

Review

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Microvesicles and exosomes in pulmonary hypertension

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Abstract

Pulmonary hypertension is a serious disorder with a high morbidity and mortality rate. The juxtaposition of endothelial cells and smooth muscle cells maintains vascular homeostasis. Vascular injury results in endothelial dysfunction, leading to impaired vascular relaxation, cell proliferation, and altered immune and metabolic states. In addition, injury induces pulmonary arterial endothelium and other cells to release increased levels of extracellular vesicles, including exosomes and microparticles that may be involved in enhancing the proliferation of apoptosis-resistant smooth muscle cells. These extracellular vesicles carry proteins, lipids, RNA, miRNA, chemokines cytokines and modulate immune function, inflammation, embryogenesis, regenerative processes, and serve as intercellular messengers. Importantly, mesenchymal stem cells-derived extracellular vesicles exert inhibitory effects on inflammation and restore homeostasis. This article reviews the pathophysiological role of extracellular vesicles in pulmonary hypertension.

Keywords: Endothelial cells, extracellular vesicles, mesenchymal cells, pulmonary hypertension

INTRODUCTION

Pulmonary hypertension (PH) is a serious complication of a number of systemic diseases including cardiovascular, respiratory and hematological disorders, autoimmune diseases, genetic mutations, and as yet unidentified causes. Based on the underlying disease, PH can be classified into five major groups. The term pulmonary arterial hypertension (PAH) is applied to diseases in group 1, which includes idiopathic and heritable PAH (IPAH, HPAH), and PAH associated with congenital heart defects, inflammation, autoimmune diseases and drug toxicity. A number of genetic mutations are also associated with PAH.



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Table 1. Pulmonary hypertension classification (based on Ref #1)

Gr. 1 PAH	Idiopathic and heritable PAH, PAH associated with CHD, inflammatory, autoimmune diseases, drug toxicity, genetic mutations, HIV, portal hypertension, Gr 1' - pulmonary capillary hemangiomatosis, pulmonary veno-occlusive disease, Gr 1'' - Persistent PH of the newborn
Gr. 2 PH	Left ventricular diseases (congenital and acquired)
Gr. 3 PH	Chronic lung diseases, hypoxia, developmental lung diseases
Gr. 4 PH	Chronic thromboembolic pulmonary hypertension
Gr. 5 PH	Miscellaneous systemic disorders including hematological, myeloproliferative, metabolic and thyroid diseases, and chronic renal failure

PAH: pulmonary arterial hypertension; PH: pulmonary hypertension

Pulmonary veno-occlusive disease and pulmonary capillary hemangiomatosis are assigned subcategory 1' and persistent PH of the newborn is designated as 1''. The remaining four groups are designated as PH. Group 2 includes PH associated with left ventricular diseases and pulmonary venous hypertension. Included in group 3 are chronic lung diseases, alveolar hypoventilation disorders and developmental lung diseases while group 4 includes chronic thromboembolic PH. Finally, group 5 includes PH associated with miscellaneous diseases such as myeloproliferative, hematological, thyroid and renal diseases^[1] [Table 1].

During the sixth World Symposium on PH, the mean pulmonary artery pressure threshold was reduced to > 20 mmHg from \geq 25 mmHg, and pulmonary vascular resistance maintained as > 3 Wood units^[2]. This change is based on the evaluation of 47 studies from 13 countries that showed normal mean pulmonary artery pressure rarely exceeded 20 mmHg, irrespective of age^[3]. The survival time for patients with PAH (Gr. 1) without treatment is reported to be 2.8 years^[4]. Modern treatment has improved the quality of life and 3-year survival in these patients is now around 58%-67%^[5,6].

Despite such treatment, the underlying vascular pathology continues to worsen^[7]. Regardless of the underlying disease, pulmonary endothelial cell (EC) disruption/dysfunction plays a pivotal role in the pathogenesis of PH. ECs maintain vascular homeostasis and regulate vascular tone, cell permeability, inflammation, and coagulation through several mediators such as nitric oxide (NO), endothelium-derived hyperpolarization factor, endothelin-1 (ET-1), cell adhesion molecules, cytokines and chemokines. Endothelial dysfunction, alterations in the expression of NO, ET-1, caveolin-1, serotonin, inflammatory cytokines and chemokines, and disordered proteolysis of the extracellular matrix all contribute to the pathogenesis of PAH^[8,9]. The increased expression of cytokines such as interleukin-1 (IL-1) and IL-6^[10,11] and chemokines such as CX(3)CL1 (fractalkine) and CCL5, also known as RANTES^[12,13], have all been observed in both human and experimental PH. Furthermore, plexiform lesions contain perivascular infiltration with inflammatory T and B cells^[14,15]. Disruption or dysfunction of endothelial caveolin-1 (a major protein constituent of caveolae) associated with the activation of proliferative molecules such as tyrosine tyrosine phosphorylated signal transducer and activator of transcription 3 (py-STAT3), B cell lymphoma-extra-large (Bcl-xL) and β -catenin leads to smooth muscle cell (SMC) proliferation, medial hypertrophy and PH^[16,17].

SMCs are a heterogeneous cell population which exhibit different proliferative, inflammatory, and extracellular matrix production changes during vascular remodeling. In addition, the extension of pericytes into non-muscularized arteries has been documented in PH^[18]. Pericytes are heterogeneous cells in origin. The close interactions between pericytes and ECs are important for paracrine signaling involved in vascular development and stability. In addition, pericytes modulate immune responses^[19]. It has recently been shown that the dysfunctional EC in IPAH may partly contribute to the increased pericyte coverage

in distal pulmonary arteries, through the EC-derived fibroblast growth factor 2 (FGF-2) and IL-6^[20]. Pericyte-specific upregulation of CXCR (C-X-C chemokine receptor)-7 and transforming growth factor- β receptor II (TGF- β RII) in patients with PAH are considered critical for their proliferation/migration capacities and myogenic potentials. During the early phase, pericyte numbers increase in a CXCL (C-X-C motif chemokine ligand)-12-dependent manner and later, the activation of the TGF- β signaling pathway induces pericytes to differentiate into smooth muscle-like cells^[21]. Furthermore, reduced endothelial-pericyte interactions result in progressive loss of small vessels in PAH. Increased expression of pyruvate dehydrogenase kinase 4 (*PDK4*) gene and protein in PAH pericytes correlated with reduced mitochondrial metabolism, higher rates of glycolysis, and hyperproliferation. Reducing PDK4 levels improved endothelial-pericyte interactions, restored mitochondrial metabolism, and reduced cell proliferation^[22]. These studies underscore the importance of EC and pericyte interactions in maintaining vascular homeostasis.

The disruption/apoptosis of ECs and accompanying endothelial caveolin-1 loss followed by enhanced expression of caveolin-1 in SMCs, proliferation of antiapoptotic ECs and neointima formation have all been reported in experimental PH and human PAH^[23-26]. In a monocrotaline (MCT) + hypoxia model, the enhanced expression of caveolin-1 revealed the presence of tyrosine 14-phosphorylated caveolin-1 (p-cav-1) and the loss of polymerase 1 and transcription factor also known as cavin-1^[25]. Cavin-1 maintains the shape of caveolae and stabilizes caveolin-1 in caveolae. The loss of cavin-1 is indicative of the flattening of caveolar structure. Cavin-1 knockout mice exhibit pathological lung changes such as remodeled pulmonary vessels, PH and right ventricular hypertrophy (RVH). In addition, these mice have an altered metabolic phenotype with insulin resistance^[27]. It is worth noting here that in cancer, p-cav-1 has been shown to inactivate the growth inhibitory function of the caveolin-1 scaffolding domain and facilitate cell migration^[28-30]. These studies indicate that the disruption of endothelial caveolin-1 and dysfunction of SMC caveolin-1 participate in the progression of PH. In addition, other factors such as vascular endothelial growth factor (VEGF), epidermal growth factor, transforming growth factor β (TGF β), matrix metalloproteinases, bone morphogenic protein receptor type 2 (BMP2) and Notch1 have all been implicated in the pathophysiology of PAH^[31]. Thus, a large number of deregulated transcription factors and proliferative pathways participate in the pathobiology of PH. Recent studies have shown that extracellular vesicles (EVs) may have an important role in the pathogenesis of PH.

EVs

EVs have been isolated from body fluids such as blood, urine, saliva and cerebrospinal fluid. Initially EVs were thought to be a means for cells to get rid of unwanted components. Currently, they are identified as important mediators of intercellular communication. EVs participate in the exchange of lipids, proteins and genetic material between cells, modulate immune, inflammatory and regenerative processes, and maintain homeostasis. EVs are released from a variety of cells including platelets, erythrocytes, leukocytes, and ECs maintain their different compositions and function. Most cell types generate EVs that play important roles in various biological processes, including embryogenesis, tissue regeneration and immunomodulation. They regulate the transfer of biological information both locally as well as remotely^[32-35]. EVs include exosomes (30-130 nm, in diameter), microparticles (MPs, also known as microvesicles, 100-1000 nm) and apoptotic bodies (50-4000 nm). Apoptotic bodies are generated following activation of the apoptotic pathway and cell death.

Exosomes

For exosome formation, endosomal membrane invagination captures cytosolic components within intraluminal vesicles. Early endosomes then mature into late endosomes and accumulate intraluminal vesicles, known as multivesicular bodies in their lumen. These multivesicular bodies either fuse with lysosomes for degradation or with the plasma membrane and are released into the extracellular space as exosomes^[36,37] [Figure 1].

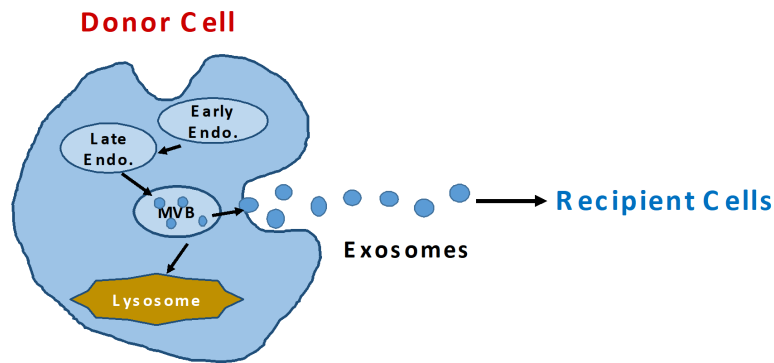


Figure 1. Early endosomes (Early Endo) mature into late endosomes (Late Endo) and accumulate intraluminal vesicles known as MVB. MVB release exosomes into the extra cellular space, or fuse with lysosome for degradation. These exosomes are then incorporated into recipient cells. MVB: multivesicular bodies

Reticulocytes were the first cells shown to release exosomes containing transferrin receptor during maturation^[38]. The main components of exosome membranes are lipids and proteins enriched with lipid rafts. In addition, they contain mRNAs, microRNAs (miRNAs), and other non-coding RNAs. The interaction between the transmembrane proteins of exosomes and the signaling receptors of target cells could either be direct, or indirectly after fusion with the plasma membrane of recipient cells to deliver the content into the cytosol. In addition, exosomes internalized into recipient cells may merge into endosomes, undergo transcytosis, move across recipient cells and released into neighboring cells. Alternatively, endosomes fused with engulfed exosomes mature into lysosomes and undergo degradation^[39]. EVs are engulfed into the cells via endocytosis. The lipid rafts, the known sites for endocytosis, are the specific microdomains within the plasma membrane that contain high concentrations of cholesterol and glycosphingolipids. Caveolins and flotillins are enriched with these lipid rafts. Interestingly, siRNA-mediated knockdown of caveolin-1 and flotillin-1, but not clathrin heavy chain, results in the inhibition of EV internalization^[40]. However, caveolin-1 localized in the plasma membrane negatively regulates exosome uptake, partly through the suppression of ERK1/2 signaling activation^[41]. *CAV1* gene knockout results in reduced caveolin-1 protein and impaired EV uptake. However, *CAV1* knockout in mouse embryonic fibroblast cells resulted instead, in increased EV uptake^[42]. Thus, EV uptake may depend on the cell type and its pathophysiological state. In addition, Rab proteins are essential regulators of EV transport^[43]. Endothelial exosomes are also involved in vascular development as they incorporate and transfer Delta-like 4 (Dll4) to neighboring ECs, resulting in inhibition of notch signaling and increased vascular branch formation^[44].

MPs

MPs are shed from various cell types as small fragments. In 1967, MPs shed during coagulation were regarded as platelet dust. Studies over the years have since shown that these MPs participate in cellular signaling, homeostasis, vascular injury and coagulation^[45]. MPs are formed by the plasma membrane pinching off and encapsulating cytosolic components, and they maintain surface markers and receptors of the plasma membranes of the parent cells [Figure 2].

They efficiently exchange biological information between cells and thus, participate in intercellular communication. In the healthy state, circulating MPs originate mainly from platelets and, to a smaller extent, from leukocytes, erythrocytes, granulocytes, monocytes, lymphocytes and ECs. Increased circulating MPs are biomarkers of vascular injury and inflammation. Cells exposed to different stimuli such as shear stress, physical agonists, pro-apoptotic stimuli or injury release MPs contributing to EC dysfunction in cardiovascular diseases^[46]. Stimulation of ECs by cytokines, reactive oxygen species, plasminogen activation inhibitor, thrombin or C-reactive protein leads to the formation of endothelial MPs.

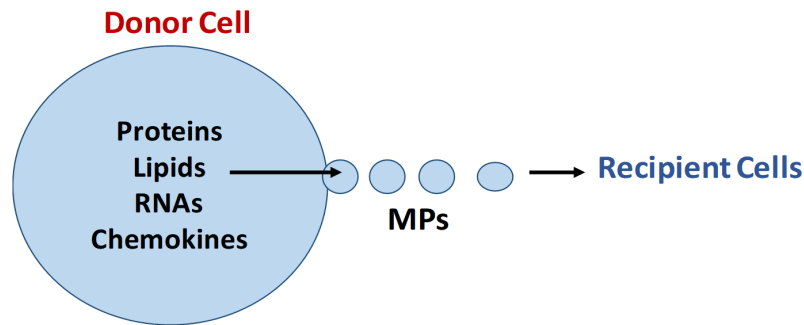


Figure 2. MPs are formed by the plasma membrane pinching off and encapsulating cytosolic components. MPs: microparticles

MPs play an important role in inflammation, EC function and cellular survival^[47]. Circulating MPs impair the atheroprotective function of ECs, in part, by decreasing NO synthesis. Increased levels of circulating MPs are useful biomarkers of vascular injury and a predictor of adverse cardiovascular events and mortality in patients with atherosclerotic disease. Atherosclerotic lesions contain a large number of MPs of leukocyte, SMC, EC and red blood cell origin^[48], and even patients with subclinical atherosclerosis exhibit increased levels of circulating leukocyte-derived MPs^[49].

In addition, MPs promote coagulation, inflammation and alter angiogenesis and apoptosis in ECs. Studies indicate that MPs induce EC dysfunction by disrupting NO release. Endogenous NO inhibits the release of endothelial MPs upon stimulation with C-reactive protein through a tetrahydrobiopterin-dependent mechanism^[50]. Endothelial MPs deliver C-reactive protein and participate in inflammation and coagulation^[51]. MPs from T-lymphocytes depress endothelial nitric oxide synthase (eNOS) activity and increase oxidative stress in ECs; however, T-lymphocytes carrying morphogen sonic hedgehog increase NO production^[46]. Furthermore, the release of MPs carrying lytic complement C5b-9 complex protects ECs from complement-induced lysis. MPs from leukocytes stimulate pro-inflammatory genes in ECs *in vitro*, resulting in the release of cytokines and leukocyte-EC adhesion molecules. On the other hand, MPs from polymorphonuclear leukocytes contain annexin-1, a functionally active anti-inflammatory protein. *In vivo*, annexin-1 inhibits the interaction between leukocytes and ECs^[52]. In *in vitro* studies, the inhibition of endothelial MP release has been shown to induce caspase-3 accumulation, leading to cellular apoptosis. Thus, endothelial MP release could protect EC apoptosis by reducing caspase-3 levels^[53]. Pulmonary microvascular ECs have higher levels of basal cAMP and pulmonary ECs release cAMP containing EVs. These EVs are thought to facilitate compartmentalized cAMP signals and thus, may strengthen the endothelial barrier^[54]. *In vitro* studies have shown that MPs derived from platelets stimulate cell proliferation, migration, and tube formation in ECs. In addition, metalloproteinases contained in endothelial MPs regulate proteolytic activity on the matrix and elicit angiogenesis. Furthermore, MPs derived from endothelial progenitor cells may induce apoptosis-resistant cell proliferation^[55]. MPs contain the cell surface protein and cytoplasmic components of the original cell, and exhibit phosphatidylserine on the surface that accounts for their procoagulant character. MPs from T cells induce EC dysfunction by altering NO and prostacyclin pathways. T-lymphocyte-derived MPs reduce eNOS expression and vascular relaxation, and exhibit increased expression of caveolin-1^[56].

EVs participate in physiological as well as pathological conditions depending on their cargo. EV biogenesis is dysregulated in pathological conditions. Release of EVs can induce inflammation, angiogenesis and thrombosis, and are implicated in many diseases such as cancer, chronic obstructive pulmonary disease, atherosclerosis and PH. During degranulation, activated polymorphonuclear leukocyte-derived exosomes (CD63⁺/CD66b⁺) acquire surface-bound neutrophil elastase that is resistant to α 1-antitrypsin (α 1AT). In chronic obstructive pulmonary disease and bronchopulmonary dysplasia (BPD), polymorphonuclear

leukocyte-derived exosomes acquire surface-bound elastase that degrade extracellular matrix^[57]. In sickle cell disease, inflammasome-dependent shedding of platelet EVs carrying IL-1 β and caspase-1 activate neutrophils and other vascular cells to form large platelet-neutrophil aggregates that occlude pulmonary arteries. The level of circulating platelet EVs correlate with disease severity. Increased circulating platelets and erythroid MPs were observed in untreated children with sickle cell disease; hydroxyurea therapy, however, normalized MP levels^[58,59]. *In vitro* studies have shown that high arterial stress results in platelet activation and release of prothrombotic EVs in the blood^[60]. In cell culture studies, platelet MPs have been shown to induce SMC proliferation in a platelet derived growth factor-independent manner^[61]. In addition, circulating platelet-derived EVs induced increased production of cytokines (IL-1, IL-6, and IL-8), leading to immune modulation, endothelial dysfunction and remodeling. In contrast, neutrophil-derived EVs enhance the biosynthesis of specialized pro-resolving mediators^[62]. MPs from patients with metabolic syndrome decrease NO production in ECs in culture, independent of oxidative stress. Furthermore, injection of MPs in mice resulted in reduced eNOS synthesis and impaired vascular relaxation^[63]. In addition, tissue factor containing MPs are proinflammatory mediators between increased glucose levels and diabetic vasculopathy^[64]. In cancer, EVs promote tumor growth, metastasis and resistance to therapy; and the activation of oncogene signaling pathway can increase EV production. These EVs transfer oncoproteins and facilitate tumor angiogenesis, immunosuppression and metastasis^[65,66]. *In vitro* studies have shown large EVs carrying caveolin-1 in cancer cells to be involved in metastasis^[67]. Thus, the function of MPs depends on the cell types that they originate from and their pathophysiological state.

Mesenchymal stem cells (MSC) are the best cell types for tissue regenerative therapies. They can be readily derived, propagated and differentiated into a variety of cell types. They modulate several biological functions such as tissue repair, downregulation of inflammatory responses, and modulation of the immune system. MSC-derived exosomes have protective effects on ischemia-reperfusion injury via inhibition of cell apoptosis and inflammatory responses^[68,69]. Furthermore, treatment with multipotent stem cells showed strong regenerative capabilities in animal models of myocardial ischemia^[70], stroke^[71], and diabetes^[72]. Recent studies have suggested that MSC-derived EVs containing miRNAs might promote cell and tissue repair, and regeneration^[73]. Similar to the biological activities of MSC, MSC-derived exosomes recover and regenerate the damaged tissues and restore homeostasis^[74]. MSC-exosomes reduce oxidative stress and prevent adverse remodeling in hearts subjected to ischemia-perfusion^[75]; and placental MSC-derived exosomes promote new blood vessel formation and angiogenesis within the placenta under low oxygen conditions^[76]. Interestingly, female bone marrow derived MSCs exhibit higher therapeutic efficacy compared with male MSCs in reducing neonatal hyperoxia-induced lung inflammation, vascular remodeling and PH in a rat model of BPD. Female MSCs express higher levels of VEGF and IL-10, and are better in attenuating PH and improving pulmonary vascular remodeling. The effects on angiogenesis and alveolarization however, were similar in female and male MSC treatment^[77]. In an ischemia-reperfusion injury model, female MSC infusion revealed a greater degree of myocardial recovery compared to male MSC. The protective effect of female MSCs appears to be related to lower levels of TNFR1^[78]. In addition, 17 β -estradiol-treated cardiac stem cells increased the expression of VEGF and SDF-1, and decreased caspase3 resulting in improved myocyte survival after acute ischemia perfusion injury^[79]. These studies suggest that the superior cell survival effect of female MSCs may be dependent on estrogen levels. In contrast, male muscle-derived stem cells display better cartilage regeneration potential compared with their female counterparts^[80]. Thus, these gender differences may be tissue-specific and female MSCs appear to provide better protection in cardiovascular and lung diseases.

PULMONARY HYPERTENSION AND EXTRACELLULAR VESICLES

Altered immunity, platelet activation, vascular inflammation, endothelial dysfunction and thromboembolic complications are well known features of PH. Platelet MPs have been shown to roll over ECs to deliver CCL5^[81]. Importantly, CCL5 is a chemo-attractant for monocytes and T cells. In severe PAH, ECs

were found to be the major source of CCL5^[13]. Circulating MPs have also been shown to stimulate ICAM expression in pulmonary arterial ECs during the late stage in a Sugen + hypoxia model of PH^[82]. Increased levels of platelet-derived MPs, defined as CD31⁺/CD41⁺, and endothelium-derived MPs as CD31⁺/CD41⁻, were observed in IPAH, HPAH and “associated” PAH compared to controls^[83]. Recent studies have shown increased levels of circulating MPs of platelet (CD31⁺/61⁺), leukocyte (CD11b⁺) and endothelial (CD62E⁺) origin in patients with PH. Furthermore, significantly increased endothelial MP levels were detected in patients with thrombo-embolic PH compared to non-embolic patients, indicating increased endothelial dysfunction in the former^[84]. In addition, elevated levels of endothelial MPs, but not leukocyte MPs, prior to treatment are associated with adverse clinical events. Increased CD62e⁺ levels are associated with an inflammatory state^[85]. EVs from animals with PH induce endothelial dysfunction. MPs from hypoxic rats impair EC-dependent relaxation in pulmonary arteries and aorta via reducing NO production and increasing oxidative stress^[86]. MPs bearing active tissue factor and CD105 (endoglin) were reported to be elevated in patients with PAH. Interestingly, in patients with PAH, a further increase in endothelium-derived CD105 MPs was observed in pulmonary arterial blood compared with venous blood. Furthermore, patients in functional class III and IV were found to have higher levels of MPs bearing active tissue factor^[87]. In addition, increased levels of circulating endothelial (CD62E-E-selectin) and CD3 (T cell)-derived EVs were observed in patients with different forms of PH^[88]. Interestingly, *in vitro* studies have revealed that upon exposure to injury, pulmonary arterial ECs release increased amounts of exosomes that induce apoptosis-resistant pulmonary arterial SMC proliferation^[89].

Disturbed blood flow has been shown to acutely induce both endothelial activation and apoptosis, resulting in the release of MPs from activated (CD62E⁺) and apoptotic (CD31⁺/CD42b⁻) ECs^[90]. Increased endothelial MPs have been reported in adult patients with congenital heart defects (atrial and ventricular septal defects) especially with ‘associated’ PAH. These endothelial MPs may contribute to inflammation, leading to endothelial dysfunction, impaired vasodilatation and inhibit angiogenesis via p39 MAPK^[91]. However, no differences in endothelial MP expression were observed in children with congenital heart defects with or without associated PAH^[92].

Apoptotic ECs release exosomes containing tumor susceptibility gene 101 and translationally controlled tumor protein (TCTP) with antiapoptotic function. Vascular SMCs, upon exposure to these nanovesicles, exhibit increased resistance to apoptosis and ERK1/2 activation. Silencing TCTP blocks the resistance to apoptosis and ERK1/2 activation^[93]. TCTP is also a potent mediator of inflammation. In patients with IPAH, HPAH and in a Sugen + hypoxia model, increased levels of TCTP were found in blood outgrowth EC. Knockdown of TCTP resulted in increased apoptosis in these cells in *in vitro* studies. These authors have further shown increased levels of blood outgrowth EC-derived exosomes and MPs in patients with PAH associated with BMPR2 mutation^[94,95]. Upregulation of miR143-5p has been reported in pulmonary arterial SMCs from experimental PH and human PAH. Pulmonary arterial SMC-derived exosomes exhibited enhanced expression of miR143-5p, which induced pro-migratory and pro-angiogenic effects on ECs^[96].

CD39, an ectonucleoside triphosphate diphosphohydrolase, is expressed in the lipid raft domain in plasma membranes of cells including ECs, monocytes and lymphocytes, and functions as an anti-inflammatory and thromboregulatory factor. The absence of CD39 on ECs results in increased susceptibility of ECs to stimulation^[97]. Importantly, ECs in IPAH exhibit downregulation of CD39. Furthermore, suppression of CD39 in *in vitro* studies results in apoptosis resistant pulmonary arterial ECs, and an increased ATP niche that stimulates SMC proliferation and migration^[98]. Visovatti *et al.*^[99] reported the presence of increased circulating endothelial and platelet MPs with CD39 on the surface in patients with IPAH. In addition, ATPase and ADPase activities were increased. In PAH, endothelial dysfunction/disruption and/or apoptosis is the underlying pathological event. Therefore, it is likely that CD39 is lost from ECs, which

could then facilitate proliferation of anti-apoptotic ECs. The loss of endothelial caveolin-1, PECAM-1, vWF and vascular endothelial cadherin, which is indicative of endothelial disruption, has been described in experimental and human PAH^[16,24,100]. Furthermore, increased levels of endothelial MPs carrying vascular endothelial cadherin and PECAM-1 were shown to be associated with hemodynamic severity of PAH^[101]. In addition, BMPR2 loss has been reported in experimental PH and in patients with IPAH without the BMPR2 mutation, and to a lesser degree, in patients with “associated” PAH^[102,103]. Interestingly, the loss of endothelial caveolin-1 and oxidative stress leads to reduced BMPR2 expression, increased TGFβ-derived Smad 2/3 signaling and pulmonary vascular remodeling^[104]. Oliveira *et al.*^[105] recently showed that in the Sugen + hypoxia model of PH in rats and human PAH, endothelial caveolin-1 loss accompanied by increased plasma levels of caveolin-1 EVs and TGFβ, indicating that the endothelial caveolin-1 loss contributes to increased TGFβ signaling, leads to EC proliferation, vascular remodeling and PAH. Caveolin-1 appears to be a plasma biomarker of vascular injury and a key determinant of TGFβ-induced vascular remodeling. It is possible then that the increased levels of EVs containing caveolin-1 in plasma could, in part, be responsible for enhanced expression of caveolin-1 in SMCs observed in IPAH, HPAH and PAH associated with drug toxicity and congenital heart defects^[23,24,100,106].

Recent studies have shown that human pulmonary arterial ECs can efficiently incorporate EVs transmitted by human pulmonary arterial SMCs and translate their mRNA cargo. These EVs enriched in Zeb1 and TGF-β superfamily ligands contribute to endothelial mesenchymal transition (EndMT), thus facilitating disease progression^[107]. However, partial EndMT is a physiological process necessary for angiogenesis. In partial EndMT, ECs do not separate from their neighboring cells^[108]. **Figure 3** (as shown on page 9) depicts the inter-relationship between ECs and SMCs, and the role played by EVs in PH.

Interestingly, cigarette smoking results in the release of endothelial EVs with spermine enrichment both on the surface as well as in the cytosol and activates a Ca²⁺-sensing receptor leading to pulmonary vasoconstriction, SMC proliferation and PH^[109]. These results strongly support endothelial injury and disruption underlying the release of EVs. Depending on the cargo, EVs participate in EC-SMC crosstalk in physiological or pathological conditions.

PH, MSC AND MESENCHYMAL EVS

A number of studies have shown the beneficial effects of MSC therapy in experimental models of PH. Intravenous treatment with adipose-derived MSCs improved MCT-induced PH in rats. In addition, adipose-derived MSCs in co-culture with MCT-treated human pulmonary arterial ECs exhibit increased cell proliferation and expression of VEGF^[110]. Bone marrow-derived MSCs over-expressing eNOS attenuated MCT-induced PH in rats^[111], and MSCs expressing increased hemoxygenase (HO)-1 reversed hypoxia-induced PH in mice^[112]. In addition, transplantation with bone marrow-derived MSCs transduced with prostacyclin synthase, and therapy with adiponectin gene modified adipose MSCs significantly attenuated MCT-induced PH, RVH, pulmonary vascular thickening and survival in rats. In *in vitro* studies, the inhibitory effect of adiponectin on the proliferation of pulmonary arterial SMCs obtained from rats with MCT-induced PH was shown to be dependent on the regulation of the AMPK/BMP/Smad pathway^[113,114]. Interestingly, intravenous administration of bone marrow-derived MSCs from donor rats with MCT-induced PH to the recipient rat with MCT-induced PH resulted in attenuation of PH and RVH, and normalization of right ventricular function. Bone marrow-derived MSCs from MCT rats produced more VEGF compared to controls^[115]. In addition, adipose tissue-derived MSC therapy in rats with shunt flow-induced hyperkinetic PAH was attenuated via increased expression of hepatocyte growth factor and eNOS promoting angiogenesis in the injured lungs^[116].

Importantly, female bone marrow-derived MSCs were found to attenuate MCT-induced PH and RVH in mice better than male MSCs. Female MSCs had increased expression of glyceraldehyde-3-phosphate

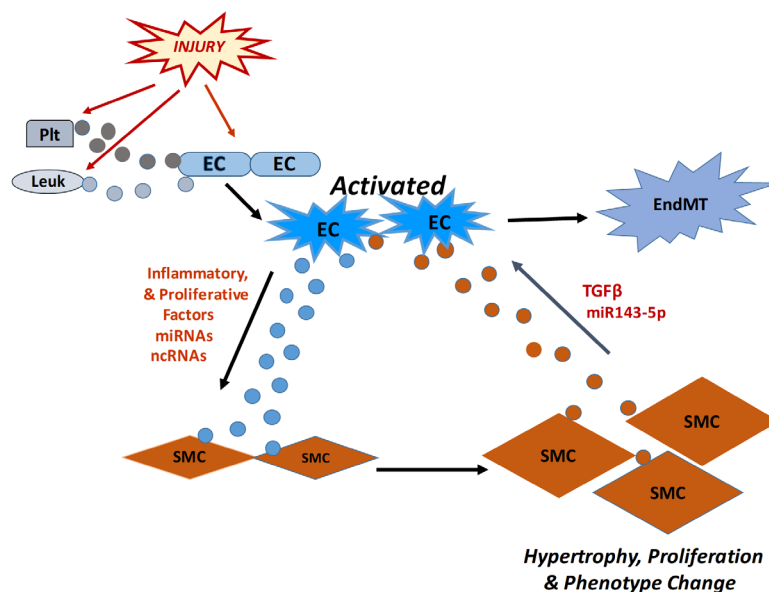


Figure 3. This figure depicts the possible mechanism of injury resulting in dysregulation of a number of vascular and inflammatory cells. Injury (inflammatory, oxidant, drug toxicity, increased pulmonary flow) activates Plt, Leuk and EC to produce increased amounts of EVs. EVs from platelets and leukocytes induce EC dysfunction. Endothelial EVs contain inflammatory and proliferative factors, and miRNAs are incorporated in SMCs, leading to hypertrophy, proliferation and phenotype change. These SMCs produce EVs containing TGFβ, miRNAs and other factors that are incorporated into ECs, leading to EndMT. EC: endothelial cell; EVs: extracellular vesicles; SMCs: smooth muscle cells; EndMT: endothelial mesenchymal transition; Plt: platelets; Leuk: leukocytes; TGFβ: transforming growth factor β

dehydrogenase that regulates (Ca^{2+})_i signal associated function, which might be responsible for the superior function of female bone marrow-derived MSCs^[117]. Furthermore, in an ischemia-reperfusion model, rat hearts treated with female MSCs demonstrated significantly greater recovery of left ventricular pressure compared to male MSC treated hearts. Importantly, male MSCs produced higher levels of TNFα and less VEGF than female MSCs^[118].

EVs from plasma and lung homogenates from mice with MCT-induced PH have been shown to induce PH and RVH in healthy mice. Interestingly, exosomes but not microvesicles from MCT-mice injured wild type mice; MSC-induced exosomes also prevented and reversed MCT-induced PH. Furthermore, exosomes from MCT-treated mice and patients with IPAH revealed increased expression of miRs-19b, -20a, -20b, and -145. In contrast, MSC-exosomes exhibited increased levels of anti-proliferative and anti-inflammatory miRs (-34a, -122, -124, -127)^[119]. Chen *et al.*^[120] have shown that both bone marrow-derived MSCs and MSC-EVs ameliorated MCT-induced PH and RVH, indicating that a cell free approach to stem cell therapy is effective. In addition, adipose-derived exosomes have been shown to transfer miR125a to ECs to promote angiogenesis by suppressing angiogenic inhibitor Dll4 and facilitate repair^[121]. Renin-angiotensin involvement in the pathogenesis of PH is well documented. Liu *et al.*^[122] have reported that bone marrow-derived MSC-microvesicles attenuated MCT-induced PH and RVH in rats accompanied by increased levels of ACE2 mRNA in lung tissue, increased plasma levels of Ang-(1-7) and decreased ACE compared with controls. Interestingly, the administration of adipose-derived MSC-EVs was shown to inhibit neointima formation in the vein graft model, accompanied by a significant decrease in the expression of IL-6, monocyte chemoattractant protein-1 and phosphorylation of Akt, Erk1/2^[123]. In mice with hypoxia-induced PH, intravenous treatment with MSC-derived exosomes attenuated PH and RVH, reduced STAT3 activation and upregulated the miR-17 superfamily of miRNAs. In addition, treatment with MSC exosomes increased the level of miR204. It is worth noting here that the expression of miR204 in the lungs is low in patients with PAH. These authors have further shown in *in vitro* studies that the activation

of py-STAT3 in human pulmonary arterial SMCs in response to hypoxia is inhibited by exosomes derived from the human umbilical cord^[124]. Klinger *et al.*^[125] have recently reported the prevention and reversal of the Sugen+ hypoxia model of PH in rats by MSC EVs. Similar to the cancer phenotype, in PAH, cells undergo a metabolic shift towards glycolysis and lactic acid formation which enables sustained ATP production and uncontrolled cell growth. MSC exosomes increase glucose oxidation and prevent a shift to glycolysis and mitochondrial damage. In addition, exosomes inhibit SIRT4 expression upstream of pyruvate dehydrogenase and glutamate dehydrogenase that contribute to the improvement of mitochondrial function^[126]. Thus, MSC-derived EVs can have beneficial effects on the pathophysiology of PH and mitochondrial function.

PH is a frequent and serious complication in preterm infants with BPD, a chronic lung disease. Treatment with conditioned media from cultured mouse bone marrow-derived MSCs showed significant improvement in hyperoxia-induced BPD in mice. It reversed hyperoxia-induced lung parenchymal pathological changes and PH^[127]. Chang *et al.*^[128] treated nine preterm infants (gestational age 25.3 ± 0.9 weeks) with intra-tracheal transplantation of human umbilical cord blood-derived MSCs. At 7 days after treatment, these infants had no adverse effects, and the severity of BPD was observed to be low. In addition, tracheal aspirates revealed lower levels of IL-6, IL-8, metalloproteinase-9, TNF α and TGF β 1. These studies showed the beneficial effects of MSCs on lung development.

In summary, EVs play a significant role in the pathophysiology of PH. Under normal conditions, EVs produced by different cells modulate immune responses, participate in intercellular communication and maintain homeostasis. Increased levels of EVs observed in PH are indicative of endothelial injury. These EVs facilitate cell proliferation, inflammation, and progression of the disease. MSCs and MSC-derived EVs are capable of modulating immune responses, repairing injured tissues and have regenerative properties. The beneficial effects of MSCs and MSC-EVs, including some genetically modified MSCs have been reported in several experimental models of PH. Treatment with MSC-EVs (naïve or genetically modified) may have an advantage over cell therapy.

DECLARATIONS

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Contributed to discussion: Dorai T

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Ethical approval and consent to participate

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Consent for publication

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