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MESENCHYMAL STROMAL CELLS

Cell type–dependent variation in paracrine potency determines therapeutic efficacy against neonatal hyperoxic lung injury

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Abstract

Background aims. The aim of this study was to determine the optimal cell type for transplantation to protect against neonatal hyperoxic lung injury. To this end, the *in vitro* and *in vivo* therapeutic efficacies and paracrine potencies of human umbilical cord blood–derived mesenchymal stromal cells (HUMs), human adipose tissue–derived mesenchymal stromal cells (HAMs) and human umbilical cord blood mononuclear cells (HMNs) were compared. *Methods.* Hyperoxic injury was induced *in vitro* in A549 cells by challenge with H_2O_2. Alternatively, hyperoxic injury was induced in newborn Sprague-Dawley rats *in vivo* by exposure to hyperoxia (90% oxygen) for 14 days. HUMs, HAMs or HMNs (5×10^5 cells) were given intratracheally at postnatal day 5. *Results.* Hyperoxia-induced increases in *in vitro* cell death and *in vivo* impaired alveolarization were significantly attenuated in both the HUM and HAM groups but not in the HMN group. Hyperoxia impaired angiogenesis, increased the cell death and pulmonary macrophages and elevated inflammatory cytokine levels. These effects were significantly decreased in the HUM group but not in the HAM or HMN groups. The levels of human vascular endothelial growth factor and hepatocyte growth factor produced by donor cells were highest in HUM group, followed by HAM group and then HMN group. *Conclusions.* HUMs exhibited the best therapeutic efficacy and paracrine potency than HAMs or HMNs in protecting against neonatal hyperoxic lung injury. These cell type-dependent variations in therapeutic efficacy might be associated or mediated with the paracrine potency of the transplanted donor cells.

Key Words: *bronchopulmonary dysplasia, cell transplantation, hepatocyte growth factor, vascular endothelial growth factor*

Introduction

Despite recent improvements in neonatal intensive care medicine [1], bronchopulmonary dysplasia (BPD), a chronic lung disease in premature infants that requires prolonged ventilatory support and oxygen supplementation, remains a major cause of mortality and long-term respiratory morbidity among premature infants [2,3]. Few effective treatments are available against BPD; therefore, the development of a new therapeutic modality to improve the outcome of this intractable disease is urgently needed.

Recently, the transplantation of various types of stem/progenitor cells has shown potential in the prevention and treatment of neonatal hyperoxic lung injury [4–9]. Of the various types of stem/progenitor cells, mesenchymal stromal cells (MSCs) are most often used in cell transplantation studies using animal models of BPD [6,9–15]. Although bone marrow (BM) is the best characterized source of MSCs, the practical use of BM is limited because of its highly invasive acquisition process [16]. Adipose tissue (AT), which can be harvested less invasively and in larger quantities than BM, is one possible alternative source of MSCs. However, the *in vivo* therapeutic efficacy of AT-derived MSCs in protecting against hyperoxic lung injury has not yet been tested. Umbilical cord blood (UCB) is considered another promising source for human MSCs because of its ready availability and the lack of significant ethical concerns. However, a long expansion time is required to obtain MSCs from UCB [17]. Thus, UCB

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mononuclear cells (MNCs), which contain high levels of primitive, multipotent stem/progenitor cells [18], might be a suitable alternative source for UCB-derived MSCs in protecting against BPD [19].

To the best of our knowledge, the therapeutic efficacies of various stem cells in protecting against neonatal hyperoxic lung injury have not yet been directly compared. The aim of the present study was to determine the best cell type for protecting against neonatal hyperoxic lung injury. To this end, the therapeutic efficacies of human UCB-derived MSCs, AT-derived MSCs and UCB MNCs in protecting against both *in vitro* and *in vivo* neonatal hyperoxic lung injury were directly compared. We also evaluated whether cell type−dependent variations in therapeutic efficacy were associated with or mediated by paracrine potency by determining the amounts of growth factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) produced by the transplanted donor cells.

Methods

Cell preparation

Human UCB was collected from umbilical veins after full-term newborn delivery. Informed consent was obtained from all pregnant mothers before UCB collection. From the single donor UCB, MSCs were isolated and expanded as previously described [20,21]. The differentiation potentials and immunophenotypic results of this process have also been previously described [11,20,22−24]. No changes in the karyotype was observed until 11 passages of UCB-MSCs (Supplementary Figure 1). Human AT-derived MSCs were isolated from AT that had been obtained from three adult females undergoing elective liposuction procedures. All patients provided informed consent. AT was isolated using collagenase (type I, Sigma) [25]. Fluorescence-activated cell sorter analysis revealed that both UCB-derived and AT-derived MSCs were positive for typical MSC antigens (CD73 and CD105) but negative for hematopoietic antigens (CD14, CD34 and CD45) by passage 6 (Figure 1A, Table I). To compare the differentiation potentials of the human UCB-derived MSCs and the AT-derived MSCs into mesodermal cells *in vitro*, MSCs (passage 6) were cultured in induction media for bone, cartilage and fat differentiation (Figure 1B). Both UCB-derived MSCs and AT-derived MSCs showed the potential to differentiate into bone, cartilage and fat, as evidenced by the resultant alkaline phosphatase activity, safranin O staining and numbers of lipid vacuoles, respectively. Human UCB-derived MNCs were isolated by centrifugation of UCB on a Ficoll-Hypaque gradient (density 1.077 g/cm^3, Sigma). The separated MNCs were washed and suspended as previously described [24].

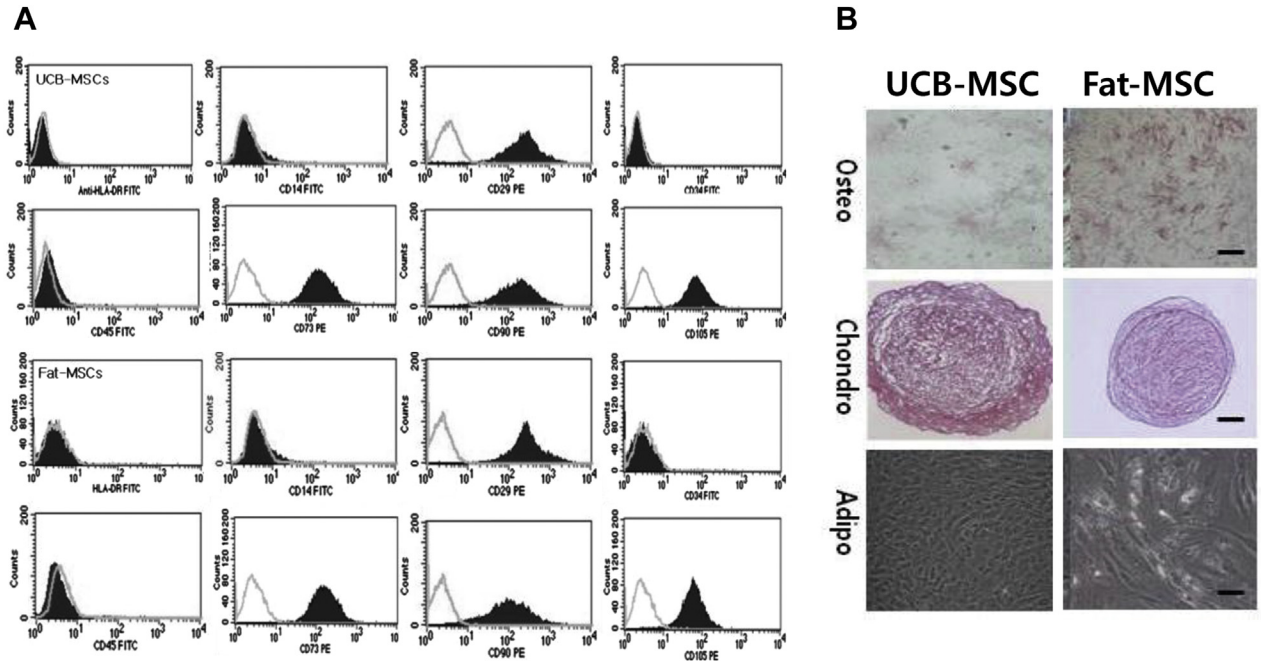

Figure 1. Comparison of the multi-lineage differentiation capacities and immunophenotypes of MSCs obtained from UCB or AT (labeled "Fat" in the figure). (A) Differentiation potentials of UCB-MSCs and AT-MSCs cultured under induction conditions. In each population, osteogenic (osteo), chondrogenic (chondro) and adipogenic (adipo) differentiation was examined by alkaline phosphatase live staining, safranin O staining and enumeration of lipid vacuoles, respectively (scale bar: 50 μm). (B) Flow cytometry analysis demonstrating the expression of various surface antigens on UCB MSCs and AT MSCs.

Table I. Fluorescence-activated cell sorter analysis of MSCs.

Marker	HLA-DR	CD14	CD34	CD45	CD29	CD73	CD90	CD105
UCB MSC	−	−	−	−	+	+	+	+
AT MSC	−	−	−	−	+	+	+	+

HLA, human leukocyte antigen; −, less than 5%; +, more than 95%.

In vitro cell culture

A549 cells (human alveolar basal epithelial cells) were purchased from the Korea Cell Line Bank. A549 cells (5×10^3 cells/well) were seeded into 96-well plates in 100 μL of RPMI 1640 medium containing 10% fetal bovine serum (Gibco) and cultured for 24 h at 37°C. To generate oxidative stress, cells were treated with 100 μmol/L H_2O_2 for 60 min as previously described [26]. Then, H_2O_2-treated A549 cells were incubated in complete medium (CM) alone, CM with 1×10^3 human UCB-derived MSCs, CM with 1×10^3 human AT-derived MSCs or CM with 1×10^3 human UCB MNCs for 24 h. The resultant supernatants were preserved at −70°C until enzyme-linked immunosorbent assay (ELISA) was performed. A colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium] assay (Dojindo Molecular Technologies) was used to evaluate cell viability according to the manufacturer's protocol. Relative viabilities were determined by normalizing to 0% (no cells) and 100% (untreated cells) controls.

Animal work

The Animal Care and Use Committee of Samsung Biomedical Research Institute (Seoul, Korea) approved all animal procedures in this study. This study was also conducted in accordance with all institutional and National Institutes of Health guidelines for laboratory animal care. Timed pregnant Sprague-Dawley rats (Orient Co.) spontaneously delivered newborn rat pups as previously described [11]. Newborn rats were randomly allocated to five experimental groups: normoxia control (NC) ($n = 18$), hyperoxia control (HC) ($n = 20$), hyperoxia with transplantation of human UCB-derived MSCs (HUM) ($n = 22$), hyperoxia with transplantation of human AT-derived MSCs (HAM) ($n = 18$) and hyperoxia with transplantation of human UCB MNCs (HMN) ($n = 17$). Normoxic rats were maintained in normal room air, whereas hyperoxic rats were raised in hyperoxic chambers (90% oxygen) from birth until postnatal day (P) 14. Nursing mother rats were rotated daily between hyperoxia and room-air litters to prevent oxygen toxicity in the dams. On P5, 5×10^5 human UCB-derived MSCs, AT-derived MSCs or UCB MNCs in 0.05 mL normal saline (0.9% NaCl) were transplanted intratracheally [11–13]. Cell dose for transplantation used in this study has been referred to our previous study [12]. An equal volume of normal saline was administered to the control groups in the same manner. On P7 and P14, rat pups were sacrificed under deep pentobarbital anesthesia (60 mg/kg, intraperitoneal), and lung tissue was harvested for morphometric and biochemical analyses as previously described [11–13].

Morphometry

The degree of alveolarization was estimated using the mean linear index (MLI) as previously described [11,27–30]. A minimum of three sections per sample and a minimum of six fields per section were evaluated randomly in a blinded manner.

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

Cell death in lung tissue was assessed using immunofluorescent terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining with an *in situ* cell death detection kit (S7110 ApopTag, Chemicon). TUNEL staining was performed according to the manufacturer's protocol as previously described [11,27–30]. TUNEL-positive cells from 10 nonoverlapping fields (magnification ×200) from each condition were counted by investigators who were blinded to the treatment groups.

Immunohistochemistry

Immunofluorescent staining was used to detect von Willebrand factor (vWF), an indicator of angiogenesis, and ED-1, a marker of alveolar macrophages, in deparaffinized 5-μm lung sections. Primary antibodies were diluted as follows: anti-vWF, 1:200 (endothelial cell markers, rabbit polyclonals, Dako); anti-CD68 and anti-ED-1, 1:100 (monocyte/macrophage markers, mouse monoclonals, Millipore). Secondary antibodies were diluted as follows: fluorescein isothiocyanate–conjugated anti-rabbit immunoglobulin, 1:200 (green fluorescence, Dako); Alexa Fluor 488 conjugated anti-mouse immunoglobulin, 1:100 (green fluorescence, Molecular Probes).

A minimum of three sections per rat and a minimum of six fields per section were randomly chosen for evaluation. The amount of vWF present in each section was estimated by measuring the light signal intensity of the immunofluorescent staining with Image J (U.S. National Institutes of Health). Numbers of ED-1 positive cells were determined by manual counting. These assessments were performed by an investigator who was blinded to the study groups.

ELISA

Frozen lung tissue was homogenized in cold buffer (50 mmol/L Tris-HCl, pH 7.4) supplemented with 1 mmol/L ethylenediaminetetraacetic acid, 1 mL ethylene glycol tetraacetic acid, 1 mmol/L phenylmethanesulfonyl fluoride or phenylmethylsulfonyl fluoride, 42 mmol/L KCl and 5 mmol/L $MgCl_2$. To remove cellular debris, homogenized lung tissue was spun by centrifugation at 8000 *g* for 20 min at 4°C. The total protein contents in the supernatants were then measured according to the Bradford method. Supernatant levels of interleukin (IL)-1α, IL-1β and IL-6 and tumor necrosis factor-α were measured using the Multiplex MAP ELISA Kit (Millipore) according to the manufacturer's protocol. The amounts of VEGF protein were determined using human-specific and rat-specific VEGF Quantikine ELISA kits (R&D Systems), and the levels of HGF protein were measured using human-specific and rat-specific HGF ELISA kits (Uscn Life Science) according to the manufacturer's protocols. The levels of both human and rat VEGF and HGF were measured in the lung tissue of rats into which human donor cells had been transplanted. However, only the levels of human VEGF and HGF were measured in the supernatants of cocultured human A549 cells and human donor cells.

Statistical analyses

Data are expressed as mean ± SD. Continuous variables were compared between groups using one-way analysis of variance with the least significant difference post hoc test. A P value of <0.05 was considered significant. SPSS version 18.0 (IBM) was used for all analyses.

Results

Mortality and body weight

The mortality rates of the hyperoxic groups were not significantly different (23%, 15%, 16% and 13% in the HC, HUM, HAM and HMN groups, respectively). Regarding body weight growth after P9, slower weight gain was observed in the hyperoxic rats. Moreover, significant improvement of this growth retardation was observed in the HUM group (P13 and P14) but not in the HAM or HMN groups (data not shown).

Protective effects of cell transplantation in vitro and in vivo

In cultured A549 cells, H_2O_2 exposure significantly reduced cell survival. This oxidative stress—induced cell death was significantly improved by co-treatment of UCB-derived MSCs or AT-derived MSCs but not by UCB-derived MNCs, with more attenuation in the UCB-derived MSCs treated group compared with the AT-derived MSCs treated group (Figure 2A).

Representative photomicrographs showing the histopathological differences and morphometric analysis evidenced by MLI of the rat lungs in each experimental group at P14 are shown in Figure 2B and 2C. Impaired alveolar growth, as evidenced by fewer alveoli, larger alveoli and heterogeneous alveolar size, was observed in the HC group compared with the NC group. Significantly higher MLI, which is indicative of impaired alveolarization, observed in the HC group compared with the NC group. Moreover, hyperoxic lung injury represented by high MLI was attenuated in both the HUM and HAM groups but not in the HMN group, with more attenuation in the HUM group than in the HAM group (Figure 2B,C).

The significant reduction in the amount of vWF staining in the HC group compared with the NC group, which is indicative of impaired angiogenesis, was significantly improved in the HUM group but not in the HAM or HMN groups (Figure 3).

TUNEL and ED-1 staining

The numbers of TUNEL-positive and ED-1 positive cells were significantly higher in the HC group compared with the NC group (Figure 4). These hyperoxia-induced increases in the numbers of TUNEL-positive and ED-1 positive cells were significantly attenuated in the HUM group but not in the HAM or HMN groups.

Inflammatory cytokines

The levels of inflammatory cytokines such as IL-1α, IL-1β, IL-6 and tumor necrosis factor-α were significantly elevated in rat lung tissue from the HC group compared with the NC group (Figure 5). This hyperoxia-induced increase in inflammatory cytokine

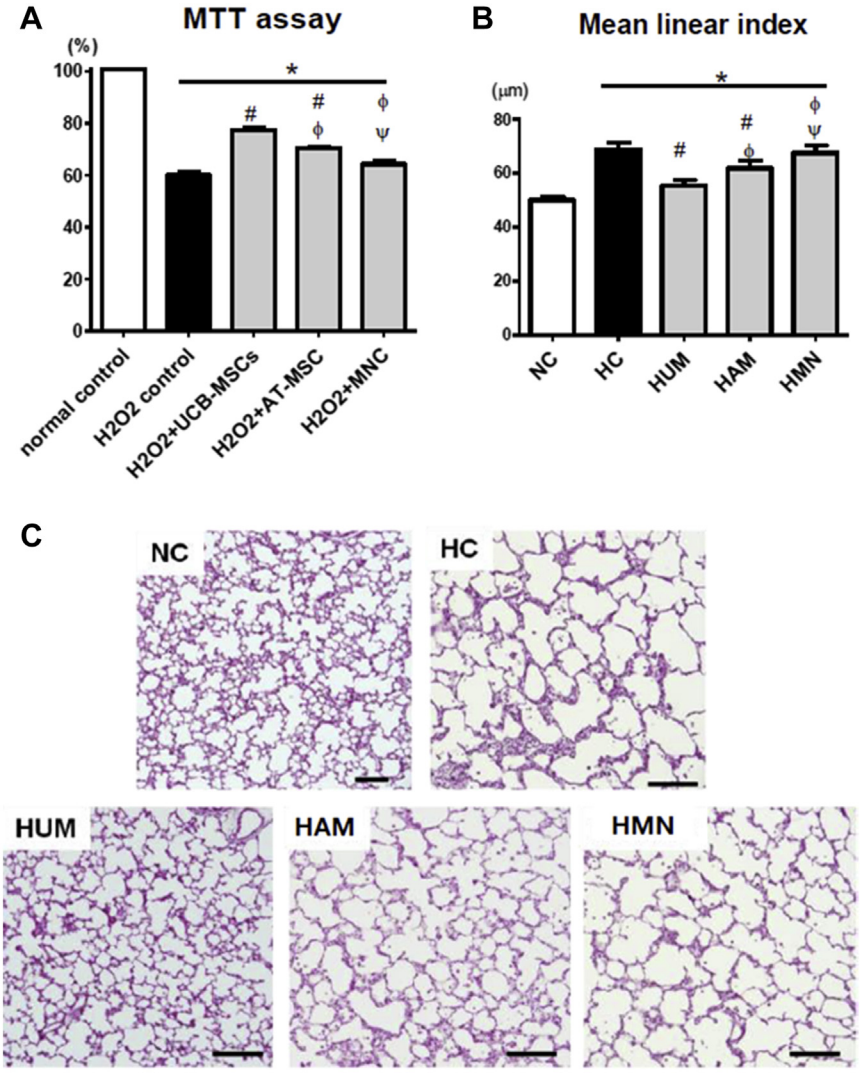

Figure 2. Cell viability in A549 cell culture with oxidative stress by 1 h H_2O_2 exposure *in vitro* and the degree of alveolarization of P14 rat lung exposed to prolonged hyperoxia *in vivo*. (A) The cell viability was quantified with MTT assay after H_2O_2 treatment for 60 min in A549 lung epithelial cell culture. (B) Degrees of alveolarization of the lung sections of P14 rat, as assessed by the mean linear intercept. (C) Representative optical microscopy photomicrographs of lung sections stained with hematoxylin and eosin (scale bar = 25 μm). Data are presented as means ± SEM. $^{*}P < 0.05$ compared with the normal control or NC group; $^{\#}P < 0.05$ compared with the H_2O_2 control or HC group; $^{\Phi}P < 0.05$ compared with the H_2O_2+UCB-MSCs or HUM group; $^{\Psi}P < 0.05$ compared with the H_2O_2+AT-MSC or HAM group.

production that was observed in the HC group was generally significantly attenuated in the HUM group but not in the HAM or HMN groups. However, the increase in IL-1β was significantly attenuated in the HUM, HAM and HMN groups, with higher attenuation observed in the HUM group than HMS or HMN group.

VEGF and HGF levels

Human VEGF and HGF levels were significantly reduced in the culture medium of A549 cells after H_2O_2 exposure (Figure 6A). These H_2O_2-induced decreases in the levels of VEGF and HGF were significantly recovered by co-treatment of UCB-derived MSCs, AT-derived MSCs or UCB-derived MNCs, with higher VEGF levels in the MSC-treated groups compared with the MNC-treated group, and a higher HGF level in the UCB-derived MSC-treated group compared with the AT-derived MSC- or UCB-derived MNC treated group.

In rat lung tissue, the levels of rat VEGF and HGF were significantly reduced in the HC group compared with the NC group on both P7 and P14 (Figure 6D,F). This hyperoxia-induced decrease in the level of rat VEGF was significantly attenuated in the HUM, HAM and HMN groups. This attenuation was greater in the HUM and HAM groups compared with the HMN group on P7 and in the HUM and HAM

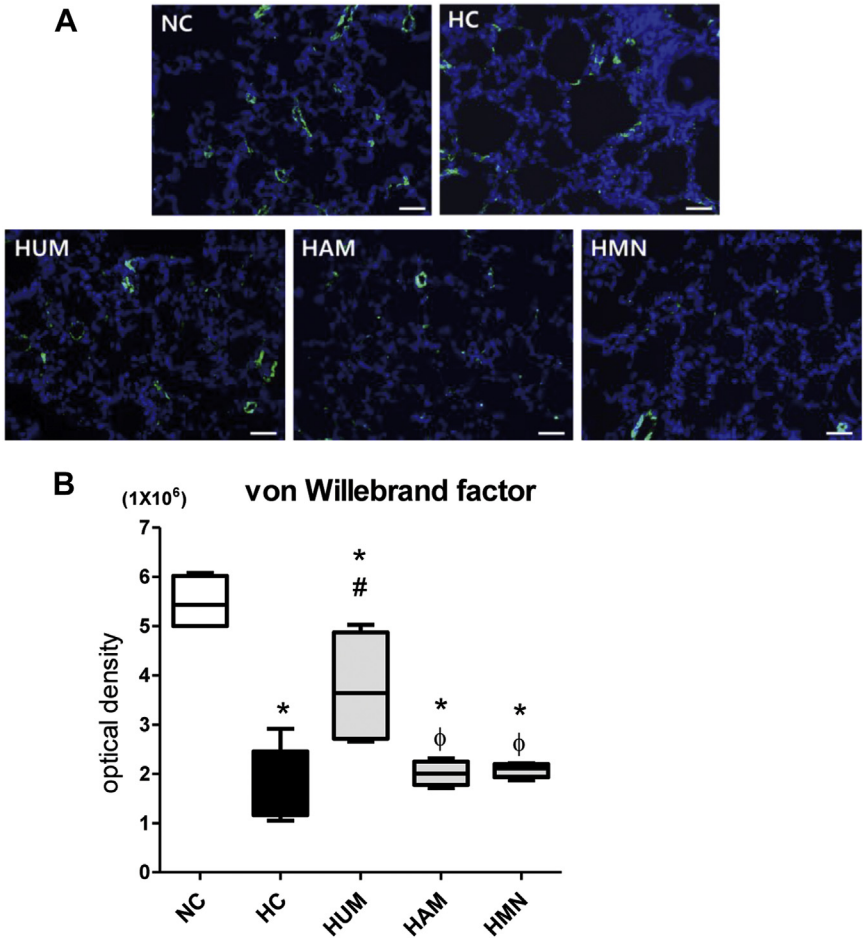

Figure 3. Evidence of pulmonary angiogenesis in P14 rat pups. (A) Representative immunofluorescence photomicrographs of vWF staining in the lungs of P14 rats. vWF was labeled with the fluorescent marker 5(6)-carboxy-fluorescein diacetate N-succinimidyl ester (green), whereas nuclei were labeled with 4′,6-diamidino-2-phenylindole (blue; scale bars = 50 μm). (B) Mean light signal intensity of vWF immunofluorescence staining per high power field. Data are expressed as medians and 10th to 90th percentiles. $^{*}P < 0.05$ compared with the NC group; $^{\#}P < 0.05$ compared with the HC group; $^{\Phi}P < 0.05$ compared with the HUM group.

groups, but not in the HMN group, on P14. Human VEGF that had been secreted from transplanted donor cells was detected in both the HUM and HAM groups on P7 but not on P14; human VEGF was not detected in the HMN group on any day. The hyperoxia-induced decrease in the level of rat HGF was significantly attenuated in the HUM group on both P7 and P14 but not in the HAM or HMN groups. Human HGF was detected in the HUM group on P7 but not on P14; human HGF was not detected in the HAM or HMN groups on any day.

Discussion

Determining the most appropriate cell type and cell source is crucial for future successful clinical translation of cell-based therapies into protection against BPD. In addition to the ease with which they can be obtained and the absence of any significant ethical concerns, UCB-derived MSCs also exhibit some biological advantages compared with BM-derived and AT-derived MSCs, such as low immunogenicity and a higher proliferative capacity [31]. In the present study, although AT-derived MSCs and UCB MNCs provided partial and minimal protection, respectively, against H_2O_2-induced cell death *in vitro* and hyperoxic lung injury *in vivo*, human UCB-derived MSCs best attenuated these damages. For example, human UCB-derived MSCs were the most effective in preventing impaired alveolarization and angiogenesis, increased apoptosis and an inflammatory response. We previously demonstrated the safety and feasibility of transplantation of human UCB-derived MSCs for treating BPD in a phase I clinical trial [32]. Cumulatively, our findings suggest that human UCB-derived MSCs are the most suitable cell source for future clinical use in protecting against BPD.

Understanding the mechanism through which transplantation of human UCB-derived MSCs, compared with AT-derived MSCs or UCB MNCs, best protects lung tissue against neonatal hyperoxic

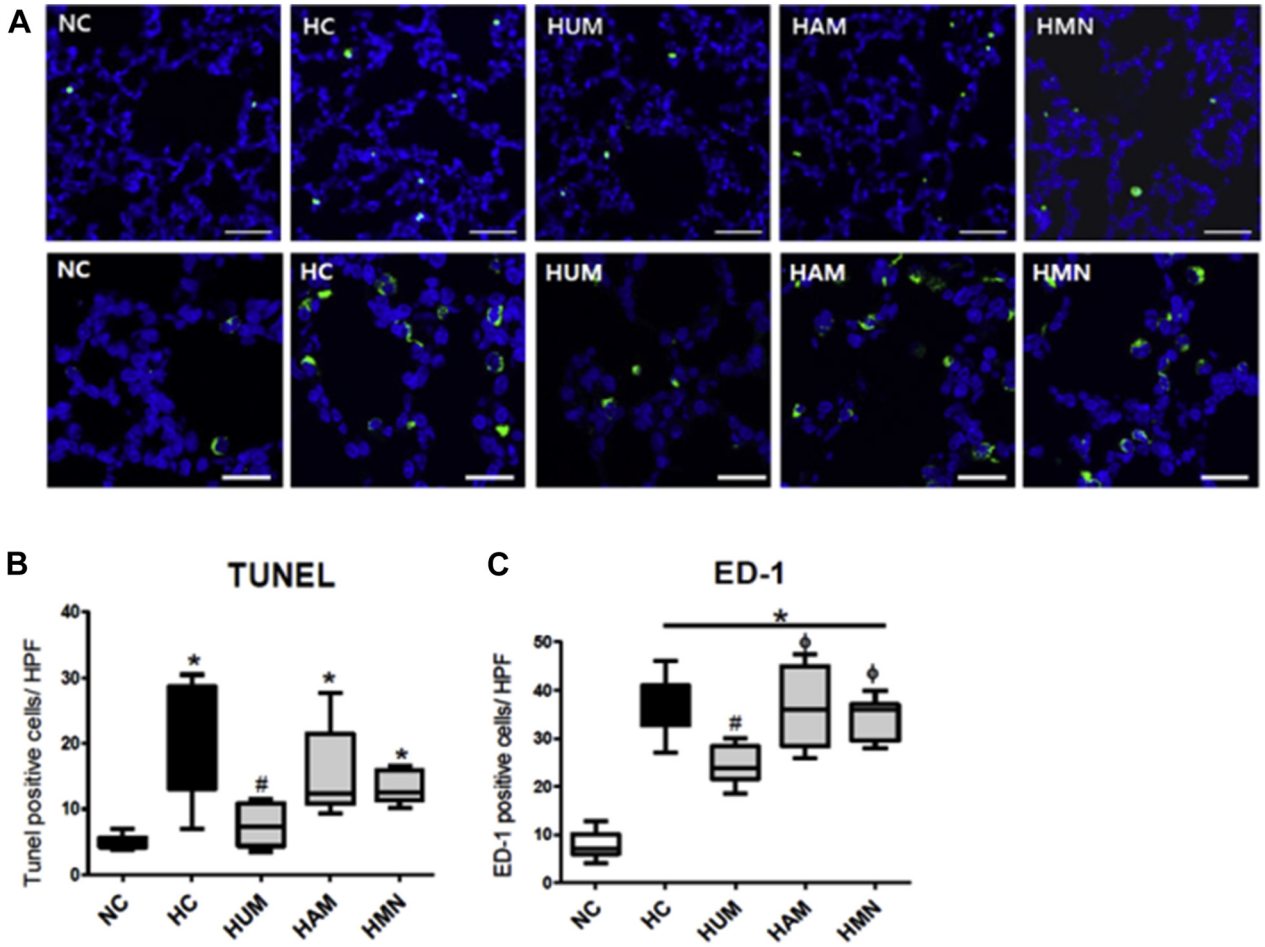

Figure 4. *TUNEL*-positive cells and pulmonary macrophages in the distal lungs of P14 rat pups. (A) Representative photomicrographs of TUNEL-positive cells and ED-1-positive cells in lung sections from P14 rats. TUNEL was labeled with fluorescein isothiocyanate–conjugated (green) and ED-1-positive alveolar macrophages were labeled with 5(6)-carboxy-fluorescein diacetate N-succinimidyl ester (green). Cell nuclei were labeled with 4′,6-diamidino-2-phenylindole (blue) (scale bar; 25 μm). (B) Numbers of observed TUNEL-positive cells per high power field. (C) Numbers of ED-1-positive cells per high power field. Data are presented as means ± SEM and medians and 10th to 90th percentiles. $^{*}P < 0.05$ compared with the NC group; $^{\#}P < 0.05$ compared with the HC group; $^{\Phi}P < 0.05$ compared with the HUM group.

lung injury is of particular interest. Previous studies have shown that the protective effects of cell transplantation are primarily mediated by paracrine rather than regenerative mechanisms [11–13,15,33]. Growth factors secreted by donor cells, such as VEGF and HGF, are known to be key paracrine mediators

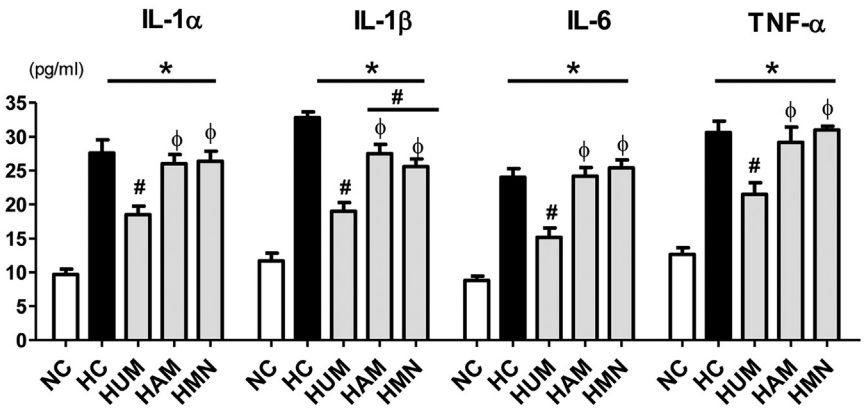

Figure 5. Measurements of the levels of lung inflammatory cytokines by ELISA. The levels of IL-1α, IL-1β, IL-6, and tumor necrosis factor-α in the lungs of P14 rats are shown. Data are expressed as means ± SEM. $^{*}P < 0.05$ compared with the NC group; $^{\#}P < 0.05$ compared with the HC group; $^{\Phi}P < 0.05$ compared with the HUM group.

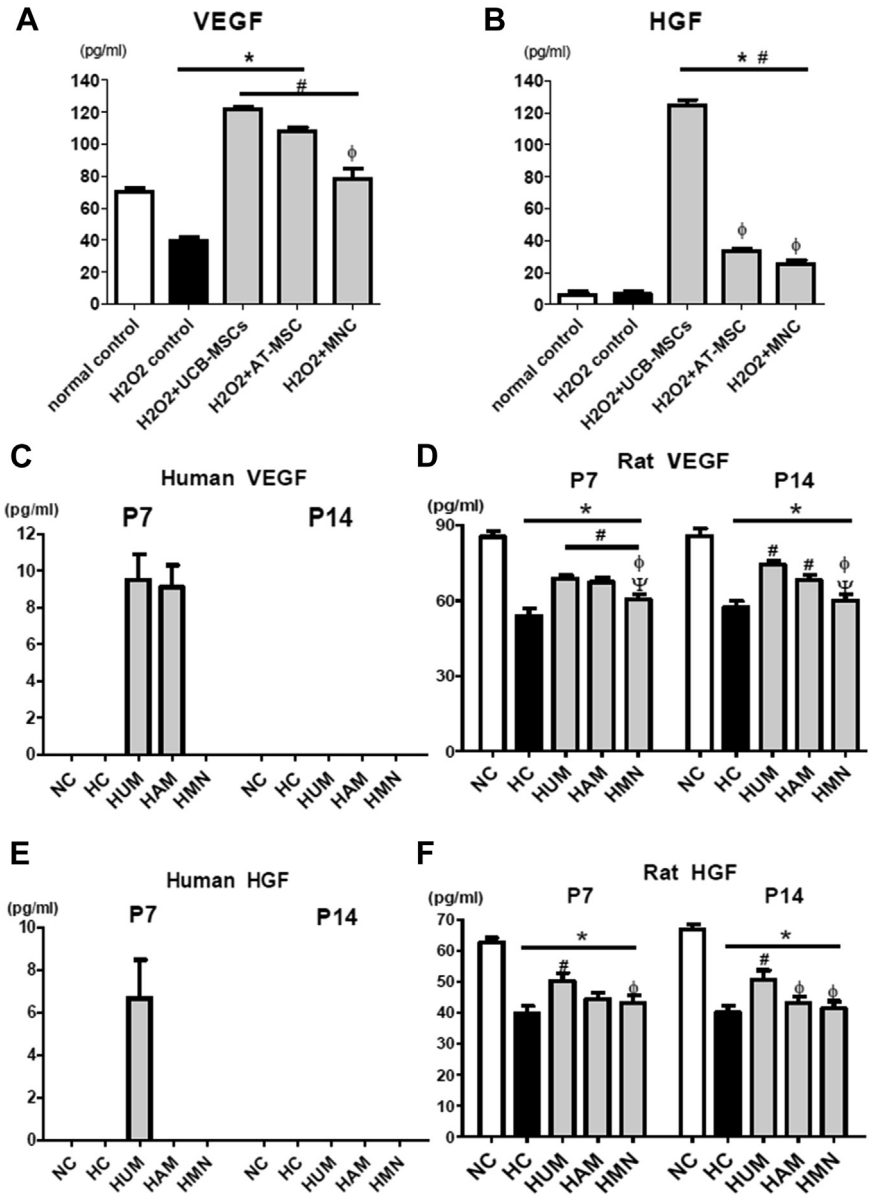

Figure 6. Expression levels of VEGF and hepatocyte growth factor HGF measured by ELISA *in vitro* and *in vivo*. Human A549 cells were treated with H_2O_2 for 1 h to induce oxidative stress and co-cultured with human UCB MSCs, AT MSCs or MNC. The levels of (A) human VEGF and (B) HGF were measured in the culture supernatant. The levels of human VEGF (C) and rat (host) VEGF (D) in the rat lung exposed to prolonged hyperoxia were measured on P7 and P14. The levels of human HGF (E) and rat (host) HGF (F) in the rat lung were also measured on P7 and P14. Data are expressed as means ± SEM. $^{*}P < 0.05$ compared with the normal control or NC group; $^{\#}P < 0.05$ compared with the H_2O_2 control or HC group; $^{\Phi}P < 0.05$ compared with the H_2O_2+UCB-MSCs or HUM group; $^{\Psi}P < 0.05$ compared with the HAM group.

that protect against neonatal hyperoxic lung injuries such as increased inflammation, oxidative stress and apoptosis, and impaired angiogenesis and alveolarization [13,15,34]. In the present study, the protective effects of stem cell therapy against hyperoxic lung injury positively correlated with the levels of human VEGF and HGF produced by the transplanted donor cells. Thus, the best protection was associated with the highest levels of paracrine growth factors and was observed with the transplantation of human UCB-derived MSCs, partial protection was observed with moderate levels of paracrine growth factors in the group receiving AT-derived MSCs and minimal protection was observed with the lowest levels of paracrine growth factors in the group receiving UCB MNCs. Overall, these findings suggest that the therapeutic efficacy of stem cell therapy is associated with or mediated by the paracrine potency of the transplanted donor cells [35,36]. Moreover, our data also suggest that the levels of paracrine

growth factors such as VEGF and HGF, which are secreted by transplanted stem cells, are potential potency biomarkers for the transplanted stem cells [15,34,37].

In our previous study [15] and in the present study, the upregulation of growth factors was primed by the transplanted MSCs and then sustained by the rat host tissue. Although the hyperoxia-induced decreases in the levels of VEGF and HGF were significantly attenuated in the present study, particularly by the transplantation of human UCB-derived MSCs, the levels of VEGF and HGF were still significantly lower than those in the normoxia group on both P7 and P14. Interestingly, overexpression of VEGF and/or HGF in MSCs has been reported to significantly enhance the therapeutic efficacy of stem cell–mediated neural and cardiac repair [38,39]. However, further studies are necessary to determine whether overexpression of growth factors such as VEGF and/or HGF enhances the beneficial effects of MSC transplantation in this BPD model.

In addition to human UCB-derived MSCs [31], MSCs derived from other birth-associated tissue, such as Wharton's jelly and the umbilical cord, have also exhibited higher cell proliferation and increased secretion of chemokines, proinflammatory proteins and growth factors compared with adult AT-derived or BM-derived MSCs [40]. Donor age has been shown to have a negative impact on MSC expansion and differentiation potential, even when the stem cells originate from the same source, that is, AT [41,42] or BM [43]. These findings suggest that the improved paracrine potency and therapeutic efficacy observed in this study of human UCB-derived MSCs in protecting against neonatal hyperoxic lung injury compared with AT-derived MSCs or UCB MNCs might be primarily attributable to the donor age. Thus, this higher efficacy may be universal to MSCs derived from birth-associated tissue. Overall, these findings suggest that donor age must be considered when aiming to develop clinically successful stem cell therapies, and that fetal-derived or newborn-derived MSCs might be the best stem cell source for treating BPD [44].

Because of the long duration (4 weeks) of the isolation, *ex vivo* expansion and characterization of MSCs [17], the relatively short therapeutic time window for treating BPD could easily be missed [13,32]. Umbilical cord blood is a rich source of MNCs and contains high levels of primitive, multipotent stem/progenitor cells [18]. Because of the short timeframe in which they can be obtained, UCB MNCs might be a good alternative cell source for MSCs for therapies designed to protect against BPD [19]. We found in the present study that human UCB MNCs were not protective against neonatal hyperoxic lung injury. However, because of to the heterogenous population of MNCs, it should be considered that inevitable deviation at comparison of cell dosages between MSCs and MNCs may be introduced.

In contrast to our results of the present study, Monz *et al.* demonstrated that UCB-derived MNCs significantly attenuated hyperoxic lung injury in a double hit mouse model of BPD [19]. The discrepancies between our work and that of Monz *et al.* might be attributable to differences in animal species, the extent of hyperoxic exposure and the dose of MNCs. Specifically, in the Monz *et al.* study, newborn mice were exposed to 75% oxygen, and 2×10^5 cells were given to mice weighing approximately 3 g; in contrast, rats were maintained in 90% oxygen, and 5×10^5 cells were given to rat pups weighing approximately 8 g in our study. Although UCB MNCs can easily be obtained in a short period of time, it is difficult to quantify the exact amount of therapeutically effective stem and/or progenitor cells within each batch of MNCs. Moreover, stem cells for human trials must be prepared in compliance with good manufacturing practice, which requires a manufacturing license [32,45]. Overall, these findings suggest that human UCB MNCs might not be a suitable source for "off-the shelf" clinical applications for treating BPD.

A previous report [31] displayed that transplantation of bone marrow–derived MNCs in the sham normal mice did not induce functional or histological changes in the lungs. Moreover, cord blood T cells have experienced little or no antigen exposure and thus present phenotypically and functionally more naive characteristics compared with peripheral blood–derived cells [25]. Monz *et al.* [19] also proved improved histologic changes after allogenic transplantation of human UCB-MNCs in the neonatal mice model of lung injury. On the basis of these findings, the concerns related with the immunogenicity of UCB-derived MNCs may seem to be less significant in delineation of comparative efficacy between MNCs and MSCs transplantation, although this still must be considered.

In summary, human UCB-derived MSCs were the most effective cells in attenuating hyperoxic lung injury in newborn rats, AT-derived MSCs were partially effective and UCB MNCs were not effective. The cell type-dependent variations in therapeutic efficacy were associated with or mediated by the paracrine potencies, that is, the amounts of the growth factors such as VEGF and HGF that were produced by the transplanted donor cells. These findings suggest that human UCB-derived MSCs might be the optimal cell source for future clinical use in protecting against BPD in premature infants.

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Disclosure of interest: Samsung Medical Center and MEDIPOST Co., Ltd., own issued or filed patents for "Method of treating lung diseases using cells separated or proliferated from umbilical cord blood" in the names of the inventors, Yun Sil Chang, Won Soon Park and Yoon Sun Yang. In addition, Soo Jin Choi declares a potential conflict of interest arising from paid employment.

References

[1] Stoll BJ, Hansen NI, Bell EF, Shankaran S, Laptook AR, Walsh MC, et al. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. Pediatrics 2010;126:443–56.

[2] Bhandari A, Panitch HB. Pulmonary outcomes in bronchopulmonary dysplasia. Semin Perinatol 2006;30:219–26.

[3] Bland RD. Neonatal chronic lung disease in the post-surfactant era. Biol Neonate 2005;88:181–91.

[4] Fung ME, Thebaud B. Stem cell-based therapy for neonatal lung disease: it is in the juice. Pediatr Res 2014;75:2–7.

[5] Vosdoganes P, Lim R, Moss TJ, Wallace EM. Cell therapy: a novel treatment approach for bronchopulmonary dysplasia. Pediatrics 2012;130:727–37.

[6] van Haaften T, Byrne R, Bonnet S, Rochefort GY, Akabutu J, Bouchentouf M, et al. Airway delivery of mesenchymal stem cells prevents arrested alveolar growth in neonatal lung injury in rats. Am J Respir Crit Care Med 2009;180:1131–42.

[7] Park HK, Cho KS, Park HY, Shin DH, Kim YK, Jung JS, et al. Adipose-derived stromal cells inhibit allergic airway inflammation in mice. Stem Cells Dev 2010;19:1811–8.

[8] Alphonse RS, Vadivel A, Fung M, Shelley WC, Critser PJ, Ionescu L, et al. Existence, functional impairment, and lung repair potential of endothelial colony-forming cells in oxygen-induced arrested alveolar growth. Circulation 2014; 129:2144–57.

[9] Balasubramaniam V, Ryan SL, Seedorf GJ, Roth EV, Heumann TR, Yoder MC, et al. Bone marrow-derived angiogenic cells restore lung alveolar and vascular structure after neonatal hyperoxia in infant mice. Am J Physiol Lung Cell Mol Physiol 2010;298:L315–23.

[10] Pierro M, Ionescu L, Montemurro T, Vadivel A, Weissmann G, Oudit G, et al. Short-term, long-term and paracrine effect of human umbilical cord-derived stem cells in lung injury prevention and repair in experimental bronchopulmonary dysplasia. Thorax 2013;68:475–84.

[11] Chang YS, Oh W, Choi SJ, Sung DK, Kim SY, Choi EY, et al. Human umbilical cord blood-derived mesenchymal stem cells attenuate hyperoxia-induced lung injury in neonatal rats. Cell Transplant 2009;18:869–86.

[12] Chang YS, Choi SJ, Sung DK, Kim SY, Oh W, Yang YS, et al. Intratracheal transplantation of human umbilical cord blood-derived mesenchymal stem cells dose-dependently attenuates hyperoxia-induced lung injury in neonatal rats. Cell Transplant 2011;20:1843–54.

[13] Chang YS, Choi SJ, Ahn SY, Sung DK, Sung SI, Yoo HS, et al. Timing of umbilical cord blood derived mesenchymal stem cells transplantation determines therapeutic efficacy in the neonatal hyperoxic lung injury. PLoS One 2013;8:e52419.

[14] Ahn SY, Chang YS, Kim SY, Sung DK, Kim ES, Rime SY, et al. Long-term (postnatal day 70) outcome and safety of intratracheal transplantation of human umbilical cord blood-derived mesenchymal stem cells in neonatal hyperoxic lung injury. Yonsei Med J 2013;54:416–24.

[15] Chang YS, Ahn SY, Jeon HB, Sung DK, Kim ES, Sung SI, et al. Critical role of VEGF secreted by mesenchymal stem cells in hyperoxic lung injury. Am J Respir Cell Mol Biol 2014;51:391–9.

[16] Mosna F, Sensebe L, Krampera M. Human bone marrow and adipose tissue mesenchymal stem cells: a user's guide. Stem Cells Dev 2010;19:1449–70.

[17] Kogler G, Critser P, Trapp T, Yoder M. Future of cord blood for non-oncology uses. Bone Marrow Transplant 2009;44:683–97.

[18] Mayani H, Alvarado-Moreno JA, Flores-Guzman P. Biology of human hematopoietic stem and progenitor cells present in circulation. Arch Med Res 2003;34:476–88.

[19] Monz D, Tutdibi E, Mildau C, Shen J, Kasoha M, Laschke MW, et al. Human umbilical cord blood mononuclear cells in a double-hit model of bronchopulmonary dysplasia in neonatal mice. PLoS One 2013;8:e74740.

[20] Jang YK, Jung DH, Jung MH, Kim DH, Yoo KH, Sung KW, et al. Mesenchymal stem cells feeder layer from human umbilical cord blood for *ex vivo* expanded growth and proliferation of hematopoietic progenitor cells. Ann Hematol 2006;85:212–25.

[21] Kim JY, Kim DH, Kim DS, Kim JH, Jeong SY, Jeon HB, et al. Galectin-3 secreted by human umbilical cord blood–derived mesenchymal stem cells reduces amyloid-beta42 neurotoxicity in vitro. FEBS Lett 2010;584:3601–8.

[22] Kim ES, Chang YS, Choi SJ, Kim JK, Yoo HS, Ahn SY, et al. Intratracheal transplantation of human umbilical cord blood–derived mesenchymal stem cells attenuates *Escherichia coli*–induced acute lung injury in mice. Respir Res 2011;12:108.

[23] Lee JK, Lee MK, Jin HJ, Kim DS, Yang YS, Oh W, et al. Efficient intracytoplasmic labeling of human umbilical cord blood mesenchymal stromal cells with ferumoxides. Cell Transplant 2007;16:849–57.

[24] Yang SE, Ha CW, Jung M, Jin HJ, Lee M, Song H, et al. Mesenchymal stem/progenitor cells developed in cultures from UC blood. Cytotherapy 2004;6:476–86.

[25] Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 2006;24:1294–301.

[26] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.

[27] Jeon GW, Sung DK, Jung YJ, Koo SH, Choi SH, Chang YS, et al. Granulocyte colony stimulating factor attenuates hyperoxia-induced lung injury by down-modulating inflammatory responses in neonatal rats. Yonsei Med J 2011;52: 65–73.

[28] Chang YS, Kim YJ, Yoo HS, Sung DK, Kim SY, Kang S, et al. Alpha-phenyl-N-tert-butylnitrone attenuates hyperoxia-induced lung injury by down-modulating

inflammation in neonatal rats. Exp Lung Res 2009;35:234–49.
[29] Shim JW, Chang YS, Park WS. Intratracheal administration of endotoxin attenuates hyperoxia-induced lung injury in neonatal rats. Yonsei Med J 2008;49:144–50.
[30] Lee JH, Sung DK, Koo SH, Shin BK, Hong YS, Son CS, et al. Erythropoietin attenuates hyperoxia-induced lung injury by down-modulating inflammation in neonatal rats. J Korean Med Sci 2007;22:1042–7.
[31] Jin HJ, Bae YK, Kim M, Kwon SJ, Jeon HB, Choi SJ, et al. Comparative analysis of human mesenchymal stem cells from bone marrow, adipose tissue, and umbilical cord blood as sources of cell therapy. Int J Mol Sci 2013;14:17986–8001.
[32] Chang YS, Ahn SY, Yoo HS, Sung SI, Choi SJ, Oh WI, et al. Mesenchymal stem cells for bronchopulmonary dysplasia: phase 1 dose-escalation clinical trial. J Pediatr 2014;164:966–972 e6.
[33] Ahn SY, Chang YS, Sung DK, Sung SI, Yoo HS, Lee JH, et al. Mesenchymal stem cells prevent hydrocephalus after severe intraventricular hemorrhage. Stroke 2013;44:497–504.
[34] Deuse T, Peter C, Fedak PW, Doyle T, Reichenspurner H, Zimmermann WH, et al. Hepatocyte growth factor or vascular endothelial growth factor gene transfer maximizes mesenchymal stem cell-based myocardial salvage after acute myocardial infarction. Circulation 2009;120:S247–54.
[35] Khubutiya MS, Vagabov AV, Temnov AA, Sklifas AN. Paracrine mechanisms of proliferative, anti-apoptotic and anti-inflammatory effects of mesenchymal stromal cells in models of acute organ injury. Cytotherapy 2014;16:579–85.
[36] Henning RJ, Sanberg P, Jimenez E. Human cord blood stem cell paracrine factors activate the survival protein kinase Akt and inhibit death protein kinases JNK and p38 in injured cardiomyocytes. Cytotherapy 2014;16:1158–68.
[37] Lehman N, Cutrone R, Raber A, Perry R, Van't Hof W, Deans R, et al. Development of a surrogate angiogenic potency assay for clinical-grade stem cell production. Cytotherapy 2012;14:994–1004.
[38] Lee HJ, Kim KS, Park IH, Kim SU. Human neural stem cells over-expressing VEGF provide neuroprotection, angiogenesis and functional recovery in mouse stroke model. PLoS One 2007;2:e156.
[39] Zisa D, Shabbir A, Suzuki G, Lee T. Vascular endothelial growth factor (VEGF) as a key therapeutic trophic factor in bone marrow mesenchymal stem cell-mediated cardiac repair. Biochem Biophys Res Commun 2009;390:834–8.
[40] Amable PR, Teixeira MV, Carias RB, Granjeiro JM, Borojevic R. Protein synthesis and secretion in human mesenchymal cells derived from bone marrow, adipose tissue and Wharton's jelly. Stem Cell Res Ther 2014;5:53.
[41] Choudhery MS, Badowski M, Muise A, Pierce J, Harris DT. Donor age negatively impacts adipose tissue-derived mesenchymal stem cell expansion and differentiation. J Transl Med 2014;12:8.
[42] Dos-Anjos Vilaboa S, Navarro-Palou M, Llull R. Age influence on stromal vascular fraction cell yield obtained from human lipoaspirates. Cytotherapy 2014;16:1092–7.
[43] Kretlow JD, Jin YQ, Liu W, Zhang WJ, Hong TH, Zhou G, et al. Donor age and cell passage affects differentiation potential of murine bone marrow–derived stem cells. BMC Cell Biol 2008;9:60.
[44] Pievani A, Scagliotti V, Russo FM, Azario I, Rambaldi B, Sacchetti B, et al. Comparative analysis of multilineage properties of mesenchymal stromal cells derived from fetal sources shows an advantage of mesenchymal stromal cells isolated from cord blood in chondrogenic differentiation potential. Cytotherapy 2014;16:893–905.
[45] Bieback K, Kinzebach S, Karagianni M. Translating research into clinical scale manufacturing of mesenchymal stromal cells. Stem Cells Int 2011;2010:193519.

Supplementary data

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