Microvesicles Derived from Human Umbilical Cord Mesenchymal Stem Cells Stimulated by Hypoxia Promote Angiogenesis Both In Vitro and In Vivo

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Although mesenchymal stem cells (MSCs) have been increasingly trialed to treat a variety of diseases, the underlying mechanisms remain still elusive. In this study, human umbilical cord (UC)-derived MSCs were stimulated by hypoxia, and the membrane microvesicles (MVs) in the supernatants were collected by ultracentrifugation, observed under an electron microscope, and the origin was identified with the flow cytometric technique. The results showed that upon hypoxic stimulus, MSCs released a large quantity of MVs of ~100 nm in diameter. The MVs were phenotypically similar to the parent MSCs, except that the majority of them were negative for the receptor of platelet-derived growth factor. DiI-labeling assay revealed that MSC-MVs could be internalized into human UC endothelial cells (UC-ECs) within 8 h after they were added into the culture medium. Carboxyfluorescein succinimidyl ester-labeling technique and MTT test showed that MSC-MVs promoted the proliferation of UC-ECs in a dose-dependent manner. Further, MVs could enhance in vitro capillary network formation of UC-ECs in a Matrigel matrix. In a rat hindlimb ischemia model, both MSCs and MSC-MVs were shown to improve significantly the blood flow recovery compared with the control medium ($P < 0.0001$), as assessed by laser Doppler imaging analysis. These data indicate that MV releasing is one of the major mechanisms underlying the effectiveness of MSC therapy by promoting angiogenesis.

Introduction

MESENCHYMAL STEM CELLS (MSCs) are characterized by their highly proliferative activity, regulatory actions on nearly all the immune cells, and ability to differentiate into at least 3 lineages, including osteoblasts, chondrocytes, and adipocytes [1–3]. MSCs have been discovered in virtually all adult and extraembryonic tissues, including compact bone, tendon, adipose, placenta, and umbilical cord (UC) [4,5], since bone marrow MSCs were first described in detail [1]. Therefore, MSCs have been utilized in many clinical trials for a wide range of therapeutic applications, such as immune-associated disorders, including graft-versus-host disease and autoimmune diseases, bone and cartilage repair, cardiac infarction, neurological degenerative diseases, liver failure, diabetes, and other conditions in which tissue damage is potentially or definitely involved [6–9].

Of note, the transplanted MSCs are unable to survive in vivo as a result of death cues, including hypoxia, inflammatory cytokines, and proapoptotic factors, no matter they

are systemically [10–13] or locally administered [14,15]. Therefore, a paracrine mechanism has been considered to explain MSC therapy in the tissue damage in organs such as the heart, liver, brain, lungs, kidneys, and pancreas [16–18]. However, the nature of the factors responsible for the beneficial paracrine effects of MSCs remains elusive.

It has been proposed that death signals might play an active role in the repair of the damaged tissues [19,20], and microvesicles (MVs) released by the injured cells are one of the important actors mediating the crosstalk with the surrounding cells [21–23]. A pioneering work from Camussi and his colleagues demonstrates that MVs derived from human bone marrow MSCs are as effective as MSCs in protecting the kidney from glycerol-induced acute injury in immunodeficient mice [24]. A recent study from the same group proves that MSC-MVs can also protect against ischaemia-reperfusion-induced acute and chronic kidney injury [25]. These investigations suggest that the beneficial effect of MSCs is, at least in part, due to the release of MVs [22,23,26]. To detail the functional activities of MSC-MVs, human

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UC-MSCs were stimulated by hypoxia, and the angiogenesis-promoting effects of MSC-MVs were investigated. The results here imply that MSC-MVs could promote blood vessel formation both in vitro and in vivo, providing some novel information about the mechanisms of MSC therapy.

Materials and Methods

Cell culture

Human UCs were harvested after obtaining informed consent, according to the Ethics Guidelines for Research Involving Human Subjects or Human Tissue from the General Hospital of Air Force. UC-MSCs were cultured and identified as previously described in our lab [27]. Briefly, UCs were cut into small pieces and digested with collagenase type I. The cells were cultured in minimum essential medium alpha (alpha-MEM) containing 10% fetal bovine serum (FBS) selected from lots (Stem Cell Co.), and the adherent cells were passaged and identified by surface marker analysis and in vitro differentiation assays [27]. MSCs at passages 3–5 were used in all the experiments below.

Meanwhile, UC endothelial cells (UC-ECs) were prepared from the UCs. In brief, the veins were washed twice with phosphate-buffered saline (PBS), and the ECs were flushed out after digestion with 0.25% trypsin for 30 min at room temperature. UC-ECs were seeded onto fibronectin-/gelatin-coated plastic dishes in Dulbecco's modified Eagle medium (DMEM) containing 20% FBS, vascular endothelial growth factor (50 ng/mL), and basic fibroblast growth factor (bFGF, 10 ng/mL). The ECs at passage 2 were used in the experiments as described below.

Hypoxic stimulation and apoptosis detection

UC-MSCs were harvested by enzyme digestion, and the cell viability was >98% as detected by the trypan blue exclusion test. Aliquots of 5×10^5 cells suspended in alpha-MEM were plated into culture dishes of 150 mm in diameter. The cells were then cultured under the following 4 conditions: (1) with 10% FBS in normoxia; (2) serum free in normoxia; (3) with 10% fetal in hypoxia; and (4) serum free in hypoxia. For normoxic conditions, the cells were cultured in an atmosphere containing 5% CO₂, while for hypoxia, the cells were maintained in a gas mixture composed of 94% N₂, 5% CO₂, and 1% O₂. The cultures were maintained for 72 h. The cell supernatants were collected after gentle shaking, and the adherent cells were washed with PBS for twice and digested with 0.05% trypsin and 1 mM ethylenediaminetetraacetic acid for 5 min at room temperature. The digestion was neutralized with the cell supernatants, and the cells were collected by centrifugation for 6 min at a speed of 500 g. After washing with PBS, the cells were reacted with the Annexin V-fluorescent isothiocyanate apoptosis detection kit (Biosea Biotechnology) according to the manufacturer's instructions. At least 10,000 events were collected by FACSCaliber (BD Biosciences), and the data were analyzed with WinMdi 2.9 software.

MV harvest

Aliquots (5×10^5 cells) of UC-MSCs were plated into 150-mm dishes in alpha-MEM containing 10% FBS (Stem Cell Co.). The serum was ultracentrifuged (100,000 g, 1 h) to

eliminate MV contamination before its use. Cell culture was maintained under a hypoxic condition (1% O₂) for 72 h. In some cases, UC-MSCs were incubated with DiI dye (5 μ M, Sigma) in a serum-free medium for 30 min at 37°C. The cells were washed in PBS and maintained under hypoxia as described above. MVs were isolated from the culture supernatants according to the protocol described previously [28]. Briefly, cell supernatants were centrifuged to discard large debris, followed by filtration through a 0.2- μ m Super Membrane (Pall Life Sciences). After additional centrifugation at 100,000 g for 1 h at 4°C, the pellet was washed twice in the apop buffer containing 5 mM KCl, 1 mM MgCl₂, and 136 mM NaCl to eliminate protein contamination [29]. MVs were then resuspended in 50 μ L apop buffer for one Ultra-Clear™ tube (14 $\times$ 89 mm; Beckman), split into aliquots of 5 μ L, and stored at –80°C for the use in the experiments below.

MV identification

The phenotypic profile was determined by a bead-based flow cytometric technique according to the protocol described previously with a slight modification [28]. In brief, MSC-MVs were bound to aldehyde/sulfate latex beads (4 μ m; Molecular Probes; Invitrogen) suspended in an MES buffer (0.025 M MES, 0.154 M NaCl, pH 6.0), followed by antibody incubation and data collection by a flow cytometry. Single beads and bead doublets were gated, and the relative fluorescence was analyzed with WinMdi2.9 software [28].

The amount of MVs was determined by measuring total MV-associated proteins, using the Bradford method, and BSA (Sigma-Aldrich) for the standard curve [29]. Also, MVs were observed directly under a transmission electron microscope (Hitachi H-7650), and the photos were taken at a magnification of 10,000.

Internalization of MSC-MVs into UC-ECs

UC-ECs at passage 2 were plated onto fibronectin-/gelatin-coated dishes and maintained at 37°C overnight for plastic adherence. DiI-labeled MSC-MVs at a concentration of 10 μ g/mL were then added, and the culture was maintained under normoxia or hypoxia as described above. At different time points, the cells were observed with a fluorescent microscopy, and also, UC-ECs were harvested by trypsin digestion, washed with PBS twice at a speed of 500 g for 5 min, and the relative fluorescence intensity was analyzed after the events were collected by FACSCalibur.

Carboxyfluorescein succinimidyl ester-labeling technique

A carboxyfluorescein succinimidyl ester (CFSE) dye dilution assay was used to observe the cell proliferative status as previously reported [30]. Briefly, human UC-ECs were pre-labeled by CFSE dye, seeded onto plastic dishes, and cultured in alpha-MEM containing 1% FBS with different doses of MSC-MVs or 10 ng/mL of bFGF. The labeled cells cultured in alpha-MEM containing 5% and 10% FBS were served as the positive controls. The culture was maintained in hypoxia or normoxia for 72 h. The cells were harvested by enzyme digestion, washed in PBS, and the events were collected by FACSCalibur. The data were analyzed by ModFit software (Verity Software House). Using the proliferation

wizard basic model of this software, CFSE-labeled cell populations were distributed into 10 generations of cell division after the parent histogram had been curved from the CFSE-labeled cells before culture.

MTT test

MTT test was performed as routinely described. Briefly, aliquots of UC-ECs (2000 cells) were seeded into a 96-well tissue plate. MTT was then added at different time points, and the culture was maintained for 4 h. The formazan was dissolved in dimethyl sulfoxide, and the optical density (OD) at a wavelength of 490 nm was assessed.

Tube formation assay

Aliquots of UC-ECs (1×10^5) were seeded onto Matrigel-coated wells of a 24-well plate and cultured in 1% fetal calf serum-supplemented DMEM in the presence of MSC-MVs at the indicated concentrations or 10 ng/mL of recombinant human basic fibroblast growth factor (PeproTech) for 24 h. Triple wells were set for each concentration, and the cells were maintained in hypoxia or normoxia. Tube formation was examined by a phase-contrast microscopy (MOTIC AE21), and the number of the network structure was quantified by randomly selecting 5 fields per well as previously described [31,32].

Rat model of hind-limb ischemia

All the animal experiments were carried out according to the approved institutional protocols from the Guide for the Care and Use of Laboratory Animals provided by Beijing Institute of Radiation Medicine. Female Wistar rats, aged 10–12 weeks and weighed around 170 grams, were divided into 6 groups ($n=6$ for each group). The hindlimb ischemia model was created by permanent ligation of the right femoral artery. The ligation was close to the iliac arteries at the proximal end and to the bifurcation to the saphenous and popliteal arteries at the distal end. Then, the femoral artery was cut between the 2 ligation ends. apop buffer, 10^6 MSCs, or graded doses of MVs in a total volume of 200 μ L were intramuscularly injected 24 h after ligation of the vessels with 5 separate injections into femoral biceps, semitendinous, semimembranous, and adductor muscles.

Laser Doppler blood flow detection

Laser Doppler perfusion imaging was performed in anesthetized rats that had been settled on a heating plate with PeriscanPIM (Perimed), and the blood flow perfusion of region of interest was analyzed with LDPIwin2.3 software. The ratio of right (ischemic) to left (nonischemic) leg was calculated as previously described [33]. The rats that had received the artery ligation without any treatment for 2 days were served as test controls.

Histological examination

After laser Doppler blood flow (LDBF) detection, the adductor muscles from some rats were removed, fixed in 10% formalin, embedded in paraffin, cut into sections of 5 μ m in thickness, and stained with hematoxylin and eosin. Histological examination was performed by a pathologist who was blind to the test groups.

Statistical analysis

All values are expressed as the mean $\pm$ SEM. Analysis of data was completed by analysis of variance with *post hoc* Fisher's least significant difference to adjust for multiple group comparisons. All statistical analysis was completed using the Statistical Package for the Social Sciences (SPSS). A P -value < 0.05 was considered as significant.

Results

UC-MSCs released MVs after hypoxic stimulation

To observe if UC-MSCs could release MVs under hypoxia, the status of UC-MSCs after stimulation was first evaluated by Annexin-V/PI staining. UC-MSCs were cultured under different conditions, and all the cells in culture, including those in the supernatants and those attached relatively loosely and tightly to the plastic, were collected for flow cytometric analysis. The results showed that compared to the cells maintained in a medium containing 10% FBS in normoxia, hypoxia alone did not induce MSC apoptosis, while cells cultured in a serum-free medium under hypoxia had a tendency to adopt the apoptotic pathway (Fig. 1A).

Subsequently, MSC-MVs were collected by ultracentrifugation, and the total protein contents were assessed. Three individual experiments showed that the total protein content was $402.48 \pm 178.12 \mu\text{g}/10^8$ MSCs in normoxia, significantly lower than that of cells in hypoxia ($2270.26 \pm 574.14 \mu\text{g}/10^8$, $P=0.01349$) and that of cells upon serum-free stimulation alone ($1715.616 \pm 516.12 \mu\text{g}/10^8$ cells, $P=0.00106$). The highest content was collected from MSCs under a serum-free and hypoxic condition ($3036.73 \pm 375.45 \mu\text{g}/10^8$). It seemed that hypoxia or serum-free culture stimulated MSCs to release MVs.

Under an electron microscope, MSC-MVs took a round morphology with a transparent center, and their diameters were around 80–150 nm, all of which were typical for MVs described elsewhere [28,29] (Fig. 1B). Bead-based flow cytometric analysis showed that MSC-MVs were highly positive for CD29, CD44, and CD73, partially positive for the beta-chain of the receptor for platelet-derived growth factor (PDGFR), and negative for CD31 and CD45. The results here suggested that the MVs were originated from MSCs (Fig. 1C). Interestingly, though the majority of the parent cells were positive for PDGFR-beta, only a small part of MSC-MVs were positive for this receptor.

MSC-MVs could be internalized by UC-ECs

To investigate the possible functions of MSC-MVs, DiI-labeled MVs were added into the UC-EC culture to observe whether these vesicles could be internalized into UC-ECs. MSCs were firstly labeled with DiI dye (Fig. 2A), and MVs were harvested from the labeled MSCs. The fluorescence of UC-ECs in normoxia (Fig. 2B, D) or hypoxia (Fig. 2C, E) was observed at different time points after the addition of DiI-MVs, using a fluorescent microscope and flow cytometry. The results showed that DiI-MVs could be easily internalized into ECs under hypoxic or normoxic conditions, and nearly all the cells became DiI positive after coincubation of 8 h (Fig. 2D, E). Further, it seemed that the internalization processes in hypoxia and normoxia were not obviously different.

FIG. 1. Umbilical cord mesenchymal stem cells (UC-MSCs) shed microvesicles (MVs). (A) Representative flow cytometry results show that cells in serum-free medium (SF) under hypoxia (Hypo) were inclined to apoptosis compared with the cells normoxia (Norm). X-axis represents Annexin-V-fluorescent isothiocyanate, and Y-axis indicates propidium iodide (PI). (B) Morphological features of MVs under electron microscope; bar: 100 nm. (C) Identification of MSC-MVs by flow cytometry. Upper panel: washed beads (left) or MV-coated beads after reaction with isotype antibodies (right) were collected, and the single beads (green) and doublets (pink red) were gated for fluorescence analysis. MVs: MSC-MVs, MSCs: the parent MSCs. X-axis: forward scatter corner (FSC). Y-axis: phycoerythrin-conjugated antibodies against the antigens of choice. The positive and negative proportions are indicated. The results are representative of 2 individual experiments. Color images available online at www.liebertpub.com/scd

MSC-MVs promoted the growth of ECs

To observe whether MSC-MVs could stimulate UC-EC growth, CFSE dye dilution assay was used to evaluate how many cells had experienced division, and how many cell cycles they had experienced, after the cells were cultured for 72 h in the presence of graded concentrations of MSC-MVs. Using the proliferation wizard basic model of ModFit software, CFSE-labeled cell populations were distributed into 10 generations of cell division. The higher the generation of a cell, the lower the CFSE fluorescence intensity was (i.e., a shift to the left for the corresponding fluorescent histogram peak). The amplitude or height of each histogram represents the proportion (%) of cells in a given generation. Analysis showed that though UC-ECs from 3 subjects grew at different rates, the results definitely supported the proliferation-promoting effect of MSC-MVs on ECs in a dose-dependent manner (Fig. 3A). Chi-square analysis showed that 1 μ g/mL of MSC-MVs was able to stimulate the proliferation of ECs

FIG. 2. The fluorescence of DiI-labeled MSCs and MSC-MV-treated endothelial cells (ECs). MSCs were labeled with DiI dye (A) and umbilical cord ECs (UC-ECs) were cultured in normoxia (B, D) or hypoxia (C, E) in the presence of DiI-MVs for 2 h (B, C) and 8 h (D, E). The photographs were taken under an ordinary light (left column) or a fluorescent light (middle column). The typical histograms from flow cytometric tests are also indicated (right column). In the histograms, X-axis indicates the relative fluorescence of DiI, and Y-axis represents number of events; the hollow histograms represent the controls, and the solid ones indicate the tested cells. The results are representative of 2 separate experiments. Color images available online at www.liebertpub.com/scd

from samples 1 and 2, and MSC-MVs at a concentration of 10 μ g/mL promoted the growth of ECs at a comparable rate of cells cultured in the presence of 10 ng/mL of bFGF. Further, the proportions of 50 μ g/mL group at generation 4 were significantly greater than those of bFGF and 5% FBS groups ($P < 0.001$ for each sample). The percentages of 100 μ g/mL group at generation 4 were higher than those of 10% FBS group ($P < 0.001$ for each sample).

To confirm the results above, UC-ECs were cultured in the presence of graded doses of MSC-MVs, and MTT assay was performed at time points of 24, 48, and 72 h. Consistently, the results showed that compared with control cells, ECs grew significantly faster in the presence of MSC-MVs at a concentration of 1 μ g/mL ($P < 0.05$ at all the observed time points). After culturing for 72 h, the highest OD490 value was from wells in the presence of MSC-MVs of 100 μ g/mL, followed sequentially by 50 μ g/mL, 10% FBS, 10 ng/mL bFGF, 5% FBS, MSC-MVs of 10 μ g/mL, 1 μ g/mL, and medium control. The data here support the conclusion that MSC-MVs stimulate the proliferation of UC-ECs in a dose-dependent manner (Fig. 3B).

FIG. 3. MSC-MVs promoted the proliferation of UC-ECs. **(A)** Carboxyfluorescein succinimidyl ester (CFSE)-labeled human UC-ECs from 3 samples were cultured in the presence of MSC-MVs at graded concentrations for 72 h. The proportions of the cells that had experienced different divisions were analyzed by flow cytometry technique. Cells that were maintained in 1% fetal bovine serum (FBS) were served as sham controls (ctr) and those in the presence of 5% FBS and 10% FBS were served as positive controls. Three main portions are indicated, including histograms with the colors of *pink* (generation 2), *white blue* (generation 3), and *yellow* (generation 4). X-axis represents CFSE intensity, and Y-axis represents the number of the events. **(B)** MTT assay shows the stimulation effect of MSC-MVs on the proliferation of UC-ECs. Y-axis: optical density at a wavelength of 490 nm. The results here were from ECs of 2 subjects. Color images available online at www.liebertpub.com/scd

MSC-MVs promoted *in vitro* tube formation of ECs

Tube formation of UC-ECs in Matrigel was observed to evaluate if MSC-MVs promote *in vitro* angiogenesis under hypoxia and normoxia. Microscopic observation found that tube-like formation of ECs occurred around 6 h of culture, and the number of tube-like structure reached to its maximal at about 16 h. Therefore, the network structure was counted at the time point of 24 h. The results showed that MSC-MVs stimulated tubulogenesis of UC-ECs in hypoxia

and normoxia in a dose-dependent manner (Fig. 4A, B). As shown in Fig. 4C, further statistical analysis indicated that 10 $\mu\text{g}/\text{mL}$ of MSC-MVs could greatly increase the structure number compared with DMEM alone ($P < 0.01$). Further, those of 100 $\mu\text{g}/\text{mL}$ of MVs and bFGF groups were comparable ($P > 0.05$). It was also found that in the presence of MSC-MVs (10 $\mu\text{g}/\text{mL}$), the number of cells in hypoxia was significantly higher than that in normoxia ($P = 0.008$). The results suggested that MSC-MVs promote angiogenesis *in vitro*, and that this activity seems greater in hypoxia.

FIG. 4. MSC-MVs promoted tube formation of UC-ECs in a dose-dependent manner. ECs were incubated in Matrigel in the presence of graded doses of MSC-MVs in hypoxia or normoxia. Typical morphology of the tube-like structure is shown in rows **(A)** (hypoxia) and **(B)** (normoxia). **(C)** Y-axis represents the structure number per field, and X-axis indicates different groups. The results are representative of those from 2 individual experiments.

FIG. 5. Representative Laser Doppler images of the rat hindlimbs. Laser Doppler blood flow was performed 2 days after the artery ligation (ctr) without any treatment or 2 weeks after operation (groups as indicated). The colors that correspond to the blood perfusion are shown. Color images available online at www.liebertpub.com/scd

MSC-MVs promoted new vessel formation in a limb ischemic model

To further determine the angiogenic activity of MSC-MVs, a rat hindlimb ischemia model was developed, and MSC-MVs, MSCs, or apop buffer was injected intramuscularly into the ischemic zones. Two weeks after treatment, the LDBF technique was used to evaluate the recovery of the blood flow. The results showed that though partial recovery of the blood flow was observed in sham controls, HU-MSCs or MSC-MVs dramatically improved this process (Fig. 5). To alleviate the errors made by the detection procedure, the ratio of mean blood perfusion in region of interest from the

ischemic to that from the nonischemic hindlimb of the same rat was calculated. The results showed that the mean ratio of 10 μ g MV group (0.51 ± 0.05) was not significantly different from that of sham control (0.50 ± 0.06 , $P = 0.5390$), while that of 50 μ g MV rats was significantly increased (0.74 ± 0.06 , $P < 0.0001$). The ratio of 100 μ g rats (0.92 ± 0.06) was comparable to that of the MSC group (0.95 ± 0.08 , $P = 0.5781$). Of note, blood flow recovered to the normal range after 3 weeks in all the tested rats. However, it was found that blood flow recovery was more rapid in MSC- or MSC-MV-treated rats, indicating the angiogenesis-promoting activity of MSCs and MSC-MVs.

Two weeks after operation, the adductor muscles of the rats were removed and histologically examined. Generally, 2 kinds of typical histological changes could be observed, including complete muscle fiber bunches and inflammatory granulomatosis that occurred due to tissue necrosis. Histological examination showed that small blood vessel tissue necrosis could be readily perceived in the granulomatosis area, and the capillary network could be observed between the muscle fiber bunches. Infiltration of inflammatory cells and necrotic tissues were found in the control rats (Fig. 6A). In the MV- and MSC-treated muscles, the capillary network seemed to be found more easily (Fig. 6B).

Discussion

MVs were firstly described as exosomes derived from reticulocytes for eliminating antiquated molecules such as transferrin receptors [34]. Recently, it is recognized that MVs could be shed by all cells in resting and activated conditions [26]. Human bone marrow MSCs have also the ability to produce MVs in the resting state [35]. In the present study, it was proved that human UC-MSCs could discharge MVs either, especially upon hypoxic stimulation. Membrane dye-labeling test showed that the MVs could be incorporated into human UC-ECs, as those into tubular epithelial cells reported previously [35]. The incorporation was rapid, as nearly all ECs became positive for DiI after exposure to MSC-MVs for 8 h. Previous data showed that internalization of MVs derived from human bone marrow MSCs into murine epithelial cells peaked between 12 and 24 h [35], seemingly slower than the observation in this study. The mechanisms underlying

FIG. 6. Typical histological changes of the adductor muscles. (A) Inflammatory granulomatosis; (B) muscle fiber bunches. Arrowhead: small vessels; Arrow: capillaries. Bar: 25 μ m. Color images available online at www.liebertpub.com/scd

internalization remain unclear and should vary depending on the diverse original cell types or even MVs released from the same cell type under different conditions. A recent report suggests that cytoskeleton proteins such as ADP-ribosylation factor 6 on the surface of MVs might contribute to this process [36].

In agreement with a previous report on platelet MVs [32], our results also indicated that like MSCs, MVs from human UC-MSCs promoted the recovery of blood flow in an ischemic model of limbs. The proangiogenic activity of MVs derived from brain tumor cells was also reported [37], and MSC-MVs were found to protect the kidneys in a rat ischemia-reperfusion model [25]. Previous data have also demonstrated that MVs derived from other cell types, including platelets [32], endothelial progenitors [38–41], tumor cells [37,42], and ES-derived MSCs [43], have the proangiogenic activity both in vitro and in vivo.

It is generally accepted that transplanted MSCs promote angiogenesis mainly by the paracrine mechanism [44]. However, it remains largely unknown about which of the compositions released by MSCs contribute to the angiogenesis-promoting activity. Previous data have suggested that MV proteins such as sonic hedgehog [45] and PDGFR [46] might contribute to the proangiogenic activity. However, our results indicated that MSC-MVs were only partially positive for this receptor, appealing further investigations to clarify the complexity. Also, several reports indicate that the horizontal transfer of mRNA and micro-RNAs from MVs to the target cells is the mechanism of action [38–41,43,35,47]. Recent studies point out that miR-126, miR-296, and let-7 micro-RNA family might play a critical role in the angiogenesis-promoting effect of MVs [40,43]. Therefore, it is possibly reasonable to postulate that transfer of mRNA, micro-RNA, and proteins as well may occur, and this process might be attributable to the intricate biological activities of MVs [48].

The findings here that MSC-MVs enhanced the proliferation of ECs might be one of the critical processes in the new vessel formation, as collateral arteries grow from pre-existing anastomoses (arteriogenesis), is the main pathway in the reparative process of arterial occlusion [49]. Nevertheless, the detailed principles on the functionality of MSC-MVs need further investigations to be clarified, and the composition should be analyzed in the coming experiments to find out if the similar mechanisms exist in MSC-MVs as those discovered in the studies with MVs from other cell types.

Further, it still remains unknown about the difference of MVs derived from hypoxia-stimulated MSCs and the resting MSCs. Cells release MVs upon activation and during cell growth. In hypoxia, MSCs proliferate faster than in those in normoxia [50–52]. UC-MSCs were induced to express hypoxia-inducible factors (HIFs) and energy metabolism-associated molecules, including glucose transporter 1, lactate dehydrogenase, and pyruvate dehydrogenase kinase isozyme 1 upon hypoxia stimulation [51,52]. The induction of HIF expression in MSCs might enhance their proangiogenic activity by secreting vascular endothelial growth factor (VEGF) and erythropoietin [53,54], the 2 main HIFs that may involve the neoangiogenesis. Previous data have demonstrated that cancer and ECs in hypoxia can secrete MVs containing angiogenic molecules, including interleukin (IL)-8, VEGF, leukemia inhibitory factor, oncostatin M IL-11,

matrix metalloproteinase-9 [55], heparin-binding EGF-like growth factor [37], and vascular cell adhesion molecule [56]. Further, recent studies have proved that MVs derived from the injured lungs could induce hematopoietic cells to express pulmonary epithelial cell-specific genes [57,58], and MV sources are the important determinants in the cell fate modulation [59]. Detailed investigations are needed to understand if it is the case for MSC-MVs.

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Author Disclosure Statement

All the authors disclose that no competing financial interests exist.

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