

VEGF secreted by mesenchymal stem cells mediates the differentiation of endothelial progenitor cells into endothelial cells via paracrine mechanisms

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Abstract. Stem cell therapy is a promising treatment strategy for ischemic diseases. Mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs) adhere to each other in the bone marrow cavity and in *in vitro* cultures. We have previously demonstrated that the adhesion between MSCs and EPCs is critical for MSC self-renewal and their multi-differentiation into osteoblasts and chondrocytes. In the present study, the influence of the indirect communication between EPCs and MSCs on the endothelial differentiation potential of EPCs was investigated, and the molecular mechanisms underlying MSC-mediated EPC differentiation were explored. The effects of vascular endothelial growth factor (VEGF), which is secreted by MSCs, on EPC differentiation via paracrine mechanisms were examined via co-culturing MSCs and EPCs. Reverse transcription-quantitative polymerase chain reaction and western blot analysis were used to detect the expression of genes and proteins of interest. The present results demonstrated that co-culturing EPCs with MSCs enhanced the expression of cluster of differentiation 31 and von Willebrand factor, which are specific markers of an endothelial phenotype, thus indicating that MSCs may influence the endothelial differentiation of EPCs *in vitro*. VEGF appeared to be critical to this process.

These findings are important for the understanding of the biological interactions between MSCs and EPCs, and for the development of applications of stem cell-based therapy in the treatment of ischemic diseases.

Introduction

Chronic ischemic disease, including Buerger's disease, diabetic foot ulcers and ischemic heart disease, seriously impair the patients' quality of life. Chronic ischemic diseases are primarily treated with pharmacological agents, surgery and endovascular intervention (1). However, currently available treatment strategies do not achieve optimal effects when the integrity of the vascular outflow tract is compromised. In addition, the application of these treatments is limited to high-risk patients, despite surgical procedures inevitably increasing vascular damage. Endothelial cells are involved in the mechanisms underlying vascular injury repair (2), and transplantation of stem cells in patients with ischemic disease has been revealed to be successful (3-5). Mesenchymal stem cells (MSCs) are a subtype of somatic stem cells that originate from the bone marrow. MSCs are characterized by multipotent differentiation into various cell lineages, including osteoblasts, chondrocytes, and adipocytes (6-8). Asahara *et al* (9) initially described endothelial progenitor cells (EPCs), which are the predecessors of endothelial cells and mainly originate from the bone marrow. EPCs can be recruited and mobilized in the serum in response to local stimulation and cell-cell interactions: EPCs differentiate into endothelial cells to participate in angiogenesis and tissue lesion repair (10,11). Therapeutic strategies based on vascular stem cells are currently under research for the treatment of several clinical conditions (12).

Previous studies from our lab have reported that MSCs and EPCs adhere to each other in the bone marrow cavity and *in vitro* (13,14), and that this mutual adhesion is important for the biological functions of both cell types. EPCs have been demonstrated to promote the differentiation of MSCs into osteoblasts (15); however, the effects of MSCs on the endothelial differentiation of EPCs have yet to be elucidated.

Cell differentiation is a result of selective gene expression. Cell differentiation pathways include extracellular and

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intracellular signal transduction, and the role of regulatory transcription factors is crucial (16,17). Extracellular signals, including bone morphogenetic protein 2 and growth factors, interact with cell-surface receptors to initiate cellular differentiation through the regulation of transcription factors (18). Previous studies have suggested that the entire differentiation repertoire of a given multipotent stem cell may theoretically be specified by a single determining factor that is located at the top of a regulatory hierarchy (19,20). Previous research on the interaction between MSCs and endothelial cells has demonstrated the formation of microvessel-like structures (21). The interactions between MSCs and endothelial cells are regulated by paracrine factors, including vascular endothelial growth factor (VEGF) (22), which is a potent angiogenic factor. VEGF-induced mobilization of bone marrow-derived EPCs has been reported to enhance EPC differentiation *in vitro* and to potentiate corneal neovascularization *in vivo* (23).

Therefore, the present study aimed to investigate whether MSC-derived VEGF may mediate the differentiation of EPCs into endothelial cells and to explore the regulatory roles of paracrine pathways in this process. Cluster of differentiation (CD)31 and von Willebrand Factor (vWF) were used as specific markers for an endothelial phenotype (24,25).

Materials and methods

Cell source and ethical approval. All experimental protocols used in the present study were reviewed and approved by the Animal Care and Use Committee of Shihezi University (Shihezi, China). A total of 24 male C57BL/6J mice (wild-type; age, 6 weeks; weight, 28–35 g) were purchased from Xinjiang Medical University (Ürümqi, China; certificate no. SYXK [Xin] 2010-0001). Mice were maintained in the Animal Facility of Shihezi University (Shihezi, China) under controlled conditions (temperature, 20°C; humidity, 55±5%; 12-h light/dark cycles), with free access to food and water and were used as a cell source. The technique that was used to harvest and culture all cell types was the same, except for the materials and the culture media that were used. All cells used in subsequent experiments were the third generation.

Isolation and culture of murine bone marrow MSCs (BMMSCs). BMMSCs were isolated using the technique reported in our previous studies (13,14). Briefly, bone marrow cells were collected from 6-week-old wild-type male C57BL/6 mice euthanized by cervical dislocation. The cells were cultured in low-glucose Dulbecco's modified Eagle's medium (LG-DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 100 U/ml penicillin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), streptomycin sulfate (100 µg/ml; Sigma-Aldrich; Merck KGaA), and 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C in a 5% CO₂ humidified incubator. Following 72 h of adhesion, non-adherent cells were removed, and adherent cells were cultured for an additional 7 days with a single change of medium on the third day. Adherent cells were then retrieved by trypsin digestion.

Aliquots of cells (1×10⁶) were incubated for 20 min at 4°C with phycoerythrin (PE)-conjugated anti-stem cells antigen (Sca)-1 (cat. no. 108107; dilution, 1:40),

fluorescein isothiocyanate (FITC)-conjugated anti-CD29 (cat. no. 102205; dilution, 1:50), peridinin chlorophyll protein (Per CP)-conjugated CD45 (cat. no. 202220; dilution, 1:20) and allophycocyanin (APC)-conjugated anti-CD11b (cat. no. 201809; dilution, 1:100; all from BioLegend, Inc., San Diego, CA, USA). Acquisition was performed by fluorescence-activated cell sorting (FACS) using a FACS Aria flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and analysis was performed using FACSDiva software version 6.1.3 (BD Biosciences). The sorted CD29⁺/Sca-1⁺/CD45⁺/CD11b⁺ cells were enriched by further culture in LG-DMEM containing penicillin, streptomycin sulfate and 10% FBS at 37°C in a 5% CO₂ humidified incubator.

Isolation and characterization of mouse bone marrow EPCs (BMEPCs). Bone marrow cells were collected and cultured as aforementioned. Cell aliquots (1×10⁶) were incubated for 20 min at 4°C with the following anti-mouse antibodies: APC-conjugated anti-CD11b (dilution, 1:100; BioLegend, Inc.), FITC-conjugated anti-CD31 (cat. no. 102506; dilution, 1:50; BioLegend, Inc.), Per CP-conjugated anti-CD144 (cat. no. 46-1441-82; dilution, 1:50; eBioscience; Thermo Fisher Scientific, Inc.) and PE-conjugated anti-CD133 (cat. no. 141203; dilution, 1:40; BioLegend, Inc.). Acquisition was performed using a FACS Aria flow cytometer, and data were analyzed using FACSDiva software version 6.1.3. Sorted CD133⁺/CD31⁺/CD144⁺/CD11b⁺ cells were enriched by further culture in endothelial growth basal medium (EBM)-2 (Lonza Group, Ltd., Basel, Switzerland).

Experimental groups and induction culture conditions. A total of 3 experimental groups were used: i) Single culture group, where EPCs were seeded in a 6-well plate at a density of 5×10⁵ cells/plate. This group was used as a negative control. ii) Co-culture group, where MSCs were plated into the upper and EPCs into the lower chambers of transwell inserts (Corning Incorporated, Corning, NY, USA) at a density of 5×10⁵ cells/insert. iii) VEGF group, where EPCs (5×10⁵ cells) were seeded in a 6-well plate, in medium supplemented with recombinant VEGF (20 ng/ml; PeproTech, Inc., Rocky Hill, NJ, USA). This group was used as a positive control. The cell culture medium used in the experiments was EBM-2 supplemented with 5% FBS (Hyclone; GE Healthcare Life Sciences), 100 U/ml penicillin (Sigma-Aldrich; Merck KGaA) and 100 µg/ml streptomycin (Sigma-Aldrich; Merck KGaA). Cells were incubated for 48 h at 37°C in a 5% CO₂ humidified incubator. The alterations in cell morphology were observed under an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Immunofluorescence. EPCs (1×10⁵ cells) were washed 3 times in PBS, fixed in 4% paraformaldehyde for 15 min at room temperature, and blocked in 3% BSA (Sigma-Aldrich; Merck KGaA) to block non-specific binding sites at 37°C for 1 h. Cells were then incubated with the following primary antibodies for 4 h at 37°C: Rabbit polyclonal anti-CD31 (cat. no. ab28364; 1:50; Abcam, Cambridge, MA, USA) and goat polyclonal anti-vWF (cat. no. ab11713; 1:100; Abcam). Following washing 3 times with PBS, cells were incubated with secondary FITC-conjugated goat anti-rabbit immunoglobulin (Ig)G (cat.

no. TA130021; 1:100; OriGene Technologies, Inc., Beijing, China,) and secondary FITC-conjugated rabbit anti-goat IgG (cat. no. TA130029; 1:200; OriGene Technologies, Inc.) at 37°C for 1 h. Cells were then washed and soaked in ddH₂O 3 times and examined under a DM IL Leica fluorescence microscope (Leica Microsystems GmbH). Experiments were performed in duplicate and repeated at least 3 times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cultured EPCs (1x10⁶ cells) were washed twice with ice-cold PBS. Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA purity was determined by the ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}) using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA), where a range of 1.8-2.0 was considered suitable for cDNA synthesis. Total RNA (200 ng) was reverse transcribed into cDNA using a QuantiTect Reverse Transcription kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. The temperature protocol was as follows: Initial denaturation at 70°C for 5 min, then 4°C for 2 min, followed by 42°C for 1 h and 70°C for 5 min. qPCR was performed on cDNA using the QuantiNova SYBR Green PCR kit (Qiagen, Inc.), according to the manufacturer's protocol. The thermocycling conditions were as follows: Initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 10 sec and at 55°C for 30 sec. The reaction included 10 μ l SYBR Green PCR mix, 2 μ l primers, 3 μ l cDNA (500 ng) and 5 μ l DNase/RNase free water to a final reaction volume of 20 μ l. The results were analyzed using Bio-Rad CFX96 Manager software version 3.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Data were collected after each annealing step. β -actin was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. The primer sequences and the sizes of the amplified fragments were as follows: CD31 (114 bp), forward 5'-CCACAGAGCCAGCAGTA TG-3', reverse 5'-TGACCACTCCAATGA CAACC-3'; vWF (108 bp), forward 5'-TGCCTCAGTGGG AGAAAGAT-3', reverse 5'-CAGGTTTGTGCTCTGCTT GA-3'; and β -actin (203 bp), forward 5'-TTCCTTCTTGGG TATGGAAT-3' and reverse 5'-GAGCAATGATCTTGATCT TC-3'. Experiments were performed in triplicate and repeated 3 times. Results were quantified using the 2^{- $\Delta\Delta C_q$} method (26).

Western blot analysis. Cultured EPCs (1x10⁶ cells) were washed twice with ice-cold PBS. Total proteins were extracted from EPCs using radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.) containing phenylmethylsulfonyl fluoride (Thermo Fisher Scientific, Inc.). Protein concentration was measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of extracted protein samples (40 μ g) were separated by 6% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked by incubation in TBS containing 3% BSA (Sigma-Aldrich; Merck KGaA) for 2 h at room temperature. Membranes were incubated overnight at 4°C with the following primary antibodies: polyclonal rabbit anti-CD31 (cat. no. ab28364; 1:500; Abcam) and polyclonal goat anti-vWF (cat. no. ab11713; 1:1,000; Abcam), with gentle agitation. After washing with TBS, the membrane was incubated for 2 h at

room temperature with goat anti-rabbit (cat. no. ZB-2301; 1:10,000), rabbit anti-goat (cat. no. ZB-2306; 1:10,000) or goat anti-mouse (cat. no. ZB-2305; 1:20,000; all from OriGene Technologies, Inc.) horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized using enhanced chemiluminescence (GE Healthcare Life Sciences). β -actin (cat. no. NB600-501; 1:1,000, Novus Biologicals, LLC, Littleton, CO, USA) was used as an endogenous control for normalization. Blots were semi-quantified by densitometry using Gel-Pro Analyzer software version 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). Experiments were performed in triplicate and repeated 3 times.

Detection of VEGF levels using ELISA. MSCs that were at passage 3 were cultured at a density of 1x10⁶ cells/plate in serum- and growth factor-free LG-DMEM without FBS for 24 h at 37°C in an atmosphere containing 5% CO₂. The medium was subsequently collected, passed through a 0.22 μ m filter, and the concentration of VEGF in the MSC conditioned media (MSC^{CM}) was measured using an ELISA kit (cat. no. E-EL-M1292c; Elabscience Biotechnology Co., Ltd., Wuhan, China) according to the manufacturer's protocol. The absorbance at 450 nm was measured using a Bio-Rad Model 3550-UV microplate reader (Bio-Rad Laboratories, Inc.). Experiments were performed in triplicate and repeated 3 times.

The culture medium of the cells from the 3 experimental groups was also collected, following 48 h of culture, passed through a 0.22 μ m filter, and used for VEGF detection by ELISA as aforementioned.

VEGF blocking assay. A total of 4 experimental groups were used: i) LG-DMEM group, where EPCs (5x10⁵) that were at passage 3 were seeded in LG-DMEM without FBS for 48 h at 37°C in a 5% CO₂ humidified incubator; ii) MSC^{CM} group, where EPCs (5x10⁵) that were at passage 3 were seeded in MSC^{CM} for 48 h at 37°C in a 5% CO₂ humidified incubator; iii) MSC^{CM} with mouse IgG group, where EPCs (5x10⁵) that were at passage 3 were seeded in MSC^{CM} with mouse IgG (cat. no. ZDR-5109; 100 ng/ml; OriGene Technologies, Inc.) for 48 h at 37°C in a 5% CO₂ humidified incubator; iv) MSC^{CM} with anti-VEGF antibody group, where EPCs (5x10⁵) that were at passage 3 were seeded in MSC^{CM} with anti-VEGF antibody (cat. no. ab9570; 100 ng/ml; Abcam) for 48 h at 37°C in a 5% CO₂ humidified incubator.

Statistical analysis. Statistical analyses were conducted using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean $\pm$ standard error of the mean. The statistical significance of the differences between groups was assessed using an unpaired Student's t-test for pair-wise comparisons or a one-way analysis of variance followed by Dunnett's post hoc multiple comparisons test. P<0.05 was considered to indicate a statistically significant difference.

Results

MSCs promote the endothelial differentiation of EPCs. We have previously reported that MSCs and EPCs adhere to each other in the bone marrow cavity and *in vitro* (13,14), and that

this mutual adhesion is important for the biological functions of both cell types. EPCs have also been reported to promote the differentiation of MSCs into osteoblasts (15). Therefore, the effects of MSCs on EPC differentiation were investigated in the present study. EPCs and MSCs were co-cultured in transwell inserts in the absence of direct cellular interactions. Fig. 1 demonstrated the morphological alterations that were observed in differentiated EPCs. Cells in the single culture group appeared to be irregularly shaped (Fig. 1A), whereas cells in the co-culture group displayed a cobblestone endothelial-like appearance (Fig. 1B). These findings suggested that MSCs may promote the endothelial differentiation of EPCs *in vitro*.

VEGF is implicated in EPC differentiation. Previous studies (21-23) have suggested a role for VEGF in MSC-promoted EPC differentiation. EPCs that were cultured in the presence of VEGF (20 ng/ml) stimulation were used as a positive control in the present study. As presented in Fig. 2, following 48 h of culture, EPCs cultured alone appeared to be irregularly shaped (Fig. 2A), whereas cells in the co-culture and VEGF groups demonstrated a cobblestone appearance (Fig. 2B and C).

To assess the phenotypes of the differentiated cells, the expression of the endothelial cell markers CD31 and vWF was examined (Fig. 3). EPCs in the single culture group were revealed to weakly express CD31 (Fig. 3A) and vWF (Fig. 3D), whereas EPCs in the co-culture and VEGF groups highly expressed CD31 (Fig. 3B and C) and vWF (Fig. 3E and F). The expression of specific endothelial markers was quantified using RT-qPCR and western blotting. RT-qPCR demonstrated that the mRNA expression levels of CD31 and vWF were significantly upregulated in the co-culture and VEGF groups compared with in cells from the single culture group (Fig. 3G). Similarly, western blot analysis revealed that CD31 and vWF protein expression was significantly enhanced in MSC-co-cultured and VEGF-treated cells compared with in the single culture group (Fig. 3H and I). In addition, the protein expression levels of CD31 and vWF were significantly increased in the co-culture group compared with in the VEGF group (Fig. 3H and I). These results suggested that VEGF may promote the differentiation of EPCs into endothelial cells *in vitro*.

MSCs secrete VEGF. On the basis of the aforementioned findings, we investigated whether MSCs may secrete VEGF, which may mediate EPC differentiation. VEGF levels in MSC^{CM} were measured using ELISA. As presented in Fig. 4, VEGF levels were significantly upregulated in MSC^{CM} compared with in serum- and growth factor-free LG-DMEM. A previous study from our group (15) demonstrated that EPCs secreted VEGF; in combination with the present results, it may be hypothesized that the MSC-promoted EPC differentiation may be related to VEGF secretion by MSCs.

VEGF levels are increased in co-culture conditioned media. Based on the aforementioned findings, the culture medium from each of the 3 experimental groups was used to measure VEGF levels via ELISA. Compared with the single culture group, VEGF levels were significantly upregulated in conditioned

media from the co-culture and VEGF groups (Fig. 5). No statistically significant differences in VEGF levels were detected between the co-culture and VEGF groups (Fig. 5). These results also suggested that MSC-secreted VEGF may mediate the endothelial differentiation of EPCs *in vitro*.

Anti-VEGF inhibits the differentiation of EPCs. To further investigate whether MSC-secreted VEGF may promote the endothelial differentiation of EPCs, cells were treated with a VEGF neutralizing antibody or mouse IgG (Fig. 6). EPCs were cultured in LG-DMEM, MSC^{CM}, MSC^{CM} with mouse IgG, and MSC^{CM} with anti-VEGF antibody for 48 h. The expression of CD31 (Fig. 6A-C) and vWF (Fig. 6E-G) appeared to be enhanced in the MSC^{CM} groups compared with in the LG-DMEM group. However, EPCs cultured in MSC^{CM} with anti-VEGF antibody were demonstrated to weakly express CD31 (Fig. 6D) and vWF (Fig. 6H) compared with EPCs cultured in MSC^{CM} and MSC^{CM} with IgG. RT-qPCR revealed that CD31 and vWF mRNA expression levels were significantly upregulated in the MSC^{CM} group compared with in the LG-DMEM group (Fig. 6I and J), whereas treatment with IgG did not appear to affect CD31 and vWF mRNA expression. However, CD31 and vWF mRNA expression levels were significantly downregulated in EPCs cultured in MSC^{CM} and treated with the anti-VEGF antibody (Fig. 6I and J). Western blot analysis revealed similar effects in CD31 and vWF protein expression (Fig. 6K and L). The present results demonstrated that anti-VEGF significantly inhibited the protein expression of CD31 and vWF in EPCs. These findings suggested that VEGF may promote the *in vitro* differentiation of EPCs into endothelial cells.

Discussion

Cell differentiation is a highly significant process for living organisms, and it results in the optimized formation of various cell populations with specialized biological functions. VEGF is a cytokine that has been implicated in numerous cell differentiation pathways and regulatory mechanisms: VEGF is critical for determining the fate of differentiating cells and controlling the differentiation process (21,27,28). The various cellular functions of VEGF result from its ability to initiate a diverse, complex and integrated network of signaling pathways through its main receptor, the kinase insert domain receptor: VEGF can stimulate cell differentiation, proliferation, migration and survival (29,30). Previous studies have reported that VEGF was critical for the differentiation of endothelial cells, and that nitric oxide was an important effector of the biological actions of VEGF (31-33). In addition, VEGF has been reported to induce the differentiation of mouse multipotent adult progenitor cells into endothelial cells (34,35), through a mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 signaling pathway-mediated mechanism (36). These findings suggested that VEGF may influence cell differentiation through complex interactions and various signal transduction pathways.

The differentiation of EPCs into endothelial cells has been suggested to be critical for endothelial repair (2). Stem/progenitor cell differentiation is closely related to the cellular microenvironment (19,20), and VEGF may be implicated in

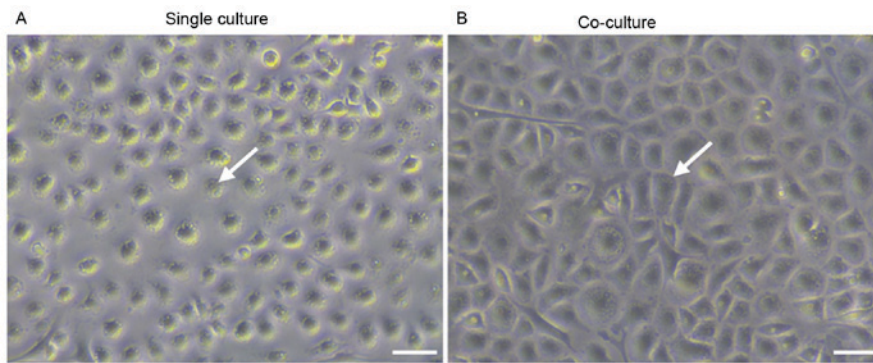


Figure 1. MSCs promote the differentiation of EPCs into endothelial cells *in vitro*. Single culture group, EPCs were seeded in 6-well plates alone. Co-culture group, EPCs were seeded in the bottom chambers of transwell inserts and MSCs were seeded in the upper chambers. Following culture for 48 h, (A) EPCs in single culture appeared as elongated sprouts, whereas (B) cells in co-culture demonstrated a cobblestone appearance. Representative photomicrographs indicated that MSCs promoted the endothelial differentiation of EPCs. Scale bar, 100 μ m. MSC, mesenchymal stem cell; EPC, endothelial progenitor cell.

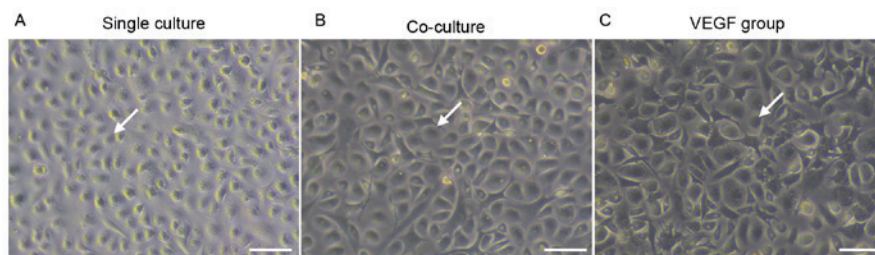


Figure 2. VEGF promotes the differentiation of EPCs into endothelial cells similar to MSCs *in vitro*. Following 48 h of culture, (A) EPCs cultured alone were irregularly shaped, whereas (B) cells co-cultured with MSCs or (C) stimulated with VEGF exhibited a cobblestone appearance. Scale bar, 100 μ m. VEGF, vascular endothelial growth factor; EPC, endothelial progenitor cell; MSC, mesenchymal stem cell.

the differentiation mechanisms. In the present study, MSCs were demonstrated to regulate the endothelial differentiation of EPCs, through the secretion of paracrine factors, including VEGF. EPCs were co-cultured with MSCs for 48 h *in vitro* and the observed morphological alterations suggested that MSCs may be implicated in the differentiation of EPCs into endothelial cells. Phase-contrast microscopy was used to examine the effects of VEGF on EPC morphology: Cells in the single culture group appeared to be irregularly shaped, whereas cells in the co-culture and VEGF groups displayed a cobblestone endothelial-like appearance.

To assess the phenotypes of differentiated cells, the expression of the endothelial cell markers CD31 and vWF (24,25) was examined. Immunofluorescence, RT-qPCR, and western blotting results revealed that CD31 and vWF expression levels were significantly upregulated in the co-culture and VEGF groups compared with the single culture group, thus suggesting that VEGF may have similar effects to the presence of MSCs on EPC differentiation. Furthermore, MSCs were demonstrated to secrete VEGF, in accordance with our previous report (15).

VEGF levels in the conditioned media from the 3 experimental groups were also measured: VEGF levels were significantly increased in the co-culture group compared with the single culture group. To further investigate the roles of VEGF during EPC differentiation, MSC^{CM} were neutralized with an anti-VEGF antibody. Anti-VEGF was demonstrated to inhibit the effects of secreted VEGF in MSC^{CM} on the endothelial differentiation of EPCs. These findings suggested that

VEGF may promote EPC differentiation to endothelial cells *in vitro*.

VEGF may be among the several cytokines that influence EPC differentiation. VEGF expression was not revealed to be significantly different between the co-culture and VEGF groups; however, the expression of the endothelial cell markers CD31 and vWF was significantly increased in the co-culture group compared with in the VEGF group. In addition, blocking the influence of VEGF in MSC^{CM} was revealed to diminish the effects of MSC^{CM} on EPC differentiation. EPC differentiation was not abolished, possible due to the presence of other cytokines secreted by MSCs in the medium, which may exert synergistic effects on EPC differentiation. Guo *et al* (37) reported that EPCs derived from CD34⁺ cells could differentiate into endothelial-like cells, and that differentiation was induced by the addition of basic fibroblast growth factor or platelet-derived growth factor-BB. Previous studies have also demonstrated that VEGF was critical for the *in vitro* differentiation of EPCs or hematopoietic stem cells into endothelial cells (38-40). These findings suggested that several MSC-released cytokines may have additive or synergistic effects on EPC differentiation pathways. Therefore, compared with single cytokine approaches, therapeutic strategies based on the combination of MSCs with EPCs may be more effective in enhancing tissue repair in humans.

Several kinds of seed cells have been used in applications of tissue engineering to promote angiogenesis in ischemic diseases. MSCs and EPCs have favorable biological characteristics that make them suitable for use in tissue engineering

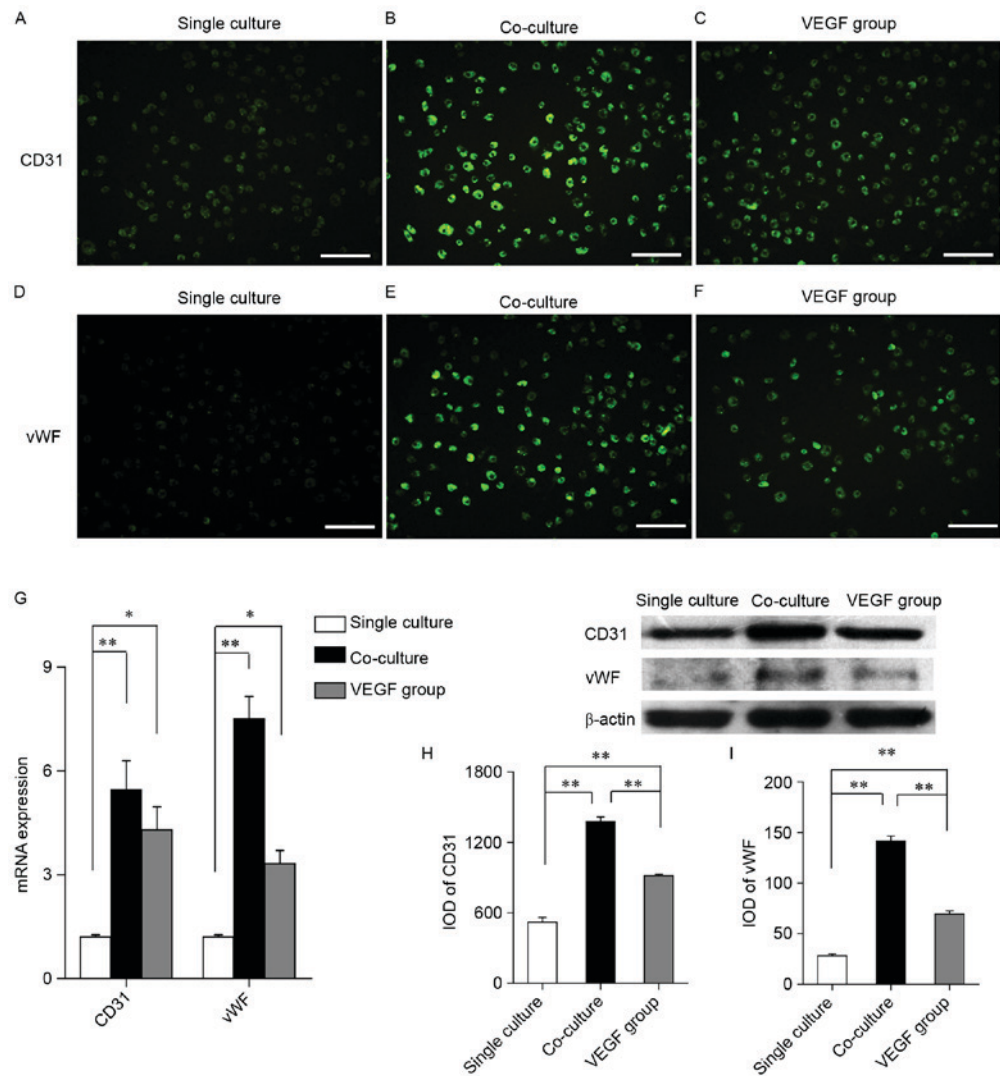


Figure 3. VEGF and MSCs upregulate the expression of endothelial cell differentiation markers in EPCs *in vitro*. (A-C) Immunofluorescence staining demonstrating CD31 expression in the (A) single culture, (B) co-culture and (C) VEGF groups. (D-F) Immunofluorescence staining demonstrating vWF expression in the (D) single culture, (E) co-culture and (F) VEGF groups. Scale bar, 50 μ m. (G) Reverse transcription-quantitative polymerase chain reaction was used to assess the mRNA expression of CD31 and vWF in the single culture, co-culture and VEGF groups. (H and I) Western blotting revealed (H) CD31 and (I) vWF protein expression. Data are expressed as the mean $\pm$ standard error of the mean. *P<0.05 and **P<0.01, as indicated. VEGF, vascular endothelial growth factor; MSC, mesenchymal stem cell; EPC, endothelial progenitor cell; CD, cluster of differentiation; vWF, von Willebrand factor; IOD, integrated optical density.

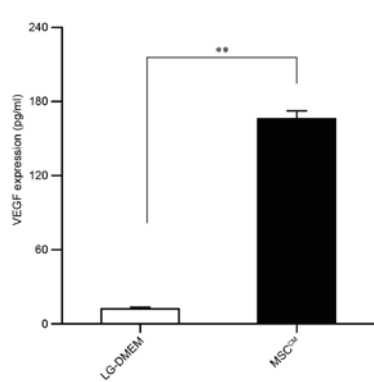


Figure 4. MSCs secrete VEGF. VEGF levels in MSC^{CM} and LG-DMEM were determined using ELISA. VEGF levels were significantly increased in MSC^{CM} compared with in LG-DMEM. These results indicated that MSCs could secrete VEGF. Data are expressed as the mean $\pm$ standard error of the mean. **P<0.01, as indicated. MSC, mesenchymal stem cell; VEGF, vascular endothelial growth factor; MSC^{CM}, MSC conditioned media; LG-DMEM, low-glucose Dulbecco's modified Eagle's medium.

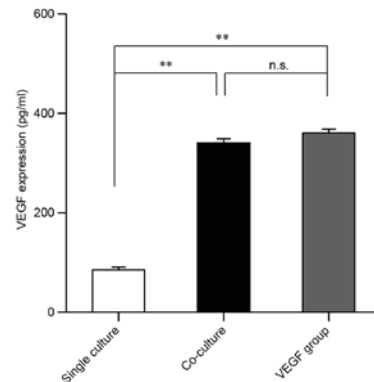


Figure 5. VEGF levels are upregulated in conditioned media from the co-culture and VEGF groups. ELISA was used to assess VEGF levels in conditioned media from the single-culture, co-culture and VEGF groups. VEGF levels in the co-culture and VEGF groups were significantly higher compared with in the single culture group. Data are expressed as the mean $\pm$ standard error of the mean. **P<0.01, as indicated. VEGF, vascular endothelial growth factor; n.s., not significant.

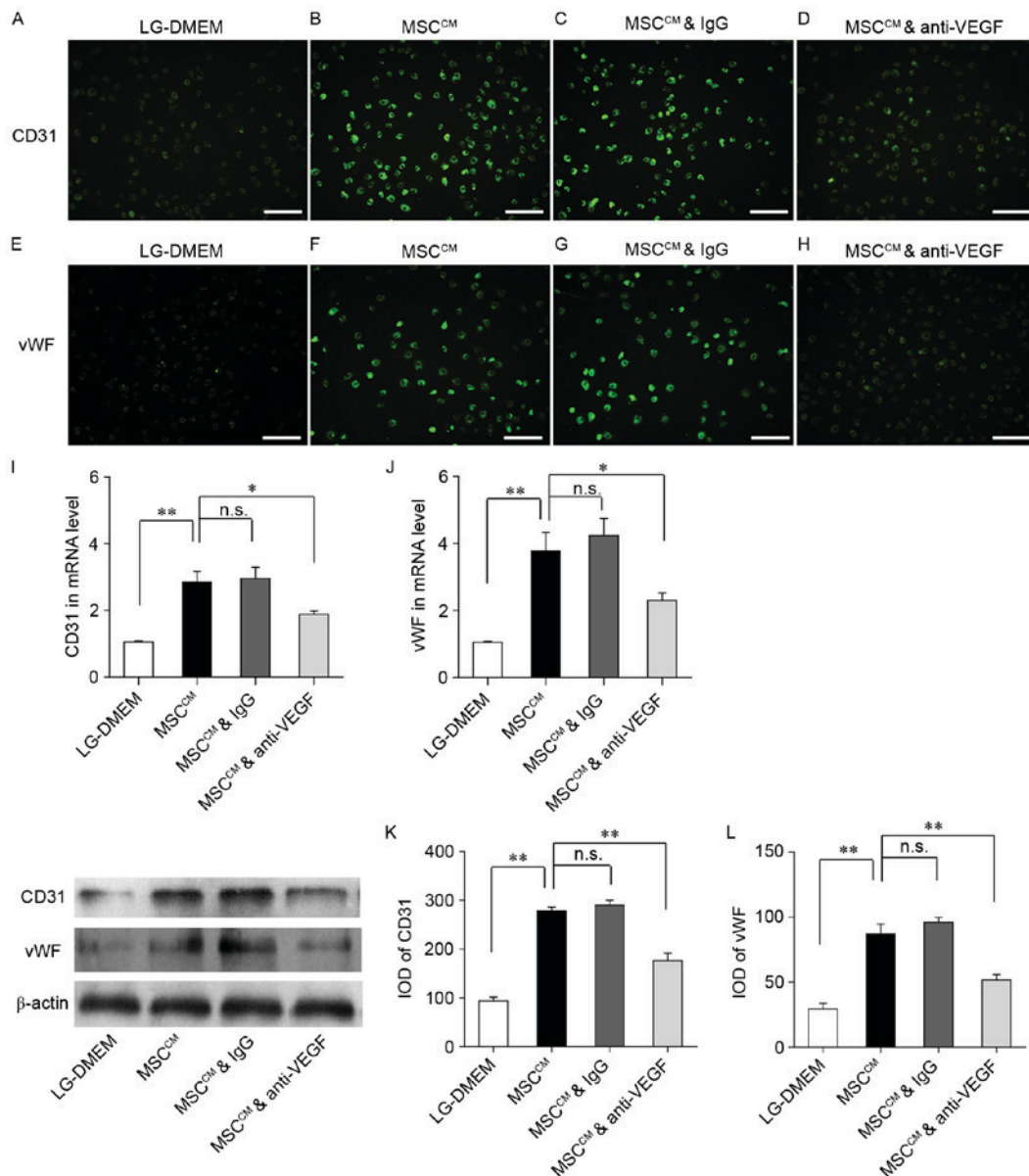


Figure 6. Anti-VEGF neutralizing antibody inhibits the expression of endothelial markers in differentiating EPCs *in vitro*. A VEGF neutralizing antibody (100 ng/ml) was added to MSC^{CM}. MSC^{CM} with mouse IgG (100 ng/ml) was used as the control. EPCs were cultured in LG-DMEM, MSC^{CM}, MSC^{CM} with IgG and MSC^{CM} with anti-VEGF antibody for 48 h. (A-D) Immunofluorescence staining demonstrating CD31 expression in the (A) LG-DMEM, (B) MSC^{CM}, (C) MSC^{CM} with IgG and (D) MSC^{CM} with anti-VEGF groups. (E-H) Immunofluorescence staining demonstrating vWF expression in the (E) LG-DMEM, (F) MSC^{CM}, (G) MSC^{CM} with IgG and (H) MSC^{CM} with anti-VEGF groups. Scale bar, 50 μm. (I and J) Reverse transcription-quantitative polymerase chain reaction was used to assess the expression of CD31 and vWF mRNA in the LG-DMEM, MSC^{CM}, MSC^{CM} with IgG and MSC^{CM} with anti-VEGF groups. (K and L) Western blotting demonstrated the protein expression levels of (K) CD31 and (L) vWF in the LG-DMEM, MSC^{CM}, MSC^{CM} with IgG and MSC^{CM} with anti-VEGF groups. Data are expressed as the mean ± standard error of the mean. *P < 0.05, **P < 0.01, as indicated. VEGF, vascular endothelial growth factor; EPC, endothelial progenitor cell; MSC, mesenchymal stem cell; MSC^{CM}, MSC conditioned media; Ig, immunoglobulin; LG-DMEM, low-glucose Dulbecco's modified Eagle's medium; CD, cluster of differentiation; vWF, von Willebrand factor; n.s., not significant; IOD, integrated optical density.

applications: They are able to migrate to injured tissues, and they are able to differentiate into various cell phenotypes according to the type of tissue in which they reside (41). In addition, MSCs demonstrate potent self-renewal properties, and can secrete various cytokines that may exert a greater or synergetic impact on the biological function of EPCs compared with one type of cytokine. Furthermore, MSCs are more easily available compared with cytokines (42,43). Therefore, therapeutic approaches based on the co-culture of EPCs with MSCs may have potential to be successfully applied in tissue engineering and regenerative medicine strategies.

In conclusion, the results of the present study suggested that the presence of MSCs may enhance the *in vitro* endothelial differentiation of EPCs, through a paracrine mechanism that may involve the secretion of cytokines, including VEGF, instead of direct cell-cell contacts. Further studies are required to explore the molecular mechanisms that mediate EPC differentiation and to elucidate the exact roles of MSCs in the differentiation processes. As endothelial cells can participate in endothelial repair and angiogenesis following ischemia, the findings of the present study may promote the understanding of tissue repair mechanisms, and may lead to the development

of novel strategies for therapeutic interventions aimed at ischemic diseases.

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