequences of BMPR-II Mutation

Mutations in the *BMPR2* gene have been found in ≈70% of families with PAH (73,74). In addition, up to 25% of patients with apparently sporadic IPAH harbor mutations (75).

Normal BMP/TGF-β signaling. BMPs are the largest group of cytokines within the TGF-β superfamily (76). BMPs are now known to regulate growth, differentiation, and apoptosis in a diverse number of cell lines (77). The TGF-β superfamily type II receptors are constitutively active serine/threonine kinases. BMPR-II initiates intracellular signaling in response to specific ligands (78). Ligand specificity for different components of the receptor complex may have functional significance to the tissue-specific nature of BMP signaling (79,80). Recently, BMP9 was identified as a ligand that signals through a complex comprising BMPR-II and ALK-1 (81). This important finding might provide a mechanism for the rare occurrence of severe PAH in some families with hereditary hemorrhagic telangiectasia due to ALK-1 mutations (82). After ligand binding, the type II receptor phosphorylates a glycine-serine–rich domain on the proximal intracellular portion of an associated type I receptor (usually BMPR-IA [ALK-3] or BMPR-IB [ALK-6]). Activated type I receptors in turn phosphorylate cytoplasmic signaling proteins known as Smads, which are responsible for TGF-β superfamily signal transduction (83). BMPs signal through a restricted set of receptor-mediated Smads (R-Smads), Smads-1, -5, and -8, which must complex with the common partner Smad (Co-Smad), Smad-4, to translocate to the nucleus. Switching off Smad signaling in the cell is achieved by Smad ubiquitination and regulatory factors (Smurfs) (84) and by recently identified Smad phosphatases (85).

The consequences of BMPR2 mutation for BMP/TGF-β signaling. The mechanism by which BMPR-II mutants disrupt BMP/Smad signaling is heterogeneous and mutation specific (86). Of the missense mutations, substitution of cysteine residues within the ligand binding or kinase domain of BMPR-II leads to reduced trafficking of the mutant protein to the cell surface. At least for the ligand binding domain mutants, the mistrafficking can be rescued with chemical chaperones, resulting in improvements in Smad signaling (87). In contrast, noncysteine mutations within the kinase domain reach the cell surface but fail to

activate Smad-responsive luciferase reporter genes. Many mutations lead to nonsense-mediated mRNA decay of the mutant transcript, leading to a state of haploinsufficiency. PASMCs from mice heterozygous for a null mutation in the *BMPR2* gene are also deficient in Smad signaling (88,89). Thus, haploinsufficiency or missense mutation leads to a loss of signaling by the Smad1/5 pathway in response to BMP2 and BMP4. However, marked siRNA knockdown of BMPR-II leads to increased Smad signaling in response to some ligands, for example, BMP7 (80,89). This effect is mediated by increased signaling through the ActR-II receptor. In PASMCs, BMPR-II appears to mediate growth inhibition and differentiation, whereas ActR-II mediates osteoblastic differentiation (90).

Studies of BMP signaling cells and tissues from PAH patients. In the lung, BMPR-II is highly expressed on the vascular endothelium of the PAs (91) and at a lower level in PASMCs and fibroblasts. The expression of BMPR-II is markedly reduced in the pulmonary vasculature of patients with mutations in the BMPR-II gene (91). BMPR-II expression is also reduced in the pulmonary vasculature of patients with IPAH in whom no mutation in the *BMPR2* gene was identified. A reduction in the expression of BMPR-II may be important to the pathogenesis of PAH, whether or not there is a mutation in the gene. In addition, since the level of BMPR-II expression in familial cases was considerably lower than predicted from the state of haploinsufficiency, this suggests that some additional environmental or genetic factor may be necessary to further reduce BMPR-II expression below a threshold that triggers vascular remodeling.

Phosphorylation of Smad1/5 is also reduced in the pulmonary arterial wall of patients with underlying BMPR-II mutations and in patients with IPAH with no identifiable mutation (92). The response of PASMCs to BMP ligands depends to some extent on the anatomical origin of cells. The serum-stimulated proliferation of cells harvested from the main or lobar PAs tends to be inhibited by TGF-β1 and BMPs 2, 4, and 7 (92). Indeed, BMPs may induce apoptosis in these cells (93). The growth inhibitory effects of BMPs have been shown to be Smad1 dependent (92). In contrast, in PASMCs isolated from PAs of 1 to 2 mm diameter, BMPs 2 and 4 stimulate proliferation (92). This pro-proliferative effect of BMPs is dependent on the activation of ERK1/2 and p38MAPK. Both Smad and MAPK pathways are activated to a similar extent in cells from both locations, but the integration of these signals by the cell differs. This integration may be at the level of an important family of transcription factors, the inhibitors of DNA binding (Id genes) (94).

The response of vascular ECs to BMPs is dependent on the specific BMP ligand. Endothelial cells proliferate, migrate, and form tubular structures in response to BMP4 and BMP6 (95). In addition, BMPs in general protect endothelial cells from apoptosis (96). Interestingly, BMP9, which acts through BMPR-II and ALK-1, seems to inhibit

PAEC proliferation. Knockdown of BMPR-II with siRNA increases the susceptibility of PAECs to apoptosis (96).

The contrasting effects of BMPs in pulmonary vascular ECs and the underlying PASMCs provide a hypothesis for pulmonary vascular damage and remodeling in familial PAH. A critical reduction in BMPR-II function in the endothelium may promote increased endothelial apoptosis, which compromises the endothelial barrier. This would allow ingress of serum factors and stimulate activation of vascular elastases. High rates of apoptosis in the endothelium could favor the development of apoptosis-resistant clones of ECs and lead to plexiform lesion formation. In the underlying media, PASMCs already compromised in their ability to respond to the growth-suppressive effects of BMPs are exposed to growth factors stimulating proliferation.

BMP signaling in rodent models of PAH. Reduced mRNA and protein expression of BMPR-II have been reported in the lungs of animals with experimental PH (97,98). In the monocrotaline rat model, adenoviral delivery of BMPR-II through the airways failed to prevent PH (99). However, targeted gene delivery of BMPR-II to the pulmonary endothelium did significantly reduce PH in chronically hypoxic rats (100).

Studies in knockout mice reveal the critical role of the BMP pathway in early embryogenesis and vascular development (101). However, heterozygous BMPR-II +/– mice survive to adulthood with no discernable phenotype (88). When heterozygotes are exposed to lung overexpression of interleukin-1β (102) or chronically infused with 5-HT (88), they develop more PH compared with wild-type littermates. Thus, BMPR-II dysfunction increases the susceptibility to PH when exposed to other environmental stimuli. The relatively low penetrance of PAH within families supports a "two-hit" hypothesis, in which the vascular abnormalities are triggered by accumulation of genetic and/or environmental insults in a susceptible person.

Transgenic mice overexpressing siRNA targeting BMPR-II exhibit ≈10% of the normal levels of BMPR-II during development. These mice survive but do not develop spontaneous PAH. Intriguingly, they display a phenotype suggestive of hereditary hemorrhagic telangiectasia, with vascular ectasia and anemia (103). Conditional overexpression of a dominant negative kinase domain mutant BMPR-II in vascular SMCs of adult mice causes increased pulmonary vascular remodeling and PH (104). Conditional knockout of endothelial BMPR-II in adult mice has also been shown to predispose to PH (105).

The Extracellular Matrix

The extracellular matrix (ECM) not only represents a substrate for tissue morphogenesis, but also instructs almost all forms of cell behavior at the biophysical and biochemical levels through interactions with multiple receptors, including heterodimeric integrins composed of α and β subunits (106). Importantly, major qualitative and quantitative

changes in the ECM underscore a number of human pathologies, including cancer and PAH. Functional differentiation of the breast epithelium relies upon contact with an appropriate basement membrane by β1 integrins that promote both proper cell polarity and patterns of gene expression (107). Similarly, the underlying ECM dictates whether human stem cells will differentiate into adipocytes or osteoblasts (108). In this instance, differentiation relies upon cytoskeletal tension generated by RhoA and ROCK. Many studies highlight the critical importance of understanding the reciprocal relationships between the ECM and signaling pathways, such as Rho GTPases. The connections between integrins, ECM ligands, and actin-based microfilaments inside the cell are indirect and are linked through scaffolding proteins, such as talin, paxillin, and α-actinin (106). These scaffolds activate or recruit numerous signaling molecules, including focal adhesion kinase and Src kinase family members, which then phosphorylate their substrates (109).

Tenascin-C in PAH. Tenascin-C, a large ECM glycoprotein, is expressed within the medial SMC layer of injured and remodeling PAs from hypertensive animals (110) and humans (111,112). It surrounds proliferating PASMCs within arteries from hypertensive individuals (110,111). Furthermore, tenascin-C promotes PASMC proliferation and survival. For example, exogenous tenascin-C protein amplifies the SMC proliferative response to soluble growth factors, including epidermal growth factor and basic fibroblast growth factor (110), by promoting clustering and activation of receptor tyrosine kinases, such as epidermal growth factor receptors (113). Moreover, studies using isolated PASMCs and PAs from monocrotaline-exposed hypertensive rats revealed that suppression of tenascin-C using an antisense approach induces SMC apoptosis and regression of pulmonary vascular lesions (114).

Origins of the Myofibroblast in PAH

Pulmonary hypertension is characterized by cellular changes in the walls of PAs. Virtually all of these changes are characterized by increased numbers of cells expressing α-SM actin (115). It has been thought that the SM-like cells that express α-SM actin and accumulate in vascular lesions were derived from the expansion of resident vascular SMCs or adventitial fibroblasts. However, new data suggest other possible sources of α-SM actin-expressing cells (SM-like cells and/or myofibroblasts) in various vascular diseases. Circulating progenitor cells can assume an SM-like phenotype (116). Resident vascular progenitor cells have also been demonstrated to express SM-like characteristics in several vascular injury states (117). Finally, the possibility that both epithelial and endothelial cells have the capability of transitioning into a mesenchymal or SM-like phenotype has been raised.

Endothelial-mesenchymal transition. The term endothelial-mesenchymal transition (EnMT), rather than transformation or transdifferentiation, relates to epithelial biology, where the process of epithelial-mesenchymal transition has been more thoroughly investigated. Epithelial-mesenchymal transition is a process in which epithelial cells lose cell-to-cell contacts and polarity and undergo dramatic remodeling of the cytoskeleton (118), with repression of epithelial markers. Concurrently, cells begin to express mesenchymal antigens, including FSP-1, α-SM actin, fibronectin, and types I and III collagens, and manifest a proliferative and migratory phenotype. The transition of epithelial cells toward a mesenchymal phenotype occurs during embryonic development, and recent data suggest that epithelial-mesenchymal transition is important in cancer biology. A role for epithelial-mesenchymal transition during tissue injury leading to organ fibrosis is also becoming clear.

Less is known regarding EnMT than epithelial-mesenchymal transition. However, several groups have provided evidence that EnMT is critical in aortic and PA development (119). Endothelial cells labeled at an early stage of development appear later (at the onset of SMC differentiation) in the subendothelial space of the developing aorta and express α-SM actin (120). Morphologic studies in human embryos suggest that endothelial-like cells may give rise to SMC during the maturation of both PAs and veins (121). Findings in experimental wound repair have suggested that EnMT may also take place in the adult. Similarly, microvascular ECs transition into mesenchymal cells in response to chronic inflammatory stimuli (122). A role for EnMT in the neointimal thickening observed in transplant atherosclerosis and restenosis has also been suggested (120).

Endothelial cells from a variety of vascular beds retain the capability of transitioning into mesenchymal or even SM-like cells under several culture conditions (119). Endothelial cells derived from the adult bovine aorta convert to spindle-shaped α-SM actin-expressing cells when treated with TGFβ-1(123). Human dermal microvascular ECs can be induced to transform into myofibroblasts in vitro, after long-term exposure to inflammatory cytokines (124). Recent studies have demonstrated that hypoxia is also capable of inducing transdifferentiation of PAECs into myofibroblast or SM-like cells in a process regulated by myocardin (125).

Circulating Mesenchymal Progenitor Cells in Pulmonary Vascular Remodeling

Bone marrow-derived circulating cells, known as fibrocytes, may be a source for myofibroblast accumulation during reparative processes in the lung (126). Fibrocytes are mesenchymal progenitors that coexpress hematopoietic stem cell antigens, markers of the monocyte lineage, and fibroblast products. They constitutively produce ECM components as well as ECM-modifying enzymes and can further differentiate into myofibroblasts. These cells can contribute to the new population of fibroblasts and myofibroblasts that emerge at tissue sites during normal or aberrant wound

healing, in ischemic or inflammatory fibrotic processes, and as part of the stromal reaction to tumor development (127).

The fibrocyte may differentiate into mature mesenchymal cells in vivo. Differentiation of fibrocytes into myofibroblastlike cells occurs where there is increased production of TGFβ-1 and/or endothelin. In these settings, fibrocytes or fibrocyte precursor cells demonstrate downregulation of leukocytic markers (e.g., CD34 and CD45) with a concomitant upregulation of mesenchymal markers. A causal link between accumulation of fibrocytes at injured sites and ongoing tissue fibrogenesis or vascular remodeling has been provided in animal models of pulmonary disease (116). Inhibition of fibrocyte accumulation results in reduced collagen deposition and reduced accumulation of myofibroblasts. In the chronically hypoxic rat, monocyte/fibrocyte depletion markedly attenuated pulmonary vascular remodeling (116).

The transition of any cell type including ECs, progenitor cells, fibroblasts, or even SMC into a myofibroblast becomes relevant to a better understanding of PH, as myofibroblasts can generate long-lasting constriction regulated at the level of Rho/Rho-kinase–mediated inhibition of MLC phosphatase (128). Thus, cells that have transitioned into fibroblastlike and myofibroblastlike cells may play a role in the inability of the vessel wall to dilate in response to traditional vasodilating stimuli.

Author Disclosures

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