Advancing Stem Cell Therapy for Repair of Damaged Lung Microvasculature in Amyotrophic Lateral Sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal disease of motor neuron degeneration in the brain and spinal cord. Progressive paralysis of the diaphragm and other respiratory muscles leading to respiratory dysfunction and failure is the most common cause of death in ALS patients. Respiratory impairment has also been shown in animal models of ALS. Vascular pathology is another recently recognized hallmark of ALS pathogenesis. Central nervous system (CNS) capillary damage is a shared disease element in ALS rodent models and ALS patients. Microvascular impairment outside of the CNS, such as in the lungs, may occur in ALS, triggering lung damage and affecting breathing function. Stem cell therapy is a promising treatment for ALS. However, this therapeutic strategy has primarily targeted rescue of degenerated motor neurons. We showed functional benefits from intravenous delivery of human bone marrow (hBM) stem cells on restoration of capillary integrity in the CNS of an superoxide dismutase 1 (SOD1) mouse model of ALS. Due to the widespread distribution of transplanted cells via this route, administered cells may enter the lungs and effectively restore microvasculature in this respiratory organ. Here, we provided preliminary evidence of the potential role of microvasculature dysfunction in prompting lung damage and treatment approaches for repair of respiratory function in ALS. Our initial studies showed proof-of-principle that microvascular damage in ALS mice results in lung petechiae at the late stage of disease and that systemic transplantation of mainly hBM-derived endothelial progenitor cells shows potential to promote lung restoration via re-established vascular integrity. Our new understanding of previously underexplored lung competence in this disease may facilitate therapy targeting restoration of respiratory function in ALS.

Keywords

ALS, respiratory dysfunction, lungs, microvasculature, repair

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by motor neuron degeneration in the brain and spinal cord leading to muscle atrophy and paralysis[1–3]. Regardless of disease type, sporadic or familial, ALS patients usually die within 3–5 years of diagnosis due to respiratory failure[4–6]. Respiratory complications (e.g., dyspnea or orthopnea), ensuing about 3 years from disease onset, are the most common causes of death for ALS patients[7–9]. Patients with bulbar onset typically display early respiratory problems associated with more rapid disease progression. Riluzole and edaravone (Radicava®) are the only Food and Drug Administration-approved treatment drugs[10–12]. Although these drugs have a modest survival benefit, they offer no improvement in clinical symptoms[13]. Progressive respiratory muscle weakness leads to impaired

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diaphragm function[14,15], resulting in ineffective breathing and pneumonia in ALS patients[16,17]. Moreover, a recent study[18] showed that respiratory dysfunction in ALS patients is associated with weakness of cervical spinal cord muscles. Due to these complications, palliative approaches such as tracheostomy or breathing tube to support ventilation and airway clearance are demanded[7,19,20], which may prolong survival and improve quality of life in ALS patients for at least two additional years. Since about 95% of ALS patients in the United States decline implantation of mechanical ventilation devices[6], management of respiratory failure represents a significant unmet clinical need in addition to development of effective therapeutic strategies.

Cell-based regenerative medicine has emerged as a potent therapy in a variety of neurological disorders, including ALS[21–25]. Stem cell therapy, however, has primarily targeted rescue of motor neurons from degeneration via a neuroprotective action rather than cell replacement as discussed[26–29]. For instance, intrathecal administration of bone marrow mesenchymal stem cells into ALS patients (phase I/IIa clinical trial) showed safety and efficacy in treatment by providing potential neurotrophic support to motor neurons[30]. Moreover, combining autologous adult neural stem cell treatment with T-cell vaccination might be a feasible therapeutic approach for ALS patients[31]. In addition, autologous hematopoietic stem cell transplantation into the frontal motor cortex of ALS patients was shown to be safe and well tolerated by patients[32]. Important considerations for using stem cell treatment for ALS, specifically in early stage clinical trials, are comprehensively discussed[24].

Despite intensive investigations into potential ALS treatments, strategies to restore and/or preserve respiratory function remain limited. In an effort to address this issue, Nichols et al.[33] showed that transplantation of human neural progenitor cells into the cervical spinal cord of pre-symptomatic G93A SOD1 mutant rats preserved breathing capacity and improved phrenic motor neuron survival. Also, administration of glial-restricted precursors into the cervical spinal cord of a rat model of ALS revealed increased animal survival in addition to moderating the decline in respiratory function in cell-treated rats[34]. Moreover, to our knowledge, only one phase I clinical trial examined the effect of intramedullary infusion of autologous bone marrow mononuclear cells at the thoracic level on the breathing patterns of ALS patients[35]. Results of this study demonstrated safety of the cell injection; however, no significant differences were observed in oxygen saturation or inspiratory flow after 1 year. Although preclinical and clinical studies are important for targeting respiratory function by preserving breathing capability, the invasiveness of cell injections into the spinal cord may preclude this treatment for a large population of ALS patients.

Accumulated evidence identified ALS as a neurovascular disease[36,37], which led to investigations into additional effectors in motor neuron degeneration. Our[38–41] and other[42–47] studies demonstrated impairment of microvessel endothelial cells (ECs) in the brain and spinal cord of rodent models of

disease and ALS patients, resulting in compromised blood–central nervous system (CNS) barrier (B-CNS-B) integrity. Since ECs play a key role in lung vascular development and function[48,49], it is possible that microvascular EC dysfunction in lungs may occur and lead to and/or even initiate respiratory complications in ALS.

To address therapeutic strategies for repair of the damaged endothelium in ALS, our research team focused on the effects of intravenously (iv) administered human bone marrow-derived CD34+ cells (hBM34+) or endothelial progenitor cells (hBM-EPCs) into symptomatic G93A SOD1 mutant mice. Results showed structural and functional benefits of transplanted cells on restoration of barrier competence in the CNS, leading to increased motor neuron survival[50–53]. The B-CNS-B repair was due to the widespread transplanted cell engraftment into capillary lumen in the brain and spinal cord, mainly in hBM-EPC-treated ALS mice. Taking into account that the majority of iv-transplanted cells are initially trapped in the lungs[54], this pulmonary first-pass effect could be favorable for therapeutic options in lungs. This possibility should be investigated.

In this study, we briefly explored the potential role of the microvasculature in triggering lung damage in ALS. A specific focus was to determine the effects of intravenous stem cell transplants on promoting microvasculature EC repair in the lungs. Our understanding of the underexplored lung complications in ALS may foster development of targeted therapies for restoration of respiratory function in ALS.

Respiratory Dysfunction and Lung Damage in Rodent Models of ALS

Based on notable respiratory complications in ALS patients, studies on respiratory function in animal models of disease were initiated. Respiratory dysfunction resulting in altered breathing patterns and metabolism (i.e., O_2 consumption and CO_2 production) has been shown in SOD1 mutant G93A mouse[55] and rat[33,56] models of ALS until the late disease stage, mainly by motor impairment of phrenic nerve. Also, rats with fused in sarcoma RNA-binding protein (FUS) gene transfer showed progressive motor deficiencies in association with breathing abnormalities and arterial blood acidosis[57]. Importantly, Tankersley et al.[55] demonstrated three phases of respiratory phenotype in G93A SOD1 mutant mice according to their ages: stable breathing characteristics (10–14 wk of age), breathing pattern and respiratory apparatus less efficient (16–18 wk of age), and rapid decline in ventilator function (after 18 wk of age). However, these studies or others did not evaluate the lung as a respiratory organ in an animal model of ALS. The importance of the interaction between the lung parenchyma and the airways has been comprehensively discussed[58]. The authors emphasized that the interdependence between respiratory system components such as the airways, lung parenchyma, and vasculature is imperative for the physiological respiratory
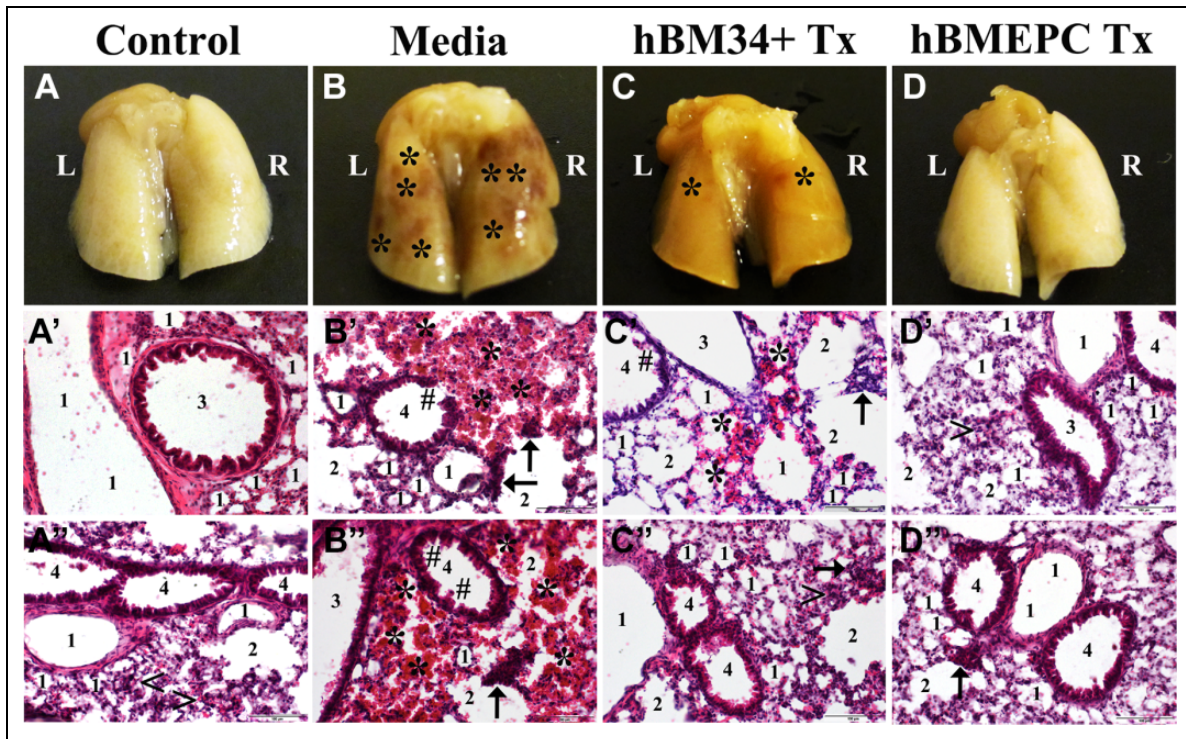
Figure 1. Characteristic of the lungs in G93A SOD1 mice at the late disease stage. Gross view of the lungs with dorsal side up showed abundant microhemorrhages (mh) in media-treated mice (B) versus controls (A). At 4 wk post-treatment, a substantial decrease of mh was noted after hBM34+ cell treatment (C) whereas no mh were found in ALS mice receiving hBM-EPCs (D). The H&E staining in left lung lobe revealed typical appearance of all lung compartments, including cellular components in control mice (A′, A″). In contrast, diffuse eosinophilic infiltration around bronchioles and bronchiolar epithelium damage were seen in media-treated mice (B′, B″). Also, ruptured capillaries near alveoli or alveolar sacs were noted. Mice treated with hBM34+ cells demonstrated some areas of eosinophilic permeation, mild damage of bronchiolar epithelium, and a few burst capillaries (C′, C″). Near normal presence of airway components was observed in ALS mice receiving hBM-EPCs (D′, D″). Only scarce ruptured capillaries around alveolar sacs were found (D″). Scale bar in A′–D″ is 100 μm. L: left lobe; R: right lobe; 1: alveolus; 2: alveolar sac; 3: bronchus; 4: bronchiole; <: typical capillary; ←: ruptured capillary; *: microhemorrhage; #: damaged bronchiolar epithelium; Tx: cell transplant; ALS: amyotrophic lateral sclerosis; hBM34+: human bone marrow-derived CD34+ cells; hBM-EPCs: human bone marrow endothelial progenitor cells; H&E: hematoxylin & eosin.

function and that the weakening of any these components contributes to "airway dysfunction and gas exchange impairment"[58].

We initiated studies to evaluate the lung status in G93A SOD1 mutant mice and effect(s) of systemically administered hBM-derived stem cells. The lungs were obtained from randomly selected ALS mice used in our previous studies[50–52]. These mice received iv hBM34+ cells or hBM-EPC transplants at equal cell doses of 1×10^6 cells/mouse at early symptomatic stage. Also, media-injected mice and mice not carrying the mutant SOD1 gene were used as controls. Cell-transplanted and media-injected animals received cyclosporine A (50 mg/mL ampules, NDC 0574-0866-10, Perrigo, Minneapolis, MN, USA) (10 mg/kg, ip) for the entire post-transplant period. All mice were sacrificed at 17 wk of age (4 wk post-cell or media administration) under pressure-controlled fluid delivery to avoid capillary rupture as described in our published papers mentioned above. The left lung lobe was removed and processed for histological analysis using a routine hematoxylin & eosin

(H&E) staining. Results showed extensive lung damage in media-treated ALS mice versus controls (Fig. 1). Numerous microhemorrhages (mh) were found in both lung lobes in these mice (Fig. 1B). Substantial reduction of mh was noted after hBM34+ cell transplantation; only a few mh were detected (Fig. 1C). In mice receiving hBM-EPCs at the same cell concentration, no mh were observed and the lung tissues were grossly similar to those in control mice (Fig. 1D).

The H&E staining confirmed the gross lung observations. Control mice showed typical morphologies of all lung compartments and cellular components (Fig. 1A′, A″). However, diffuse eosinophilic infiltration around bronchioles and bronchiolar cuboidal epithelium damage were seen in many lung patches of media-injected mice (Fig. 1B′, B″). Also, ruptured capillaries, as indicated by nucleated cell extravasation near alveoli or alveolar sacs, were noted. Mice with hBM34+ cell treatment demonstrated some areas of eosinophilic permeation, mild damage of bronchiolar epithelium, and a few burst capillaries (Fig. 1C′, C″). Near normal appearance of airway
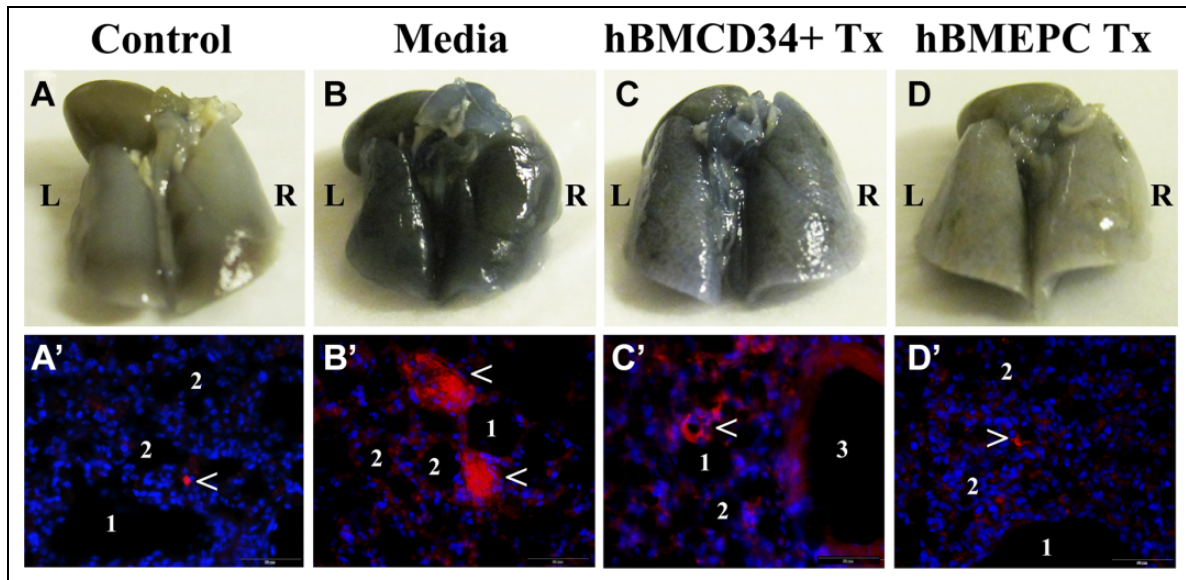
Figure 2. Characteristic of capillary permeability in the lungs of G93A SOD1 mice at the late disease stage. Gross view of the lungs with dorsal side up showed intensive blue-colored lung tissues in media-treated ALS mice (B) compared to controls (A). At 4 wk post-treatment, reduced color intensity was detected in the lungs of ALS mice receiving hBM34+ cell transplant (C). Almost similar to controls, the lung tissues were grossly observed in mice after transplantation of hBM-EPCs (D). Examination of the lung tissues in control mice demonstrated only a few spots of EB extravasation close to alveolar sacs (red, arrowhead, A′). The large areas of EB extravasation near alveoli or alveolar sacs were detected in media-treated mice (red, arrowheads, B′). In the lungs from ALS mice treated with hBM34+ cells, EB leakage from several capillaries was found (red, arrowhead, C′). Minor EB permeation from some lung capillaries was observed in ALS mice receiving hBM-EPCs (red, arrowhead, D′) similar to control mice. DAPI (blue) was used as counterstaining for cell nuclei. Scale bar in A′–D′ is 50 μm. L: left lobe; R: right lobe; 1: alveolus; 2: alveolar sac; 3: bronchiole; EB: Evans blue; Tx: cell transplant; DAPI: 4′,6-diamidino-2-phenylindole; ALS: amyotrophic lateral sclerosis; hBM34+: human bone marrow-derived CD34+ cells; hBM-EPCs: human bone marrow endothelial progenitor cells.

components and ciliated cuboidal epithelial cells were observed in ALS mice receiving hBM-EPCs; only scarce ruptured capillaries around alveolar sacs were found (Fig. 1D′, D″).

Altogether, our study results demonstrated significant lung damage in ALS mice at late symptomatic stage with notable mh by ruptured capillaries near the alveolar wall resulting in lung petechiae. Typically, numerous capillaries surrounding the alveolar walls allow rapid diffusion of O_2 into the bloodstream and CO_2 waste from blood into alveoli. This gas exchange occurs through a thin respiratory membrane (i.e., blood–gas barrier) located at the outer capillary membrane[59]. Any damage to microvessels in the lung may lead to airflow obstruction or restriction. Our novel finding on lung petechiae via microvessel damage in G93A SOD1 mutant mice near the end stage of disease may explain previously reported declines in breathing function in this animal model of ALS at the same age[55]. Importantly, intravenous transplantation of hBM-derived stem cells into symptomatic ALS mice attenuated lung hemorrhagic damage. More efficacious repair was determined by the administration of hBM-EPCs versus hBM34+ cells. Similar results on the reduction of mh in the spinal cords of symptomatic ALS mice treated with hBM stem cells were reported in our previous study[53].

Capillary Permeability in the Lungs of G93A SOD1 Mutant Mice

Our previous studies demonstrated substantial capillary leakage in the spinal cords of late-stage G93A SOD1 mutant mice and that iv transplantation of hBM34+ cells[51] or hBM-EPCs[52] significantly decreased vascular permeability as determined by the analysis of Evans blue (EB) dye extravasation into the spinal cord parenchyma. These results, along with other effects of cell transplantation, indicate functional capillary repair in the spinal cords of ALS mice.

In our current initial study, lungs were obtained from randomly selected cell-treated or media-treated G93A SOD1 mice and controls, which received EB injection as described in our above-mentioned studies. The left lung lobe was removed and processed for the examination of EB extravasation into tissue parenchyma. Gross observation of the lungs from mice at 17 wk of age showed intensive blue color in both lung lobes from media-treated ALS mice (Fig. 2B) compared to controls (Fig. 2A). Reduced color intensity was detected in the lungs of ALS mice receiving hBM34+ cell transplant (Fig. 2C). However, some blue patches were seen in the lungs of these mice. The lung tissues observed in mice after transplantation of hBM-EPCs were grossly similar to controls (Fig. 2D). Detailed examination of the lung tissues in control mice
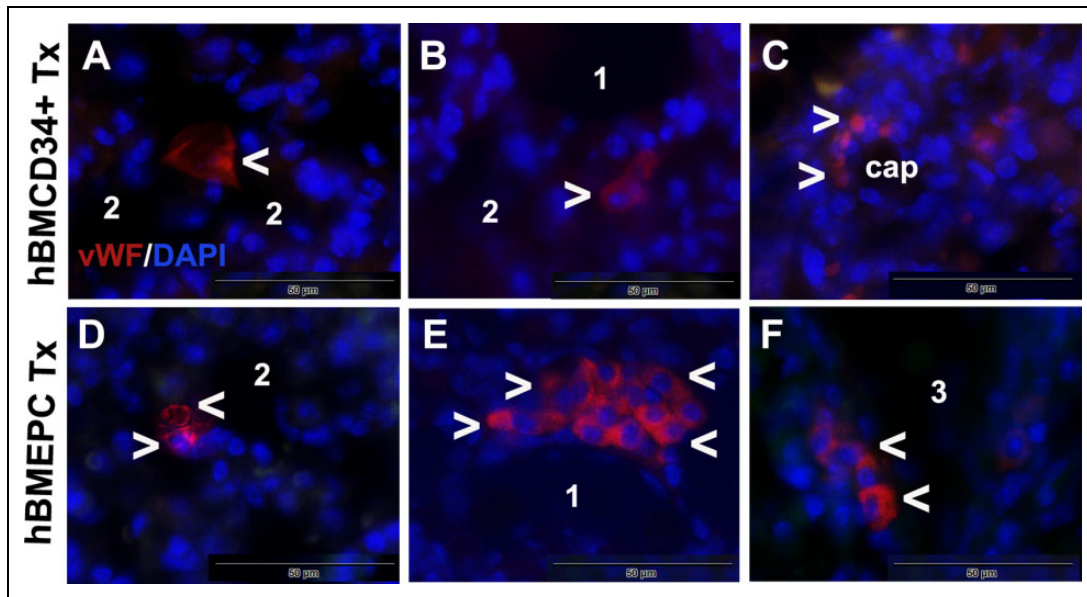
Figure 3. Cellular immunoexpression of human vWF in the lungs of G93A SOD1 mice 4 wk after cell transplantation. A few hBM34+ transplanted cells positive for vWF marker (red, arrowheads) showed epithelial-like morphology (A, B) or presented as rounded cells in capillary wall (C). A number of cells immunoexpressing vWF (red, arrowheads) with EC-like (D) or epithelial-like (E, F) morphology were detected in the lungs after hBM-EPC transplant. DAPI (blue) was used as counterstaining for cell nuclei. Scale bar in A–F is 50 μm. 1: alveolus; 2: alveolar sac; 3: bronchiole; cap: capillary; vWF: von Willibrand factor; hBM34+: human bone marrow-derived CD34+ cells; hBM-EPC: human bone marrow endothelial progenitor cell; DAPI: 4′,6-diamidino-2-phenylindole; EC: endothelial cells.

revealed only a few spots of EB extravasation close to alveolar sacs (Fig. 2A′). In contrast, large areas of permeated EB dye near alveoli or alveolar sacs were detected in media-treated mice (Fig. 2B′). In the lungs from ALS mice treated with hBM34+ cells, EB leakage from several capillaries was found (Fig. 2C′). Analogous to control mice, minor EB extravasation from some lung capillaries was observed in ALS mice receiving hBM-EPCs (Fig. 2D′).

Taken together, these study results demonstrating capillary permeability in the lungs of G93A SOD1 mutant mice at the late stage of disease supported our histological analysis described above. Since capillaries in the lungs are fenestrated, a slight amount of EB permeation into the lung parenchyma was detected in control mice. However, taking into account that the molecular size of EB is 961 Da, the observed extensive dye extravasation in the lung tissues of media-treated ALS mice may confirm significant rupture of capillaries. Also, the reduction of lung hemorrhagic damage after iv cell transplantation, mainly observed after hBM-EPC transplants, potentially resulted in functional microvessel repair as determined by decreased EB extravasation.

Cellular Immunophenotypic Analysis of Migrated Cells Into the Lungs of G93A SOD1 Mutant Mice After Intravenous Cell Transplantation

To determine that structural and functional lung improvements were related to a cell treatment effect, immunohistochemical staining of residual parts of the lung tissues from ALS mice which received hBM34+ cells and hBM-EPC transplants in our histological study was performed via detection of human anti-von Willibrand factor (hvWF) antigen as we previously described[50,52]. At 4 wk post-transplant, there were a few hvWF-positive cells in the lungs of mice treated with hBM34+ cells that displayed epithelial-like morphology and were located near the alveolar sac (Fig. 3A, B). Several rounded cells were found in the capillary wall (Fig. 3C). In the lungs of ALS mice that received hBM-EPCs, numerous cells immunoexpressing hvWF exhibited EC-like (Fig. 3D) or epithelial-like (Fig. 3D, F) cell phenotype. Interestingly, cells with epithelial morphology were detected in the bronchiolar wall (Fig. 3D).

Thus, iv-transplanted cells were found to migrate and engraft in the lung tissues of G93A SOD1 mice. More cells in the lungs were detected in ALS mice after transplantation of hBM-EPCs and may support our previous observations on advanced repair of damaged lungs via this cell type. We recently showed[60] that hBM-EPCs release angiogenic factors (vascular endothelial growth factor [VEGF] and angiogenin) in vitro, so these cells might also maintain endogenous EC function and/or promote angiogenesis in the lungs. Additionally, hBM-EPCs may release nanosized vesicles, vesicles which potentially mimic cell transplant effects and primarily act as mediators of intercellular communications[61,62]. Reportedly, human EPC-derived microvesicles (MVs) have demonstrated in vivo benefits through promotion of angiogenesis[63,64]. According to the authors, these

MVs internalized and delivered bioactive molecules into target ECs, fostering angiogenesis and/or promoting neoangiogenesis. Human EPC-derived MVs also promoted EC survival, proliferation, and development of capillary-like structures in vitro and stimulated angiogenesis in vivo using immunodeficient severe combined immunodeficiency disease (SCID) mice[64]. Further, inflammatory signs were lessened after the application of extracellular vesicles (EVs) obtained from hBM mesenchymal stromal cells (MSCs) in a mouse model of lipopolysaccharide-induced lung injury[65] or lung ischemia-reperfusion injury[66]. In a recent review, Namba[67] discussed the efficacy of conditioned media or exosomes derived from human MSCs in treating preterm infants with bronchopulmonary dysplasia. The author emphasized paracrine effects of MSCs via secretion of various bioactive substances (i.e., interleukin [IL]-6, IL-8, VEGF, extracellular matrix proteins, and exosomes), important for anti-inflammatory protection of pulmonary epithelial and microvascular ECs. Similarly to actions of MSC-derived factors, hBM-EPCs may exert their angiogenic, anti-inflammatory, and/or immunomodulatory paracrine effects for repair of damaged lungs in ALS. However, the specific paracrine humoral factors, such as cytokines, chemokines, and growth factors, mediating beneficial cell transplant effects on residential endothelial and/or epithelial cells in lungs should be determined. In our ongoing study, characteristics of EVs derived from hBM-EPCs, including proteomics, should be informative regarding vesicle contents for establishing lung homeostasis.

Conclusions and Perspectives

ALS is a multifactorial fatal disease with limited therapies. Respiratory dysfunction is the most common cause of death for ALS patients. Similarly, respiratory complications have also been identified in SOD1 mutant rodent models of ALS. Integrated function of all respiratory system components, including the lungs, is vital for proper physiological respiration. However, the lung status has not been previously explored in animal ALS models or ALS patients.

We initiated studies to evaluate the lung condition of G93A SOD1 mutant mice at the late stage of disease. Our preliminary study results demonstrated numerous mh resulting in lung petechiae in ALS mice at 17 wk of age. This lung damage occurred due to microvascular rupture, as confirmed by EB dye extravasation into the lung tissues. The lung microvasculature impairment is concurrent with our and others' findings on structural and functional capillary deficiencies within the CNS in both ALS patients and animal models of ALS. We believe that microvascular lung damage caused by EC dysfunction *initiates* respiratory complications by obstruction of breathing in ALS. However, since the lung hemorrhagic damage was observed in ALS mice at the late disease stage, evaluation of this respiratory organ should be performed, at least, in early symptomatic mice to confirm our supposition. Also, it might be necessary to examine the lungs in ALS patients for the appearance of mh in order to provide proper treatment.

Although intensive investigations of effective treatment for ALS are ongoing, development of appropriate therapeutic strategies to restore and/or preserve respiratory function is an extreme need. A few preclinical and clinical studies showed the promise of stem cell transplant approaches for targeting respiratory function by preserving breathing capability; however, invasive route of cell delivery into the spinal cord may not to be feasible for a large cohort of ALS patients. Re-establishing lung microvasculature via intravenous cell administration may be a promising minimally invasive therapeutic strategy. We showed the benefits of iv-transplanted hBM-derived stem cells into symptomatic G93A SOD1 mice by repairing CNS endothelium. In the current study, migration and enrichment of these transplanted cells in the lungs of ALS mice at 4 wk posttransplant support our suggestion. While hBM34+ cell treatment attenuated lung hemorrhagic damage, hBM-EPC transplantation demonstrated more benefits on lung repair. Differences in outcomes between these hBM-derived stem cells suggest that a restricted cell lineage, such as EPCs, versus hematopoietic CD34+ stem cells provides enhanced restorative results on damaged microvessels in the lungs of ALS mice.

Thus, our initial study demonstrated a proof-of-principle that lung microvascular damage might be an essential effector leading to respiratory dysfunction in ALS. Repair of lung capillaries via intravenous hBM-EPC transplantation is promising and may form the basis for a therapeutic approach toward lung restoration. However, studies regarding posttransplant efficiency of blood gas exchanges for proper breathing capacity and capillary integrity are needed to prove our concept that hBM-EPC treatment targets the vasculature for repair of damaged lungs in ALS. These studies will be addressed in our future investigations.

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