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Co-culture with endothelial progenitor cells promotes survival, migration, and differentiation of osteoclast precursors

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ABSTRACT

In this study, we report the effect of endothelial progenitor cells (EPCs) on the biological behavior of osteoclast precursors in vitro by establishing an indirect co-culture system of mice EPCs and RAW 264.7 monocyte cells. Results show that the survival, migration, and differentiation of osteoclast precursors were greatly enhanced when co-cultured with EPCs. These phenotypic changes coincide with the upregulation of multiple genes affected cell behavior, including phospho-VEGFR-2, CXCR4, phospho-Smad2/3, phospho-Akt, phospho-ERK1, and phospho-p38 MAPK. The results collectively suggest that EPCs could modulate the survival, migration, and differentiation potential of osteoclast precursors, thus providing new insights in understanding of correlation between angiogenesis and bone homeostasis.

1. Introduction

Bone homeostasis is maintained by a dynamic balance between bone-resorbing osteoclasts and bone-forming osteoblasts [1,2]. The osteoclast is a bone-degrading macrophage polykaryon generated from the cell–cell fusion of monocyte/macrophage precursors at or near the bone surface [3]. Old or necrotic bones must be removed by osteoclasts to provide space for osteogenic precursors before new bone formation. Osteoclast-based bone resorption precedes and leads to growth of the neovasculature [4]. Therefore, osteoclast activity is critical in regulating bone homeostasis.

Endothelial progenitor cells (EPCs) are a specialized source of pluripotent progenitors that are capable of inducing vasculogenesis and angiogenesis [5]. Vasculogenesis or angiogenesis is an important fundamental process during skeletal development, repair, and regeneration because the bone is a highly vascularized tissue [6]. Recent studies have shown that EPCs crosstalk with osteoblast precursors and tightly couple with osteoblastic bone formation [7,8]. Whether or not EPCs can also affect the function and activity of osteoclast precursors has yet to be examined so this topic is the focus of our present study.

2. Materials and methods

2.1. Isolation and culture of EPCs

All animal procedures were approved by the Institutional Animal Care and Use Committee of The Third Military Medical University. Murine bone marrow cells harvested from the tibias and femurs of C57BL/6 mice (8 wk to 12 wk old) were subjected to density gradient centrifugation in Percoll (density = 1.077 g/mL; Sigma, USA) at 1500 rpm for 20 min at room temperature. The mononuclear cells were isolated from the buffy coat between the Percoll solution and the blood plasma and cultured in endothelial cell growth medium-2 (EGM-2, Cambrex, USA) with Single Quots growth supplements (Cambrex, USA) on fibronectin-coated dishes. Cells were maintained in a humidified atmosphere at 37 °C, incubated with 5% CO_2, and digested with 0.25% trypsin plus 0.01% ethylenediaminetetraacetic acid for subculture until they reached about 80% to 90% confluence.

EPCs were characterized by immunohistochemical staining for vascular endothelial growth factor receptor 2 (VEGFR-2) (Abcam, CA, UK) and von Willebrand Factor (vWF) (Santa Cruz, USA). The cells were washed with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 15 min, followed by permeabilization with 0.1% Triton X-100 for 5 min. After blocking with 5% goat

Abbreviations: VEGFR-2, vascular endothelial growth factor receptor-2; CXCR4, C-X-C chemokine receptor type 4; TGF-β1, transforming growth factor beta 1; Smad2/3, mothers against decapentaplegic homolog 2/3; p38MAPK, p38 mitogen-activated protein kinases; ERK1/2, extracellular signal-regulated kinases1/2.

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serum at room temperature for 30 min, cells were stained with specific primary antibodies at 4 °C overnight. After rinsing with PBS three times and incubating with secondary antibodies for 2 h at 37 °C, EPCs were mounted with Vectashield mounting medium (Vector Laboratories) before observation.

Bone marrow-derived EPCs were also identified by the uptake of DiI-labeled acetylated low-density lipoprotein (DiI-ac-LDL; Molecular Probes, USA) and fluorescein isothiocyanate (FITC)-labeled Ulex Europaeus Agglutinin 1 (FITC-UEA-1; Vector, USA). Bone marrow-derived EPCs were treated with 10 mg/L Dil-ac-LDL for 4 h at 37 °C according to the manufacturer's instructions. After subsequent washing with PBS, the cells were fixed with 2% paraformaldehyde for 10 min at 4 °C. Following 1 h incubation with 10 mg/L FITC-UEA-1 at room temperature and subsequent PBS washing, cells were observed under a fluorescence microscope (Leica, Germany).

The angiogenic capacity of the early EPCs was determined by Matrigel tube-like formation assay. Matrigel (Sigma) was diluted 1:1 in 500 μL EGM-2 media in 96-well plates and polymerized by incubating at 37 °C for 1 h. EPCs (2×10^4 cells/well) were seeded onto the Matrigel and incubated in a humidified atmosphere at 37 °C with 5% CO_2. After 24 h, 3 d, and 7 d representative digital micrographs were taken.

2.2. RAW 264.7 cell culture

RAW 264.7 monocyte/macrophage cells (American Type Culture Collection, USA) were used as a cell model for osteoclast precursors and cultured in Dulbecco's modified eagle's medium (DMEM, Gibco, CA, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. The RAW 264.7 cells were kept at 37 °C in a 5% CO_2 incubator, and the medium was changed every 2 d.

2.3. EPC and RAW 264.7 cell co-culture

The co-culture system was established by inserting a transwell chamber (Millipore, Bedford, MA, USA) with 3 μm pores into 12-well plates. RAW 264.7 cells were seeded on the plates (Costar, NY, USA) at a density of 1×10^3 cells/well. The same amount of EPCs was seeded on the transwell chamber. The chamber was inserted into the wells of the plate to allow crosstalk between the two cell types. DMEM (1 mL) was added to cover both upper-layer EPCs and lower-layer RAW 264.7 cells. Half of the culture medium was changed every 2 d.

2.4. RAW 264.7 viability assay

The survival of RAW 264.7 cells was analyzed using a cell counting kit-8 (CCK-8, Beyotime, China) on days 1 through 7 and measured by microplate reader scanning at 450 nm. The attached RAW 264.7 cells co-cultured with EPCs were washed twice with PBS, carefully removed from the 12-well plates using 0.25% trypsin, and then collected by centrifugation at 1000 rpm for 5 min. For the cell survival assays, RAW 264.7 cells were cultured in 24-well plates at 2×10^3 cells per well for 24 h, treated with 10 μL of CCK-8 in every well, and incubated for 1 h at 37 °C. Measurement of the cell absorbance was performed at 450 nm.

2.5. Transwell migration assay

The migration of RAW 264.7 cells was measured using transwell inserts with a pore size of 8 μm (Costar, Corning, NY). RAW 264.7 cells were placed in the upper chamber and EPCs were seeded on the lower chamber. The cells were incubated at 37 °C and 5% CO_2 for 5 d, rinsed with PBS, and then fixed with 10% formalin for 10 min. Migrated cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI) (1:10,000) for 15 min before fluorescent images of the lower surface of the transwell membrane were captured using a microscope (Leica, Germany) at 200 × magnification. The number of cells in three 2.4 mm^2 images per membrane was respectively quantitated by counting cells with blue-stained nuclei to calculate the number of cells per mm^2, as previously described by Gordillo et al.[9].

2.6. Osteoclastic differentiation

To confirm the effect of EPCs on the osteoclastogenesis of osteoclast precursors, the number of multinucleated osteoclast-like cells in the co-culture group was compared with those in a negative control group that had RAW 264.7 cells cultured in DMEM medium with 10% fetal bovine serum (FBS) and those in a positive control group that had RAW 264.7 cells cultured with 50 ng/mL receptor activator of nuclear factor kappa-B ligand (RANKL) and 25 ng/mL macrophage colony-stimulating factor (M-CSF) (PeproTech, NJ, USA). The multinucleated osteoclast-like cells were detected by the enzyme tartrate-resistant acid phosphatase (TRAP) and identified as TRAP-positive multinucleated ($\geqslant$3 nuclei) cells. The number of multinucleated osteoclast-like cells formed in each well was quantified after a 7 d culture and presented as the mean of six wells to evaluate the osteoclast-formation activity.

2.7. Western blot

For analysis of the changes in protein abundance, protein was extracted from RAW 264.7 cells with or without EPC co-culture for Western blot. Primary antibodies for Western blot included CXCR4 (ABcam, Cambridge, MA, USA), phospho-VEGFR-2, phospho-Akt, phospho-ERK1/2, phospho-Smad2/3, phospho-p38MARK, and glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz, CA, USA). Western blot was performed according to the manufacturer's instructions.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The release of vascular endothelial growth factor-A (VEGF-A), stromal cell-derived factor-1 (SDF-1), and transforming growth factor beta 1 (TGF-β1) was measured in 10 × concentrated culture supernatants by ELISA (R&D Systems, USA), as previously described by Urbich et al.[10]. The medium of RAW 264.7 cells, EPCs, and co-culture system was respectively concentrated 10 × by centrifugation at 10,000 rpm for 20 min at 4 °C after EPCs were cultured with equal amounts of medium without supplements and FBS for 24 h.

2.9. Statistical analysis

All data are expressed as mean ± SEM. Student's *t* test was used to assess statistical significance for paired observations. Data of multiple comparisons were analyzed using ANOVA and Dunnett's post hoc test. All tests were performed by SPSS and the level of significance was defined at $p < 0.05$.

3. Results

3.1. Identification and characterization of EPCs

After 1 wk in vitro culture, bone marrow-derived EPCs exhibited a spindle-shaped morphology and tended to form cluster-like colonies (Fig. 1A). Immunohistochemistry demonstrated that EPCs were positive for VEGFR-2 (Fig. 1B) and vWF (Fig. 1C) after culturing for 7 d. EPCs were also defined by the binding of FITC-UEA-1

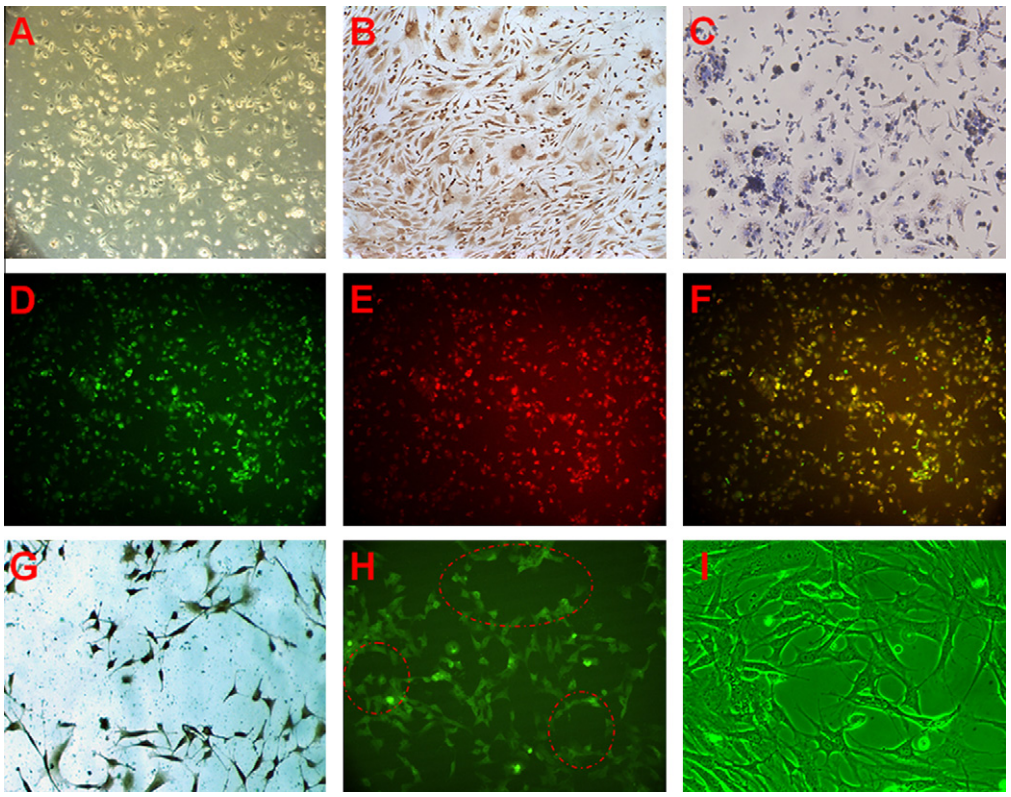

Fig. 1. Culture and characterization of EPCs (100×). (A) The morphology of EPCs after culturing for 7 d. Immunohistochemically, EPCs are strongly positive for VEGFR-2 (B) and vWF (C). FITC-UEA-1/Dil-ac-LDL double-positive fluorescence staining also characterizes bone marrow-derived EPCs. FITC-UEA-1 shows green fluorescence (D); Dil-ac-LDL display red fluorescence (E); dual-positive cells are EPCs that exhibit orange fluorescence (F). EPCs formed tube-like structures when plated on Matrigel after 24 h (G), and tended to form vascular tissue-like network in vitro from 3 d (H) to 7 d (I). The red circles indicate tube-like structures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 1D) and the uptake of DiI-ac-LDL (Fig. 1E); subsequent immunofluorescence staining showed that most EPCs were double-positive with yellow merged images (Fig. 1F). The functional capacity of EPCs was described by their ability to form tube-like structures during several days. At 24 h, tube-like structures were already present in Matrigel-coated plates (Fig. 1G). From 3 d to 7 d (Fig. 1 H and I), EPCs gradually formed vascular tissue-like network in vitro.

3.2. EPCs promote survival of RAW 264.7 cells

To confirm whether or not co-culture with EPCs can stimulate the survival of RAW 264.7 cells, the cell viability of RAW 264.7 cells was measured by CCK-8 assay every 2 d. A control group was set up without EPCs seeded on the inserted chamber (Fig. 2A). The results demonstrated no significant difference between the control and co-culture groups after 1 d of culture. After 2 d, RAW 264.7 cells in the co-culture group (Fig. 2B) grew faster than those in the control and a significant difference was observed (Fig. 2C).

3.3. EPCs promote migration of RAW 264.7 cells

The migration ability of RAW 264.7 cells was measured using transwell. After a 4 d culture, migration of RAW 264.7 cells was significantly enhanced (Fig. 2F). Fluorescence images of the lower surface of the transwell membrane demonstrated that RAW 264.7 cells stained with DAPI in the co-culture group (Fig. 2E) outnumbered those in the control (Fig. 2D). Moreover, we observed that EPCs secreted VEGF-**A**, SDF-1, and TGF-β1 in the co-culture medium (Fig. 4A) and increased the protein expression of phospho-VEGFR-2, CXCR4, phospho-Smad2/3, phospho-Akt, phospho-ERK1, and phospho-p38 MAPK in the co-cultured RAW 264.7 cells (Fig. 4B).

3.4. EPCs promote differentiation of RAW 264.7 cells

The osteoclastogenesis of RAW 264.7 cells is traditionally induced by RANKL and M-CSF. To further determine whether or not EPCs alone possess the capacity to elicit induction, we compared the number of multinucleated (⩾3 nuclei) osteoclast-like cells in three groups (Fig. 3D). From TRAP staining after 7 d of culture, the formation of large, multinucleated, TRAP-positive cells was observed. The average number of multinucleated osteoclast-like cells in the co-culture group (59.83 ± 8.50) (Fig. 3B) showed a 3.5-fold increase compared with that in the negative control group (17.33 ± 2.73) (Fig. 3A) without the EPC co-culture but remained lower than that in the positive control group (102.50 ± 6.50) (Fig. 3C).

4. Discussion

This study is the first to identify the effects of EPCs on the biological behavior of osteoclast precursors, including their survival, migration, and differentiation, in vitro. Our data suggest that bone marrow-derived EPCs provide costimulatory cytokines, including VEGF-A, SDF-1, and TGF-β1, to synergistically promote the survival and migration of RAW 264.7 cells. Aside from increases in survival and migration, we further found that EPCs directly increase the osteoclastogenetic ability of RAW 264.7 cells.

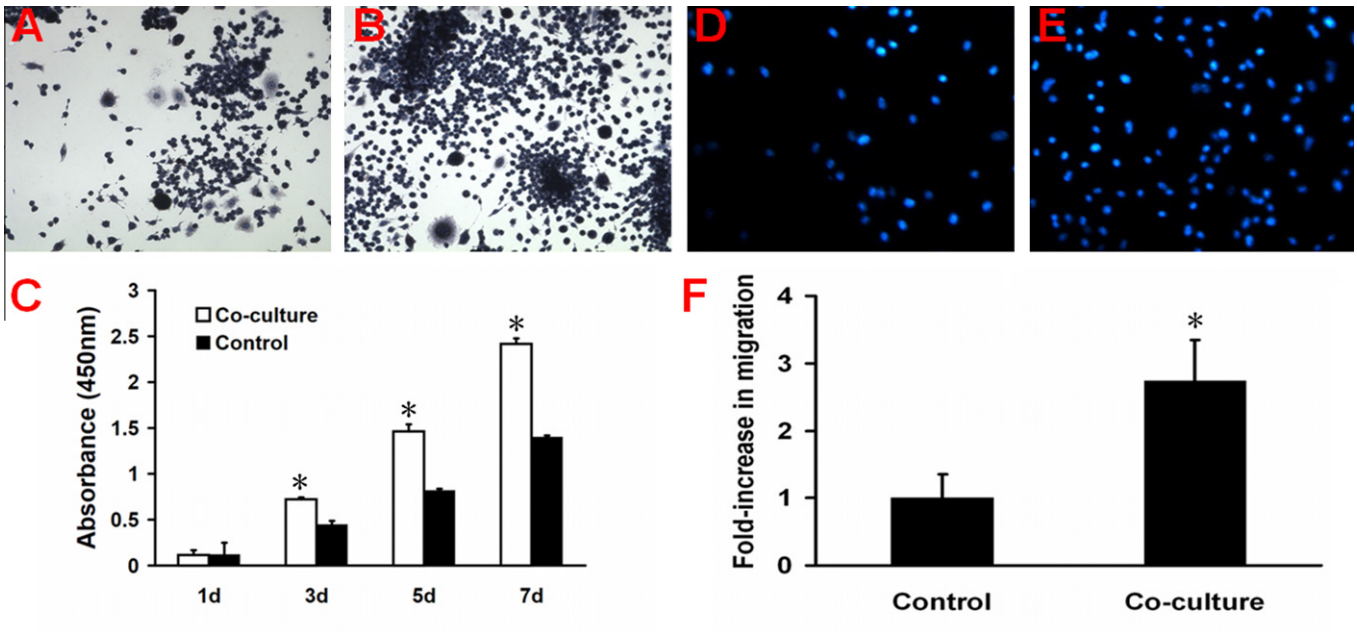

Fig. 2. EPCs promote survival and migration of RAW 264.7 cells. Representative microscopic images of the 7 d culture demonstrate that EPCs stimulated survival of RAW 264.7 cells in the co-culture group (B) compared with the control (A). (C) The absorbance at 450 nm during the Cell Counting Kit-8 assay of RAW 264.7 cells with or without EPC co-culture. Imaging of RAW 264.7 cells stained with DAPI on the lower surface of the transwell membrane indicates that EPCs enhance the migration of RAW 264.7 cells in the co-culture group (E) compared with the control group (D). The results of cell counting (F) show that co-culture with EPCs results in a 2.73-fold increase in the number of migrated RAW 264.7 cells compared with the control group. $^{*}p < 0.05$, vs. control group.

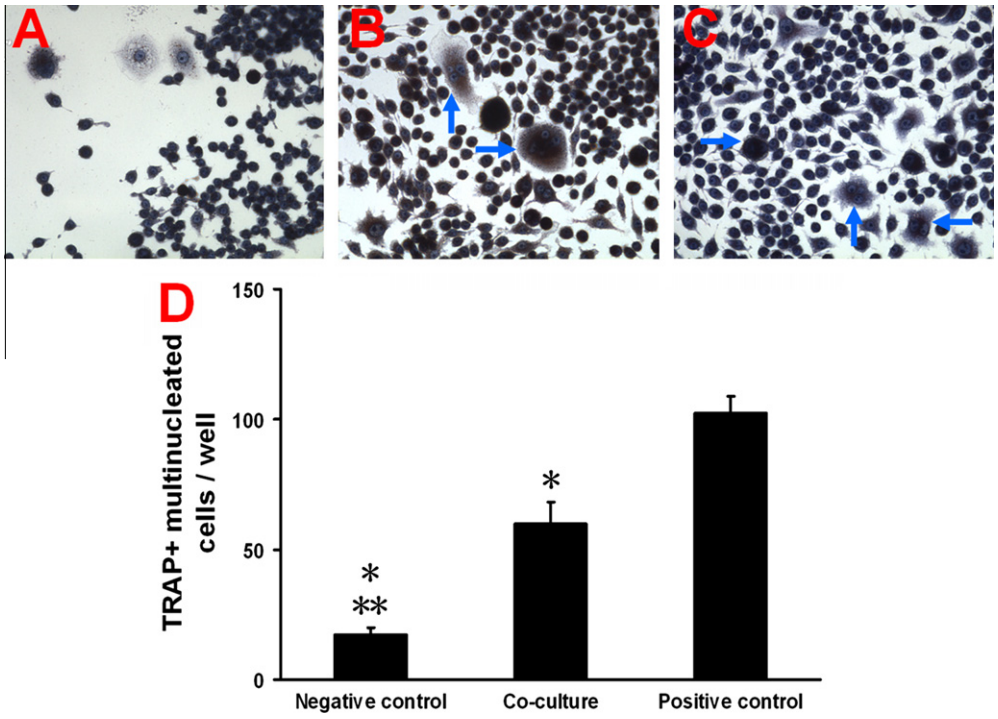

Fig. 3. EPCs promote in vitro osteoclast differentiation. Photographs of representative TRAP-positive multinucleated ($\geqslant$3 nuclei) cells in the co-culture group (B), negative control group (A), and positive control group (C) were observed at 400 $\times$ magnification. The cell number in each group was counted after the 7 d culture. Co-culture group = EPCs + RAW 264.7 cells; negative control group = RAW 264.7 cells without EPCs; positive control group = RANKL + M-CSF + RAW 264.7 cells. TRAP-positive multinucleated cells in the co-culture group are higher in number than those in the negative control group but remain lower than those in the positive control group (D). $^{*}p < 0.05$, compared with the positive control group; ** $p < 0.05$, compared with the co-culture group. The blue arrows indicate TRAP-positive multinucleated osteoclast-like cells.

EPCs regulate the survival and migration of other cell lineages, such as cardiac progenitor cells and endothelial cells [10]. In this study, the presence of EPCs positively affected the proliferative and migratory capacities of RAW 264.7 cells. The positive effects

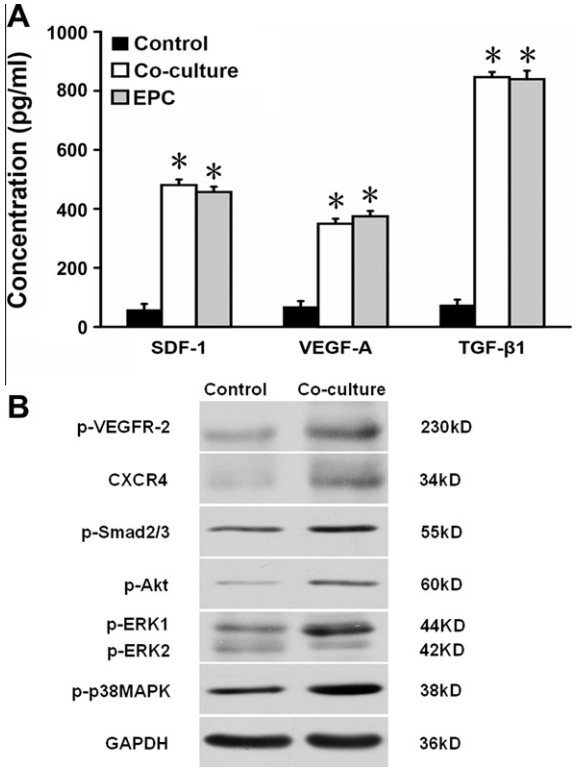

Fig. 4. Analysis of the regulators involved in positive effects of co-culture with EPCs on RAW 264.7 cells. (A) ELISA data show that EPCs can release various cytokines and growth factors into the co-culture medium, including VEGF-A, SDF-1, and TGF-β1. *Control group = RAW 264.7 cells medium; co-culture group = co-culture medium; EPC group = EPCs medium.* $^*p < 0.05$, compared with control group. (B) Increased protein expression of phospho-VEGFR-2, CXCR4, phospho-Smad2/3, phospho-Akt, phospho-ERK1, and phospho-p38 MAPK in co-cultured RAW 264.7 cells compared with the control group without EPCs.

of the presence of EPCs during co-culture on RAW 264.7 cells were confirmed by ELISA for detecting VEGF-A, SDF-1, and TGF-β1 in the co-culture medium. All of the cytokines have been proven in previous studies to have the potential to boost the survival and migration of osteoclasts or precursors. VEGF-A enhances osteoclast survival and osteoclastic bone resorption [11]. SDF-1 promotes the chemotactic recruitment, development, and survival of osteoclast precursors [12]. TGF-β1 modulates growth and differentiation in many cell types and induces osteoclast chemotaxis [13,14]. Consequently, these factors exert a strong synergistic effect on the survival and migration of RAW 264.7 cells.

In addition to increasing survival and migration, VEGF-A, SDF-1, and TGF-β1 also work synergistically to induce osteoclastogenesis. Our results show for the first time that multinucleated osteoclast-like cell can be directly induced by EPCs in the absence of RANKL and M-CSF, although the osteoclastogenetic ability of RAW 264.7 cells induced by EPCs remains weaker than that stimulated by RANKL and M-CSF. The presence of RANKL and M-CSF is required for osteoclast differentiation from monocyte or macrophage precursors [15]. A number of other cytokines and growth factors can substitute these two molecules [16]. Osteoclastic formation could be induced by other known RANKL substitutes, including TGF-β1 [17,18]. VEGF-A released by EPCs could serve as M-CSF substitutes for osteoclastogenesis [19]. SDF-1 also could upregulate the expression of a number of osteoclast activation-related genes, including RANKL, RANK, and TRAP [20]. Thus, the effect of EPCs on RAW 264.7 cells is sophisticated, depending on the cytokines and growth factors generated from EPCs.

In the present study, we also observed the up-regulation of phospho-VEGFR-2, CXCR4, phospho-Smad2/3, phospho-Akt, phospho-ERK1, and phospho-p38 MAPK expression levels in the co-cultured RAW 264.7 cells. Activation of the PI3K/Akt and MAPK/ERK pathways is associated with a series of cellular processes, such as cell survival, migration, and differentiation [21,22]. A number of investigators have shown that VEGF-A [11] and SDF-1[23] participate in cell survival and migration via the PI3K/Akt and MAPK/ERK signaling pathways. Gingery et al. reported that TGF-β1 could activate TGFβ-activated kinase 1 (TAK1)/MEK/Akt and Smad2/3 pathways to promote osteoclast survival [24]. Although the pathways for osteoclastogenesis induced by TGF-β1 have yet to be completely understood, activation of TAK1/p38 MAPK pathways may be one of the possible mechanisms underlying enhanced osteoclast formation [25]. These findings indicate that the effects of EPCs on RAW 264.7 cells are multiplied and combined with involvement in multiple signaling pathways, including PI3K/Akt, MAPK/ERK, and TGF-β/Smad.

The results obtained provide strong evidence that EPCs can promote the survival, migration, and differentiation of RAW 264.7 cells in co-culture. Our findings suggest that the survival, recruitment, and formation of osteoclast precursors modulated by bone marrow-derived EPCs may be a possible mechanism underlying the promotion of osteoclast-based bone resorption for maintaining bone homeostasis.

Acknowledgments

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