Endothelial progenitor cell-derived extracellular vesicle-mediated cell-to-cell communication regulates the proliferation and osteoblastic differentiation of bone mesenchymal stromal cells

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Abstract. Bone tissue engineering is a promising treatment strategy to increase bone regeneration. Endothelial progenitor cells (EPCs) and bone marrow stromal cells (BMSCs) are commonly used to promote vessel formation and osteoblastic differentiation in tissue engineering. Previous studies have demonstrated that EPCs regulate both proliferation and differentiation of BMSCs. However, the underlying mechanism remains unclear. Understanding this mechanism is critical to developing more effective treatments. The role of extracellular vesicles in cell-to-cell communication has attracted substantial attention. These small vesicles deliver proteins, DNA, and RNA and consequently regulate the commitment, function, and differentiation of target cells. In the present study, EPC-derived extracellular vesicles (EPC-EVs) were isolated using gradient ultracentrifugation and ultrafiltration and the influence of EPC-EVs on BMSC osteoblastic differentiation and proliferation was examined in vitro. The results indicated that EPC-EVs regulate the osteoblastic differentiation of BMSCs by inhibiting the expression of osteogenic genes and increasing proliferation in vitro. It is suggested that the results regarding the role of EPC-EVs will provide a novel way to explain the crosstalk between EPCs and BMSCs.

Introduction

Bone mesenchymal stromal cells (BMSCs) are plastic adherent cells and are characterized by their multi-potency, and the ability to give rise to colony forming unit-fibroblasts (CFU-F) (1). These types of cells can differentiate into osteogenic, adipogenic and chondrogenic lineages. Therefore, BMSCs are commonly used to promote tissue regeneration.

Bone regeneration is a complicated and highly regulated process. Bones are regenerated in two distinct ways: Intramembranous ossification and endochondral ossification (2). Flat bones arise from intramembranous ossification, and long bones arise from endochondral ossification, and the mesenchyme condensation marks the beginning of both bone regeneration processes (3). In intramembranous ossification, bones are directly formed by osteoblastic differentiation of BMSCs, whereas endochondral ossification involves the cooperation of multiple cell types, during which chondrocytes mediate the growth and formation of the skeleton (4). In endochondral ossification, chondrocytes in the cartilage stop proliferating, undergo hypertrophy, and secrete type X collagen (5). Hypertrophic chondrocytes guide vessel penetration, osteoblastic differentiation of BMSCs and osteoblast migration. Finally, hypertrophic chondrocytes are programmed to undergo apoptosis, and blood vessels and osteoblasts infiltrate the cartilage matrix and subsequently achieve bone growth and regeneration. Therefore, stimulation of vessel formation and osteoblastic differentiation greatly promote bone regeneration (6).

Bone tissue engineering is a promising therapy to increase vessel formation and bone regeneration (6). An active blood vessel network is a precondition for implants to integrate with the local tissue (7). Endothelial progenitor cells (EPCs) are hematologic precursor cells, defined by Asahara et al (8), that are known for their pro-angiogenic ability (9). The high mobility of EPCs enables the cells to migrate to trauma sites and stimulate neovascularization (10). Therefore, EPCs are often attached to implants to increase vessel penetration. However, the effects of EPCs remain controversial. Duttenhoefer et al (11) identified that EPCs may negatively regulate osteoblastic differentiation of BMSCs in vitro. In contrast, Goerke et al (12) have argued that EPCs support bone regeneration by stimulating vessel formation. Therefore, understanding the communication between EPCs and BMSCs will aid the development of future tissue engineering treatments.

Extracellular vesicles (EVs) are a group of vesicles that include apoptotic bodies, exosomes and microvesicles (13), which are released by almost all cells in the body, including reticulocytes (14), dendritic cells (15), B cells (16), tumor cells (17), mast cells (18), T cells (19), epithelial cells (20) and endothelial cells (21). EVs are widely distributed throughout the body (22) and serve crucial roles in cell-to-cell communication.

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communication. These vesicles can be internalized by the recipient cells through endocytosis (23), and then release functional proteins, DNA and RNA (24). Via this method, EVs can effectively transport and deliver content between cells and regulate homeostasis.

In the present study, the role of EPC-derived EVs in the regulation of osteoblastic differentiation and proliferation of BMSCs was examined. The results demonstrated that EPC-derived EVs can enter BMSCs through endocytosis and release their cargo in the Golgi apparatus, thereby modulating differentiation and proliferation of BMSCs in vitro.

Materials and methods

Cell culture. Mouse BMSCs were isolated from 7-8-week-old male C57BL/6 mice (n=3, weight: 20 g, 12-h light/dark cycle, free access to food and water) supplied by the Shanghai Jiaotong University Affiliated Sixth People's Hospital and housed at 22°C in 50% humidity according to established protocols. The use of all samples was approved by and was conducted in accordance with the Ethical Committee of Shanghai Jiaotong University Affiliated Sixth People's Hospital (Shanghai, China). Briefly, BMSCs were collected according to current protocols (25). Cells from passages 3-5 were used in the experiments. BMSCs were cultured with Dulbecco’s modified Eagle’s medium (DMEM; cat. no. 12571071) and 10% foetal bovine serum (cat. no. 10099141) (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified environment with 5% CO₂.

EPCs isolated from the bone marrow of 7-8-week-old C57BL/6 mice were obtained from BioChain (cat.no. 7030031). These cells express various endothelial markers, including CD31, CD105, vascular endothelial growth factor receptor 1 and neuropilin-1, and have the spindle morphology common to EPCs. These cells are considered to be late-stage EPCs due to the fact that they are negative for CD133, an early stage endothelial progenitor marker. All EPCs were cultured with DMEM and 10% foetal bovine serum at 37°C in a humidified environment with 5% CO₂. EPCs were treated with EPC-derived EVs (5 µg/ml) when the cells reached 70-80% cell confluency. Following 4 h culture, the cells were washed three times with PBS and then fixed with 4% PFA. The endoplasmic reticulum (ER-Tracker Red; cat. no. L12492) (all from Life Technologies; Thermo Fisher Scientific, Inc.) was stained according to the manufacturer’s protocols. The cell nuclei were stained with DAPI according to the manufacturer’s protocol (cat. no. BD5010; Bioworld Technology, Inc., St. Louis Park, MN, USA).

CFU-F assay. BMSCs were diluted in DMEM with 10% FBS and seeded into a 6-well plate at 100 cells/well and treated with EPC-CM (EPC-CM group) and BMSC culture medium (BMSC). Following 14 days of culture at 37°C in a humidified environment with 5% CO₂, the cells were washed three times with 1X PBS and fixed by addition of ice-cold 100% ethanol. The cells were stained with 0.1% crystal violet solution for 10 min at room temperature. Images were captured by light microscopy.

Alizarin Red staining and MTT. Cells were fixed in 70% ethanol for 30 min and rinsed with double-distilled H₂O and then stained with 40 mM Alizarin Red S (cat. no. 130223; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the manufacturer’s protocols and were then added to BMSCs in the culture medium. A total of 1x10⁵ BMSCs were seeded in each well of a 6-well plate. Subsequently, BMSCs were treated with EPC-derived EVs (5 µg/ml) when the cells reached 70-80% cell confluency. Following 4 h culture, the cells were washed three times with PBS and then fixed with 4% PFA. The endoplasmic reticulum (ER-Tracker Red; cat. no. E34250; Golgi apparatus (Golgi-RFP; cat. no. C10593) and lysosomes (Lyso-Tracker Red; cat. no. L12492) (both from Life Technologies; Thermo Fisher Scientific, Inc.) were stained according to the manufacturer’s protocols. The cell nuclei were stained with DAPI according to the manufacturer’s protocol (cat. no. BD5010; Bioworld Technology, Inc., St. Louis Park, MN, USA).

Western blotting. The cells were washed three times with PBS and lysed in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM using a BCA Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

Electron microscopy. Collected EVs were fixed in 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature. Fixed EVs were placed onto a formvar-coated grid and dried at room temperature for 20 min. Subsequent to being washed with PBS, the EVs were fixed in 1% glutaraldehyde for 5 min, washed with water and stained with saturated aqueous uranyl oxalate for 5 min at room temperature. EVs were then embedded in 0.4% uranyl acetate and 1.8% methylcellulose and incubated on ice for 10 min. The excess liquid was then removed. The grid was dried at room temperature for 10 min and viewed at a magnification of x20,000 using an electron microscope (Philips CM 120; Medical Systems B.V., Eindhoven, The Netherlands).

Confluent differentiation and proliferation of BMSCs

For the osteogenic differentiation assay, the osteogenic medium (OM) contained 0.05 mM ascorbate-2 phosphate, 10 mM β-glycerophosphate, and 50 ng/ml dexamethasone, and 10 mM -glycerophosphate. BMSCs were stained with DAPI according to manufacturer’s protocol. Following 14 days of culture at 37°C in a humidified environment with 5% CO₂, the cells were washed three times with 1X PBS and fixed by addition of ice-cold 100% ethanol. The cells were stained with 0.1% crystal violet solution for 10 min at room temperature. Images were captured by light microscopy.

For the CFU-F assay, BMSCs were continuously treated with EPC-CM, EPC-deprived extracellular vesicles (5 µg/ml in all experiments, EPC-EV), EV-depleted EPC-CM (noEV) and BMSC culture medium (BMSC-group).

For the osteogenic differentiation assay, the osteogenic medium (OM) contained 0.05 mM ascorbate-2 phosphate, 10⁻⁸ dexamethasone, and 10 mM β-glycerophosphate. BMSCs were divided into the following 4 groups: EPC-CM + OM, EPC-EV + OM group, noEV + OM and the OM group.

Extracellular vesicle isolation. EVs were isolated from the supernatant of EPC-CM following culturing for 24 h according to current ultracentrifugation protocols (26). Briefly, the EPC culture medium was collected, centrifuged at 500 x g for 30 min at room temperature to remove dead cells and then at 16,500 x g for 20 min at 4°C. This was followed by filtration through a 0.22 µm filter to eliminate cell debris. Then, EVs underwent ultracentrifugation (Beckman Ti70 rotor; Beckman Coulter, Inc., Brea, CA, USA) at 120,000 x g for 2 h at 4°C. The protein content of EVs was measured.
NaCl, 1% (w/v) Nonidet P-40, 0.1% (w/v) SDS, and 1% (w/v) sodium deoxycholate supplemented with phenylmethylsulfonyl fluoride (Shen Neng Bo Cai Corporation, Shanghai, China). The lysates were incubated on ice for 30 min and then centrifuged at 9,000 x g for 10 min at 4°C to precipitate the debris. The protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Then, 30 µg of the protein lysates were separated using 10 and 18% (w/v) SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto PVDF membranes (Roche Diagnostics, Basel, Switzerland). Membranes were blocked with 5% non-fat dry milk in Tris-buffer saline with Tween-20 (TBST) for 2 h and probed with primary antibodies diluted in TBST containing 5% milk at 4°C overnight. Primary antibodies used included rabbit anti-mouse alkaline phosphatase (ALP) antibodies (cat no. ab108337; 1:1,000), rabbit anti-mouse osteocalcin (OCN) antibodies (cat no. ab93876; 1:1,000), rabbit anti-mouse osteopontin (OPN) antibodies (cat no. ab8448; 1:500), rabbit anti-mouse runt-related transcription factor-2 (RUNX-2) antibodies (cat no. ab23981; 1:1,000) and rabbit anti-mouse GAPDH (cat no. ab9485; 1:1,000) (all from Abcam, Cambridge, MA, USA). Membranes were washed and incubated with horseradish peroxidase conjugated goat anti-rabbit secondary antibody (cat no. A0208; 1:1,000; Beyotime Institute of Biotechnology, Haimen, China) for 1 h at room temperature. Targeted proteins were visualized using an enhanced chemiluminescence (ECL) detection system (ChemiDoc™ XRS+ imaging system; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Osteogenic gene expression in BMSCs at day 14 was measured using RT-qPCR for the following marker genes: ALP, RUNX2, OPN and OCN in BMSCs. Total RNA was extracted using the TRizol reagent (Thermo Fisher Scientific, Inc.) and qualified by absorbance at 260 nm. cDNA was synthesized from 1 µg of RNA by Takara RNA PCR kit (Takara Bio, Inc., Otsu, Japan) following the manufacturer's protocols. Real-time PCR was performed using a SYBR Green Master Mix (Takara Bio, Inc.) on ABI StepOnePlus system (Applied Biosystems; Thermo Fisher Scientific, Inc.) SYBR Premix Ex Taq, 12.5 µl; PCR forward primer, 1 µl; PCR reverse primer 1 µl; diethylpyrocarbonate water, 8.5 µl; DNA, 2 µl. The cycling conditions were: 95°C for 30 sec (1 cycle); 95°C for 5 sec, then 60°C for 30 sec (40 cycles). Primer sequences used were: ALP forward, GCCGGCCAGACCAAAGATAG; RUNX2 forward, CCCCTCTAGCCAGACAGATGC and reverse, AAGGCCCCAGTCAGACTCGT and reverse, GGTAGTGAG TTTCCTCTGTCGCGC; OCN forward, GCCAGCTGAGCAGCTCG; β-actin forward, CAGCAGATTGTCGCGCGT GACAT and reverse, GGACTCGTACATCTCTCTCTGTCG. The relative expression level of genes was normalized to the value of GAPDH by the $2^{-\Delta\Delta C_q}$ method (26), allowing the calculation of differences in gene expression using the ABI software.

**Statistical analysis.** CFU-F analysis, Alizarin staining, MTT and RT-qPCR analyses were repeated three times, and data are presented as the mean ± standard deviation. Differences among the results were assessed using Bonferroni’s multiple comparison tests, and statistical significance was analysed using SPSS, version 20.0 software (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**EPC-CM inhibits osteogenic differentiation and promotes proliferation of BMSCs.** To examine the potential influence of EPC-derived EVs on BMSCs, whether EPC-CM could regulate the osteoblastic differentiation and proliferation of BMSCs was measured. The results indicated that the OM group had an increased number of calcium deposits compared with that of the EPCs-CM + OM group (Fig. 1A). The OD ratio of both groups at day 14 also confirmed the results (Fig. 1B; P=0.003). Furthermore, it was identified that the BMSCs in the EPCs-CM group formed an increased number of CFU-Fs compared with the OM group (Fig. 1; P<0.0038). Altogether, these results demonstrate that EPC-CM inhibits osteoblastic differentiation and

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**Figure 1.** (A) Alizarin Red staining and CFU-F assay for 14 days. (B) The OM group (0.93±0.02) exhibited a significant increase in calcium deposition, as compared with the EPC-CM + OM group (0.47±0.03) (P<0.05, P=0.0003). There was an increased number of CFU-Fs in the EPC-CM group (49±3.1) when compared with the BMSC medium group (14±1.2; P<0.05, P=0.000027). "P<0.001 and ""P<0.0001. CFU-F, colony forming unit-fibroblasts; OM, osteogenic medium; EPC, endothelial progenitor cell; CM, conditioned medium; BMSC, bone marrow stromal cell.
stimulates cell proliferation of BMSCs. These results confirm the results of an earlier report (11).

**EPC-derived extracellular vesicle isolation.** Prior to examining the function of EPC-derived extracellular vesicles (EPC-EVs), it was determined whether EPC-EVs are isolated and can enter BMSCs. The EPC-EVs were isolated according to a commonly used ultracentrifugation protocol and analysed using electron microscopy (Fig. 2A, red arrows) (27). In addition, CD63, a common marker of EVs, was used in western blot analysis to further confirm that EVs are purified from EPC-CM (Fig. 2A) (28). Subsequently, it was examined whether the EPC-EVs were absorbed by the BMSCs. Therefore, the collected EVs were labelled with PKH67 (green), a stable green fluorescent cell membrane linker. Subsequently, the PKH67-stained vesicles were added to the BMSC culture medium. Following a 4 h incubation, the cells were fixed and labelled with diverse organelle-specific dyes (red) and were imaged and analysed with confocal microscopy. As presented in Fig. 2B, the PKH67-labelled EVs (green dots) were absorbed by the BMSCs and presented in the endoplasmic reticulum (ER-Tracker Red), Golgi apparatus (Golgi-RFP) and lysosomes (Lyso-Tracker Red). These results indicated that EPC-EVs fused with the cells and were degraded by the Golgi apparatus, releasing their cargo along the way. However, it was not possible to demonstrate an association between EPC-EVs and lysosomes, which have been suggested to digest EVs (29).

**EPC-derived extracellular vesicles inhibit osteogenic differentiation of BMSCs.** Because EPC-EVs are absorbed by BMSCs, the function of EPC-EVs was investigated using...
osteoblastic differentiation assays. Alizarin Red staining demonstrated that there was a marked decrease in calcium deposits in the EPC-CM + OM group and EPC-EV + OM group compared with the noEV + OM group and OM group on days 7 and 14 (Fig. 3A). Western blot analyses further confirmed that osteoblastic differentiated-related proteins were downregulated in the EPC-CM + OM group and EPC-EV + OM group (Fig. 3B). Accordingly, the expression of osteoblastic markers and osteogenic genes, including ALP, RUNX2, OPN and OCN, were additionally decreased in the EPC-CM + OM and EPC-EV + OM groups. Compared with the EV-group, the expression levels of ALP, OCN, OPN and RUNX2 were decreased by 2.4-, 2.0-, 3.1- and 1.6-fold, respectively, in the OM group vs. the EPC-EV + OM group; "****P<0.0001. OM, osteogenic medium; ALP, alkaline phosphatase; OCN, osteocalcin; OPN, osteopontin; RUNX2, runt-related transcription factor-2; EPC, endothelial progenitor cell; CM, conditioned medium; EV, extracellular vesicle; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 3. (A) The macroscopic Alizarin Red staining for the OM-group, the EPC-CM + OM group, EPC-EV + OM group, noEV + OM group and OM group at day 1 and day 14. (B) Western blot analysis of ALP, OCN, OPN and RUNX2 at day 14. (C) The expression of ALP, OCN, OPN and RUNX2 increased by 1.9-, 3.8-, 3.2- and 2.4-fold, respectively, in the OM group vs. the EPC-EV + OM group; "****P<0.0001. OM, osteogenic medium; ALP, alkaline phosphatase; OCN, osteocalcin; OPN, osteopontin; RUNX2, runt-related transcription factor-2; EPC, endothelial progenitor cell; CM, conditioned medium; EV, extracellular vesicle; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Discussion

EPCs have been demonstrated to induce vessel formation in vivo and in vitro and are commonly used in tissue
engineering to promote bone regeneration (30). EPCs exhibit good vessel formation when coated with matrigel (31) and attenuate limb ischaemic mouse models (32). However, EPCs have a controversial influence on the proliferation and osteoblastic differentiation of BMSCs. Duttenhoefer et al (11) demonstrated that BMSCs co-cultured with EPCs exhibit a significantly reduced level of osteogenic gene expression and less calcium deposition. Furthermore, Wen et al (33) established an EPC/BMSC indirect Transwell culture system, and identified that co-cultured BMCs proliferate more than mono-cultured MSCs. In the present study, the osteoblastic differentiation of BMSCs treated with EPC-derived EVs was inhibited, whereas the number of CFU-Fs was increased.

Although the osteoblastic differentiation of BMSCs is inhibited by EPC-EVs, the exact mechanism remains unclear. EVs deliver a large variety of cargo, including proteins, DNA and RNA. The proteins and RNA in EVs serve critical roles in regulating the differentiation and function of recipient cells (34). Spectrometry data of EVs have identified over 4,000 different proteins in the exosomes (35). Although the proteins differ substantially according to their origin and have different functions, some proteins are shared by all types of EVs (36). These shared proteins are associated with cell-to-cell communication. Trafficking-associated proteins, including heat shock protein (HSP) 70 and HSP90, and cytoskeletal proteins, including myosin, actin and tubulin, are widely distributed in EVs (37). Additionally, studies have demonstrated that small RNAs in EVs also have an influence on target cells (38,39). Koppers-Lalic et al (34) reviewed the known small RNAs in EVs and noted that the functional RNAs are critical in the regulation of cell commitment, differentiation and activity. Therefore, profiling of proteins, DNA and RNAs in EPC-derived EVs will aid in the exploration of communication between EPCs and BMSCs.

The confocal microscopy data indicated that the green round-like fluorescence (red arrow) in the EVs was internalized, transported to and degraded by the Golgi apparatus, the lumen of which was stained red (white arrow). These results suggested that the cargo in the EVs was released in the Golgi apparatus rather than in the lysosomes. However, the exact mechanism of EV degradation remains controversial. Baixauli et al (40) observed that EVs that are internalized by cells may fuse with either the lysosomes or the multivesicular bodies. Alvarez-Erviti et al (41) proposed that lysosomal dysfunction in SH-SY5Y cells is associated with increased EV release, thus indicating a critical role of lysosomes in EV degradation. Previous studies have demonstrated that other cell organelles may also serve a prominent role in EV degradation. Campanella et al (42) identified that EVs are transported to the Golgi apparatus by HSP60 located in tumor cells. A previous study additionally demonstrated that the Golgi apparatus is key to EV degradation (43). Therefore, how the cells degrade the EVs requires further investigation.

In conclusion, EPC-CM inhibits osteogenic gene expression and calcium deposition in BMSCs in vitro. EPC-derived EVs are one of the major regulators in EPC-CM. Osteoblastic differentiation of BMSCs was inhibited by EPC-derived EVs, whereas BMSC proliferation was increased. BMSCs treated with EPC-derived EVs exhibited fewer calcium deposits however increased CFU-Fs. Western blot analysis and RT-qPCR results also demonstrated that osteoblastic differentiation was inhibited. In addition, it was observed that EPC-EVs were delivered to and degraded by the Golgi apparatus rather than by the lysosomes in BMSCs. The present study demonstrated that EPCs communicate and regulate BMSCs through EPC-derived EVs and provides a foundation for further exploration of the communication between EPCs and BMSCs.

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