Endothelial and Smooth Muscle Cell Interactions in the Pathobiology of Pulmonary Hypertension

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Abstract

In the pulmonary vasculature, the endothelial and smooth muscle cells are two key cell types that play a major role in the pathobiology of pulmonary vascular disease and pulmonary hypertension. The normal interactions between these two cell types are important for the homeostasis of the pulmonary circulation, and any aberrant interaction between them may lead to various disease states including pulmonary vascular remodeling and pulmonary hypertension. It is well recognized that the endothelial cell can regulate the function of the underlying smooth muscle cell by releasing various bioactive agents

such as nitric oxide and endothelin-1. In addition to such paracrine regulation, other mechanisms exist by which there is cross-talk between these two cell types, including communication via the myoendothelial junctions and information transfer via extracellular vesicles. Emerging evidence suggests that these nonparacrine mechanisms play an important role in the regulation of pulmonary vascular tone and the determination of cell phenotype and that they are critically involved in the pathobiology of pulmonary hypertension.

Keywords: paracrine; myoendothelial junction; microvesicles; vasoconstriction; vascular remodeling

In the pulmonary vasculature, endothelial cells (ECs) and smooth muscle cells (SMCs) are the key cell types involved in the regulation of vessel diameter in most of the pulmonary vascular tree and thereby they regulate pulmonary arterial pressure and total vascular resistance. ECs, which are strategically located as the innermost layer of the blood vessel and are in contact with the circulating blood, exert a delicate and constant influence on the underlying vascular smooth muscle cells (VSMCs). It is well recognized that the regulation of pulmonary vasoreactivity by the endothelium is predominantly accomplished by paracrine signaling through the release of various vasoactive agents such as nitric oxide (NO), endothelin-1 (ET-1) (1–3), and others (4–8). However, there is increasing evidence that the relationship between the EC and the SMC is not a simple one-way

interaction from the endothelium to the SMC; rather, there are complicated interactions between them (9–11). Moreover, the communication patterns are not limited to paracrine signaling; cross-talk through myoendothelial gap junctions (MEJs), extracellular vesicles (EVs), and other mechanisms is critically involved (12–20). These mechanisms operate in a complicated but co-ordinated manner to maintain the homeostasis of the pulmonary circulation. Under pathological conditions, the interaction between ECs and VSMCs may be chronically altered so that a sustained increase in vasocontractility and abnormal vascular proliferation develops, which leads to high pulmonary artery pressure, vascular remodeling, right ventricular hypertrophy, and the clinical condition, pulmonary hypertension (PH) (1, 21–24). This article discusses the recent progress in

our knowledge of the interactions between ECs and SMCs through paracrine mechanisms, MEJs, and EVs in the pathobiology of PH.

Paracrine Signaling

Paracrine signaling is the primary mechanism by which the EC and smooth muscle communicate. Here we focus on the paracrine mechanism by which endothelial-derived NO (EDNO) from the EC acts on the underlying VSMC, as well as on the interaction between EDNO and ET-1 from the EC. These vasoactive agents are key players in maintaining pulmonary vascular tone and normal vasoreactivity. The progressive vasculopathy in PH often results from an imbalance in the activities of NO and ET-1 (1–3).

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EDNO as a Paracrine Regulator

EDNO is synthesized by endothelial nitric oxide synthase (eNOS) using L-arginine and molecular oxygen as the substrates in response to shear stress and various vasoactive stimuli such as bradykinin, thrombin, and serotonin. EDNO can diffuse readily into the underlying SMCs to activate soluble guanylyl cyclase (sGC), resulting in increased production of cyclic guanosine monophosphate (cGMP) and activation of cGMP-dependent protein kinase (PKG). The cyclic GMP-PKG pathway is the primary mechanism responsible for a broad range of the biological actions of EDNO (3). EDNO may cause pulmonary vasculature to relax by decreasing the cytosolic Ca^{2+} level resulting from PKG-dependent stimulation of Ca^{2+} -activated potassium channels, which leads to membrane hyperpolarization and Ca^{2+} influx suppression (25). EDNO may also cause pulmonary vasodilatation by reducing the sensitivity of myofilaments to Ca^{2+} via the PKG-dependent phosphorylation of the regulatory subunit of myosin light chain phosphatase at threonine-695 and -852 (Thr-695 and Thr-852, respectively, human sequence), which leads to increased dephosphorylation of the myosin light chain and reduced contractility (26, 27). NO has been found to suppress the proliferation of pulmonary vascular smooth muscle through the inhibition of cell cycle progression at the G1/S transition, which results from an increased nuclear localization of p21 and p27 and decreased cyclin A expression (28). Studies also suggest that NO may exert its antiproliferative effect through the activation of PKG followed by the inhibition of mitogen-activated protein kinases (29) and by the enhanced expression of myocardin, which leads to reduced binding of E-26-like protein 1 to the serum response factor and the augmented expression of genes involved in cell proliferation (30, 31).

In patients with primary PH, pulmonary vascular resistance does not decrease in response to infused endothelium-dependent vasodilator agents, suggesting an impaired EDNO response (32). A dysfunctional EDNO-sGC-cGMP-PKG signaling pathway constitutes one of the most fundamental and initial changes in the development of PH (1, 33), resulting from aberrant

expression of eNOS (34), reduced NO production caused by eNOS uncoupling (35–38), diminished NO bioavailability caused by oxidative stress (39, 40), diminished activities of sGC (41, 42) and PKG (26, 43–46), and augmented activity of phosphodiesterase type 5 (47).

Increased oxidative stress in the pulmonary vasculature has been documented in vessels from patients with PH and in the vasculature in animal models of PH. An increased production of reactive oxygen species (ROS) may result from activated nicotinamide adenine dinucleotide phosphate reduced oxidases and dysfunctional mitochondria (48, 49). ROS can oxidize tetrahydrobiopterin, an essential cofactor of eNOS, to dihydrobiopterin, which leads to eNOS uncoupling, a process by which eNOS produces superoxide instead of NO. It has been reported that mice with deficient tetrahydrobiopterin biosynthesis developed PH even under normoxic conditions and that they showed greatly increased susceptibility to hypoxia-induced PH, because of the decreased activity but not the decreased expression of eNOS, accompanied by elevated superoxide production (35). These results suggest that uncoupling of eNOS predisposes to the development of PH. Mutations in the ALK1 gene are associated with familial PH (50–52). Mice with a heterozygous deletion of Alk1 have been found to develop PH associated with increased ROS production. The level of NO production is decreased, partially because of decreased production caused by eNOS uncoupling and partially because of a ROS-mediated reduction in NO bioavailability (53).

The protective effects of NO on PH, including its vasodilator and antiproliferative actions, are mediated mainly by activation of sGC, followed by cGMP elevation and PKG activation (3, 54). Activation of sGC is initiated by the binding of NO to the ferrous heme of the β subunit of the enzyme. Oxidation of the ferrous heme to a ferric form can result in dissociation of the heme from sGC, rendering it unresponsive to NO (42). In ovine pulmonary arterial smooth muscle cells (PASMCs) taken from a model of persistent PH of the newborn in lambs, the expression of sGC was increased but basal cGMP levels were decreased, raising the possibility of increased heme dissociation from sGC because of oxidative stress. In

this persistent PH of the newborn animal model, pulmonary vasodilatation caused by acetylcholine is reduced but that caused by cinaciguat is increased. Vasodilatation caused by acetylcholine depends on the release of EDNO and activation of sGC associated with heme in a reduced state, whereas cinaciguat activates sGC in a heme-independent manner (55). PKG is the primary effector in mediating cGMP actions. It exists in two types in mammalian tissues, and only the type I isoform is present in blood vessels. PKG type I deficiency induces PH accompanied by decreased phosphorylation of Rho A at Ser188, suggesting that a deficiency in PKG-mediated inhibition of Rho A/Rho kinase signaling contributes to the pathobiology of PH (56). Hypoxia exposure increases PKG nitration and decreases PKG activity in pulmonary vein SMCs, which is prevented by scavengers of ROS or peroxynitrite, indicating that hypoxia may suppress PKG through nitrosative modification (43). In the lung of mice with PH obtained with genetic deletions caveolin-1 and in the lung tissue of patients with idiopathic pulmonary arterial hypertension (PAH), PKG activity is impaired, resulting from nitration at Tyr345 and Tyr549 secondary to an increased formation of peroxynitrite by NO and superoxide (44). Studies also show that nitration at tyrosine 247 attenuates PKG-I α activity. An antibody against 3-NT-Y247 identifies increased levels of nitrated PKG-I α in humans with PH (57, 58). Interestingly, although cGMP is considered the sole vasoactive molecule generated by sGC, a recent study reveals that hypoxia may promote sGC to synthesize a vasoconstrictor inosine 3', 5'-cyclic monophosphate. Whether 3', 5'-cyclic monophosphate is involved in the pathobiology of PH remains to be determined (59).

Interaction between NO and ET-1

In the pulmonary vasculature, the NO signaling pathway does not function independently. It acts in co-ordination with other vasodilators and antiproliferative agents and is also counteracted by agents with vasoconstrictor and mitogenic activities. The balance of these opposing effects maintains the homeostasis of pulmonary circulation (1, 60). This is exemplified by the interaction between NO and ET-1. The underlying mechanisms

Figure 1. Altered signaling for endothelin-1 (ET-1) and nitric oxide (NO) in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) in pulmonary hypertension. ET-1 is synthesized as preproendothelin (pp-ET-1), which is cleaved to big-ET-1 by the enzyme furin convertase and then to ET-1 (134). ET-1 is released to extracellular space in response to various stimuli (63). ET-1 exerts its actions on VSMCs via endothelin A receptor (ET_A) or endothelin B receptor (ET_B), predominately the former. It may cause sustained vasoconstriction by increasing intracellular Ca²⁺ concentration resulting from inositol 1,4,5-trisphosphate-induced Ca²⁺ release from the sarcoplasmic reticulum and by sensitizing the myofilaments to Ca²⁺ through the Rho kinase (ROCK) pathway. It also promotes vascular remodeling through the activation of the mitogen-activated protein kinase (MAPK) pathway. ET-1 also causes the release of NO from ECs after binding to the ET_B receptor (134). The formation and release of ET-1 is inhibited by NO (69–72). Such action is diminished in pulmonary hypertension, in part because of reduced production of NO by uncoupled endothelial nitric oxide synthase (eNOS) (34). The uncoupled eNOS generates superoxide anions, which reduce the bioavailability of NO (53). NO can counteract the vasoconstriction and vascular remodeling caused by ET-1 action through the stimulation of soluble guanylyl cyclase (sGC) and the elevation of cyclic guanosine monophosphate (cGMP). Such effects are impaired in pulmonary hypertension (56, 134). The *thick* and *thin* arrows denote enhanced and suppressed activity, respectively. The *red* and *green* arrows denote stimulatory and inhibitory effect, respectively. L-Arg, L-arginine; O^{•−}, superoxide anion.

involve paracrine and autocrine regulations among different cell types. Because the focus of this article is the cross-talk between EC and VSMC, other cell types such as immune cells and fibroblast cells will not be discussed (11, 61). ET-1 is a 21 amino acid peptide synthesized predominantly by the EC. ET-1 is derived from a larger 203 amino acid precursor peptide named preproendothelin, which is cleaved to a smaller 38 amino acid peptide, big-ET-1, by furin convertase and then to the bioactive 21 amino acid peptide ET-1 by endothelin-converting enzyme (62). ET-1 is stored in Weibel-Palade bodies. It is released by exocytosis in response to Ca²⁺

elevation elicited by various stimuli such as mechanical stretch, hypoxia, growth factors, cytokines, and adhesion molecules. ET-1 is also shuttled continuously from the trans-Golgi network to the cell surface in secretory vesicles via the constitutive secretory pathway, which contributes to the basal vascular tone under physiological conditions (63). The peptide is released mainly (~80%) toward the albuminal side of the endothelium and it binds to two receptor types, the endothelin A receptor (ET_A) and the endothelin B receptor (ET_B) (62). Both receptor types are present in pulmonary smooth cells, with ET_A being dominant (64, 65). The activation of these receptors leads to vasoconstriction

mediated by an increase in intracellular Ca²⁺ concentration and sensitization of myofilaments to Ca²⁺. It also results in mitogenic events mediated by phospholipase C-diacyl glycerol mitogen-activated protein kinases cascade, Rho A/Rho kinase pathway, and phosphatidylinositol-3 kinase pathway (66–68). The ET-1/ET_A and ET_B activities are inhibited by the NO pathway at several levels. The mRNA expression of ET-1 and the secretion of the peptide by the EC are inhibited by NO (69–73). In the SMC, NO may attenuate ET-1 induced contraction by the inhibition of Ca²⁺ influx and Ca²⁺ release from the sarcoplasmic reticulum and by the interference with RhoA-Rho kinase signaling in a cGMP-PKG-dependent manner (26, 27). NO-induced generation of cGMP contributes to the inhibition of ET-1-induced mitogenic and hypertrophic responses (74). ET_B receptors are located predominantly on ECs. Their activation on ECs stimulates the production of NO and PGI₂. ET_B receptors also possess “clearance” functions: once ET-1 binds the receptors, the receptors are internalized and subjected to degradation by endosomes/lysosomes (75, 76). Ablation of ET_B receptors exclusively from ECs decreases the endogenous release of NO and increases plasma ET-1 (77).

ET-1 levels are increased in the lungs and plasma of patients with PAH as well as in the lungs of rats and fetal lambs with PH (78–80). The increased ET-1 levels may result in part from diminished inhibition of NO caused by reduced NO production and bioavailability (35, 37, 39, 40). The reduced NO production may also result from the decreased gene expression and protein levels of ET_B that occur in PH (80–82). ROS production is increased in human PH (49). ET_B receptors on human pulmonary artery ECs can be inactivated by oxidative modification of cysteinyl thiols in the eNOS-activating region of ET_B, resulting in decreased NO production (83). In contrast to decreased ET_B-stimulated NO production, the clearance of ET-1 by the endothelial ET_B receptor has been found to be normal or near normal in patients with PAH (84).

In patients with PH, the density of the ET_A and ET_B receptors in the distal pulmonary arteries shows a significant

Figure 2. Possible mechanism for endothelial–smooth muscle cross-talk via the myoendothelial junctions (MEJs) in pulmonary hypertension exemplified by serotonin actions. Serotonin is synthesized primarily from L-tryptophan (L-Trp) by tryptophan hydroxylase 1 (Tph1) in the ECs (99). It may diffuse to the VSMCs through MEJs and dissociate transforming growth factor β (TGF- β) from latency-associated peptide (LAP), resulting in the binding of TGF- β to its receptor (R) activin receptor-like kinase 5 (ALK5) (92, 99), which leads to increased vascular smooth muscle remodeling through the activation of similar mothers against decapentaplegic protein (SMAD) signaling (92, 99, 135) and phosphorylated TGF-associated kinase 1 (TAK1-p)–MAPK signaling pathways (136). Serotonin may also stimulate the production of superoxide anions by nicotinamide adenine dinucleotide phosphate reduced oxidase (NOX) in VSMCs (101, 102). The increased number of superoxide anions may diffuse into ECs through MEJs and may lead to eNOS uncoupling and reduced NO bioavailability and the resultant decreased activation of sGC–cGMP signaling (91). Accordingly, the antiproliferation effect of the NO–cGMP–cGMP-dependent protein kinase pathway is diminished. The *thick* and *thin* arrows denote enhanced and suppressed activity, respectively. The *red* and *green* arrows denote stimulatory and inhibitory effects, respectively.

increase. Both receptors promote the proliferation of PSMCs (85). Oxidative stress is present in PH (49, 86, 87), and ET-1 increases ROS production in PSMCs (88). Because NO signaling is compromised by oxidative stress, the increased ROS production evoked by ET-1 may promote vasoconstriction and vascular remodeling via the suppression of NO activity (1, 60). A study with fetal ovine pulmonary ECs cocultured with PSMCs showed that the eNOS promoter activity and protein levels in ECs were suppressed by SMC-derived hydrogen peroxide production stimulated by ET-1, suggesting that a feedback mechanism exists that may contribute to the already impaired NO signaling (88) (Figure 1). It is of interest that although ET-1 is synthesized primarily by ECs, PSMCs can also synthesize this peptide. Studies using PSMCs obtained from mice deficient in ET-1 and human PSMCs after

silencing of ET-1 suggest that ET-1 derived from VSMC may modulate pulmonary vascular tone and SMC proliferation (89).

Myoendothelial Junction Mechanisms

The MEJ is a distinct anatomic structure formed by the apposed membranes of the EC and the VSMC and the gap junctions between them. The gap junctions at the tip of the MEJ are composed of two hemichannels termed connexons, each composed of six connexin proteins. Currently, three different connexins (Cx 37, 40, and 43) are known to be associated with the MEJ that allow the transfer of current and small molecules ($< \sim 1.2$ kD) between the cytoplasm of these adjacent cells (14). MEJs have been observed in several species and in many vascular beds, including the

pulmonary vasculature (13, 90–92). There are more numerous MEJs in smaller- than in larger-diameter arteries (10, 13, 93). The more abundant expression of MEJs would facilitate electronic signals propagating between EC and SMC. Coincidentally, endothelium-dependent hyperpolarization is more pronounced in small- compared with large-diameter arteries (94).

In rat mesenteric arteries, ECs send cellular projections passing through the holes in the internal elastic lamina to make direct contact with the overlying VSMC. The MEJs have a bulbous or mushroom-like head on a thin stalk, usually ~ 100 – 150 nm in diameter and ~ 0.8 μ m in length, either abutting the SMC membrane or lying in indentations in the membrane, with the former observed more commonly. It is estimated that there are approximately two MEJs per SMC at the level of the internal elastic lamina in the distal mesenteric artery, and one EC can communicate with 15 to 18 SMCs (13, 95). For a particular blood vessel, the MEJ may be derived from the EC, from the SMC, or from both cell types and may meet halfway between them, as observed in canine pulmonary arteries and veins (13, 90).

Studies of the caudal and mesenteric artery of the rat show that the distance between MEJs and the homocellular endothelial gap junctions are often within < 2 μ m (13, 95). Such an arrangement is thought to render the endothelium a favorable pathway for the transmission of electrical signals along blood vessels and to facilitate the co-ordination of the vascular response (96). In the lungs of mice, the hyperpolarization of the endothelium evoked by hypoxia retrogradely propagates to upstream arterioles along the endothelial layer; this activity is absent in the preparations from mice deficient in Cx40-containing gap junctions. In mice lacking Cx40, a hypoxia-evoked increase in pulmonary arterial pressure is largely absent. Moreover, the muscularization of small pulmonary arterioles and the right ventricular hypertrophy caused by chronic hypoxia are attenuated in these mice. In the lungs of mice, Cx40 is confined to the vascular endothelium. Hence, the interendothelial Cx40-containing gap junctions appear to be critically involved in hypoxic vasoconstriction and the pathobiology of PH in this species (97). In isolated rat pulmonary arteries, sustained constriction, Rho kinase activation, and

Figure 3. ECs and smooth muscle cells (SMCs) release extracellular vesicles (EVs) and interact through the transfer of EVs. Increased circulating levels of endothelium-derived microparticles (MPs) have been documented in patients with pulmonary hypertension (PH) (a). Visovatti and colleagues (128) demonstrated increased CD39 expression and function in the circulating MPs of patients with idiopathic pulmonary arterial hypertension, which may be associated with the increased ATPase/ADPase activity in MPs (b). Tual-Chalot and colleagues (126) showed that circulating MPs from hypoxic rats can suppress endothelial-dependent vascular relaxation in rat aorta and pulmonary arteries by decreasing NO production (c). More recently, Aliotta and colleagues (127) reported that healthy mice injected with circulating or lung EVs isolated from monocrotaline-treated mice show elevated right ventricular-to-body weight ratio and pulmonary arterial wall thickness-to-diameter ratio, compared with that of mice injected with control EVs (d). Deng and colleagues (130) showed a high abundance of microRNA (miR)-143-3p in pulmonary arterial smooth muscle cell (PASM) derived exosomes and a paracrine promigratory and proangiogenic effect of these miR-143-3p-enriched PASM derived exosomes on pulmonary arterial endothelial cell (e). However, the cross-talk between ECs and SMCs through EV transfer, especially from ECs to SMCs, and the underlying molecular mechanisms remain unclear (f).

phosphorylation of myosin phosphatase targeting protein caused by hypoxia are prevented by the inhibitors of gap junctions, implying an important role for MEJs (14).

In rat lungs, Cx 37, 40, and 43 are present in the endothelium, and Cx 37 and 40 are present in the smooth muscle. Cx-mimetic peptide targeted against Cx 37 and Cx 43 eliminates the diffusion of the dye carboxyfluorescein-AM from the EC to VSMC, indicating that the MEJs formed by Cx 37 and/or Cx 43 are functional (91). In chronic hypoxia or monocrotaline-induced rat PH models, contraction of the pulmonary artery induced by serotonin and phenylephrine (an agonist of the α 1-adrenoceptor) is attenuated by the Cx-mimetic peptide blocker against Cx 37 and Cx 43 or against Cx 40, whereas

contraction of pulmonary arteries from control rats is affected to a lesser extent or is not affected (98). These results suggest that an enhanced MEJ activity may contribute to the exaggerated pulmonary vasoconstriction in PH (98). In rat small intrapulmonary arteries, the contractile and calcium responses to serotonin are reduced by the Cx-mimetic peptide against Cx 37 and Cx 43. These responses are also decreased by superoxide dismutase and catalase and are reversed by the inhibition of eNOS. Electronic paramagnetic resonance reveals that serotonin-induced superoxide anion production originates from the SMC. Hence, vasoconstrictor-induced ROS production in VSMCs may diffuse into ECs via the MEJ to reduce NO bioavailability and thus blunt

endothelial NO-dependent control of pulmonary vasoreactivity. Such a MEJ-mediated cross-talk between SMC and EC may become more pronounced in PH, in which there is often excessive ROS production (91).

There is increasing evidence that MEJs are also involved in regulating PASM phenotype (92, 99). When rat pulmonary arterial ECs (PAECs) and PSMCs are touch cocultured on a porous Transwell membrane, MEJs are formed because Cx43 has been found on projections extending into the membrane from both cell types and dye transfers from PAECs to PSMCs, but Cx43 is not present when cells were not touch cocultured. When cells are grown in non-touch-coculture systems, PAEC exhibit a more contractile-like phenotype with activated transforming growth factor (TGF)- β 1 signaling. These effects are prevented by blockers of gap junctions or by knockdown of Cx43 in PAEC. Thus, MEJs appear to act as a gateway for the PAEC-derived signals required for TGF- β -dependent PASM differentiation (92). The activation of TGF- β signaling and the induction of differentiation of touch-cocultured PSMCs are abolished by the inhibition of serotonin synthesis in PAEC. The monoamine can be detected by immunostaining in both PAECs and PSMCs in touch-coculture but not in non-touch-coculture settings. Furthermore, inhibition of gap junctions but not the serotonin transporter prevents serotonin transfer from PAECs to PSMCs (99). Therefore, serotonin, a vasoconstrictor and a potent mitogen for PSMCs (100–102), is synthesized by PAEC and transferred through MEJs into PSMCs, where it regulates cell differentiation via the TGF- β signaling pathway (99) (Figure 2). Because electrical activity and small molecules <1.2 kD can all be transferred through the MEJ, they potentially can be involved in EC-VSMC cross-talk (14). Besides ROS (14) and serotonin (14), molecules such as Ca^{2+} that affect membrane potential activities and IP_3 have been implicated as being transferred through the MEJ to affect pulmonary vasoactivities (103).

Signaling Through EVs

EVs refer to different types of membrane vesicles. Currently, they are divided into three classes primarily on the basis of their

Figure 4. Cross-talk between pulmonary arterial endothelial cells and pulmonary arterial smooth muscle cells through paracrine effect (A), MEJ (B), and EV transfer (C).

size and presumed biogenetic pathways: exosomes, microvesicles, and apoptotic bodies. Exosomes are 50–100-nm membrane vesicles of endocytic origin. They are released into the extracellular space by fusion with the plasma membrane. Exosomes contain endosome-specific proteins such as Alix and TSG101, components of microdomains in the plasma membrane such as cholesterol, ceramide, integrins, and tetraspanins, mRNA, microRNA (miRNA), and other noncoding RNAs. ExoCarta, an exosome database, provides a comprehensive list of identified exosomes (<http://exocarta.org/>) (104, 105). Microvesicles, also referred to as microparticles (MPs), especially in the cardiovascular field, are sized 20–1,000 nm. They are formed through the outward budding and separation of the plasma membrane. During their formation, microvesicles retain surface molecules from parent cells and part of their cytosolic content (proteins, RNA, miRNA) (18, 105). Apoptotic bodies are the largest vesicles of the EV, with a size of 1–5 μm . They are formed through outward blebbing of the cell membrane during the late steps of apoptosis. Apoptotic bodies contain cellular organelles, proteins, DNA, RNA, and miRNA (104–106). EVs can be released from most cell types, including ECs and VSMCs. There is evidence that EVs are able to transfer information (miRNA, proteins, etc.) to

their target cells by membrane fusion, endocytosis, or receptor-mediated binding and thus they represent an important mechanism for intercellular communication (18, 105, 107–112). EV-mediated intercellular communications are conserved evolutionarily (113). Therefore, EVs are rich sources of biomarkers for diagnosis and/or prognosis of human diseases (114–121) and provide us with potential therapeutic approaches (122, 123).

Increased circulating levels of endothelium-derived MPs have been documented in various cardiovascular diseases, including PH (18, 124) (Figure 3). In patients with PH, the levels of circulating endothelial CD31⁺ (platelet endothelial cell adhesion molecule positive)/CD41[−], CD144⁺ (vascular endothelial cadherin⁺), and CD62e⁺ (E-selectin⁺) MPs are increased compared with in control subjects. Moreover, patients with PAH exhibit higher values of platelet endothelial cell adhesion molecule positive and vascular endothelial cadherin⁺ MPs than do those with chronic obstructive pulmonary disease-related PH (124). Higher levels of endothelium-derived MPs bearing E-selectin are also noted in patients with thromboembolic PH as compared with subjects with nonthromboembolic PH (125), suggesting that the cause of the disease may influence MP levels (17). MPs are not only a biomarker of PH; they also actively contribute to the development of PH (126,

127). Visovatti and colleagues demonstrated increased CD39 expression and function in the circulating MPs of patients with idiopathic PAH, which may be associated with the increased ATPase/ADPase activity in MPs (128). The endothelium-dependent relaxation of rat pulmonary arteries is suppressed after incubation with MPs obtained from rats exposed to chronic hypoxia as compared with control arteries exposed to normoxia, accompanied by attenuated eNOS activity and increased ROS production (126). In another study, Lee and colleagues demonstrated that mesenchymal stromal cell-derived exosomes exert a pleiotropic protective effect on the lung and inhibit vascular remodeling and hypoxic PH, with suppression on the STAT3/miR-17 level and induction of the miR-204 level in the lung (129). Moreover, a recent study reported that healthy mice injected with EVs isolated from monocrotaline-treated mice showed elevated right ventricular-to-body weight ratio and pulmonary arterial wall thickness-to-diameter ratio, compared with that of mice injected with control EVs, providing direct *in vivo* evidence that EVs contribute to pulmonary vascular remodeling and PH (127). The cross-talk between ECs and PASCs via EVs in the pulmonary vasculature is demonstrated in a recent study by Deng and colleagues (130). This study showed that migration and angiogenesis of PAECs are induced not only by exosome-derived miR-143 but also by coculture of PAECs with PASCs under conditions in which direct cell-cell contact is prevented. The miR-143-enriched exosomes derived from PASCs are internalized by PAECs, which leads to increased EC migration and angiogenesis. This study also showed that miR-143 is up-regulated in the pulmonary vasculature of murine models of PH and in patients with PH. Genetic deletion of miR-143 or pharmacological inhibition of miR-143 in mice prevented the development of hypoxia-induced PH. Hence, cross-talk between ECs and PASCs via miR-143-enriched exosomes may be involved in the pathogenesis of PH under *in vivo* conditions.

Conclusions

Paracrine regulation and cell-to-cell communication via myoendothelial junctions and EVs represent three distinct

mechanisms by which cells communicate (Figure 4), each with unique features, so a complicated and diversified regulation of cellular interactions can be accomplished. In paracrine regulation, the signaling molecules are secreted into the extracellular environment and form concentration gradients according to the distance between the cells, thus resulting in a graded influence on the nearby cells. The outcome of the recipient cells is affected by their distance from the communicating cells as well as by the local environment. In contrast, the signals transferred through myoendothelial injunctions are restricted to the cells connected by the junctions. Thus, cell-to-cell communication is more direct and localized via these junctions. EVs have been shown to contain a variety of cargo not typically thought to be released by viable cells, including mRNA and miRNA, so transfer of genetic information is emerging as a new way of intercellular communication (105, 109, 111). Such a mechanism has been implicated in the interaction between the ECs and pulmonary VSMCs and is involved in the development of PH (130).

In the lung, the myriad interactions between ECs and SMCs play a major role in the maintenance of homeostasis in the pulmonary circulation. Aberrant cross-talk between them may lead to various lung

diseases including PH. In the past decade, substantial knowledge has been gained regarding the paracrine regulation by EC of the pulmonary vascular contractility and SMC phenotype. The feedback control of ECs by VSMCs is also recognized increasingly as being critically important to the integrity of pulmonary vascular function. These paracrine mechanisms are exemplified by EDNO and ET-1, as discussed in this article. In contrast, our knowledge of nonparacrine regulation of the pulmonary circulation is sparse. For instance, in the systemic vasculature, the contractile phenotype of VSMC may regulate the activity of eNOS via an increased transfer of inositol 1, 4, 5-trisphosphate through MEJs followed by increased Ca^{2+} release from the endoplasmic reticulum localized in the MEJs. Meanwhile, the diffusion of EDNO generated at the MEJ to the overlying VSMC is regulated by the redox status of iron in Hb α that is richly expressed at the MEJ. This mechanism has not been explored in the pulmonary vasculature (10). Regarding the EV-mediated interaction between ECs and VSMCs, available evidence supports the notion that they may be critically involved in the pathobiology of PH. However, the related mechanisms are largely underexplored (17). In addition to the MEJs and EVs discussed in this article, other nonparacrine mechanisms,

such as Notch signaling, in which the binding of ligands to Notch receptors occurs through direct cell contact, may operate in the interaction between pulmonary ECs and VSMCs (131). Notch signaling plays an important role during vascular development, and defects in this pathway may promote the development of PH (132, 133). Interestingly, a recent study showed that miRNA in VSMCs may modulate the functions of EC through tunneling nanotubes formed between these two cell types (20). It clearly indicates the complexity of EC–VSMC communication. This area of research is full of many opportunities for us to explore and to find a better understanding of the pulmonary vascular function and new therapeutic clues for PH. It is worth mentioning that it is often difficult to determine which cell type is more important in the pathobiology of PH because both SMC-dependent medial thickening and EC-dependent angio-obliteration are present at the same time. Therefore, to design specific pharmacotherapies, it is critical to understand the contribution of cross-talk between the two cell types and the contribution of individual cell types to the development of the disease. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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