Original Article

Exosomes secreted by endothelial progenitor cells improve the bioactivity of pulmonary microvascular endothelial cells exposed to hyperoxia *in vitro*

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Background: Paracrine factors secreted by endothelial progenitor cells (EPCs) are suggested to be responsible, in part, for the improved microvascular development in bronchopulmonary dysplasia (BPD) models. This study aims to investigate the potential role of exosomes derived from EPCs (EPC-EXOs), a component of paracrine secretion, in angiogenesis by mediating the activity of PMVECs exposed to hyperoxia.

Methods: EPCs were isolated from bone marrow of rats. EPC-EXOs were isolated by ExoQuick-TC kits from the conditioned media of EPCs. The PMVECs were divided into three groups, including the normal group, the hyperoxia group (exposed to 85% O_2) and the EPC-EXOs treatment group (exposed to 85% O_2 and EPC-EXOs with the concentration of 100 μg/mL). The activities of proliferation, migration and tube formation of PMVECs were detected at the endpoint. The mRNA and protein expression levels of VEGF, VEGFR2 and eNOS in different groups were detected by real-time quantitative PCR and western blot.

Results: We found EPC-EXOs exhibited a cup or biconcave morphology, with the size ranging from 30 to 150 nm, and positive for the characteristic exosomal surface marker proteins, CD63 and TSG101. Comparing to the control group, Hyperoxic stress impaired the proliferation, migration, and tubule formation of PMVECs, and decreased the expression of endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor (VEGF), and vascular endothelial growth factor receptor 2 (VEGFR-2) of PMVECs. Comparing to the hyperoxia group, EPC-EXOs treatment enhanced the bioactivity of PMVECs *in vitro*, and increased the expression of eNOS, VEGF and VEGFR2.

Conclusions: Our data demonstrate EPCs secrete exosomes that have independent angiogenic activity *in vitro*. This may help explain in part the protective effects of EPCs on hyperoxic injury in the developing lung vasculature and may represent a promising therapeutic strategy for BPD.

Keywords: Angiogenesis; bronchopulmonary dysplasia (BPD); endothelial progenitor cells (EPCs); exosomes; pulmonary microvascular endothelial cells (PMVECs)

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Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung disease with high morbidity and mortality in extremely preterm infants. Simplification of the pulmonary vasculature and alveolar is the pathological hallmark of BPD (1-4). Over 50 years since Dr. Northway first described this disorder, BPD has remained a critical clinical problem due to the lack of effective therapy (5).

A hyperoxic injury is one of the most commonly acknowledged etiologies of BPD, and the pulmonary capillary endothelium is the primary target cells in the initial burst in reactive oxygen species (6,7). Human infants dying from BPD, who had more days of oxygen supplementation, showed disrupted pulmonary vasculature with the decrease of angiogenesis-related factors, including VEGF, Flt-1, and TIE-2 (8). Hyperoxia exposure is a widely employed injurious stimulus to induce preclinical BPD model, resulting in inhibited distal microvasculature formation and an imbalance of pro- and anti-angiogenic factors in developing lungs (9-11).

Recent insights into the application of stem/progenitor cells to restore the lung development have provided us with the hope to find a new therapy for BPD. Endothelial progenitor cells (EPCs), precursors of endothelial cells, have been shown to promote the repair of damaged blood vessels in various disease models (12). In BPD rat model, exogenous EPCs transplanted through the peripheral vessels can migrate and engraft into the lungs of neonatal rats, improving vascularity and airspace development (13). Recent studies have reported that paracrine components of late EPCs also exert protection to BPD models by improving the growth of endothelial cells and alveolar cells, resulting in better lung structure and function (14,15). More recently, researchers have found that exosomes, the extracellular, membrane-bound nanoparticles that serve as an important paracrine factor, carry proteins, mRNA, and miRNA of EPCs and exchange their cargo with the endothelial cells of the target organ, serving as an additional mechanism for intercellular communication (16-18). Studies revealed that EPC-EXOs could transfer to endothelial cells, thus enhancing the function of endothelial cells to induce vascular formation and organ regeneration in various diseases (19-21).

However, the effect of EPC-EXOs on hyperoxic BPD model remains unclear; consequently, in this study, we established a hyperoxic cellular model of PMVECs via increasing the ambient oxygen concentration throughout the culture period, mimicking the process of BPD. Here, we attempt to investigate the potential role of EPC-EXOs in angiogenesis by mediating the activity of PMVECs under an external oxidative challenge, expecting to find an alternative therapy for BPD.

Methods

Cell culture and study design

The animal experiments were approved by the Animal Ethics Committee of the Children's Hospital of Fudan University (No. 201687). The surgery was performed under anesthesia to minimize suffering.

PMVECs were purchased from Be Na Culture Collection Co., Ltd. (category No. BNCC338210; Beijing, China). PMVECs were cultured in DMEM culture media (Gibco, NY, USA) supplemented with 10% FBS (Gibco) and 100 U/mL Penicillin-Streptomycin Solution (Gibco). PMVECs were randomly divided into three groups: the control group, the hyperoxia group, and the EPC-EXOs group. The control group was exposed to normoxic gas (21% O_2/5% CO_2/74% N_2), the hyperoxic group was exposed to high O_2 gas (85% O_2/5% CO_2/10% N_2), the EPC-EXO group was exposed to high O_2 gas and EPC-EXOs (85% O_2/5% CO_2/10% N_2 & 100 μg/mL exosomes). The plates of hyperoxia groups were placed in an airtight acryl box with moist tissues to maintain humidity, with O_2 continually being infused into the box. The oxygen concentration in the box was continuously measured by monitor.

Isolation and identification of EPCs from rat bone marrow

The methods were performed as previously described with some modification (13). Briefly, mononuclear cells were isolated from the bone marrow of 10 days' old SD rats using density gradient centrifugation with separation medium (Sigma, St. Louis, MO, USA). The isolated cells were placed into plates pre-coated with fibronectin (Sigma) and cultured in EGM-2MV (Lonza, Allendale, NJ, USA). After 48 h, non-adherent cells were discarded. Between the 7th and 10th days, cells were harvested for characterization of EPCs. The capacity to uptake Dil-ac-LDL and combined FITC-UEA-1 of the isolated cells were analyzed by fluorescence microscope. The expression of CD34 and VEGFR2 in EPC was assayed by immunocytochemistry with antibodies of anti-CD34 and VEGFR2 (Santa Cruz, CA, USA).

Isolation and identification of EPC-EXOs

When EPCs were 80% confluent, the cells were changed with the medium of EGM-2MV supplemented with 5% exosome-depleted FBS media supplement for an additional 48 h. The culture medium of EPCs was obtained and centrifuged at 2,000 ×g for 15 min to eliminate dead cells and cellular debris. The obtained medium underwent centrifugation at 10,000 ×g for 60 min, and the supernatant was then filtrated with 0.22 μm filter to remove cellular debris and large particles further. Exosomes were isolated using exosome precipitation solution (Exo-Quick; System Bioscience, MO, USA) following the manufacturer's instructions. The exosome pellet was resuspended with PBS and stored at –80 ℃ for subsequent study.

The ultrastructure and size distribution of exosomes were analyzed by transmission electron microscopy and NanoSight (Malvern, UK) respectively. The characteristic exosomal surface marker proteins of CD63 and TSG101 (Abcam, Cambridge, UK) were determined by western blotting.

Internalization assay of EPC-EXOs

Purified exosomes were labeled with a PKH67(green) kit (Sigma-Aldrich) according to the manufacturer's instructions. The labeled exosomes were suspended with PBS and incubated with PMVECs. After cells were fixed, they were visualized under a fluorescence microscope.

Proliferation assay

To investigate the effect of EPC-EXOs on cell proliferation, PMVECs were seeded on 6 cm culture plates with a density of 5×10^5 cells. The plates were exposed to the indicated oxygen concentration. Pictures were taken every 24 h using a phase-contrast microscope. Cells numbers of three randomly chosen fields of every plate were counted using Image J software at the end of timepoint.

Tube formation assay

To assay *in vitro* angiogenesis, PMVECs (10,000 cells/well) were seeded onto 96-well plates coated by Matrigel and incubated in different conditions. The culture medium was changed every 24 h and cells were incubated for 72 h. The capillary-network formation was monitored using an inverted microscope. The number of the nodes in three randomly chosen fields of every plate was examined.

Migration assay

PMVEC migration was analyzed using 8.0 μm Transwell inserts (Corning, 3422). In detail, 900 μL of DMEM medium was added to the lower chamber, while 1×10^5 PMVECs in 200 μL of DMEM medium deprived of FBS were seeded on the upper chamber of Transwell inserts. Seventy-two hours after incubation in different conditions, suspension cells were removed, and cells on each insert were removed with cotton swabs. The membranes were fixed with 4% paraformaldehyde for 15 min at room temperature. The migrated cells were stained with Crystal Violet Staining Solution (Solarb, China) for 15 min, then rinsed with PBS three times, and counted under the microscope in five microscopic random fields.

qRT-PCR analysis

PMVECs (5×10^5 cells per well) were seeded in 6 cm plates and cultured in DMEM media supplemented with exosomes in indicated oxygen concentration for 48 h. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized from 1 μg of total RNA by using the PrimeScriptTM RT reagent Kit with gDNA (Takara Biotechnology, Japan). Then, qRT-PCR was performed with a Roche LightCycler 480 System with SYBR Premix ExTaqTM II (Takara Biotechnology, Japan). β-ACTIN was used as a housekeeping gene for internal normalization. Primers used in the amplification reaction were synthesized as the following: VEGF: forward, 5'-GCAGATGTGACAAGCCAAGG-3', and reverse, 5'-GATGGTGGTGTGGTGGTGAC-3'; VEGFR2: forward, 5'-ACAGCATCACCAGCAGTCAG, and reverse, 5'-GATGCTCCAAGGTCAGGAAG-3'; eNOS: forward, 5'-ACTATGGCAACCAGCGTCCT, and reverse, 5'-CTCGTGGTAGCGTTGCTGAT-3'; β-ACTIN: forward, 5'-TGTCACCAACTGGGACGATA, and reverse, 5'-GGGGTGTTGAAGGTCTCAAA-3'. After validation of amplification efficiencies of target genes and the internal control gene, quantification of target gene expression was calculated using a $2^{-\Delta\Delta CT}$ method.

Western blotting analysis

After different treatments, proteins from PMVECs were obtained with lysis buffer supplemented with protease

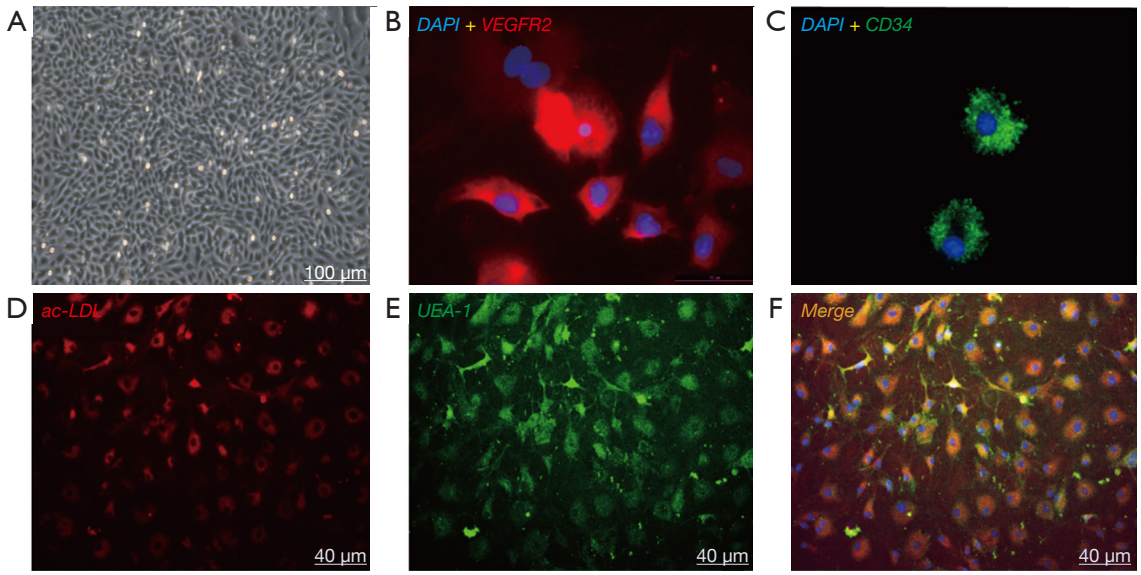

Figure 1 Characterization of EPCs from rat bone marrow: (A) EPCs showed a typical cobblestone-like morphology. Scale bar =40 μm; (B,C) immunostaining of cell-specific antigens of CD34 and VEGFR2 on EPCs. Scale bar =50 μm; (D,E,F) testing of Dil-ac LDL uptake and FITC-UEA-1 binding revealed that most of the cells were double Dil-acLDL/FITC-UEA-1 positive. Scale bar =40 μm.

inhibitor cocktail (Thermo Scientific, FL, USA). Cell debris was removed by centrifugation at 12,000 ×g, for 30 min at 4 ℃. Then, the cell lysates were separated by SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked by incubating with 10% dry milk for 2 h and then incubated with primary antibodies against VEGF, VEGFR2, and eNOS in a concentration of 1:2,000 at 4 ℃ overnight. All the antibodies were purchased from Abcam (Cambridge, UK). GAPDH (1:4,000; Sigma-Aldrich, St. Louis, MO, USA) was used to normalize protein loading. After being washed thoroughly, membranes were incubated with horseradish peroxidase-(HRP) conjugated IgG (1:5,000; Cell Signaling Technology, MA, USA) for 2 h at room temperature. Blots were then developed with enhanced chemiluminescence developing solutions and quantified by Image J software.

Statistical analysis

All experiments were performed in triplicate. The data were shown as mean ± standard deviation (SD). Differences were analyzed using one-way analysis of variance (ANOVA) with SPSS 22.0, followed by post hoc. Bonferroni testing for between-group differences. P values <0.05 were considered statistically significant.

Results

Identification of rat bone marrow-derived EPCs

Bone marrow-derived mononuclear cells were isolated by density gradient centrifugation and cultured in endothelial-specific conditions. The cells developed the typical endothelial-like cobblestone morphology at day 7 (*Figure 1A*). Immunostaining results showed that EPC colonies expressed endothelial lineage markers of VEGFR-2 and CD34 (*Figure 1B,C*). They demonstrated the ability to uptake Dil-ac-LDL and bind FITC-UEA-1 (*Figure 1D,E,F*).

Identification and characterization of exosomes from EPCs

Exosomes isolated from EPCs demonstrated biconcave or cup-shaped morphology under transmission electron microscopy (*Figure 2A*). Nanosight analysis showed that the size of EPC-EXOs was approximately 50–150 nm (*Figure 2B*). Western blotting exhibited that exosomal marker proteins CD63 (exosomal surface marker protein) and TSG101 (exosomal luminal protein) were present in these exosomes (*Figure 2C*). To further investigate whether the EPC-EXOs could be transferred into endothelial cells, exosomes were labeled with PKH67 and incubated with PMVECs *in vitro*. The uptake was confirmed by

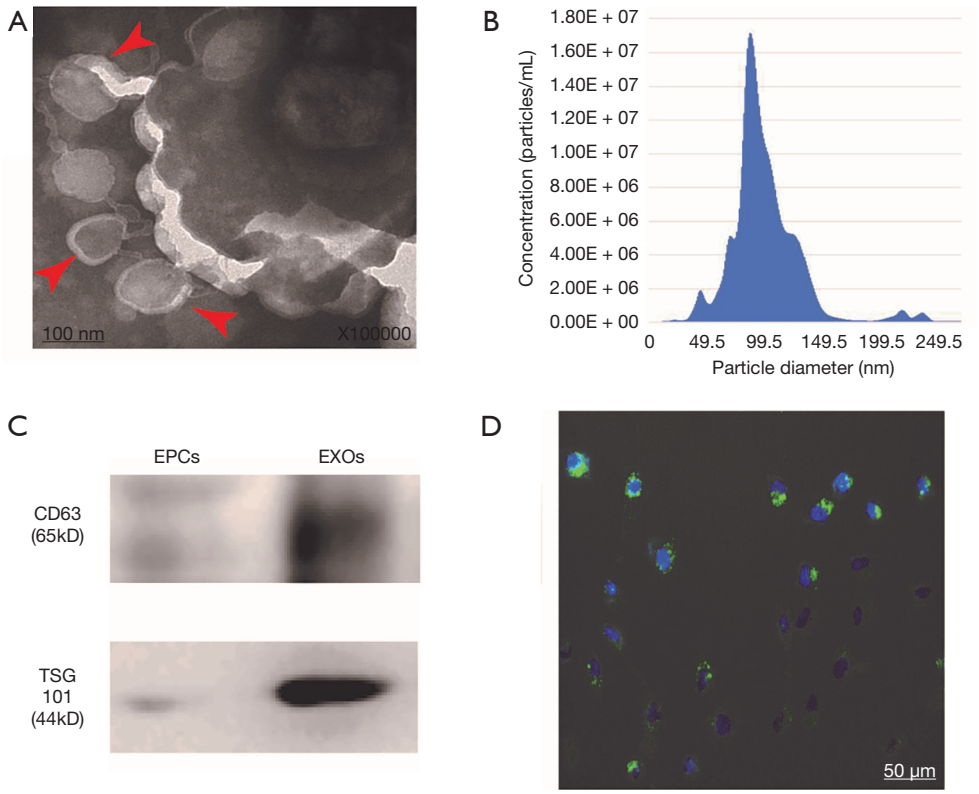

Figure 2 Characterization of EPC-EXOs. (A) EPC-EXOs exhibited a cup or biconcave morphology under transmission electron microscopy; (B) particle size distribution and concentration of EPC-EXOs measured by NTA. Scale bar =100 nm; (C) Western blotting analysis of exosomal surface marker proteins (including TSG101 and CD63) in EPC-EXOs; (D) internalization of PKH67-labeled EPC-EXOs (green) into PMVECs. Scale bar =50 μm.

fluorescence microscopy. After 12 h, over 95% of PMVECs were PKH67 positive (*Figure 2D*).

EPC-EXOs improved the angiogenic activity of PMVECs in increased oxygen risk

Effects of EPC-EXOs on PMVECs proliferation

There was a significant difference in the proliferation capacity of PMVECs among the three groups (*Figure 3A*). Compared to the control group, hyperoxia significantly inhibited PMVEC proliferation, as indicated by cell density and manual cell counting (362±9.20 *vs.* 87.0±7.17, cells/field, P<0.001). EPC-EXOs improved PMVEC proliferation compared to the hyperoxia group (180±5.62 *vs.* 87±7.17, cells/field, P<0.001).

Effects of EPC-EXOs on PMVEC migration

Transwell assay was used to determine the migration of PMVECs after treatment by exosomes. There was a significant difference among the three groups (*Figure 3B*). In comparison with normal group, it was shown that hyperoxia exposure weakened the motility of PMVECs (248±11.6 *vs.* 55.3±10.3, cells/field, P<0.001), as indicated by the number of cells in the Transwell assay. EPC-EXOs could promote the migration of PMVECs compared to the H group (144±13.7 *vs.* 55.3±10.3, cells/field, P<0.001).

Effects of EPC-EXOs on PMVEC tube formation

To further explore the pro-angiogenic role of EPC-EXOs on PMVECs *in vitro*, a tube formation assay was performed. There was a significant difference among the three groups (*Figure 3C*). Results showed tube formation of PMVECs was significantly impaired in the hyperoxia group as determined by the number of nodes, in comparison to the normal group (840±86.5 *vs.* 253±65.0, nodes/field, P<0.001). The PMVECs could not form any tubes but cell clusters. EPC-EXOs improved the vascular formation ability of PMVECs (595±65.8 *vs.* 253±65.0, nodes/field, P<0.001).

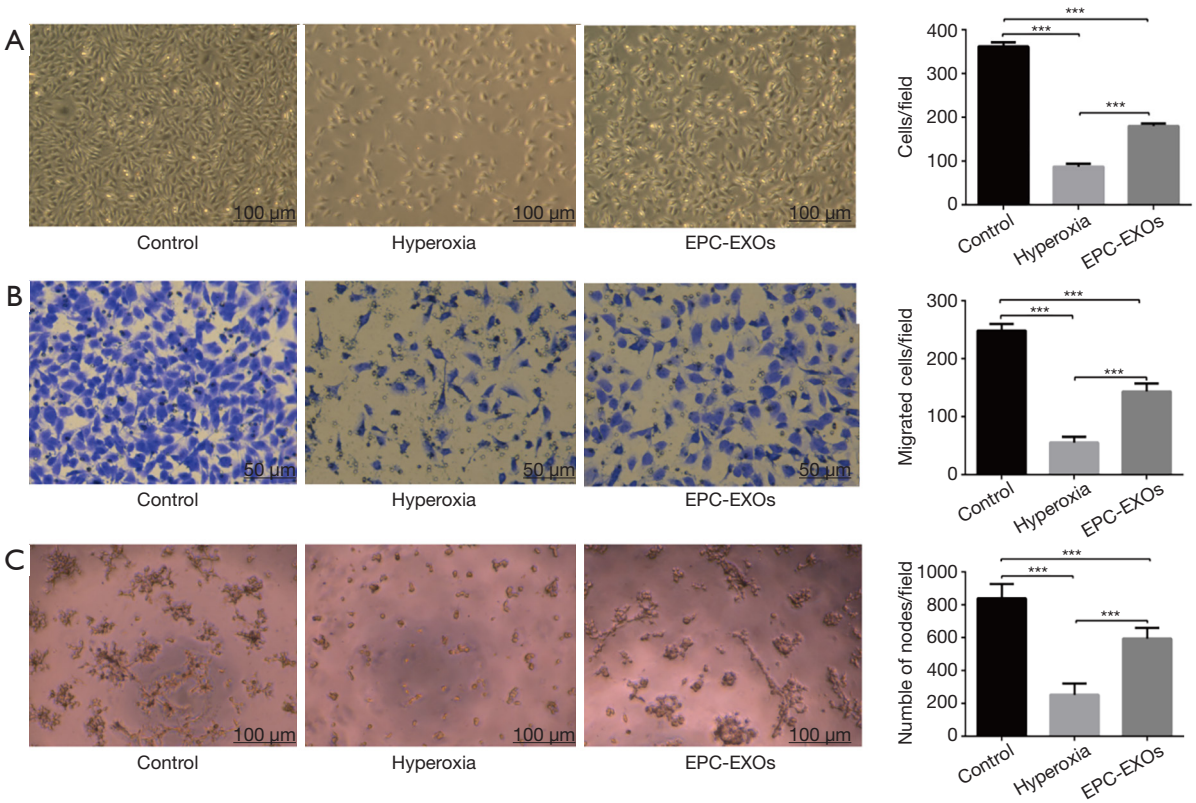

Figure 3 Internalized EPC-EXOs functional assays. (A) EPC-EXOs promoted PMVECs proliferation as analyzed by manual cell counting. Scale bar =100 μm; (B) EPC-EXOs stimulated the migration of PMVECs. Scale bar =50 μm; (C) EPC-EXOs enhanced the tube formation of PMVECs *in vitro*. Scale bar =100 μm. ***, P<0.001.

EPC-EXOs improved angiogenesis-related molecules expression of PMVECs in increased oxygen risk

To test whether angiogenesis-related molecules expression was altered by internalization of EPC-EXOs, the mRNA and protein levels of eNOS, VEGF, and VEGFR-2 were evaluated by qRT-PCR and western blot analysis. There was a significant difference among the three groups. As shown in *Figure 4*, hyperoxic stress down-regulated the mRNA and protein levels of eNOS, VEGF and VEGFR-2. After stimulated with EPC-EXOs, the levels of eNOS, VEGF and VEGFR-2 were up-regulated (*Figure 4A*,*B*).

Compared to the control group, the VEGF mRNA expression was significantly decreased in the hyperoxia groups (1.00±0.11 *vs.* 0.13±0.02, P<0.001), and the VEGF mRNA in the EPC-EXO treatment group was higher than that of the hyperoxia group (0.48±0.10 *vs.* 0.13±0.02, P<0.05). Compared to the control group, the VEGF protein expression was significantly decreased in the hyperoxia group (0.89±0.06 *vs.* 0.47±0.07, P<0.05), and the level of VEGF protein in EPC-EXOs treatment group was higher than that of the hyperoxia group, though the difference was not statistically significant.

Compared to the control group, VEGFR2 mRNA and protein expressions were significantly decreased in hyperoxia (1.00±0.09 *vs.* 0.28±0.07, 1.77±0.14 *vs.* 0.40±0.14, P<0.001, respectively) and the EPC-EXOs] groups (1.00±0.09 *vs.* 0.39±0.03, 1.77±0.14 *vs.* 0.61±0.18, P<0.001, respectively). The levels of VEGFR2 mRNA and protein in the EPC-EXO group were higher than those of the hyperoxia group, though the difference was not statistically significant. Compared to the control group, eNOS mRNA and protein expressions were decreased in the hyperoxia group (1.00±0.04 *vs.* 0.50±0.09, 1.00±0.20 *vs.* 0.40±0.13, P<0.01, respectively) and the EPC-EXO group (1.00±0.04 *vs.* 0.75±0.13, 1.00±0.20 *vs.* 0.89±0.05, P>0.05, respectively). The levels of eNOS mRNA and protein in the EPC-EXOs group were higher than those

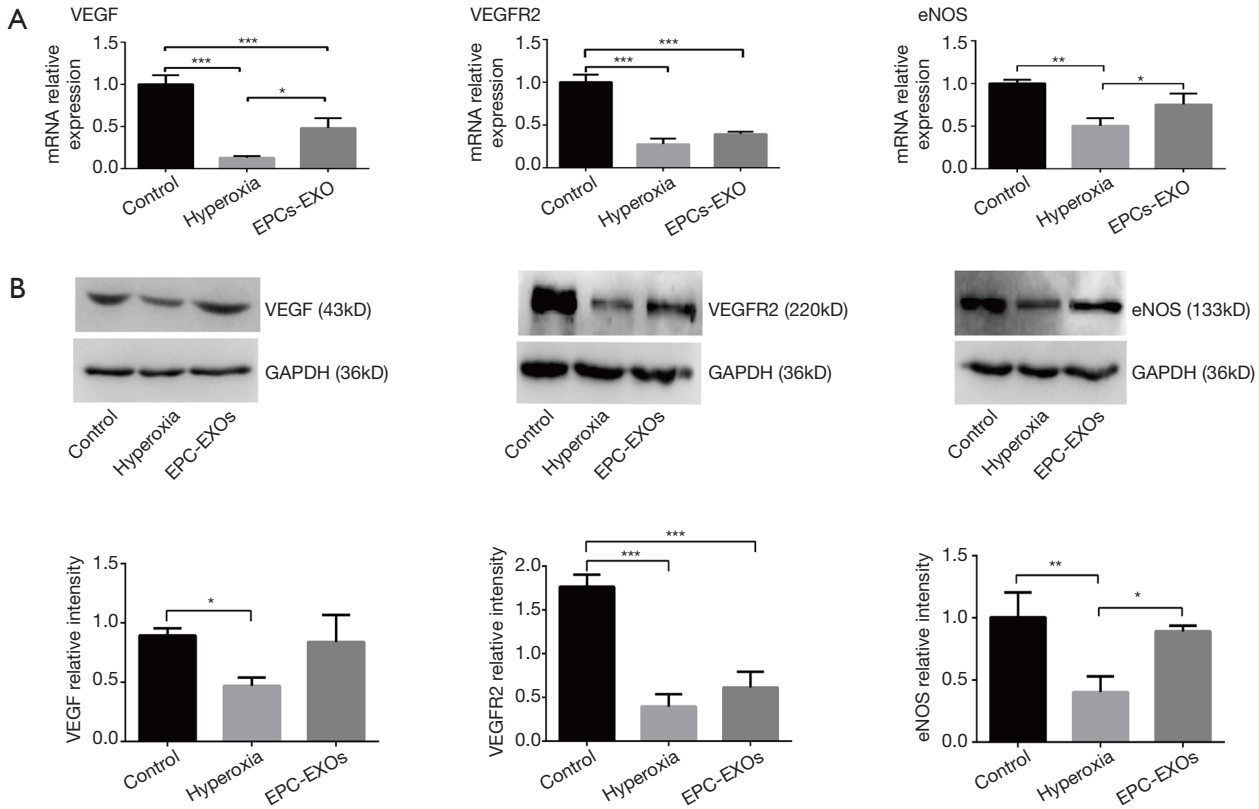

Figure 4 EPC-EXOs on the expression of angiogenesis-related genes in vascular endothelial cells. (A) qRT-PCR analysis of the mRNA levels of angiogenesis-related genes in PMVECs; (B) Western Blot analysis of the protein levels of angiogenesis-related genes in PMVECs. *, P<0.05; **, P<0.01; ***, P<0.001.

of the hyperoxia group (0.75±0.13 *vs.* 0.50±0.09, 0.89±0.05 *vs.* 0.40±0.13, P<0.05, respectively).

Discussion

Our results revealed that hyperoxia exposure impaired the capacity to proliferate, migrate, and form the vessel-like structures of PMVECs, and downregulated the expression of angiogenesis-related molecules, including VEGF, VEGFR2, and eNOS. We also found that EPC-EXOs preserved the proliferation, migration, and tube formation of PMVECs under oxidative stress and increased the expression of VEGF, VEGFR2, and eNOS, which were important angiogenesis-related molecules during lung development.

Oxidative stress may induce lung endothelial cells injury by vastly complex cellular and molecular pathways. It has been reported that hyperoxia exposure directly causes oxidative injury to endothelial cells, damaging the organelles (22), inhibiting cell cycles (23-25), and interfering with the expression of growth factors associated with vascular development (10,26), which are key events of angiogenesis. We found that hyperoxia exposure disturbed the bioactivities of PMVECs, particularly in that they could not form any tubes *in vitro* but only cell clusters, which might reflect the malformation or scarceness of the microvascular structures in BPD. Important growth factors associated with angiogenesis, including VEGF/VEGFR2 and eNOS were decreased in PMVECs exposed to hyperoxia, which was consistent with the above studies. Many studies revealed that lung vascular endothelium had a particular susceptibility to hyperoxia, and capillary endothelial damage is more extensive and typically occurs earlier than damage to the alveolar epithelium in preclinical hyperoxic lung injury models (27,28).

Taken together, PMVECs dysfunction by hyperoxia exposure may be associated with pulmonary blood vessel loss and vascular remodeling in BPD.

EPC and/or its paracrine factors-based therapies have

emerged as a promising treatment option for various diseases and an area of intense research interest. Recently, the effects of extracellular vesicles derived from EPCs have been explored in cardiovascular and renal diseases (29,30). The exosomes are important extracellular vesicles. In this study, we found EPC-EXOs exhibited a cup or biconcave morphology, with the size ranging from 30 to 150 nm and positive for the characteristic exosomal surface marker proteins of CD63 and TSG101, which were morphologically similar in size and shape to exosomes described in previous reports, carrying known exosomal protein markers. Immunofluorescence assays confirmed that PKH67-labeled exosomes could be taken up into the PMVECs. Taken together, these observations showed that EPC-EXOs could be successfully isolated and efficiently transferred to PMVECs.

We found that EPC-EXO treatment induced the angiogenic activity of PMVECs cultured under external oxidative challenge *in vitro*. We observed that EPC-EXOs better preserved PMVEC proliferation and migration compared to the hyperoxia group. The PMVECs could not form any tubes but only cell clusters in the hyperoxia group. After EPC-EXO treatment, the vascular formation ability of PMVECs improved. Overall, EPC-EXOs showed protective effects in angiogenesis-related processes including proliferation, migration, and tube formation on PMVECs exposed to hyperoxia. Other studies also showed that EPC-EXO treatment could improve survival, proliferation, and tube formation capability of endothelial cells of various types under different stresses (20,21,31).

In this study, EPC-EXO treatment increased the expression of angiogenic factors of PMVECs, including VEGF/VEGFR2 and eNOS, which were downregulated in the hyperoxic group. VEGF/VEGFR2s are important signals for angiogenesis during lung development, and eNOS/NO pathway is associated with BPD (32). VEGF phosphorylates VEGFR-2 and its downstream signaling activates eNOS. Blocking this pathway augmented smoking-induced oxidative stress and inflammatory responses leading to endothelial dysfunction, indicating its key role in the regulation of endothelial cell migration, proliferation, and survival under oxidant stress (33,34). This suggests that EPC-EXOs may contribute therapeutic benefits to PMVECs by modulating the imbalance of pro-angiogenic factors induced by hyperoxia exposure. Li also reported various critical pro-angiogenic genes of human microvascular endothelial cells cultured in normal condition, including eNOS, interleukin-8, angiopoietin-1, and E-selectin, were significantly up-regulated after stimulation with EPC-EXOs (31). A recent report revealed that additional pathways might play a role in EPC-EXO-mediated angiogenesis, showing that EPC-EXOs suppressed lung vascular leakage in septic mice and attenuated the increases in plasma levels of LPS-induced high mobility group box 1 and vascular cell adhesion molecule 1 in human microvascular endothelial cells (35). Interestingly, exosomes from mesenchymal stem cells, another alternative stem cell for treating BPD, has been shown to promote microvascular and airspace development in hyperoxic BPD mouse model by blunting pro-inflammatory signaling and immune responses in the hyperoxic lung via modulation of lung macrophage phenotype (36). Thus, EPC-EXOs appear to have promise in facilitating vascular repair in BPD model induced by hyperoxia.

There are limitations to this study. Firstly, the exact cargo of EPC-EXOs is still incompletely understood, and the exact molecular mechanisms of their therapeutic action on PMVECs need to be elucidated further. Secondly, the current research is a cellular study *in vitro*; the protective effect of EPC-EXOs on BPD animal models should be verified further.

In summary, our study showed that EPC-EXOs are active components of EPC paracrine secretion, which promotes angiogenesis by improving endothelial cell functions like proliferation, tube formation, and cell migration, when under oxidative challenge. These effects are associated with the expression of VEGF, VEGFR2, and eNOS. The exact mechanism and study *in vivo* should be considered further.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The animal experiments were approved by the Animal Ethics Committee of the Children's Hospital of Fudan University (No. 201687).

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