Published in final edited form as:

Stem Cells. 2011 January ; 29(1): 99–107. doi:10.1002/stem.548.

Mesenchymal Stromal Cells Expressing Heme Oxygenase-1 Reverse Pulmonary Hypertension

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Abstract

Pulmonary arterial hypertension (PAH) remains a serious disease, and, while current treatments may prolong and improve quality of life, search for novel and effective therapies is warranted. Using genetically-modified mouse lines, we tested the ability of bone marrow-derived stromal cells (MSCs), to treat chronic hypoxia-induced PAH. Recipient mice were exposed for five weeks to normobaric hypoxia (8%–10% O₂), MSC preparations were delivered through jugular vein injection and their effect on PAH was assessed after two additional weeks in hypoxia. Donor MSCs derived from wild-type (WT) mice or Heme Oxygenase-1 (HO-1) null mice (Hmox1^{KO}) conferred partial protection from PAH when transplanted into WT or Hmox1^{KO} recipients, whereas treatment with MSCs isolated from transgenic mice harboring a human HO-1 transgene under the control of surfactant protein C promoter (SHO1 line) reversed established disease in WT recipients. SHO1-MSC treatment of Hmox1^{KO} animals, which develop right ventricular (RV) infarction under prolonged hypoxia, resulted in normal RV systolic pressure, significant reduction of RV hypertrophy and prevention of RV infarction. Donor MSCs isolated from a bitransgenic mouse line with doxycycline-inducible, lung-specific expression of HO-1 exhibited similar therapeutic efficacy only upon doxycycline treatment of the recipients. *In vitro* experiments indicate that potential mechanisms of MSC action include modulation of hypoxia-induced lung inflammation and inhibition of smooth muscle cell proliferation. Cumulative, our results demonstrate that MSCs ameliorate chronic hypoxia – induced PAH and their efficacy is highly augmented by lung-specific HO-1 expression in the transplanted cells, suggesting an interplay between HO-1 dependent and HO-1 independent protective pathways.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Keywords

lung; hypoxia; mesenchymal stem cell; transgenic mouse

Introduction

Pulmonary arterial hypertension (PAH) is characterized by arteriolar wall remodeling, elevated pulmonary artery pressure, and right ventricular hypertrophy (RVH). Although currently there is no cure for this disease, treatment has improved during the past decade, offering both relief from symptoms and prolonged survival. Improving treatment strategies requires a thorough understanding of the molecular mechanisms underlying the pathophysiology of PAH and progress in the field has been significant, albeit slow, given the multifactorial nature of the disease. Research investigating the prevention of PAH or the treatment of established disease using animal models has explored the application of elastase inhibitors ¹, inhibition of tyrosine kinase ², inhalation of carbon monoxide (CO) ³ and the transplantation of endothelial-like progenitor cells expressing endothelial nitric oxide synthase (eNOS) ⁴.

Bone marrow cells can generate diverse non-hematopoietic cell types ⁵ and their efficacy in repairing tissue injury has been assessed in a number of systems. Transplantation studies in rodents, using whole donor bone marrow, or the plastic-adherent cell fraction, termed marrow stromal cells, multipotent stromal cells or mesenchymal stem cells (MSCs) have reported on the potential contribution of these multipotent cells in lung repair ⁶⁻⁸. Initial studies in mice provided evidence for trans-differentiation of transplanted MSCs into alveolar epithelium, with bleomycin injury apparently enhancing MSC engraftment in the lung ⁹⁻¹¹, although overall engraftment rates were subsequently revised to be significantly less than originally estimated. MSC treatment has also been reported to confer protection in rat models of PAH induced by monocrotaline ¹²⁻¹⁴ and other models of lung injury ^{10, 11, 15-17} and trans-differentiation of MSCs into airway epithelial cells has been suggested as a potential therapeutic application for cystic fibrosis ¹⁸. We have recently reported that MSC treatment can prevent lung damage in a neonatal mouse model of hyperoxia-induced bronchopulmonary dysplasia (BPD) in spite of low overall levels of donor cell engraftment and, importantly, we found that the *in vitro* MSC secretome can also confer protection, indicating that the therapeutic action of MSCs involves a significant paracrine component ¹⁹.

The inducible Heme Oxygenase-1 isoform (HO-1), responds to multiple stressors, including hypoxia, hyperoxia, acidosis, shear stress, and reactive oxygen species ²⁰⁻²³ and HO-1 activity restores homeostasis in many pathologic states by exerting anti-inflammatory, anti-apoptotic or anti-proliferative effects on diverse cell types ²⁴. By degrading the pro-oxidant heme and generating the anti-oxidant bilirubin, HO-1 protects against oxidative injury. The second enzymatic product, CO, stimulates guanylyl cyclase and increases intracellular levels of cGMP, an important regulator of vascular tone and smooth muscle proliferation. We have previously reported on the essential role of HO-1 in cardiovascular adaptation to hypoxic stress, and its protective function against lung inflammation, pulmonary hypertension, and right ventricular failure: Up-regulation of endogenous HO-1 can prevent hypoxia-induced PAH in the rat ²⁵ and transgenic mice harboring a human HO-1 transgene with lung epithelium - specific expression are protected from the development of hypoxia-induced elevation in pulmonary artery pressure, RVH, and vascular remodeling ²⁶. Significantly, HO-1 modulates the transient inflammatory response induced in the lung very early upon hypoxic exposure which triggers the molecular cascades leading to subsequent pathology ²⁶⁻²⁸. HO-1 deficient (Hmox1^{KO}) mice subjected to chronic hypoxia exhibit

exaggerated dilatation of the right ventricle with organized mural thrombi not observed in wild-type (WT) hypoxic animals ²⁹, suggesting that Hmox1^{KO} cardiomyocytes specifically develop a maladaptive response to hypoxia and subsequent pulmonary hypertension. Supplementing Hmox1^{KO} animals with exogenous CO or bilirubin reveals distinct protective actions of these two HO-1 enzymatic products on the heart and pulmonary vasculature ³⁰.

In the present study we combined the two protective approaches, MSC treatment and HO-1 overexpression, and systematically assessed the efficacy of genetically-engineered MSCs on treating established PAH in a mouse model. We transplanted MSCs from WT donors or transgenic donors designed to overexpress a human HO-1 transgene in the lung epithelium into either WT or Hmox1^{KO} recipients prior to or after five weeks of hypoxic exposure. We report that MSC treatment can both prevent and reverse PAH induced by chronic hypoxia and the efficacy of this treatment is highly augmented if the donor MSCs have the ability to overexpress HO-1 in the lung epithelium, apparently upon transdifferentiation. Although MSC treatment conferred protection in both WT and Hmox1^{KO} recipients, only WT animals exhibited complete prevention or reversal of established disease, highlighting the important role of endogenous HO-1 activity in protecting the lung. The MSC protective functions augmented by lung epithelial HO-1 expression include modulation of inflammatory mediators at the onset of hypoxia and anti-proliferative action on vascular smooth muscle cells.

Materials and Methods

MSC isolation and transgenic animal lines

Bone marrow-derived MSCs were isolated from 6–8 week old mice and their differentiation potential was verified prior to transplantation, as previously described ¹⁹. Donor animals were either WT FVB/n or belonged to one of the following transgenic lines: animals with constitutive, lung-specific expression of the human HO-1 transgene (SH01 line) ²⁶; Hmox1^{KO} (a strain harboring a disruption of the murine Hmox1 locus) ²⁹; or bitransgenic mice with Doxycycline (Dox)-inducible, lung-specific HO-1 expression (CC77 line). The latter line was derived from a cross between a line harboring a human HO-1 transgene under the control of the tetracycline operator (E. Vergadi and M.S. Chang et al., manuscript under review) and a line expressing the rtTA transcriptional activator under the control of the Clara Cell secretory protein (CCSP) gene promoter ³¹ (a kind gift of Dr. J.A. Whitsett). The human HO-1 transgene expression in line CC77 was induced by the addition of 1mg/ml Dox in the water. All animal protocols were approved by the Children's Hospital Boston Animal Care and Use Committee.

Pulmonary arteriolar wall remodeling

Randomly selected vessels with a diameter up to 100 μ m in 4 μ m hematoxylin-eosin (H&E)- or α -smooth muscle actin (α -SMA)- stained sections were captured at 400 $\times$ magnification. Medial wall thickness was measured as previously described ²⁶ using the NIH ImageJ program and expressed as an index of vessel diameter as follows: (2 $\times$ medial wall thickness)/external diameter.

Smooth muscle cell proliferation assay

Conditioned media from either MSCs or mouse lung fibroblasts (MLFs) were produced for 24 hours under serum-free conditions followed by differential centrifugation at 420 $\times$ g for 10 minutes and 12,000 $\times$ g for 30 minutes to discard cells and debris, respectively. Clarified conditioned media were concentrated by ultrafiltration using a 10 kDa filter unit and protein concentration was measured by Bradford assay (Bio-Rad). For proliferation assay, primary

rat pulmonary artery smooth muscle cells (PASMCs) were inoculated at a concentration of 2×10^3 cells per well on 96 well plates in 100 μ L DMEM containing 5% fetal bovine serum (FBS) and incubated for 24 hours under standard culture conditions. After serum starvation for two days in DMEM containing 0.1% FBS, cells were treated with either vehicle, MSC-CM, or MLF-CM for 30 minutes, then 5% FBS was added to each well. After 48 hours, cell proliferation reagent WST-1 (Roche), which is cleaved by mitochondrial dehydrogenases in metabolically active cells to form formazan dye, was directly applied to the cells followed by further incubation for three hours. The absorbance for solubilized dark red formazan was determined by using a microplate reader.

Statistical Analysis

All values are expressed as means $\pm$ SD. Comparison between different groups was performed by one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism (5.0) (GraphPad, La Jolla, CA). Significance was considered at p values less than 0.05.

See Online Data Supplement for expanded Materials and Methods including information on: MSC isolation and the hypoxia-induced PAH mouse model, quantitative PCR and sequence of the PCR primers, probes, and reaction conditions, Y-FISH and immunohistochemistry, and assessment of microvessel density.

Results

Transplantation of genetically-engineered MSCs reverses hypoxia-induced PAH in WT mice

In order to determine the efficacy of MSC treatment in reversing hypoxia-induced pulmonary hypertension and to assess the contribution of HO-1 activity in the process, we delivered MSCs through jugular vein injection to mice after 5 weeks of hypoxic exposure and measured hemodynamic, vascular, and immunologic parameters after an additional two weeks in hypoxia. At the end of the 7-week hypoxic exposure, WT mice receiving PBS or WT-MSCs exhibited elevated RVSP whereas the RVSP of WT mice receiving SH01-MSCs was within the normal range (Figure 1, panel A). The Fulton's index (RV/[LV+S]), an index of RVH, in mice receiving SH01-MSCs was significantly lower than that of the hypoxic controls receiving PBS and not significantly different from that of normoxic animals (Figure 1, panel B). Interestingly, the Fulton's index of animals receiving WT-MSCs showed significant improvement when compared with PBS recipients, suggesting that mechanisms inherent to MSCs can ameliorate RVH independently of the hHO-1 transgene overexpression. Nevertheless, the presence of the hHO1 transgene in SH01-MSCs was required for complete reversal of disease.

As a control experiment, we transplanted WT mice with MLFs under the conditions described above. There was no improvement in RVSP or RVH development under chronic hypoxia (Figure S1, panels A and B), in accord with the results obtained with PBS-treated animals.

Endogenous HO-1 expression is essential for reversal of RVH by MSC treatment

Upon transplanting Hmox1^{KO} animals which develop more severe pulmonary and cardiovascular injuries under hypoxia^{29,30}, we observed significantly elevated RVSP in PBS recipients, as expected. As in the case of WT recipients, RVSP of Hmox1^{KO} mice treated with SH01-MSCs returned to the normal range, and, although WT-MSCs improved RVSP levels, they were not as efficacious as SH01-MSC treatment (Figure 1 panel C). In contrast to WT recipients, the Fulton's index of Hmox1^{KO} mice treated with either SH01-

MSCs or WT-MSCs remained within the pathologic range despite a significant improvement compared to the PBS-treated controls, especially in the group treated with SH01-MSCs (Figure 1, panel D). Combined, these results suggest that the degree of amelioration of RVSP and RVH in hypoxic animals is associated with the ability of donor MSCs to over-express the hHO-1 transgene in recipient lung as well as the presence of an intact Hmox1 locus (i.e. endogenous HO-1 activity) in the WT recipients.

SH01-MSC treatment reverses vascular remodeling and prevents RV dilatation & thrombus in HO-1 deficient animals

Compared with normoxic controls, marked medial wall thickening in pulmonary arterioles was observed after chronic hypoxia in both WT (Figure 2) and Hmox1^{KO} animals (Figure S2). This remodeling was reversed only with SH01-MSC treatment, as evidenced by H & E and α -SMA staining of the pulmonary arterioles in both the WT animals (Figure 2, panels A–F), and the Hmox1^{KO} mice (Figure S2, panels A–D). In order to assess other possible vascular effects imparted by MSC treatment, we have quantified the number of blood vessels per microscope field and the percent area per field representing blood vessels. No difference was found in overall blood vessel density between SH01-MSC- treated and PBS-treated groups (data not shown).

We have previously reported that hypoxic Hmox1^{KO} mice manifest exaggerated maladaptive response to chronic hypoxia, presenting significant dilatation of the RV compared with hypoxic WT animals. In addition, a majority of Hmox1^{KO} animals develop RV mural thrombus after five weeks of chronic hypoxic exposure²⁹. In the current study we observed a similar proportion of mural thrombus formation in Hmox1^{KO} animals that received PBS and in some recipients of WT-MSCs, but, most significantly, we observed no thrombus in any of the animals receiving SH01-MSCs (Figure S2, panel E), the latter also presenting a reduction of the hypoxia-induced RV dilatation. This prevention of thrombus formation, in the context of only partial reversal of Fulton's index (Figure 1, panel D) indicates that MSC treatment is able to efficiently reverse the RV injury precipitated by systemic hypoxia, a maladaptive response we have suggested to be at least partially independent of the increased pulmonary vascular resistance in Hmox1^{KO} mice³⁰.

Enhancement of the efficacy of MSC treatment requires epithelial specificity of hHO-1 transgene expression

The increased efficacy of SH01-MSCs over WT-MSCs in reversing PAH implies that donor MSCs acquire the ability to express the hHO1 transgene by transdifferentiating into pneumocytes competent to activate the SP-C promoter. We sought to confirm this hypothesis by using MSC donors from an independent transgenic strain, CC77, in which the hHO1 transgene is under the control of a Dox-inducible system. In these bitransgenic mice, hHO1 expression conforms to the expression profile of the CCSP-promoter-rtTA transgene, specifically, the expected expression in Clara Cells but also the apparent ectopic expression in Type II alveolar epithelium³¹. In the presence of Dox, the hHO-1 expression profiles in the lungs of SH01 animals and the lungs of CC77 animals share significant overlap. CC77 animals in the absence of Dox supplementation behave as WT animals.

CC77-MSCs were delivered to WT mice and animals were subsequently exposed to hypoxia for two weeks and supplied with Dox in the drinking water to induce the hHO1 transgene. Dox supplementation efficiently protected CC77-MSC recipients from both, increases in RVSP and RVH, whereas, in the absence of Dox, RVSP measurements were similar to PBS recipients and the degree of RVH, although improved, remained over the normal range (Figure 3). Thus, in the absence of Dox, the efficacy of CC77-MSCs in preventing PAH paralleled the decreased efficacy of WT-MSCs in reversing disease after 5 weeks of

hypoxia. These results independently validate the importance of hHO-1 expression in augmenting MSC treatment. Aware of the reports summarized in Whitsett & Perl³² that chronic administration of high dosages of Dox may affect pulmonary parameters in certain studies, we administered Dox to one of the PBS control groups and we observed no effect of Dox treatment on hypoxia-induced PAH (data not shown).

Our results indicate that transplantation of MSCs capable of expressing the hHO-1 transgene in either a constitutive (SH01) or inducible (CC77) manner in the lung epithelium can achieve complete prevention, as well as reversal of established PAH in WT mice. This suggests that, although MSC transplantation *per se* can partially ameliorate PAH pathology, the ability of donor MSCs to express a lung-specific hHO-1 transgene can greatly augment their efficacy. Most relevant, since hHO-1 transgene activation is restricted to pulmonary epithelium in our model, transdifferentiation of donor cells is apparently obligatory for the increased efficacy to occur.

Transplantation, clearance and engraftment of donor MSCs

We examined the fate of transplanted MSCs in the recipient lung by assessing Y chromosome DNA levels through qPCR to trace male donor cells in female recipients. As reported by others^{33, 34}, a rapid clearance of donor MSCs from the lung was observed and this occurred independently of normoxic or hypoxic exposure of the recipients. Male donor cells represented approximately 0.05% of the total cells in the lung of female recipients 48 hours post-injection, and, within a week post-injection, that value decreased by two orders of magnitude (Figure 4A). At two weeks post-injection, lungs from hypoxia-treated mice had retained six-fold more donor MSCs than lungs of normoxic mice (Figure 4B). The observed difference is statistically significant, and it may indicate enhanced engraftment in the injured lung. Nevertheless, the physiologic significance remains to be elucidated, given the low number of retained donor cells, and pending the complete characterization of the pneumocytes the donor cells may have transdifferentiated into.

Expression levels of the hHO-1 transgene in the lungs of WT recipients, although readily detectable by qPCR, were assessed to be three to four orders of magnitude less than the levels present in the lungs of SH01 or CC77 animals supplemented with Dox, where the transgene is widely expressed in the entirety of alveolar epithelium. This is in accord with the low number of donor cells retained in the lung. Interestingly, when expression levels of the hHO-1 transgene, derived from lungs of animals transplanted with SH01-MSCs, were normalized to the levels of Y chromosome at each corresponding time point post-injection, a clear increase of hHO-1 transcriptional activity per resident donor cell was observed seven days post-injection, possibly indicating a time-dependent transdifferentiation of donor SH01-MSCs into pneumocytes (Figure 4C). When CC77-MSCs were transplanted, expression of the hHO-1 transgene on day seven was dependent on the presence of Dox (Figure 4D) independently buttressing the notion that hHO-1 was activated in pneumocytes. Overall, the above data indicate that, although a small number of donor cells remain in the recipient lung, at least a fraction of these resident cells has acquired the ability to activate either the SP-C or the CCSP promoter through transdifferentiation.

Transdifferentiation of donor MSCs to alveolar type II epithelium

In order to detect the distribution of resident donor cells in the lung, we used fluorescent *in situ* hybridization (FISH) staining for Y chromosome sequences. Rare male donor cells were detectable in the recipient female lung (Figure S3A, Green) and specificity of the signal was verified by observing the same positions using a red fluorescence filter (Figure S3A, Red). In addition, serial confocal scanning at 0.3 μ m increments indicated that the Y-FISH signal was indeed within the observed cell (Figure S3B). Furthermore, using

immunohistochemistry and confocal microscopy on the lungs of recipients injected with GFP⁺ MSCs, we detected expression of SP-C, a type II pneumocyte-specific marker within resident GFP⁺ cells, indicating trans-differentiation of MSCs to type II alveolar epithelium (Figure 5A, B). Using confocal microscopy, we merged a red-stained GFP⁺ cell (Figure 5C) with green-stained SP-C granules from the same cell (Figure 5D) generating a yellow signal (Figure 5E), and demonstrating that granular SP-C expression is within the transplanted cell. In addition, we produced 3D digital reconstructions from a series of confocal images taken at 0.2 μ m increments in the Z-axis through the region of interest. The optical dissection and orthogonal reconstruction of the 3D confocal images further confirmed that the expression of type II pneumocyte-specific SPC was indeed within the transplanted GFP⁺ cell (Figure 5F).

The apparent phenotypic conversion of MSCs to lung epithelial type II pneumocytes together with the hHO-1 expression data (Figure 4) would suggest activation of the SP-C promoter driving the hHO-1 transgene in donor MSCs derived from the SH01 line. Thus, transplanted MSCs represent a minor fraction of the total lung cell population, but some of these resident cells appear to have trans-differentiated into lung alveolar epithelium and significantly augment the efficacy of MSC treatment by overexpressing HO-1.

Transplanted MSCs modulate hypoxia-induced lung inflammation

We have previously reported that hypoxia triggers an early inflammatory response in the lung and when this response is suppressed by the anti-inflammatory action of HO-1, vascular remodeling is suppressed and the animals are protected from hypoxia-induced PAH²⁶. Delivery of SH01-MSCs, but not of control MLFs, suppressed the hypoxia-induced inflammation by inhibiting macrophage accumulation in the bronchoalveolar lavage (BAL) fluid of transplanted animals (data not shown). We assessed whether HO-1 expression contributed to the anti-inflammatory actions of MSCs by quantifying lung cytokine mRNA levels in WT mice treated with CC77-MSCs and exposed to hypoxia for four days in the presence or absence of Dox supplementation (Figure 6). In this early time period, hypoxia greatly increased the expression of the pro-inflammatory cytokines, CCL2 (monocyte chemoattractant protein-1, MCP-1), and interleukin (IL)-6 in the lung, and MSC treatment inhibited this inflammatory response. The expression of the anti-inflammatory cytokine, IL-10, was unaffected by hypoxia, but MSC treatment resulted in a dramatic increase of IL-10 expression. Expression of IL-1 receptor antagonist (IL1RN) was also decreased 6-fold by hypoxia, and MSC treatment ameliorated this suppression (data not shown). The immunomodulatory function of MSCs was independent of the induction of hHO-1 expression, however, in all cases there was a trend of hHO-1 expression augmenting MSC efficacy. For example, Dox treatment of hypoxic CC77-MSC recipients resulted in IL-6 levels not significantly different from normoxic controls and in the case of CCL2, there was a statistical significance between the -Dox and the +Dox CC77-MSC groups (Figure 6).

MSC conditioned media inhibit smooth muscle cell proliferation

Chronic hypoxia results in excess smooth muscle cell layers around the distal pulmonary arterioles. This remodeling contributes to enhanced vasoconstriction and elevations in pulmonary vascular resistance characteristic of PAH. Since cultured vascular smooth muscle cells (VSMCs) exposed to hypoxia manifest increased proliferation in response to a mitogenic stimulus³⁵ with increased lifespan³⁶ compared to normoxic controls, excess VSMC growth and survival may underlie the vascular remodeling of hypoxia-induced PAH. To investigate the paracrine effects of MSCs on smooth muscle cell proliferation, we treated PASMC cultures with conditioned media from cultured MSCs (MSC-CM) and measured cell proliferation, using MLF-conditioned media (MLF-CM) as controls. MSC-CM, but not MLF-CM, inhibited serum-induced smooth muscle cell proliferation (Figure 7A), and the

effect was dose-dependent (Figure 7B). These data demonstrate that the MSC secretome includes, in addition to immunomodulators impacting on lung inflammation, additional factors with anti-proliferative activity that may suppress vascular remodeling by their action on VSMCs.

Discussion

Pulmonary hypertension is a condition associated with a variety of pulmonary disorders, including alveolar hypoxia. A major therapeutic challenge in PAH is to reverse lung vascular remodeling and prevent RV failure, the latter being the final pathway leading to death in these patients. Hypoxia exposure in mice is associated with an early peak in lung inflammatory cell accumulation, coupled with marked induction of proinflammatory cytokines and chemokines that subsides within a week of hypoxic exposure²⁶. However, circulating monocytes/fibrocytes recruited at that early time may contribute significantly to the structural remodeling and persistent vasoconstriction of the pulmonary circulation weeks later³⁷, even if the alveolar inflammatory profile subsequently adjusts to reflect adaptation to a chronic hypoxic state. We have previously reported that transgenic mice with constitutive, lung-specific overexpression of HO-1 do not manifest the early inflammatory peak and are protected from PAH upon continued exposure to hypoxia²⁶.

It has not been obvious whether the protective function(s) of HO-1 reside solely with early immunomodulation, or if HO-1 overexpression can reverse established disease once severe hypoxia-induced PAH has developed. In this study we investigated both the prevention as well as reversal of PAH using WT and Hmox1^{KO} animals exposed to chronic hypoxia. Mice were transplanted with MSCs from transgenic lines that have been engineered to overexpress the hHO-1 transgene in the lung epithelium under either a constitutive (SH01 line) or a conditional (CC77 line) promoter. It is relevant to note that in the CC77 line, the CC10-promoter-driven rtTA is expressed in Clara cells and, ectopically, in Type II cells³¹. Activation of the hHO-1 transgene upon Dox administration follows the rtTA specificity of expression and therefore, in both systems, hHO-1 overexpression in the recipient lung requires phenotypic conversion of donor MSCs to lung epithelial cells. We have tested both SH01 and CC77 donor MSC cultures to verify that *in vitro* expression of hHO-1 was absent (results not shown). Therefore, the observed expression of hHO-1 in the recipient lung and augmentation of MSC efficacy must reside with the rare donor cells transdifferentiating into lung epithelium. Fusion of donor MSCs and recipient epithelial cells is apparently not the major mechanism of hHO-1 activation. Engraftment and transdifferentiation were further verified by examining Y-FISH FITC signals and using confocal techniques to ensure signal specificity. Accordingly, in WT mice, the combination of CC77-MSCs and Dox treatments prevented the development of PAH just as SH01-MSC treatment reversed disease in WT recipients. Only recipients treated with Dox were fully protected, whereas CC77-MSC treatment without Dox conferred partial protection from hypoxia-induced injury, similar to results obtained with WT-MSC transplantation. The validation of the results from both donor transgenic lines clearly indicates that, although MSCs possess innate mechanisms conferring protection against PAH, hHO-1 overexpression by rare cells engrafted in the lung epithelium augments their efficacy and restores RVSP to normal range. In this context, it is important to note that only SH01-MSC treatment was sufficient to eliminate the formation of mural thrombus that is a characteristic response of Hmox1^{KO} exposed to chronic hypoxia. Similar to the low level of lung engraftment, rare MSCs were detected in the hearts of recipient animals two weeks post-injection (data not shown). Since the hHO-1 transgene cannot be activated in the heart, the observed protection cannot be the result of cardiac HO-1 overexpression. This suggests that the cardiac protection observed in our system is the indirect result of lowered lung vascular resistance or due to paracrine factors secreted into

the circulation by lung-resident donor cells³³, but the nature of mechanisms involved in cardiac protection merits further investigation.

MSC engraftment in the lung occurs more rarely than certain original reports assessed, and paracrine functions are now recognized to be a major therapeutic component of MSC therapy in certain models of lung disease^{15, 17, 19, 33}. In our model we observed a rapid clearance of infused MSCs within two days post-transplantation, in line with reports estimating the level of engraftment to be less than 0.01%³⁸. Our results also parallel studies showing that intratracheal MSC administration producing rare engraftment in pulmonary and cardiac tissues, can attenuate monocrotaline-induced PAH and endothelial dysfunction and that transplantation of MSCs constitutively overexpressing eNOS can improve RV impairment and increase survival^{12–14}.

Although full reversal of hypoxia-induced PAH in WT mice was associated with the potential of MSCs to over-express hHO-1 in the recipient lung upon transdifferentiation, accurate assessment of relative hHO-1 mRNA levels at different time points after transplantation is challenging in the context of total lung RNA, since they are orders of magnitude less than endogenous mRNAs. Nevertheless, we observed an intriguing correlation between levels of hHO-1 mRNA and levels of Y-chromosome by deriving an index of hHO-1 expression per resident donor cell. The peak of hHO-1 mRNA observed at seven days post-injection suggests that a cohort of resident MSCs activated epithelial promoters at that time point, presumably subsequent to transdifferentiation into Type II cells. We can only speculate that the decrease in hHO-1 mRNA per resident MSC after day 7 may be the result of further differentiation of most donor-derived Type II cells into Type I cells. More importantly, our data show that a transient peak in hHO-1 expression augments the efficacy of MSC treatment, preventing PAH development in WT mice, inhibiting RV thrombus in *Hmox1*^{KO} mice, and reversing established disease in WT animals.

This apparent paradox of a prominent physiologic effect in the absence of significant MSC engraftment or sustained detectable levels of hHO-1 expression, may result from the potent immunomodulating properties of the MSC secretome as others have also postulated^{15, 17, 33}. Accordingly, the bolus of MSC paracrine factors tilts the recipient's immune system balance towards an anti-inflammatory equilibrium that persists even in the presence of a continuing hypoxic insult. Indeed, using the BPD model, we have reported that a single injection of MSC-CM protects the recipient animals for up to two weeks of hyperoxia¹⁹. In this report, we suggest that the MSC bolus modulates the inflammatory response to hypoxia, by decreasing the levels of pro-inflammatory cytokines, CCL2 (MCP-1) and IL-6, and increasing the level of anti-inflammatory IL-10. We and others have shown that these cytokines are regulated by hypoxia and participate in cascades leading to the development of lung vascular disease in various models of PAH^{26, 39–41}. Since hypoxia-induced lung inflammation peaks within the first week of hypoxia, a transient increase in lung HO-1 levels during this time, combined with the paracrine immunomodulatory action of MSCs, synergize to prevent the later development of PAH.

We note that, unlike the bleomycin injury mouse model or the monocrotaline rat model of PAH, the acute hypoxia-induced pro-inflammatory cytokine release and cellular infiltrate have subsided after five weeks of hypoxia, the time we performed MSC transplantation in our PAH reversal model. Nevertheless, MSC therapy ameliorated lung vascular disease and, as in the prevention model, HO-1 overexpression augmented MSC efficacy, completely reversing disease in WT animals. These findings point to additional cytoprotective actions of MSCs and HO-1 on the lung vasculature that may be independent of their immunomodulating effects. We have previously reported³⁵ that HO-1 and its enzymatic product, CO, inhibit cell cycle progression, proliferation, and migration of smooth muscle

cells and, as we show in this report, MSCs secrete factor(s) that strongly inhibit the proliferative effect of mitogens on VSMCs. Combined, the growth-inhibitory actions of HO-1-derived CO and the anti-proliferative factors in the MSC secretome apparently act synergistically on VSMCs to reverse lung vessel wall remodeling and reduce PAH. It is also tempting to speculate that additional cytoprotective actions of MSCs include their ability to alter the pool size and reparative capacity of tissue-specific progenitor/stem cells. In this context, it is relevant to mention that studies on our hyperoxia-induced BPD model suggests that MSC treatment can increase both the overall number of Bronchioalveolar Stem Cells (BASCs) and the number of junctions scoring positive for these progenitors⁴². Intriguingly, a characteristic of these progenitors is their ability to activate both the CC10 and SP-C promoters, therefore, both the hHO-1 transgenes we used in these studies should be expressed in BASCs. This supports the speculation that protective actions of MSCs and/or HO-1 on the injured lung go beyond immunomodulatory effects and include direct inhibition of smooth muscle cell growth or, potentially, augmentation of the function of cell types responsible for maintaining pulmonary homeostasis.

Summary

We used several model systems to investigate the effects of MSC transplantation on hypoxia-induced pulmonary hypertension and derive additional insights into potential mechanism(s) of MSC action. Results from multiple genetically-modified animal lines that include constitutive and inducible lung epithelial cell-specific overexpression of the human HO-1 transgene demonstrate that a small fraction of donor cells can trans-differentiate into lung alveolar epithelium and significantly augment the efficacy of MSC treatment by overexpressing HO-1. Importantly, the likely paracrine actions of MSCs include the previously recognized immunomodulatory effects on lung inflammation and, as we report here, potent antiproliferative effects on pulmonary VSMC growth as well as potential cardioprotective effects. Exploiting the unique characteristics of MSCs as vehicles of gene delivery, modulators of hypoxia-induced inflammation, and inhibitors of VSMC growth may provide more effective approaches to not only preventing but also reversing diseases of the pulmonary and systemic vasculature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The technical expertise of Anne Silkowski and the invaluable assistance of Stephanie Giannetto and Sarah Gately in preparing the manuscript are much appreciated.

This work was supported by NIH RO1 HL055454 (SK & SAM) and NIH RO1 HL085446 (SK & SAM). RB and MA were supported by NIH T32 HD007466 (SK).

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Figure 1.

MSC transplantation reverses established PAH. RVSP and Fulton's index measurements are shown for recipient WT (panels A and B) or Hmox1^{KO} mice (panels C and D). SH01-MSCs reversed RVSP elevations and RVH in WT recipients and significantly reduced PAH in Hmox1^{KO} animals, whereas WT-MSCs had only a partial ameliorative effect. Individual animal data are represented by filled circles, and group means by horizontal lines. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

Figure 2.

MSC transplantation reverses lung vascular remodeling in hypoxic WT recipients. Increased medial wall thickness is evident in representative pulmonary arterioles of hypoxic animals receiving PBS (panels B & E) compared to normoxic animals (panels A & D) and complete regression of medial hypertrophy is observed in animals that received SHO1-MSCs (panels C & F). Panels A–C show H & E staining, and D–F α-SMA staining, of the corresponding treatment groups. Panel G quantifies the medial wall thickness index for each group. At least three sections of each lung were used and vessels of comparable size were photographed and analyzed. Data are expressed as means ± SD. Statistical analysis as in Figure 1 legend.

Normoxia: 72 vessels from 4 WT-mice; PBS group: 71 vessels from 6 mice; and SHO1-
MSC group: 108 vessels from 8 mice.

Figure 3.

Conditional expression on hHO-1 augments MSC efficacy in preventing PAH. (A) RV pressure measurements of WT mice treated with PBS, or transplanted with CC77-MSCs and then exposed to hypoxia for two weeks without Dox, or CC77-MSCs plus Dox in the drinking water. (B) Fulton's index measurements of the same hypoxic WT mice after corresponding treatment, as indicated.

Figure 4.

Levels of transplanted MSCs and hHO-1 mRNA in the recipient lung. Time course of Y chromosome levels after male MSC transplantation in female recipients (A) and comparison between normoxic and hypoxic lungs 14 days post-injection (B). Multiplex real-time PCR assays were performed using murine Y-chromosome and GAPDH-specific primers. The results of engraftment were expressed as ratio between Y-chromosome DNA and GAPDH DNA. (C) Time course of human HO-1 transgene mRNA levels in whole lung of WT mice transplanted with SH01-MSCs. (D) Relative hHO-1 mRNA in recipient mouse lungs transplanted with CC77-MSCs after seven days of hypoxia in the absence (–) or presence (+) of Dox treatment.

Figure 5.

Type II pneumocyte-specific SP-C expression in a GFP⁺ cell. (A) Black SP-C granules indicate endogenous Type II pneumocytes (arrowheads) in the lung of a PBS recipient. (B) Black SP-C granules in an endogenous Type II pneumocyte (arrowhead) as well as a GFP⁺ cell which appeared to have assumed Type II pneumocyte morphology (arrow). A GFP⁺ cell was labeled with Alexa Fluor 594 (red in panel C), while SP-C granules were labeled with Alexa Fluor 488 (green spots in panel D) from the same cell (arrow). Confocal microscopic co-localization of SP-C expression within GFP⁺ cells was evident by merging of these serial dual fluorescence images to generate yellow spots (E). (F) Z-stack of 42 images with 0.2 μ m increment (original magnification 600 $\times$). Red/magenta framed panel to the right indicates optical dissection and orthogonal reconstruction of the selected cell along the vertical plane (indicated by a red line), while green/turquoise-blue framed panel above indicates optical dissection and orthogonal reconstruction of the selected cell along the horizontal plane (indicated by a green line). In both cases, the yellow granular SP-C can be identified within the GFP⁺ cell.

Figure 6.

MSC transplantation modulates the hypoxia-induced lung inflammation. Cytokine mRNA levels for CCL2, IL-6, and IL-10 were measured by quantitative RTPCR in lungs of animals transplanted with CC77-MSCs and exposed to hypoxia for four days in the presence or absence of Dox to induce human HO-1 transgene expression. Data expressed as means $\pm$ SD. n= 4–5 animals per condition. For CCL2, *p<0.001 versus normoxic group; $\dagger$ p<0.01 versus PBS-hypoxia; $\dagger\dagger$ p<0.001 versus PBS-hypoxia; $\ddagger$ p<0.05 versus CC77-MSC - Dox treatment group. For IL-6, *p<0.05 versus normoxic group; $\dagger$ p<0.05 versus PBS-

hypoxia; ^{††} p<0.001 versus PBS-hypoxia. For IL-10, *p<0.01 versus normoxic group; [†] p<0.05 versus PBS-hypoxia; ^{††} p<0.001 versus PBS-hypoxia.

Figure 7.

MSC-CM inhibit pulmonary artery smooth muscle cell proliferation. (A) Cultured PASMCMs were serum-deprived for 48 hours followed by treatment with MSC-CM or MLF-CM in the presence of FBS and their proliferation rate was quantified relative to treatment with FBS alone. * $p < 0.001$ compared with -FBS or +MSC-CM treatment. (B) Dose-response effect of MSC-CM on PASMCM proliferation. Experiment was repeated twice in triplicate. * $p < 0.05$ compared with non-MS-CM treated controls. Data are presented as means $\pm$ SD. Statistical analysis as in Figure 1 legend.