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Original Article

Therapeutic effects of umbilical cord blood derived mesenchymal stem cell-conditioned medium on pulmonary arterial hypertension in rats

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ABSTRACT

Introduction: Human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) may have multiple therapeutic applications cell based therapy with one being for treatment of pulmonary artery hypertension. As low survival rates and potential tumorigenicity of implanted cells could undermine the mesenchymal stem cell-based therapy, we chose to investigate the use of conditioned medium (CM) from a culture of MSC cells.
Material and methods: CM was prepared by culturing hUCB-MSCs in three-dimensional spheroids. In a rat model of PAH induced by monocrotaline, we infused CM or the control unconditioned culture media via tail-vein of 6-week-old Sprague-Dawley rats.
Results: Compared with the control unconditioned media, CM infusion reduced the ventricular pressure, the right ventricle/(left ventricle + interventricular septum) ratio, and maintained respiratory function in the treated animals. Also the number of IL-1α, CCL5 and TIMP-1-positive cells increased in lung samples and the number of TUNEL-positive cells decreased significantly in the CM treated animals.
Discussion: From our in vivo data in the rat model, the observed decreases in the TUNEL staining point to a potential therapeutic benefit of the CM in ameliorating PAH-mediated lung tissue damage. Increased IL-1α, CCL5 and TIMP-1 levels may play important roles in this regard.
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1. Introduction

Pulmonary artery hypertension (PAH) is a progressive chronic disease with a high mortality rate.[1] PAH has a complex disease mechanism, but its cardinal signs are an elevation of pulmonary artery pressure, right ventricular (RV) hypertrophy and arteriolar wall remodeling.[2] The pulmonary vascular is also remodeled with increased pulmonary vascular resistance and over-proliferation of pulmonary artery endothelial cells.[3,4] There is also damage to the pulmonary microvasculature impacting the flow blood from the heart to the lungs.[5] Although current treatments may prolong and improve quality of life for the patients, the long-term prognosis for PAH is poor with a 2–3 year survival at the diagnosis.[1]

Autologous implantation of bone marrow mononuclear cells (BM-MNCs), known to be enriched in mesenchymal cells (MSCs), has demonstrated safety and effectiveness in therapeutic angiogenesis.[6] A number of studies have also indicated a therapeutic benefit from bone marrow derived MSCs in increasing respiratory function in animal models of PAH.[7,8] In separate studies, human umbilical cord blood-derived MSCs have also improved lung function in animal models of PAH and in addition in a number PAH patients tested.[9,10]

In previous studies, we demonstrated the neuroprotective potential of various conditioned media namely human adipose tissue-derived stem cell-conditioned medium (hADSC-CM) and human neural stem cell-conditioned media (hNSC-CM) to treat rats with stroke and Huntington's disease.[11,12] We also investigated

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gene expression changes by microarray analysis after injection of human umbilical-cord blood derived MSCs (hUCB-MSCs) into rats in an experimental model of PAH.[13] Based on our findings from that study, we undertook an investigation to assess the feasibility and safety of conditioned medium from hUCB-MSCs (hUCB-MSC-CM) in the same rat PAH model and to test the hypothesis that the conditioned media from these cells may lead to improved lung function in the affected rats. Here we elaborate on our results and demonstrate that the conditioned media provide a therapeutic benefit in the rat model of PAH tested. As there are certain advantages in using conditioned media in lieu of using autologous whole bone marrow or umbilical cord cells as sources for mesenchymal cells (MSCs), our data may be provide a means of increasing the accessibility of MSCs to treat various disease including PAH.

2. Material and methods

2.1. Animals and pulmonary arterial hypertension (PAH) rat model

Six-week-old male Sprague-Dawley rats were used. All rats were housed in climate-controlled conditions with a 12-h light/12-h dark cycle, and had free access to food and water. The animals used in this experiment were treated according to the Principles of Laboratory Animal Care (NIH publication no. 86-23). PAH was induced by subcutaneous (sc) injection of 60 mg/kg monocrotaline (MCT) (Sigma–Aldrich, St. Louis, MO, USA) dissolved in 0.5 N HCl. The rats were grouped into a control (C) group (n = 20), injection of αMEM followed by MCT group (M group) (n = 20), and injection of MCT followed by hUCB-MSC-CM transfusion group (CM) (n = 20). αMEM and hUCB-MSC-CM (0.5 μl/h) were transfused by tail-vein administered 7 days after MCT injection.

2.2. Cell preparation and culture of hUCB-MSCs

hUCB-MSCs were obtained from Biomedical Research Institute (Seoul, Korea). Isolated human MSCs were expanded in culture as previously described.[5]

2.3. Preparation of hUCB-MSC-CM

To generate hUCB-MSC-CM spheroids,[13,14] 30 μl of cell suspension (1×10^6 cells/ml) was applied to the lid of a Petri dish containing phosphate buffered saline (PBS). After 24 h incubation, spheroids formed in the drops were retrieved. For the three-dimensional bioreactor culture, hUCB-MSC spheroids (4.2×10^7 cells) were cultured in a siliconized spinner flask (Bellco, Vineland, NJ, USA) containing 70 ml αMEM with stirring at 70 rpm. To obtain CM, the medium was changed to αMEM without serum, and the cells were cultured for 2 days.[11]

2.4. Determination of the organ weights and right hypertrophy index

The rats were weighed and observed for general appearance during the study period. The animals were sacrificed at the scheduled time. The wet weights of excised right ventricle (RV), left ventricle (LV) plus interventricular septum (IVS) (LV + IVS) were measured.

2.5. Pulmonary hemodynamics

A catheter filled with heparin saline was rapidly inserted along the incision and slowly advanced for about 5 cm to enter the pulmonary artery. The standard of pulmonary hypertension was defined as systolic pulmonary pressure (SPAP) >50 mmHg.[15]

2.6. Immunohistochemistry and Western blot analysis

Briefly, immunohistochemistry and Western bolt analysis were done according to the instructions of the manufacturer (Dako, Kyoto, Japan).

2.7. Cytokine array and gene expression in lung tissues

The lung samples were collected at termination (4 days after hUCB-MSC-CM injection) and quickly frozen in liquid nitrogen. A rat cytokine array (ARY008; R&D Systems, Minneapolis, MN, USA) was used to screen the lung homogenates according to manufacturer's instructions.

2.8. In situ TUNEL assay for lung cell apoptosis

Apoptotic cells in the tissue sections were detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling technique (TUNEL) using a commercial apoptosis kit (TACS TM TdT Kit; R&D Systems, Minneapolis, MN, USA) according to the supplier's instructions.

2.9. Statistical analyses

Results were expressed as the mean ± standard deviation. An unpaired two-tailed t-test and Mann–Whitney test were used, and a p value <0.05 was considered statistically significant. SPSS 14.0 for windows (SPSS, Chicago, IL, USA) was used for all statistical analyses.

3. Results

3.1. Changes in body and organ weights

There was a significant decrease in body weight at 14, 21 and 28 days in the monocrotaline group (M) compared to the control (C) group. However, body weight increased at 21 and 28 days in the conditioned media treated (CM) group compared to the M group. The M group also showed increased weights of the right ventricle (RV) at 21 and 28 days. The sum weight of left ventricle (LV) + interventricular septum (IVS) (LV + IVS) was not significantly different between the C, M and the CM groups at the time point tested. Ratio of RV to LV + IVS, namely RV/LV + IVS, was significantly higher at 14, 21 and 28 days in the M group compared with the C group. However, the RV/LV + IVS ratio was significantly decreased at 28 days in the CM group compared with the M group. Also LV + IVS was significantly lower in both M and CM groups compared to the C group at 14, 21 and 28 days. The lung weight was significantly increased in the M group compared with the C group at 21 and 28 days. However, the lung weight was significantly decreased in the CM group compared to the M group at 28 days (Table 1).

3.2. Cytokine profile in the lung tissues

A profile of the cytokine in the lung homogenates was made to investigate potential changes from hUCB-MSC-CM treatment (Fig. 1). CINC-1, CINC-2a/b, CX3CL1, LIX, LECAM-1, TIMP-1 and VEGF were lower in the M and CM groups, whereas TIMP-1, IL-1α and CCL5 were higher in the CM group compared to the C and M groups. CCL7 was higher in the M group, whereas CCL7 was lower in the CM group compared to the M group (Fig. 1).

3.3. Immunohistochemistry analysis

Immunohistochemistry (IHC) staining of the lung tissue revealed that TIMP-1-, IL-1α- and CCL5-positive cells were more

Table 1
Changes of body and organ weights after hUCB-MSCs-CM injection in PAH rats.

Days	Group	Body weight (g)	RV (g)	LV + IVS (g)	RV/(LV + IVS) (%)
7	Control	318.63 ± 14.78	0.132 ± 0.02	0.611 ± 0.02	0.21 ± 0.01
	M	278.50 ± 32.71	0.155 ± 0.03	0.543 ± 0.03	0.28 ± 0.02
	CM	280.46 ± 29.82	0.164 ± 0.02	0.561 ± 0.03	0.29 ± 0.02
14	Control	343.65 ± 24.52	0.156 ± 0.02	0.731 ± 0.03	0.21 ± 0.02
	M	256.71 ± 45.57*	0.234 ± 0.03	0.671 ± 0.02	0.34 ± 0.03*
	CM	271.21 ± 38.82	0.224 ± 0.04	0.699 ± 0.03	0.32 ± 0.02
21	Control	393.81 ± 24.62	0.166 ± 0.03	0.782 ± 0.03	0.21 ± 0.02
	M	249.67 ± 47.29*	0.314 ± 0.06*	0.677 ± 0.05	0.46 ± 0.05*
	CM	271.00 ± 51.55**	0.284 ± 0.05	0.631 ± 0.03	0.45 ± 0.03
28	Control	394.00 ± 41.61	0.171 ± 0.02	0.801 ± 0.03	0.21 ± 0.02
	M	229.71 ± 44.82*	0.394 ± 0.08*	0.751 ± 0.06	0.52 ± 0.07*
	CM	319.29 ± 36.62**	0.261 ± 0.06**	0.732 ± 0.04	0.35 ± 0.04**

Values are presented as means ± standard deviation. M, monocrotaline; CM, hUCB-MSCs-CM; RV, right ventricle; LV, left ventricle; IVS, interventricular septum.
* $P < 0.05$ compared with the C group.
** $P < 0.05$ compared with the M group.

prevalent in the CM group, and then followed by the M group in comparison with the C group at 28 days (Fig. 2A). These results confirmed that hUCB-MSC-CM increased the expression of certain immunomodulating cytokines (at the protein level) in the lungs of treated animals. Three weeks after hUCB-MSC-CM transfusion, TIMP-1-, IL-1α- and CCL5-positive cells were still observed at the transplanted lung area in the CM group. The increased levels of TIMP-1, IL-1α and CCL5 immunoreactivity observed in the M group were statistically significant ($p < 0.05$). The increased levels of CCL5 immunoreactivity were also significant in the CM group compared with the M group (Fig. 2B).

3.4. Western blot analysis

The protein expressions of CCL5 at 28 days were significantly increased in the M group compared to the C group. The protein expressions of TIMP-1, IL-1α and CCL5 at 28 days were significantly increased in the CM group compared to the M group (Fig. 3A). The protein expressions of Caspase-3 and Bcl-2 were significantly increased in the M group compared to the C group at 28 days. The protein expressions of Caspase-3 and Bcl-2 were significantly decreased in the CM group compared to the M group at 28 days (Fig. 3B).

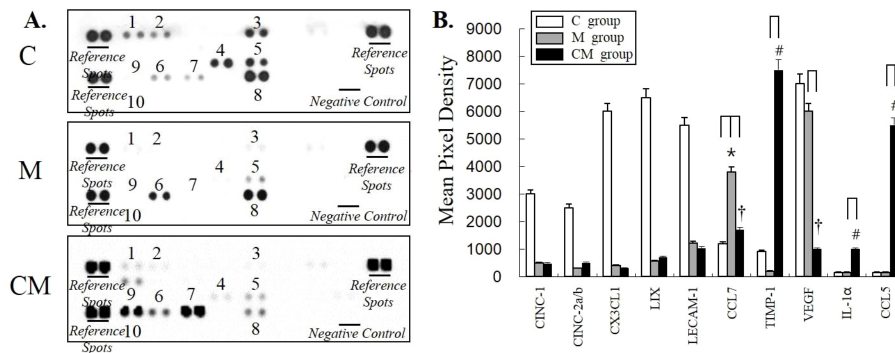

Fig. 1. Inflammatory cytokine expressions in three group. To screen whether hUCB-MSCs-CM affected local production of inflammatory cytokines by lung cells in three groups, a cytokine array was performed on lung homogenates. *$P < 0.05$ compared with the C group. †, #$P < 0.05$ compared with the M group.

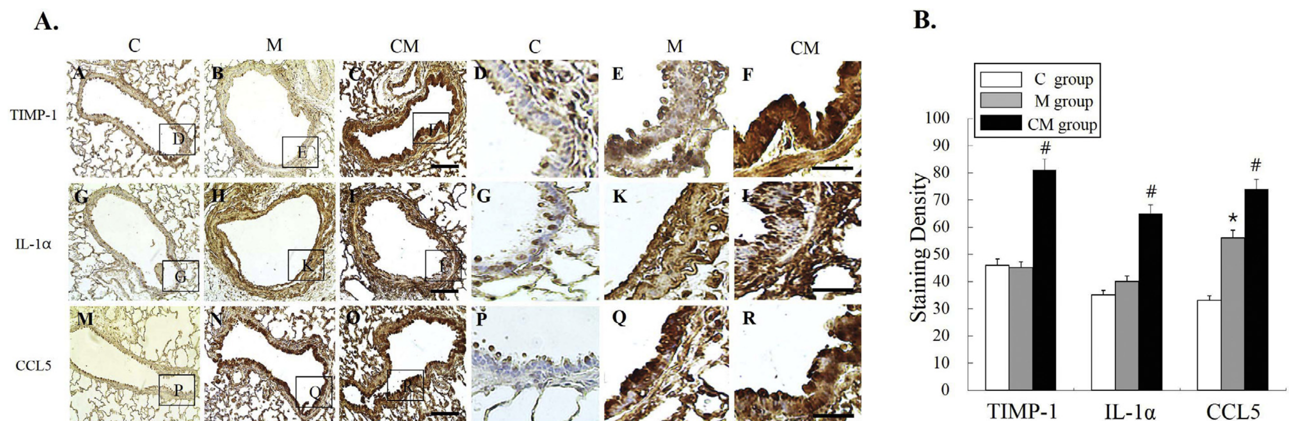

Fig. 2. Localization of IL-1α, CCL5 and TIMP-1-immunoreactive cells in the lung tissues at 28 days. Panels (A–C, G–I and M–O) are high power views of panels (D-F, J-L and P-R), respectively. *$P < 0.05$ compared with the C group. #$P < 0.05$ compared with the M group. Scale bars = 40 μm (A–C, G–I, M–O), 20 μm (D–F, J–L, P–R).

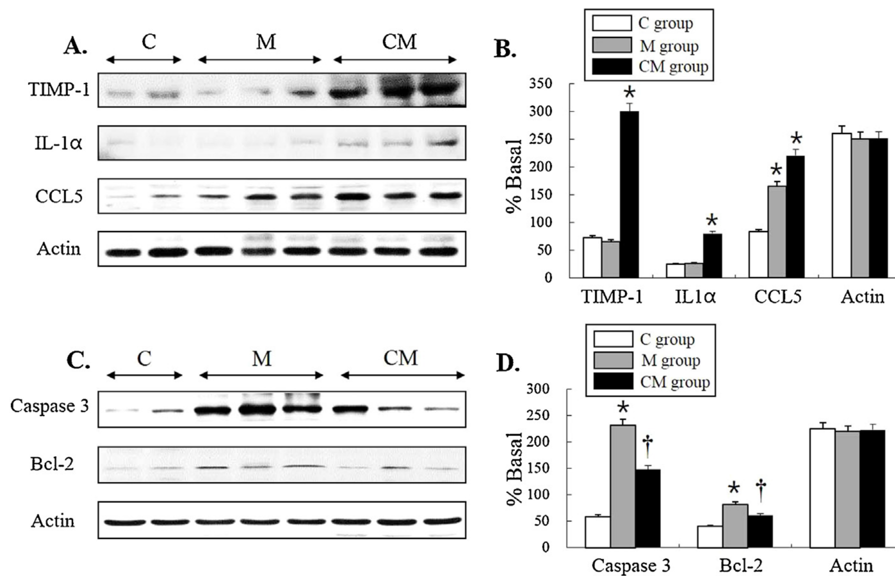

Fig. 3. Changes of IL-1α, CCL5 and TIMP-1 protein expression levels after hUCB-MSCs-CM injection in PAH rats (A). Changes of Caspase-3 and Bcl-2 protein expression levels after hUCB-MSCs-CM injection in PAH rats (B). *$P < 0.05$ compared with the C group. †$P < 0.05$ compared with the M group.

3.5. TUNEL apoptosis assay

The TUNEL staining was performed to detect apoptotic DNA in the lung tissue. The assayed C group did not have any positive staining (Fig. 4A and D). However, the M group had lung tissues with a positive TUNEL staining as seen by the presence of dark brown nuclei (Fig. 4B and E). CM group also contained cells with brown nuclei, indicating apoptotic DNA (Fig. 4C and F). Apoptotic cells were significantly more prevalent in the M group than in the C group, but they were less prevalent in the CM group than in the M group. The results indicated that hUCB-MSC-CM could attenuate apoptosis in the lung tissues of treated PAH rats.

4. Discussion

In this study, we tested the effects of infusion of CM on PAH affected lung tissue in a rat model. It was previously demonstrated that CM of hUCB-MSCs contain active levels of a number of disease modifying growth factors and cytokines.[16,17] CM of hUCB-MSCs contain sizable levels of angiopoietin, hepatocyte growth factor, interleukin-4, insulin-like growth factor, placental growth factor, vascular endothelial cell growth factor, angiogenin, stem cell factor and tyrosine hydroxylase (TH).[5,18] From our previous studies, we demonstrated neuroprotective effects of conditioned media from hADSC and hNSC in rat models of stroke and Huntington's disease[11,12] and we chose to test the CM prepared from hUCB-MSCs in a PAH rat model for therapeutic signals.

From our study, we detected relatively high concentrations of CCL5, TIMP-1 and IL-1α in hUCB-MSC-CM treated lung tissues compared with monocrotaline alone (M) and control (C) groups as confirmed by a rat cytokine array panel (Fig. 1). Lipopolysaccharide-induced CXC chemokine (also termed CXCL5) is a member of the CXC chemokine family, and is a potent neutrophil chemoattractant.[19] Tissue inhibitor of metalloproteinases-1 (TIMP-1) is a naturally occurring inhibitor of metalloproteinases[20,21] and TIMPs inhibit tumorigenesis, cellular invasion, metastasis and angiogenesis. IL-1α (and also TNF-α) are known to stimulate proliferation of endothelial cells and fibroblasts that increase the blood supply at the site of injury and repair damage.[22] IL-1α (and also TNF-α) are known to stimulate proliferation of endothelial cells and fibroblasts that increase the blood supply at the site of injury and repair damage. The numbers of TUNEL-positive cells in the lung areas were also significantly reduced by the infusion of hUCB-MSC-CM (Fig. 4). The hUCB-MSC-CM treatment was initiated 28 days after induction of PAH. Therefore, the reduction of apoptosis could be from the protective mechanisms of the hUCB-MSC-CM. For our study although no cells were implanted, our data demonstrate that an infusion of hUCB-MSC-CM can significantly reduce lung cell

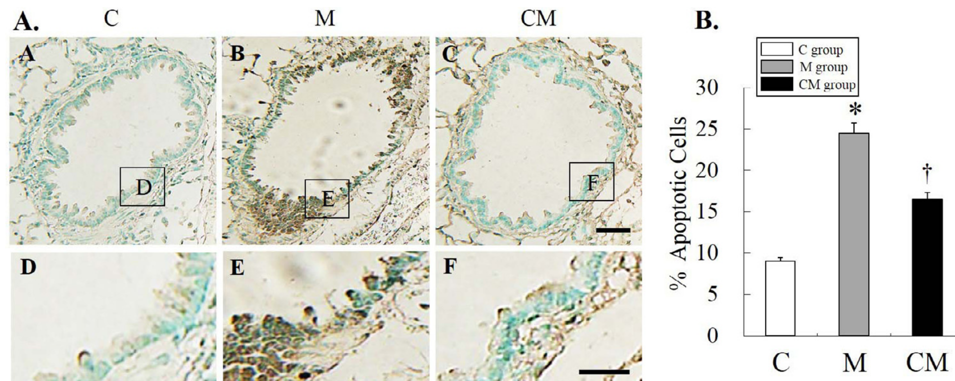

Fig. 4. TUNEL assay on lung tissues at 28 days after hUCB-MSCs-CM transfusion. Panels (A–C) are high power views of panels (D–F), respectively. *$P < 0.05$ compared with the C group. †$P < 0.05$ compared with the M group. Scale bars = 40 μm (A–C), 20 μm (D–F).

apoptosis due PAH in our rat model. The present study also revealed certain changes in chemokine, cytokine and growth factor levels after hUCB-MSC-CM transfusion in a PAH rat model. Through a complex interaction of these mediators involved in immunomodulation and inflammation we may expect a positive effect on reducing the impact of PAH on lung cells. Exactly how these cytokines and factors interact to impact the survival of the lung tissue cells remains to be explored.

5. Conclusion

Our data with factors present in hUCB-MSC-CM may present an exciting opportunity for more effective therapies. The limitations of our study included the small sample size and a short follow-up of the treated animals. Future studies with larger sample sizes and a longer duration of treatment will be required along with standardizing the quality and amount of hUCB-MSC-CM, frequency and the duration required for the treatment.

Conflicts of interest

The authors have none to declare.

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References

1. Swaminathan AC, Dusek AC, McMahon TJ. Treatment-related biomarkers in pulmonary hypertension. *Am J Respir Cell Mol Biol.* 2015;52:663–673.
2. Pereira SL, Kummerle AE, Fraga CA, et al. A novel Ca^{2+} channel antagonist reverses cardiac hypertrophy and pulmonary arteriolar remodeling in experimental pulmonary hypertension. *Eur J Pharmacol.* 2013;702:316–322.
3. Mori H, Park IS, Yamagishi H, et al. Sildenafil reduces pulmonary vascular resistance in single ventricular physiology. *Int J Cardiol.* 2016;221:122–127.
4. Fukumoto Y, Shimokawa H. Recent progress in the management of pulmonary hypertension. *Circ J.* 2011;75:1801–1810.
5. Lee JC, Kim KC, Yang YS, et al. Microarray analysis after umbilical cord blood derived mesenchymal stem cells injection in monocrotaline-induced pulmonary artery hypertension rats. *Anat Cell Biol.* 2014;47:217–226.
6. Shimamura M, Nakagami H, Koriyama H, et al. Gene therapy and cell-based therapies for therapeutic angiogenesis in peripheral artery disease. *Biomed Res Int.* 2013;2013:186215.
7. Kang H, Kim KH, Lim J, et al. The therapeutic effects of human mesenchymal stem cells primed with sphingosine-1 phosphate on pulmonary artery hypertension. *Stem Cells Dev.* 2015;24:1658–1671.
8. Guo Y, Su L, Li Y, et al. The synergistic therapeutic effect of hepatocyte growth factor and granulocyte colony-stimulating factor on pulmonary hypertension in rats. *Heart Vessels.* 2014;29:520–531.
9. Kim AK, Kim MH, Kim S, et al. Stem-cell therapy for peripheral arterial occlusive disease. *Eur J Vasc Endovasc Surg.* 2011;42:667–675.
10. Lee KB, Kang ES, Kim AK, et al. Stem cell therapy in patients with thromboangiitis obliterans: assessment of the long-term clinical outcome and analysis of the prognostic factors. *Int J Stem Cells.* 2011;4:88–98.
11. Cho YJ, Song HS, Bhang S, et al. Therapeutic effects of human adipose stem cell-conditioned medium on stroke. *J Neurosci Res.* 2012;90:1794–1802.
12. Lim HC, Lee ST, Chu K, et al. Neuroprotective effect of neural stem cell-conditioned media in in vitro model of Huntington's disease. *Neurosci Lett.* 2008;435:175–180.
13. Lee EJ, Park SJ, Kang SK, et al. Spherical bullet formation via E-cadherin promotes therapeutic potency of mesenchymal stem cells derived from human umbilical cord blood for myocardial infarction. *Mol Ther.* 2012;20:1424–1433.
14. Pereira T, Ivanova G, Caseiro AR, et al. MSCs conditioned media and umbilical cord blood plasma metabolomics and composition. *PLOS ONE.* 2014;9:e113769.
15. Golob MJ, Wang Z, Prostrollo AJ, et al. Limiting collagen turnover via collagenase-resistance attenuates right ventricular dysfunction and fibrosis in pulmonary arterial hypertension. *Physiol Rep.* 2016;4:e12815.
16. Jeong SY, Kim DH, Ha J, et al. Thrombospondin-2 secreted by human umbilical cord blood-derived mesenchymal stem cells promotes chondrogenic differentiation. *Stem Cells.* 2013;31:2136–2148.
17. Kim JY, Kim DH, Kim JH, et al. Soluble intracellular adhesion molecule-1 secreted by human umbilical cord blood-derived mesenchymal stem cell reduces amyloid-β plaques. *Cell Death Differ.* 2012;19:680–691.
18. Jin H, Sanberg PR, Henning RJ. Human umbilical cord blood mononuclear cell-conditioned media inhibits hypoxic-induced apoptosis in human coronary artery endothelial cells and cardiac myocytes by activation of the survival protein Akt. *Cell Transplant.* 2013;22:1637–1650.
19. Nicholls DJ, Wiley K, Dainty I, et al. Pharmacological characterization of AZD5069, a slowly reversible CXC chemokine receptor 2 antagonist. *J Pharmacol Exp Ther.* 2015;353:340–350.
20. Zhang LJ, Zhu ZS, Sun QR, et al. Effect of acupotomy lysis at cervical acupoints on expression of matrix metalloproteinases and tissue inhibitor of metalloproteinase 1 and changes of pulpiform nucleus ultrastructure in rats with degenerated cervical intervertebral discs. *Zhen Ci Yan Jiu.* 2015;40:352–357.
21. Bourboulia D, Stetler-Stevenson WG. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): positive and negative regulators in tumor cell adhesion. *Semin Cancer Biol.* 2010;20:161–168.
22. Kolb M, Margetts PJ, Anthony DC, et al. Transient expression of IL-1β induces acute lung injury and chronic repair leading to pulmonary fibrosis. *J Clin Investig.* 2001;107:1529–1536.