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Review

Mesenchymal stem/stromal cell therapy for pulmonary arterial hypertension: Comprehensive review of preclinical studies

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ABSTRACT

Pulmonary arterial hypertension (PAH) is a disease characterized by progressive pulmonary vascular remodeling, resulting in right-sided heart failure and premature death. Current available therapies for PAH have limited efficacy, and new therapeutic strategies need to be developed. Mesenchymal stem/stromal cells (MSCs) may offer a novel therapeutic approach to PAH. Since the first report in 2006, a number of preclinical studies have demonstrated a potential therapeutic effect of this approach, with attenuated hemodynamic and histological progression of PAH, in animal models of PAH. However, there remain several issues that should be addressed for this approach to be clinically successful. With the aim to highlight such issues, this review clarifies existing knowledge on MSC therapy for PAH in preclinical studies, including types of PAH animal models used for MSC therapy, MSC sources, and administration protocol (route, cell dose, and timing of administration). This review thereafter summarizes thoroughly and discusses the mechanism underpinning MSC therapy for PAH. For clinical success of MSC therapy, insufficient evidence of safety (*e.g.* critical risk of pulmonary embolism) and therapeutic efficacy of MSCs on established PAH with severe vascular remodeling, as well as further optimization of the MSC administration protocol, are considered as remaining issues to be addressed. In terms of the efficacy, it is controversial whether angiogenic cytokines, which are considered as one of the therapeutic mechanisms of MSC, have beneficial effect for human PAH. To address these issues, further preclinical data using more clinically-relevant animal models of PAH, such as SU5416 model, should be accumulated, whereas most preclinical studies have been conducted using monocrotaline-induced PAH model. While MSC therapy has a great potential to become a novel therapy in PAH, continuing careful preclinical research is warranted for clinical success in PAH.

Contents

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Introduction

Pulmonary arterial hypertension (PAH) is a progressive disease characterized by vascular remodeling of pulmonary arteries, resulting in elevated pulmonary vascular resistance (PVR) and right ventricular (RV) dysfunction. In clinical guidelines, developed by the European Society of Cardiology/European Respiratory Society, PAH is characterized by precapillary pulmonary hypertension (PH), which is defined by mean pulmonary artery (PA) pressure $\geq$25 mmHg, PA wedge pressure $\leq$15 mmHg, and PVR $\geq$3 Wood units at rest [1]. PAH is mainly idiopathic and heritable/familial, however it can be induced by drug and toxin exposure and be associated with other medical conditions, such as connective tissue disease, human immunodeficiency virus infection, and congenital heart disease [1]. If no treatment is given, mean survival of patients with PAH is 2.8 years [2]. During the past few decades, specific therapies for PAH have been developed, which target three signaling pathways: endothelin-1 (ET-1), prostacyclin, and nitric oxide (NO) pathways (reviewed in [3]). PAH patients treated with these targeted therapies can expect a mean survival of 7 years. However, 3-year and 5-year mortality from the time of diagnosis were still high at approximately 30% and 40%, respectively [4,5]. Lung transplantation is the only established treatment for severe PAH patients; however, there is a limited availability of donor lung and high mortality after listing for transplantation [6]. Thus, new therapeutic strategies are required to improve the prognosis for PAH patients.

Cell therapy may offer a novel therapeutic approach to PAH. Several cell types, including endothelial progenitor cells (EPCs) and mesenchymal stem/stromal cells (MSCs), have been investigated as donor cell types in preclinical studies and exhibited therapeutic benefits. Subsequently, clinical trials of EPC administration have already been conducted in PAH patients [7,8]. In contrast, no clinical trials have been conducted using MSC therapy to date, despite the therapeutic potential of MSC having been evidenced in preclinical studies similar to EPCs. As MSCs have a potential advantage over EPCs in permitting allogeneic administration without immunosuppressive reagents, MSCs may present a more attractive donor cell source for PAH therapy. This review will comprehensively summarize preclinical reports on MSC therapy for animal models of PAH, with the aim to clarify our existing knowledge and discuss issues that should be resolved before moving this approach to clinical applications.

MSC therapy for PAH

Definition and characteristics of MSCs

According to International Society for Cellular Therapy (ISCT), MSCs are characterized by the following criteria: (1) expression of a specific set of cluster of differentiation (CD) markers (CD73, CD90, and CD105), (2) lack expression of hematopoietic lineage CD markers [CD45, CD34, CD14 or CD11b, CD79α or CD19, Human Leukocyte Antigen-antigen D Related (HLA-DR)], (3) plastic adherence under standard culture conditions, and (4) an ability to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro* [9].

MSCs can be collected from bone marrow (BM), adipose tissue (AT), umbilical cord (UC), amniotic fluid, and peripheral blood. MSCs demonstrate the multimodal ability not only in terms of differentiation but also in their secretome consisting of various cytokines, chemokines, and growth factors related to multiple cellular function including angiogenesis, anti-apoptosis, and anti-inflammation (reviewed in [10,11]).

The use of the term “mesenchymal stem cell” is controversial in the scenario of cell transplantation therapy for PAH, because MSCs transplanted into the lung do not act as “stem cells” that differentiate to mesenchymal or other lineages. As proposed by the ISCT [9], “mesenchymal stromal cell” is a terminologically better description of MSCs used for cell-based therapies. As a strict distinction between mesenchymal stem or stromal cells has not been established, this review has taken an overview of all preclinical studies that have investigated mesenchymal stromal or stem cells for PAH.

Efficacy of MSC transplantation in PAH animals

Through a PubMed literature search using the terms [“pulmonary hypertension” or “pulmonary arterial hypertension”] and [“mesenchymal stem cell” or “mesenchymal stromal cell”], we found 22 original research articles on PAH animal models published between 2006 and 2018. These preclinical studies used rats (18 studies) and mice (4 studies) (Table 1, [12–33]). Among 18 rat studies, an experimental PAH model was induced by injection of monocrotaline (MCT) in 17 studies and by left-to-right shunt producing surgery in 1 study. Among 4 mice studies, PAH was induced by MCT injection in 2 studies, by exposure to hypoxia in 1 study, and by SU5146 injection following hypoxia exposure in 1 study. In this review, the other PH models, which have unusual, specific pathophysiology, *i.e.* neonatal PH due to bronchopulmonary dysplasia [34,35] and chronic thromboembolism PH (CTEPH) [36], were excluded. Among 22 studies, 21 studies have examined hemodynamic changes after MSC therapy by means of catheterization (15 studies under closed-chest, and 6 studies under open-chest). MSC administration was found to attenuate an increase in RV systolic pressure (RVSP) or PA pressure (PAP) in 16 preclinical studies (16/21 studies, 76%). In 5 studies, MSC therapy failed to reduce RVSP or PAP. Among these studies, 4 animal models presented extremely severe PAH [20] or mild PAH [24,28,32], which might affect the efficacy of MSC administration. In one study, MSC administration reduced RVSP (MSC treatment *versus* non-treatment PAH group; 28 *versus* 43 mmHg) however it did not reach statistical significance [22]. MSC attenuated RV remodeling defined by increased RV

Table 1
Preclinical studies on MSC therapy for PAH.

MSC source	MSC donor	Delivery route	Timing of injection[a]	Cell dose	Follow-up period	Hemodynamic outcome (PAH with treatment vs. PAH without treatment)		Histological outcome[b] (PAH with treatment vs. PAH without treatment)	Reference
						mPAP	RVSP		
Rat MCT model: MSC administration 3–7 days after MCT injection									
BM	Syngeneic	IV	3 days	4×10^6	18 days		Improve, 28 *vs.* 40 mmHg	Improve, 36 *vs.* 54%	[12]
BM	N/A	IV	7 days	0.5×10^6	14 days	Improve, 26 *vs.* 41 mmHg	Improve, 43 *vs.* 56 mmHg	Improve, 21 *vs.* 45%	[13]
BM	Rat, Syngeneic or allogenic	IV	7 days	1×10^6	14 days		Improve, 42 *vs.* 58 mmHg		[14]
BM	Syngeneic	IV	7 days	10×10^6	6 months	Improve, 31 *vs.* 45 mmHg[c]		Improve, 25 *vs.* 31%[c]	[15]
BM	N/A	IV	7 days	1.5×10^6	14 days	Improve, 24 *vs.* 37 mmHg		Improve, 33 *vs.* 49%	[16]
BM	N/A	IT	7 days	1.5×10^6	14 days	Improve, 26 *vs.* 37 mmHg		Improve, 33 *vs.* 49%	
UC	Human	IV	5 days	1×10^6	16 days		Improve, 29 *vs.* 47 mmHg	Improve, 18 *vs.* 32%	[17]
UC	Human	IV	7 days	3×10^6	21 days		Improve, 20 *vs.* 49 mmHg	Improve, 32 *vs.* 42%	[18]
UC	Human	IV	7 days	1.5×10^6	21 days		Improve, 15 *vs.* 49 mmHg	Improve, 35 *vs.* 42%	
UC	Human	IV	7 days	0.3×10^6	21 days		Improve, 14 *vs.* 49 mmHg	Improve, 33 *vs.* 42%	
UC	Human	IV	7 days	3×10^6	21 days		Improve, 16 *vs.* 52 mmHg	Improve, 32 *vs.* 37%	[19]
Rat MCT model: MSC administration 14 days after MCT injection									
BM	Syngeneic	IV	14 days	0.5×10^6	14 days		Not improve, 70 *vs.* 65 mmHg[c]	Not improve, 50 *vs.* 51%[c]	[20]
BM	Allogenic	IV	14 days	1×10^6	14 days		Improve, 31 *vs.* 42 mmHg		[21]
BM	Syngeneic	IV	14 days	1×10^6	21 days		Not improve, 28 *vs.* 43 mmHg	Improve, 21 *vs.* 29%	[22]
BM	Syngeneic	IT	14 days	3×10^6	21 days	Improve, 32 *vs.* 44 mmHg[c]			[23]
BM	Human	IV	14 days	0.25×10^6	14 days		Not improve, 35 *vs.* 40 mmHg[c]		[24]
UC	Human	IV	14 days	0.25×10^6	14 days		Improve, 32 *vs.* 42 mmHg	Not improve, 23 *vs.* 26%[c]	[25]
AT	Syngeneic	IV	14 days	0.1×10^6	14 days		Improve, 29 *vs.* 39 mmHg		[26]
Rat MCT model: MSC administration 21 days after MCT injection									
BM	Syngeneic	IV	21 days	3×10^6	21 days		Improve, 42 *vs.* 60 mmHg	Improve, wall thickness 38 *vs.* 42%[c]	[27]
BM	Syngeneic	IV	21 days	5×10^6	21 days	Not improve, 29 *vs.* 30 mmHg[c]		Not improve, vascular lumen ratio 33 *vs.* 34%[c]	[28]
Rat shunt model									
BM	Rat	IV	14 days[e]	$1–5 \times 10^6$	14 days	Improve, 26 *vs.* 41 mmHg	Improve, 43 *vs.* 56 mmHg	Improve, 21 *vs.* 45%	[29]
Mouse MCT model									
BM	Syngeneic	IV	3 days	2×10^6	13 days			Improve, muscularization 40 *vs.* 85%[c]	[30]
BM	Human	IV	7 days	3×10^6	21 days		Improve, 42 *vs.* 60 mmHg[c]	Improve, 40 *vs.* 53%	[31]
ESC	Human	IV	7 days	3×10^6	21 days		Improve, 32 *vs.* 60 mmHg[c]	Improve, 30 *vs.* 53%	
Mouse hypoxia model									
BM	Syngeneic	IV	35 days[f]	N/A	14 days		Not improve, 33 *vs.* 31 mmHg[c]		[32]
Mouse SU5416/Hypoxia model									
UC	Human	IV	14 days	0.5×10^6	14 days		Improve, 25 *vs.* 52 mmHg[c]	Not improve, 50 *vs.* 51%[d]	[33]

MSC, mesenchymal stem/stromal cell; PAH, pulmonary arterial hypertension; MCT, monocrotaline; BM, bone marrow; AT, adipose tissue; UC, umbilical cord blood; ESC, embryonic stem cell; IV, intravenous injection; IT, intratracheal injection; RVSP, right ventricular systolic pressure; mPAP, mean pulmonary artery pressure; N/A, not available. To show the value of PAP or RVSP, the numbers beyond a decimal point are truncated.

[a] Days from MCT injection is described if not specified elsewhere.

[b] Medial wall thickness is represented in histological outcome if not specified.

[c] The value is estimated from given figures.

[d] MSC reduces the density of fully muscularized vessels in this report [33].

[e] MSC was administered 14 days after a left-to-right shunt surgery.

[f] MSC was administered when mouse was exposed to hypoxia for 35 days.

tissue weight in 14 studies out of 18 studies that assessed this indicator (14/18 studies, 78%; no change in 4 studies). MSC also attenuated pulmonary vascular remodeling defined by increased medial wall thickness or narrowing of the vascular lumen in 16 studies (16/19 studies, 84%). Furthermore, MSC therapy improved survival in MCT model [12]. In summary, the majority of these 22 preclinical studies demonstrated promising efficacy results of MSC therapy for PAH.

Impact of MSC source on therapeutic efficacy

It is now widely believed that secretome is the most important mechanism by which MSC therapy improves the prognosis of PAH (see Section 'MSC secretome'). MSCs from different sources are likely to secrete different secretomes (reviewed in [11]). Therefore, the MSC source is likely to largely determine the therapeutic effect of MSC therapy in PAH. In the previous preclinical studies, MSCs were isolated from syngeneic BM (9 studies), allogeneic BM (1 study), human BM (2 studies), and BM from an unspecified donor (donor information not given; 4 studies), syngeneic AT (1 study), human UC blood (UC; 5 studies), and produced from human embryonic stem (ES) cells (1 study). Hence, 76% of the preclinical studies (16/22 studies) have examined MSC isolated from BM. Although it has not been clearly identified which MSC source is the most suitable for treatment of PAH, one preclinical study by Zhang and colleagues [31] provides useful information on the impact of the different MSC sources on PAH therapy. They compared MSCs isolated from human BM with those generated from human ES cells in an MCT model. As a result, ES cell-derived MSCs achieved better hemodynamic and histological improvement, due to higher levels of secreted angiogenic cytokines [vascular endothelium growth factor (VEGF), platelet derived growth factor (PDGF) and angiogenin)], compared with BM-derived MSCs [31].

All above-mentioned 5 studies, in which MSC therapy failed to improve the hemodynamic status, used BM-derived MSCs [20,22,24,28,32] (Table 1). As a result, BM-derived MSCs failed to achieve hemodynamic improvement in 31% of studies (5/16 studies). In contrast, UC-derived MSCs improve hemodynamics in all 5 studies. UC-derived MSCs have superior ability to migrate and secrete hepatic growth factor (HGF), and less senescence when compared with BM-derived MSCs [37]. Although we cannot conclude that UC-MSCs are superior to BM-MNCs from these results, it should be noted that the choice of MSC sources may influence the efficacy of MSC therapy. Further investigations are required to identify the most effective MSC sources for clinical success of MSC therapy for PAH.

Impact of the administration route on therapeutic efficacy of MSC therapy

In the previous preclinical reports, MSCs were administered either by intravenous (IV) or intra-tracheal (IT) routes. IV administration was used in most of these preclinical studies (20/22 studies), and IT administration was only employed in 1 study. In the remaining study, a direct comparison of IV or IT administration was conducted [16]. In the study by Xie et al. there were no differences in hemodynamic or histological improvement between IV and IT route and overall the two routes of administration led to comparable therapeutic efficacy [16,23]. It is likely that the preferential choice of the IV route was due to easy and practical procedure. However, IV administration of MSCs has a latent risk of pulmonary embolism [14,38]. Further information to compare the benefits and risks between the IV and IT routes should be accumulated by preclinical studies to clearly determine which route is optimal for MSC therapy in PAH.

Impact of cell-dose and timing of MSC administration on therapeutic efficacy

Optimization of the treatment protocols, in terms of the cell-number to be injected, frequency of administration, and timing of the administration, is essential for successful clinical application of MSC transplantation therapy. Lee and colleagues examined the dose and frequency of MSC administration using an MCT model at early stage, *i.e.* until 7 days after MCT injection in rats [18]. They showed that hemodynamic or histological improvement by this treatment was independent of the MSC dose used (0.3 million or 3.0 million), frequency of MSC administration (single or double administration), and the timing of administration (1 or 7 days after MCT injection) [18]. However, in this study the reason why the escalation in the MSC dose could not augment the hemodynamic or histological improvement was not clarified. As dose-dependent efficacy of MSC therapy has been proven in a clinical trial for patients with heart failure (not PAH) [39], their insistence for the dose-independent effect of MSC therapy should be further discussed. In Fig. 1, we plotted the data of the cell dose administered IV and the hemodynamic improvement measured in MCT model rats (15 plots for 12 reports in Fig. 1A, 4 plots for 4 reports in Fig. 1B; Fig. 1 does not include IT administration). This does not show any obvious relationship between the cell dose and the hemodynamic improvement. It has been proposed that increased numbers of MSCs administered intravenously may not necessarily lead to a benefit due to the risk of pulmonary embolism [14]. The MSC dose-response (beneficial and harmful) effects for PAH is a critical concern and should therefore be investigated before clinical application.

In terms of timing of MSC treatment, MSCs were administered intravenously until 7 days after MCT injection in 8 preclinical studies, all of which report that MSC attenuated hemodynamic and histological PAH progression (Table 1, Fig. 1A and B, left panel). In another 6 preclinical studies, MSCs were administered intravenously at 14 days after MCT injection, out of these studies 50% (3/6 studies) demonstrated that MSCs could not suppress the PAH hemodynamic progression (Fig. 1A, middle panel). In the remaining 2 studies, MSCs were administered intravenously at 21 days after MCT injection (Fig. 1A and B, right panel). One study demonstrated a hemodynamic and histological improvement [27], while the other study by Guo and colleagues reported that MSCs could not attenuate PAH suppression [28]. This suggests that early intervention is more potent to contribute to hemodynamic improvement. In addition, other reports demonstrated that MSCs were able to attenuate PAH progression, when MSCs were administered at an early phase of the MCT model (7 days after MCT injection), but not at a late phase of MCT model (21 days after MCT injection) [12,14]. These results suggest that MSC therapy is an effective approach for patients with early PAH, but its effect on advanced PAH may be limited.

Engraftment of transplanted MSCs in the lung

Donor cell engraftment is a key to successful MSC transplantation therapy for PAH; however, this appears to be limited using the current delivery methods. The insufficient engraftment rate is considered as one of the major hurdles for clinical application of MSC therapy [40]. IV injection has been employed in 90% of preclinical studies (20/22 studies; see Section 'Impact of the administration route on therapeutic efficacy of MSC therapy'). After IV injection of MSCs in normal mice, over 80% of cells are trapped in the lung within 15 min after administration. MSCs that are trapped in the lung form emboli in pulmonary capillaries and undergo apoptosis [41,42]. As such, the donor cell survival rate is ~20–30% 24 h after IV administration, which rapidly decreases to 0.05% by 48 h [32,42]. This trend in normal mice was also observed in normal rats, in which retention rate is only 3% and 0.7% at 24 h

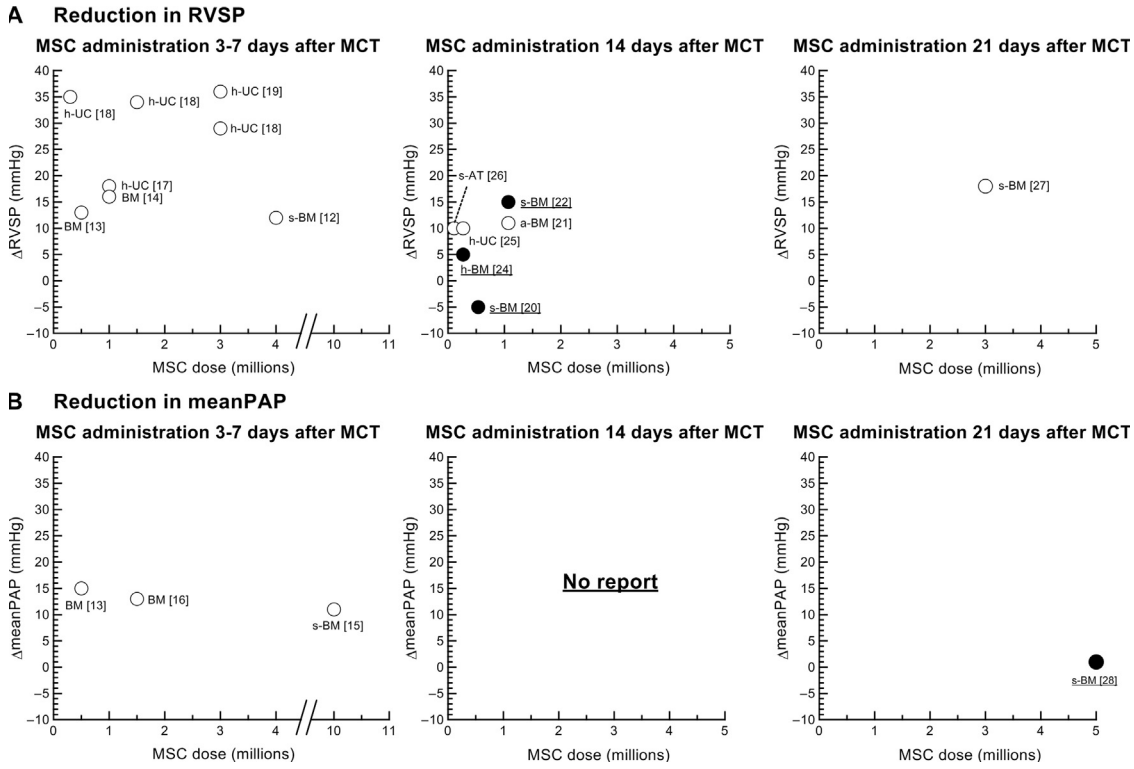

Fig. 1. Hemodynamic improvement by mesenchymal stem/stromal cell (MSC)-based therapy. Graphs represent dose of MSCs injected intravenously and reduction in right ventricular systolic pressure (RVSP) (A) or mean pulmonary artery pressure (PAP) (B) in monocrotaline (MCT)-induced pulmonary hypertension rats (total 19 plots from 16 reports). From left to right, preclinical studies in which MSCs were administered at 3–7 days, 14 days, and 21 days after MCT injection, respectively. Reduction in RVSP (ΔRVSP) and mean PAP (Δmean PAP) is calculated as the subtraction value of RVSP and mean PAP in MCT rats with MSC therapy from that without MSC therapy, respectively. Open circles show hemodynamic improvement after MSC therapy, and black circles show no hemodynamic improvement after MSC therapy. MSC source and reference are given for each data point. AT, adipose tissue-derived MSC; BM, bone marrow-derived MSC; UC, umbilical cord blood-derived MSC; a-, allogenic donor; h-, human; s-, syngeneic donor. If there is no information on donor species in articles, no description is given.

and 14 days, respectively following IV administration [20]. Takemiya and colleagues reported that MCT-treated rats exhibited a retention rate of 7.5% MSCs at 24 h after IV administration (2.5-times higher when compared with normal rats in their study), and the retention rate was maintained at 4.2% at 14 days after administration (6-times higher) [20]. Thus, PAH animals might exhibit higher MSC retention rates in the lung compared with normal animals, although these rates are not substantially high. According to two different reports, the retention rate is low at 0.25–0.375% in MCT model rats at 21 days after IV administration [12] or 0.003% in hypoxia-induced PAH mice at 14 days after IV administration [32]. Despite the limited survival of donor MSCs, MSC transplantation can treat PAH, potentially through MSC secretome (details are discussed in Section 'MSC secretome').

The IT administration is another option for MSC transplantation [16,23]. When administered via the IT route, MSCs are localized in the lung parenchyma, not in the wall of pulmonary vessels [16,23]. This finding was also observed in the preclinical studies on MSC therapy in a CTEPH model, another model of PH [36]. However, MSC retention after IT administration has not been quantitatively examined in animal models, and therefore further studies are required to quantify the engraftment rate after IT administration in PAH animal models.

Therapeutic mechanisms of MSCs in PAH

MSC secretome

MSCs have the ability to secrete multiple factors, including cytokines, chemokines, growth factors, microRNAs, and extracellular vesicles, which act on neighboring cells (paracrine effect). Given the reported ability of MSC-conditioned media (cell-free) to attenuate PAH progression in MCT model [43], the paracrine effect is now believed to be the main mechanism underpinning the therapeutic effects of transplanted MSCs to treat PAH. After transplantation, donor MSCs do secrete a certain range of factors at least for one ∼several days continuously. Secretion of beneficial factors (anti-inflammatory, vasodilative, anti-proliferative, and angiogenetic factors: Fig. 2, Table 2) will be able to unceasingly influence greater numbers of target host cells (*e.g.* ECs and SMCs), compared to the surviving donor cell number. This will be sufficient to induce histological repair and improve hemodynamics, and this effect is considered to last for a while.

Vasodilative and anti-inflammatory factors

Vasoconstriction and inflammation are key pathological factors contributing to PAH development (reviewed in [44]). Thus, the regulation of pulmonary vascular tone and inflammation is an important approach for PAH therapy [3]. MSC therapy attenuates PAH development with increased serum NO concentration in MCT rats [22]. de Mendonça and colleagues reported that MSC therapy contributed to reduction of inflammatory cytokine (IL-6) and CD68 + macrophages in the lung, whereas anti-inflammatory cytokine (IL-10) was not changed in the lung after MSC therapy [26]. Further detailed investigation on anti-inflammatory factors in the MSC secretome for PAH therapy will be necessary.

Anti-proliferative and apoptotic factors

Pathologically, vasculopathy in PAH is induced by aberrant proliferation of vascular cells [pulmonary ECs and pulmonary artery SMCs (PASMC)] (reviewed in [44]), attracting an interest in the role of anti-proliferative and apoptotic mediators for PAH

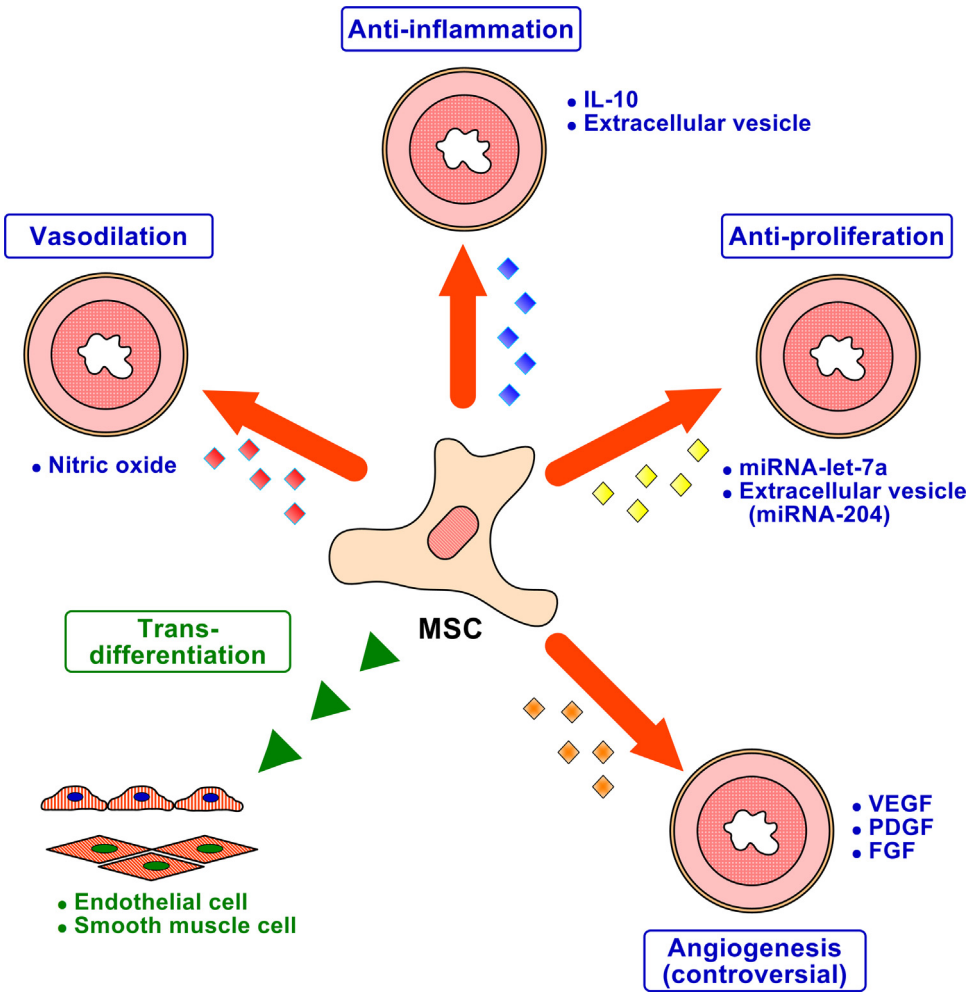

Fig. 2. Proposed mechanisms of mesenchymal stem/stromal cell (MSC)-based therapy for pulmonary arterial hypertension (PAH). Preclinical studies proposed two major mechanisms by which transplanted MSCs induce beneficial effects to treat PAH. (I) MSCs can secrete multiple factors (cytokines, chemokines, growth factors, microRNAs and extracellular vesicles), which act on neighboring cells (*i.e.* paracrine effect). With MSC secretome, MSC have multiple effects on PAH treatment through: (1) vasodilation, (2) anti-inflammation, (3) anti-proliferation, and (4) angiogenesis (although beneficial effect of angiogenetic factors is still controversial for human PAH). (II) Transdifferentiation of MSCs into endothelial cells (ECs) and smooth muscle cells (SMCs) is another potential mechanism of MSC-based therapy. However, it remains unclear whether ECs and SMCs derived from MSCs integrate into the functional pulmonary vasculature and/or contribute to the overall therapeutic efficacy. FGF, fibroblast growth factor; IL, interleukin; MSC, mesenchymal stromal/stem cell; PDGF, platelet derived growth factor; VEGF, vascular endothelium growth factor.

Table 2
Paracrine effects of MSC therapy.

Species	PAH model	Tissue	Expression	References
Vaso-dilative effect				
Rat	MCT	Serum	NO↑	[22]
Anti-inflammatory effect				
Rat	MCT	Lung	IL-10 (mRNA)→	[26]
Anti-proliferative effect				
Rat	MCT	Lung	miRNA-let-7a↑	[27]
Angiogenetic effect				
Rat	Shunt	PASMC	VEGF (IH)↑↑	[29]
Mouse	MCT	Lung	VEGF mRNA↑, FGF mRNA↑, vWF mRNA↑	[31]
Rat	MCT	Lung	VEGF mRNA→	[21]
Rat	MCT	Lung	VEGF mRNA→, PDGF mRNA→	[25]
Rat	MCT	Lung	VEGF (CA)↓	[19]
Rat	MCT	Plasma	VEGF (ELISA)↓, PDGF (ELISA)→	[26]
Rat	MCT	Lung	VEGF (IH)↓	

MSC, mesenchymal stem/stromal cell; PAH, pulmonary arterial hypertension; NO, nitric oxide; IL, interleukin; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; vWF, von Willebrand factor; PDGF, platelet-derived growth factor; IH, immunohistochemistry staining; CA, cytokine array analysis; ELISA, enzyme-linked immunosorbent assay.

therapy. Several reports on MSCs have already suggested anti-proliferative and apoptotic mediators contributing to therapeutic effects in PAH [22,26,27]. For example, MSC therapy increases expressions of Kruppel-like factor 4 (KLF4), p21, p53 protein, and miRNA-let-7a in the lung, which contributes to inhibition of aberrant PASMC proliferation [22,26,27]. KLF4 suppresses PASMC proliferation and cooperates with p53 to induce the expression of the cell cycle inhibitor, p21 [22]. miRNA-let-7a restores signal transducers and activators of transcription 3 (STAT3) and increases bone morphogenetic protein receptor type II (BMPR2) expression in PASMC, and thereby inhibits PASMC proliferation [27]. MSC-conditioned medium can inhibit *in vitro* over proliferation of PASMC [32,43], leading to attenuation of *in vivo* PAH development in an MCT model [43].

Having said this, we need to be cautious regarding the anti-proliferative effects of MSC therapy for PAH. The phase III Imatinib in Pulmonary Arterial Hypertension Randomized Efficacy Study (IMPRES) demonstrated that imatinib (a tyrosine kinase inhibitor) has a risk of serious adverse events, including anemia and subdural hematomas under concomitant anticoagulation [45], suggesting anti-proliferative strategies may have unwanted side effects. Safety and potential risks related to anti-proliferative factors in MSC therapy should therefore be further investigated.

Angiogenic factors

The beneficial effects of angiogenic cytokines released from MSCs have also been proposed. Two different research groups have demonstrated that MSC transplantation increases expression of VEGF, von-Willebrand Factor, and fibroblast growth factor (FGF) in the lung of MCT models [29,31]. However, conflicting results have been also reported, *i.e.* MSC transplantation attenuated PAH progression with reduced VEGF levels in the serum and the lung of an MCT model [19,26]. In addition, it remains arguable whether angiogenic factors are beneficial for the treatment of human PAH [26]. In particular, excessive pro-angiogenic growth factors can promote the disordered angiogenic process and lead to pathological pulmonary vascular remodeling in PAH [44,46]. Further studies will be necessary to conclude this aspect of MSC therapy in PAH.

Extracellular vesicles released from MSCs

More recently, extracellular vesicles released from MSCs have been widely investigated as potential mechanisms of MSC therapy [47–50]. Extracellular vesicles include several types of membrane vesicles of endosomal and plasma membrane origin, *i.e.* exosomes and microvesicles, respectively (reviewed in [51]). The size of each differs, with larger microvesicles ranging between 100 and 1000 nm compared to smaller exosomes, which range between 40 and 100 nm. The cargo of extracellular vesicles (exosomes and microvesicles) includes proteins, mRNAs, and miRNAs, and thus extracellular vesicles are recognized as important mediators of cell-to-cell communication [52].

IV administration of exosomes [47,49] and microvesicles derived from MSCs [48,50] attenuate the PAH progression in animal models of PAH. Lee and colleagues demonstrated that depletion of exosomes from conditioned medium of MSCs eliminates the reduction in hypoxic inflammation when injected into a hypoxia-induced PAH model [47]. They also demonstrated that IV administration of MSC-derived exosomes increases expression of miRNA-204 in the lung, accompanying attenuation of RVSP increase in hypoxia-induced PAH mice [47]. Interestingly, Aliota and colleagues reported that MSC-derived exosomes may contain unidentified protective miRNAs for PAH therapy [49]. Therefore understanding the components of extracellular vesicles released from MSCs may facilitate the establishment of future strategy of MSC-based therapy in PAH.

Transdifferentiation of donor MSCs

Transdifferentiation is a possible mechanism by which MSC-based therapy improves prognosis of PAH. *In vivo* analysis using MCT models have demonstrated that MSCs have the potential to transdifferentiate into vascular endothelial cells (ECs) and smooth muscle cells (SMCs) [15,23]. This ability of MSC to transdifferentiate into vascular cells may be an important mechanism underpinning their therapeutic effect. However, it is still unclear to what extent the ECs and SMCs derived from MSCs can integrate into the functional pulmonary vasculature and contribute to the overall therapeutic efficacy. Future preclinical studies are required to prove the contribution of transdifferentiation to the increased function of pulmonary vessels in animal models of PAH.

Remaining issues for clinical application of MSC therapy for PAH

As summarized above, there are plenty of promising preclinical data to support MSC therapy for PAH. However, a thorough search of "*ClinicalTrials.gov*", revealed no clinical trials of MSC therapy to date. Before clinical application of MSC therapy in PAH, there remain several important issues, which should be addressed through preclinical research.

Risk of pulmonary embolism after IV injection of MSCs

Administration of MSCs, particularly in the case of IV injection, has a risk to induce critical pulmonary embolism. There is an experimental report demonstrating that 25–40% of animals (normal mice) die due to pulmonary embolism after IV administration of MSCs [38]. Kanki-Horimoto and colleagues experienced animal deaths immediately after IV administration of 1 million of MSCs (there was no information on either normal or PAH animals) [14]. Although the cause of death was not clarified in their study, they suggested that administering a large number of MSCs can negatively affect survival and that the cell number to be injected intravenously should be limited. Of note, there is a clinical report detailing three patients with orthopedic diseases (without PAH) who developed pulmonary embolism after IV administration of autologous human adipose tissue-derived stem cells [53]. Therefore, it is essential to accumulate further preclinical data concerning pulmonary embolism after MSC administration in PAH animal models with severe obstructive vascular lumen.

Use of more appropriate models

Preclinical studies on MSC therapy have mostly used MCT models (19/22 studies). While the MCT model is an established animal model of PAH (reviewed in [54]), this model does not represent all aspects of human PAH [20]. Most importantly, this model does not develop occlusive neointimal and plexiform lesions. Thus, while the preventive effects of MSC therapy have been demonstrated in the preclinical studies, it remains unclear whether MSC therapy can histologically reverse established vascular remodeling, which involves abnormal proliferation of pulmonary ECs and PASMCs. Furthermore, serum VEGF concentration is reduced in the MCT model [55], in contrast to an abnormal increase in serum and endothelial level of VEGF in PAH patients [56,57].

In this regard, SU5416 model, an animal model presenting advanced vascular remodeling that is typical in late phases of PAH [58], will provide vital information concerning the effects of MSC therapy to reverse developed PAH. SU5416 model has vascular obstructive lesions, which consist of ECs highly expressing VEGF [58]. Most recently, Alencar and colleagues have reported that IV

administration of human UC-derived MSCs could decrease RVSP with reduction of the density of fully muscularized vessels in SU5416-treated mice [33]. As such, their report demonstrated potential safety and efficacy of MSC therapy in a SU5416 model, however it remains unclear whether MSCs repair the already established obstructive lesions (*i.e.* reduction in intimal thickening) in this model. Further preclinical data using SU5416 model should be accumulated.

Optimization of the protocol for MSC administration

Protocols for MSC administration need to be further optimized in terms of both safety and efficacy, before initiating clinical application of MSC therapy. Factors to be optimized include the source of MSCs (BM, AT, UC, or others; autologous versus allogeneic), cell numbers to be injected, route of transplantation (IT or IV route), and timing of the treatment (mild or severe PAH). Some questions have been partly answered by previous preclinical studies but remain uncertain. The optimized protocol of MSC administration is needed to be investigated using not only an MCT model but also SU5416 model.

Conclusions

There are a number of promising preclinical studies, which encourage development of MSC transplantation therapy as a new approach for PAH patients. However, there are important remaining issues that should be addressed before clinical application of this treatment. These include further detailed investigation on safety, therapeutic effect of MSCs on established PAH with severe vascular remodeling, and optimization of the treatment protocol. To this end, further preclinical studies using the SU5416 model should be accumulated. Although considerable efforts are required to address these issues, we believe it is worth challenging since MSC therapy has a great potential to save a large number of patients suffering PAH due to insufficient treatment options.

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