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Bidirectional juxtacrine ephrinB2/Ephs signaling promotes angiogenesis of ECs and maintains self-renewal of MSCs

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ABSTRACT

Co-transplantation of endothelial cells (ECs) and mesenchymal stem cells (MSCs) is an important strategy for repairing complex and large bone defects. However, the ways in which ECs and MSCs interact remain to be fully clarified. We found that forward ephrinB2/Ephs signaling from hBMSCs to hUVECs promoted the tube formation of hUVECs by activating the PI3K/AKT/mTOR pathway. Reverse ephrinB2/Ephs signaling from hUVECs to hBMSCs promoted the proliferation and maintenance of hBMSCs self-renewal *via* upregulation of OCT4, SOX2, and YAP1. Subcutaneous co-transplantation of ECs and MSCs in nude mice confirmed that forward ephrinB2/Ephs signaling could increase the cross-sectional area of blood vessels in the transplanted area, and reverse ephrinB2/Ephs signaling could maintain the self-renewal of transplanted hBMSCs *in vivo*. Based on these results, ephrinB2/Ephs bidirectional juxtacrine regulation between ECs and MSCs plays a pivotal role in improving the healing of bone defects by promoting angiogenesis and achieving a sufficient number of MSCs.

1. Introduction

Osteogenesis and angiogenesis are two fundamental elements in the process of bone defect healing [1–3]. Among the strategies for repairing large and complex bone defects, cell implantation alone or in combination with a scaffold are important and promising methodologies. Due to the multiple cell types involved in the cascade of bone regeneration, the implantation regimen of multiple cell types could result in better outcomes than single-cell implantation. Currently, the combinational implantation of vessel-forming endothelial cells (ECs) and bone-forming mesenchymal stem cells (MSCs) is considered a promising novel strategy for promoting the repair of critical-size bone defects [4]. A detailed understanding of the crosstalk between MSCs and ECs could help optimize joint implantation strategies.

Paracrine, microvesicle, and juxtacrine are the three main interactions between cells. Previous studies have focused on the paracrine and microvesicle crosstalk between ECs and MSCs. The osteogenic differentiation of MSCs is promoted by cytokines produced by endothelial cells, among which the bone morphology protein-2 (BMP-2) is the most recognized [5]. Angiogenesis can be enhanced by cytokines secreted from MSCs, such as vascular endothelial growth factor (VEGF) [6]. The vesicles from MSCs possess several pro-angiogenic properties [7]. However, the osculating coordination between ECs and MSCs cannot be fully explained by indirect contact. For instance, some MSCs still maintain self-renewal in bone marrow when the blood supply is sufficient [8,9]. Other regulative mechanisms between ECs and MSCs may exist. Evidence indicates direct contact between ECs and MSCs occurs *in vivo*. For example, ECs and MSCs are always in close proximity in the bone marrow [10–12]. Periocytes of blood vessels, which are in direct contact with endothelial cells, have been shown to be a pool of MSCs [13,14]. These phenomena indicate that juxtacrine regulation might exert important effects on the interaction between ECs and MSCs.

Ephrin receptors (Ephs), the largest receptor tyrosine kinase family in mammals, form a bidirectional signaling system with ephrins (which are also transmembrane proteins). The ephrins/Ephs pathway is a contact-dependent, bidirectional, and juxtacrine signaling system important in prenatal and postnatal angiogenesis in physiology and pathology [15]. However, the role and

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mechanism of ephrins/Ephs signaling in angiogenesis during bone defect healing is still unclear. Especially, the role of ephrins/Ephs in the interaction of vessel-forming ECs and bone-forming MSCs when they are co-transplanted to repair bone defects, remains to be elucidated.

The aim of this study is to clarify the functions of ephrinB2/Ephs signaling in the direct crosstalk between ECs and MSCs. Co-cultured human umbilical vein endothelial cells (hUVECs) and human bone marrow mesenchymal stem cells (hBMSCs) were used to investigate the forward ephrinB2/Ephs signaling from MSCs to ECs and the reverse ephrinB2/Ephs signaling from ECs to MSCs. The subcutaneous co-implantation of hUVECs and hBMSCs in mice was used to confirm the function of forward ephrinB2/Ephs signaling from MSCs to ECs and of reverse ephrinB2/Ephs signaling from ECs to MSCs *in vivo*. This finding will not only deepen our understanding of the involvement of ECs and MSCs in the healing of bone defects but also help standardize the clinical regimen for the combinational transplantation of ECs and MSCs for bone defect repair.

2. Materials and methods

2.1. Reagents

To prepare the pre-clustered form (cephrinB2, cEphB4) at a final concentration of 4 μg/mL [16], 40 μg rabbit anti-human Fc of immunoglobulin G (IgG) (31142; Pierce, Rockford, IL, USA) was mixed with 4 μg of ephrinB2 (E0778; Sigma-Aldrich, St. Louis, MO, USA) or 4 μg of EphB4 (E9652; Sigma-Aldrich); pre-clustered IgG (cIgG) was used as a control. Monomeric EphB4 (E9652; Sigma-Aldrich) was used as a neutralizing antibody to disconnect the cross-talk between ephrinB2 and Ephs, and human IgG was used as a control. AZD-8055 (HY-10422; MedChem Express, Monmouth Jct., NJ, USA) was used to inhibit the phosphorylation of mTOR.

2.2. Cell culture

The two human primary cell lines, hBMSCs and hUVECs, were purchased from ScienCell Research Laboratories and cultured in mesenchymal stem cell medium (MSCM; 7501; ScienCell Research Laboratories, Carlsbad, CA, USA) and endothelial cell medium (ECM; 1001; ScienCell Research Laboratories), respectively, in a humidified chamber with 5% CO_2 at 37 °C. Cells for all experiments were used at passages 3–6. The hBMSCs and hUVECs were tested and confirmed as the Supplementary Information 2 and 3 have shown.

2.3. In vitro tube formation assays

Under sterile conditions, 24-well plates were coated with 250 μL Matrigel (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) per well without introducing air bubbles. The plates sat at room temperature for at least 60 min to allow the Matrigel to gel. Next, hBMSCs + hUVECs (6×10^4 of hBMSCs and 6×10^4 of hUVECs), hUVECs*2 (12×10^4 of hUVECs), hUVECs (6×10^4 of hUVECs), and hBMSCs (12×10^4 of hBMSCs) were plated on the Matrigel. Finally, 300 μL of serum-free medium was added, and the 24-well plates were incubated at 37 °C in 5% CO_2 air incubator. Tube structures were observed under a phase contrast microscope after 4 and 8 h.

2.4. Lentivirus production and transfection

All lentivirus vectors fused with green fluorescent protein (GFP) for knockdown of *EFNB2* were purchased from Gene Pharma (Suzhou, China).

Sequence of shRNA #1:

5′-CCGGCGACAACAAGTCCCTTTGTAACTCGAGTTA-CAAAGGGACTTGTTGTCGTTTTTG-3'.

Sequence of shRNA #2:

5′-CCGGCTGGTACTATACCCACAGATACTCGAGTATCTGTGGGTA-TAGTACCAGTTTTTG -3'.

Cells transfected with scramble were treated as controls.

The lentivirus vector fused with GFP labeled with the *EFNB2* gene to upregulate *EFNB2* was purchased from Gene Pharma (Suzhou, China).

One day before lentiviral transfection, hBMSCs were seeded in a 75-cm^2 cell culture flask at a density of 15,000 cells/cm^2. Next, 100 μL of lentiviral particles (3×10^8 TU/mL; Gene Pharma, Suzhou, China) were added with 5 μg/mL polybrene (Gene Pharma) and MSCM to the cell culture for 24 h. Next, transfected cells were selected using puromycin (P8833; Sigma-Aldrich) for 3 days.

2.5. Western blot

The lysis of cultured cells was performed by RIPA lysis buffer (P0013B; Beyotime, Shanghai, China) on ice. The supernatants were gathered after centrifugation at 12,000 g at 4 °C for 20 min. The protein concentration was assessed by BCA protein assay kit (P0012; Beyotime), and whole lysates were mixed with 5 × SDS loading buffer (P0015; Beyotime) in a 1:4 ratio. The samples were heated for 5 min at the temperature of 100 °C, separated in SDS-polyacrylamide gels, and proteins were transferred to PVDF membrane. The membranes were incubated by the primary antibody overnight at 4 °C. After incubation with secondary antibody conjugated by horseradish peroxidase (HRP), autoradiograms were performed using ECL Western Blotting Substrate (32109; Pierce). The blots were recorded using x-ray film. The primary antibodies were anti-GAPDH (ab9485; Abcam), anti-ephrinB2 (ab150411; Abcam), anti-PI3K (ab86714; Abcam), anti-AKT phospho S473 (4060; Cell Signaling Technology, Boston, MA, USA), anti-AKT (4691; Cell Signaling Technology), anti-mTOR (ab32028; Abcam), anti-mTOR phospho S2481 (ab137133; Abcam), anti-SOX2 (ab92494; Abcam), anti-YAP1 (ab52771; Abcam), and anti-OCT4 (ab19857; Abcam). The secondary antibody was HRP-labeled IgG (A0208, A0216; Beyotime). GAPDH were used as the protein loading control. The protein expressions were normalized to GAPDH.

2.6. Proliferation assay

Subconfluent cells were used to perform cell proliferation assays. Cells at a density of 5×10^3 cells per well per 100 μL medium were plated in 96-well culture plates. Cells were starved in medium with 0% fetal bovine serum (FBS) for 12 h. Then, cell medium was replaced with MSCM containing 0%, 5%, or 10% FBS and the test substances. The cultures were incubated for 1, 2, or 3 days at 37 °C under 5% CO_2. Cell activity was assessed using Cell Counting Kit-8 (CCK-8) kit according to the instructions (CK04; Dojindo, Kyushu, Japan).

2.7. Alkaline phosphatase (ALP) staining and quantification

Cells cultured for 3 and 7 days were performed for alkaline phosphatase (ALP) activity and quantification. ALP staining was performed using the nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (NBT/BCIP) staining kit (C3206; Beyotime). ALP quantification was performed using the ALP assay kit according to the instructions (A059-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). ALP activity was analyzed after normalization to the total protein content.