

Co-cultivation of progenitor cells enhanced osteogenic gene expression and angiogenesis potential *in vitro*

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Abstract

Objectives: The efficiencies of osteogenesis and angiogenesis present challenges that need to be overcome before bone tissue engineering can be widely applied to clinical uses. We aimed to optimize an *in vitro* culture system to enhance osteogenesis and angiogenesis. We investigated if hematopoietic stem cells (HSCs) promoted osteogenesis *in vitro* when co-cultured with mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs).

Methods: MSC/HSC, MSC/EPC/HSC, and MSC/EPC co-cultures were incubated for 21 days. Alkaline phosphatase (ALP) activity and calcium content were analyzed to assess mineralization. Expression levels of genes encoding osteogenesis-related proteins (ALP (*ALPL*), collagen type IA (*COL1A1*), osteocalcin (*BGLAP*), and osteopontin (*OSTP*)) were also evaluated by measuring mRNA levels at day 28. Angiogenesis was evaluated by tube-formation assay.

Results: *COL1A1*, *OSTP*, *ALPL*, and *BGLAP* genes were upregulated in MSC/HSC and MSC/EPC/HSC co-cultures compared with the MSC/EPC group. Upregulation was strongest in the MSC/EPC/HSC co-cultures. There were no significant changes in ALP levels and calcium content, but ALP activity was slightly higher and calcium content was relatively lower in the MSC/EPC and MSC/EPC/HSC groups.

Conclusions: Co-culture of MSCs with HSCs or EPCs/HSCs upregulated the expression of osteogenesis-related genes but did not affect the efficiency of osteogenesis.

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Introduction

Osteogenesis has been extensively investigated with the hope of providing a helpful guide for bone engineering, and numerous experimental and preclinical reports have indicated a positive outlook for the practical application of bone engineering.^{1–3} Recent advances in seed cells, growth factors, and scaffolds have become the most prominent areas of bone tissue engineering.⁴ Studies of seed cells have been extensively documented,^{5–7} and advanced techniques for the purification and expansion of seed cells have increased the available cell numbers. However, bone tissue engineering practices have not yet been introduced to the clinic because of several limitations, such as inefficient osteogenesis, weakened differentiation, and the tendency of regenerated bone to support tumor growth.^{8,9}

Mesenchymal stem cells (MSCs) have been widely used as seed cells in preclinical studies because of their ability to differentiate into osteoblasts.¹⁰ MSCs are found in the bone marrow, adipose tissue, muscle, and peripheral blood in adults,^{11–13} with bone marrow being the preferred source of MSCs because of the reduced risk of disease transmission.¹⁴ However, MSCs represent less than 0.01% of the nucleated cells in the bone marrow, and these low numbers adversely affect the efficacy of cell-based therapy. Even after amplification in culture, the numbers of MSCs are not sufficient for therapeutic use. Moreover, a previous study found that about 50% of MSCs injected *in vivo* became inactive within 48 hours as a

result of ischemia and inflammatory reactions.¹⁵ Moreover, weakened differentiation and the tendency to form tumors during the expansion process further limit the clinical use of MSCs. Recent studies aimed at improving *in vitro* osteogenesis have focused on the co-culture of MSCs with endothelial progenitor cells (EPCs),^{16–18} which can differentiate into endothelial cells and provide vascular support for angiogenesis. Both osteogenesis and vascularization were increased in MSC/EPC co-cultures;¹⁶ however, the molecular details of how these cell types interact are unclear.

Hematopoietic stem cells (HSCs) can differentiate into osteoclasts, which play a pivotal part in bone regeneration. MSCs, HSCs, and EPCs coexist *in vivo*, and their interactions are thought to affect osteogenesis. Co-culturing MSCs and HSCs can result in their simultaneous differentiation into osteoblasts and blood cells, respectively.⁶ HSCs may thus play a pivotal role in osteogenesis when co-cultured with MSCs and EPCs.¹⁹ Here we evaluated the osteogenic and angiogenic potentials of MSC/EPC/HSC co-culture systems to improve our understanding and to optimize *in vitro* osteogenesis.

Materials and methods

Cells and culture system

MSCs and EPCs were purchased from Melero-Martin (Boston, MA, USA) and HSCs from Lonza (Bend, OR, USA). MSCs were expanded in low-glucose Dulbecco's Modified Eagle Medium

(DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Omega Scientific, Tarzana, CA, USA) and 1% penicillin–streptomycin (Thermo Fisher Scientific). HSCs were expanded in serum-free hematopoietic progenitor growth medium (Thermo Fisher Scientific) supplemented with 25 ng/mL stem cell factor, 25 ng/mL FMS-like tyrosine 2 ligand, 25 ng/mL thrombopoietin, and 1% penicillin–streptomycin (Thermo Fisher Scientific). The medium used for osteogenic induction was low-glucose DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, 2 mM L-glutamine, 100 nM dexamethasone, 0.2 nM L-ascorbic acid-2-phosphate, and 10 mM β -glycerophosphate. All cells were cultured at 37°C in a 5% CO₂, 95% humidity incubator. The medium was changed every 3 days.

Ethics approval was not required because no animal or human tissue was involved in the study.

Co-culture of MSCs, EPCs, and HSCs

Co-culture systems were prepared by combining MSCs, EPCs, and HSCs as follows: Group 1, MSC:EPC 50:50, 5×10^4 cells each; Group 2, MSC:HSC 50:50, 5×10^4 cells each; and Group 3, MSC:EPC:HSC 50:25:25, 5×10^4 MSCs, 2.5×10^4 EPCs and HSCs, each. Group 1 was used as control. The ratios were based on the literature and our preliminary experiments.^{19,20}

DNA quantitation assay

The DNA content was measured quantitatively using a commercial Quant-iT Picogreen kit (Thermo Fisher Scientific) following the manufacturer's protocol. After washing the cell layers twice with phosphate-buffered saline (PBS), 1 mL of MilliQ (Millipore, Darmstadt, Germany) was added to each sample. Serial dilutions

of double stranded DNA stock at concentrations of 0 to 2000 ng/mL were used as a standard, and 100 μ L of sample or standard was added to each well followed by 100 μ L working solution. After incubation for 5 minutes at room temperature in the dark, the fluorescence of the samples was measured at 450 nm using a microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA, USA).

Alkaline phosphatase (ALP) activity assay

ALP activity was measured by determining the absorbance at 405 nm. First, 80 μ L of sample and 20 μ L of buffer solution (0.5 M 2-amino-2-methyl-1-propanol) were mixed in 96-well plates, followed by the addition of 100 μ L of substrate solution (5 nM p-nitrophenyl phosphate) and incubation at 37°C, 5% CO₂ for 1 hour. The reaction was stopped by adding 100 μ L stop buffer (0.3 M NaOH). Serial dilutions of 4-nitrophenol were added to a final solution of 0 to 25 nmol, as standard procedure. The absorbance of the samples was measured at 405 nm using a microplate reader. ALP activity was normalized to DNA content.

Calcium quantitation assay

Mineralization was evaluated by calcium assay. Cell layers were washed twice with PBS followed by the addition of 1 mL 0.5 M acetic acid to each well. The plates were incubated on a shaker overnight at room temperature and the samples were then stored at -20°C until measurement. The working solution was prepared using a mixture of 5 mL orthocresolphthalein complexone solution, 5 mL 14.8 M ethanolamine/boric acid buffer (pH 11), 2 mL 8-hydroxyquinoline (1 g in 20 mL 95% ethanol), and 88 mL MilliQ. Serial dilutions of calcium stock (CaCl₂) at final concentrations of 0 to 100 μ g/mL were used as

standards. A total of 10 μ L of sample/standard was added to 300 μ L of working solution in a 96-well plate and incubated at room temperature for 10 minutes. The absorbance was read at 570 nm with a microplate reader.

Real-time polymerase chain reaction (PCR)

Relative expression levels of the genes encoding major osteogenic proteins (ALP (*ALPL*), collagen type IA (*COL1A*, *COL1A1*), osteocalcin (OCN, *BGLAP*), and osteopontin (OPN, *OSTP*)) were monitored, using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a reference gene. Total RNA was isolated using TRIzol (Invitrogen Corp., Carlsbad, CA, USA). cDNA was synthesized using a Primescript RT reagent kit (Thermo Fisher Scientific). Real-time PCR was performed using SYBR Premix Ex TaqTM II (Bio-Rad, Hercules, CA, USA). Human-specific oligonucleotide primers (Table 1) were designed based on nucleotide sequences from GenBank. Amplicons were generated using a two-step protocol (61°C for 30 s and 95°C for 15 s) for 45 cycles. Relative gene expression was quantified by applying the threshold cycle (Ct) and $2^{-\Delta\Delta C_t}$ was analyzed. Gel electrophoresis of PCR products was performed to validate the results.

In vitro angiogenesis assay

MSCs were cultured in low-glucose DMEM, MSCs were cultured in serum-reduced DMEM supplemented with 40 ng/mL

vascular endothelial growth factor (VEGF; CST, Boston, MA, USA), EPCs were cultured in Endothelial Basal Medium (EGM2; Lonza, Basel, Switzerland) (control group) or in serum-reduced EGM2 supplemented with 40 ng/mL VEGF, and EPCs/MSCs were co-cultured in complete or serum-reduced osteogenic medium (Sciencell, Los Angeles, CA, USA). Fluorescent staining of EPCs and MSCs was carried out using lipophilic fluorescent dyes (Vybrant-DiO and DiD; Thermo Fisher Scientific). Matrigel was added to the culture media (1:1 dilution) in a 48-well plate and left to solidify for 1 hour at 37°C. Fluorescently labeled cells were then trypsinized and seeded at 30,000 cells/well (15,000 EPCs and 15,000 MSCs in co-culture). Fluorescent images were taken after 24, 48, and 72 hours. Tube-like structures were qualified using Image-Pro Plus 6.0 (Media Cybernetics, GA, USA).

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using SPSS Statistics for Windows Version 25.0 (SPSS Inc., Chicago, IL, USA). Differences of $P < 0.05$ were considered significant.

Results

Osteogenic activity

DNA content was measured to evaluate cell numbers and proliferation (Figure 1a). DNA content increased with time in all groups. However, the trend was lower in

Table 1. Primer sequences for real-time polymerase chain reaction.

Gene	Forward primer	Reverse primer
<i>COL1A1</i>	5' TCAACGGTGCTCCTGGTGAAG 3'	5' GGACCTTGGCTACCCTGAGAA 3'
<i>OSTP</i>	5' ACGCCGACCAAGGAAAACCTC 3'	5' GTCCATAAACCCACACTATCACCTCG 3'
<i>ALPL</i>	5' ATGGGATGGGTGTCTCCACA 3'	5' CCACGAAGGGGAACCTTGTC 3'
<i>BGLAP</i>	5' ACAAGTCCCACACAGCAACTC 3'	5' CCAGGTCAGAGAGGCAGAAT 3'

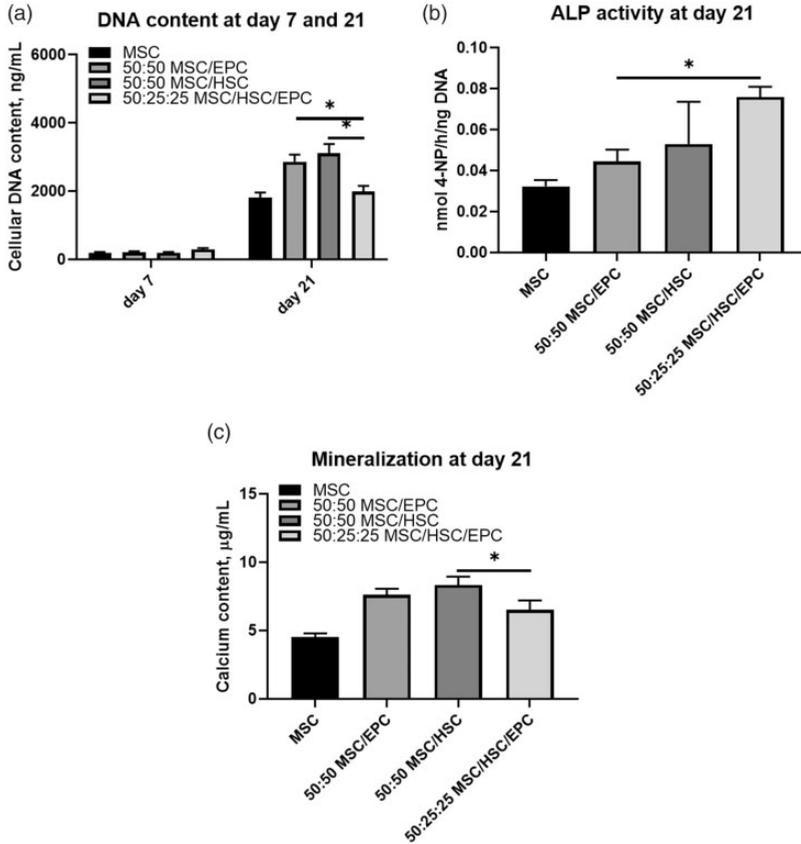


Figure 1. Verification of bone formation after 21 days in culture. For all panels, the 50:50 MSC/EPC group was used as control. (a) DNA content in each group at day 21. (b) Alkaline phosphatase (ALP) activity in each group at day 21. ALP activity and mineralization (evaluated by calcium content) were normalized to DNA content. ALP activity was increased in the 50:50 MSC/HSC and 50:25:25 MSC/HSC/EPC groups compared with the control group, but the differences were not significant. (c) Mineralization in each group at day 21. Mineralization was increased in the 50:50 MSC/EPC group and slightly decreased in the 50:25:25 MSC/HSC/EPC group. For all panels, values given as mean and standard deviation (error bars) (n = 6). *P < 0.05.

MSC, mesenchymal stem cell; EPC, endothelial progenitor cell; HSC, hematopoietic stem cell.

the co-culture groups, especially in the MSC/HSC/EPC group. DNA content was also used as a standard to normalize the data (Table 2).

ALP activity was measured after 21 days of co-culture (Figure 1b). ALP activity in the MSC/HSC/EPC group (0.074 nmol 4-nonylphenol/hour/ng DNA, 95%

Table 2. DNA content in each group at days 7 and 21.

Cell type	7 days (ng/mL)	21 days (ng/mL)
MSC:EPC	233.3 ± 44.5	2726.3 ± 375.2
MSC:HSC	256.7 ± 11.5	3168.3 ± 375.8
MSC:EPC:HSC	317.3 ± 67.0	1812.4 ± 147.3

Values given as mean ± standard deviation.

confidence interval (CI): 0.061–0.087) was higher than in either the MSC/EPC (0.046 nmol 4-NP/h/ng DNA; 95% CI: 0.036–0.056) or MSC/HSC group (0.054 nmol 4-NP/h/ng DNA; 95% CI: 0.007–0.101). However, the differences were not significant.

Mineralization was evaluated by calcium content. After 21 days of co-culture, the calcium content was higher in the MSC/HSC group (9.533 $\mu\text{g/mL}$) than in the control group (7.467 $\mu\text{g/mL}$), but lower in the MSC/HSC/EPC group (6.267 $\mu\text{g/mL}$) compared with the other groups (Figure 1c). Only the difference between the MSC/HSC and MSC/HSC/EPC groups was significant ($P < 0.05$).

Expression of osteogenesis-related genes

mRNA levels of *ALPL*, *COL1A1*, *BGLAP*, and *OSTP* were measured by quantitative

PCR after 28 days of co-culture (Figure 2). Expression levels of all four genes were lowest in the control group. *ALPL* expression was significantly lower in the control group than in the other groups ($P < 0.05$), but there was no significant difference in *ALPL* expression levels between the MSC/HSC and MSC/HSC/EPC groups. *OSTP* expression levels were significantly higher in the MSC/HSC group compared with the control group ($P < 0.05$), and significantly lower in both the MSC/EPC and MSC/HSC groups compared with the MSC/HSC/EPC group ($P < 0.05$). *COL1A1* and *BGLAP* expression levels showed similar trends.

Tube-formation analysis

Angiogenesis was evaluated by tube-formation assay. In addition to the interaction between stem cells (MSCs and EPCs),

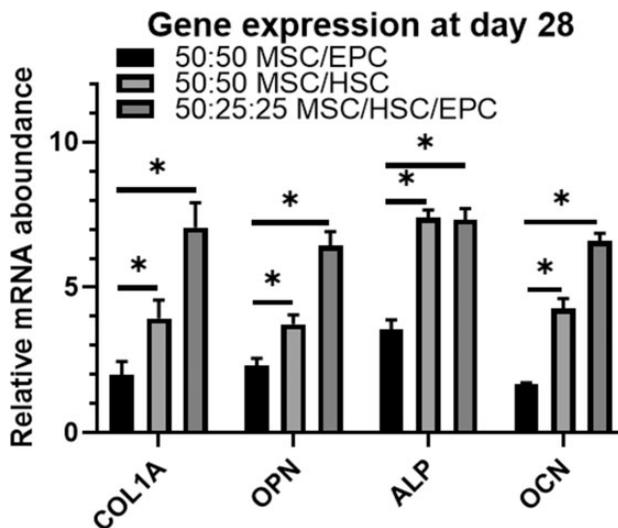


Figure 2. mRNA expression levels of genes associated with osteogenesis. Expression levels of genes encoding alkaline phosphatase (ALP), collagen type IA (COL1A), osteopontin (OPN), and osteocalcin (OCN) were measured by real-time quantitative polymerase chain reaction. The 50:50 MSC/EPC group was used as control. Expression levels of all the measured genes were significantly increased in the 50:50 MSC/HSC group and especially in the 50:25:25 MSC/HSC/EPC group compared with the control group. Values given as mean and standard deviation (error bars) ($n = 3$).
* $P < 0.05$.

MSC, mesenchymal stem cell; EPC, endothelial progenitor cell; HSC, hematopoietic stem cell.

we also investigated the roles of growth factors (VEGF) and serum nutrition in angiogenesis. Stem cells mainly developed into tube-like structures within 24 hours. Neither EPCs nor MSCs could form tube-like structures independently, but co-culturing EPCs with MSCs increased

their tube-formation abilities. However, there was no obvious difference between the groups cultured with different levels of VEGF and serum (Figure 3). These results indicated that angiogenesis was mainly affected by the interaction of EPCs and MSCs, but not by VEGF or serum.

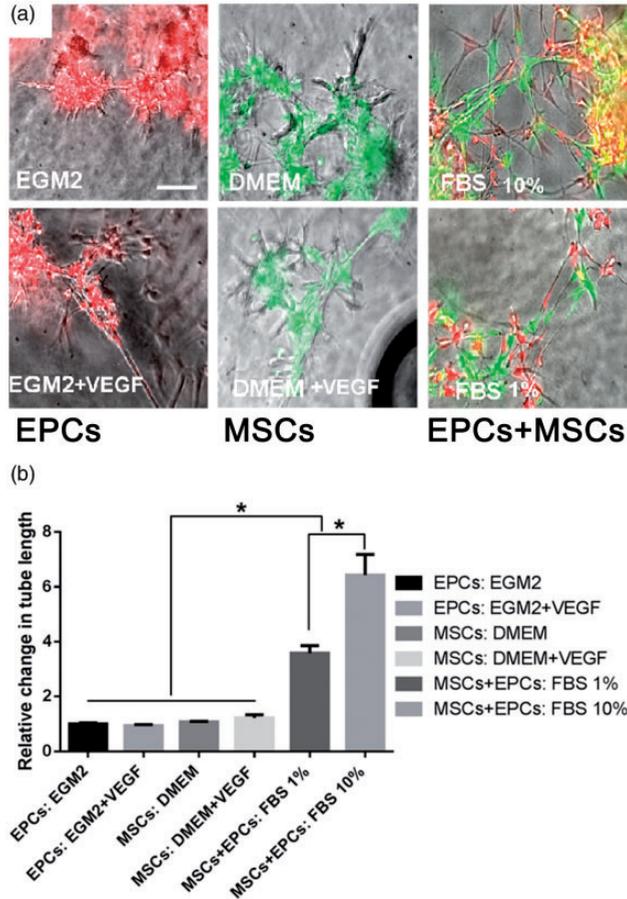


Figure 3. Effect of co-culture on formation of tube-like structures as indicator of angiogenesis. (a) Tube-formation assay was conducted after 24 hours of co-culture (15,000 EPCs and 15,000 MSCs). The addition of vascular endothelial growth factor and serum to the culture medium did not significantly affect angiogenesis. Angiogenesis was mainly affected by serum and the interaction of MSCs and EPCs. (b) Tube-like structures were detected using Image-Pro Plus 6.0. MSCs and EPCs were stained with Vybrant-DiO and DiI, respectively. Scale bar: 100 μm. *P < 0.05.

VEGF, vascular endothelial growth factor; FBS, fetal bovine serum; DMEM, Dulbecco's Modified Eagle Medium; EGM2, Endothelial Basal Medium; MSC, mesenchymal stem cell; EPC, endothelial progenitor cell.

Discussion

Extensive progress in bone tissue engineering has been achieved in recent decades, particularly in relation to cell sources, growth factors, and vascular formation.⁴ However, despite the initial optimism, limitations and challenges mean that bone tissue engineering practices have not yet proceeded to clinical practice. We therefore aimed to optimize an *in vitro* system to enhance osteogenesis and angiogenesis, to facilitate future clinical studies.

Recent studies on osteogenesis have focused on the role of stem cells.^{1,3} For example, MSCs can differentiate into osteoblasts, which secrete osteoid matrix, and co-culturing of MSCs and EPCs can promote osteogenesis.^{3,21} In addition to MSC/osteoblast combinations, HSCs and osteoclasts also contribute to osteogenesis.^{21,22} In this study, we co-cultured MSCs with combinations of HSCs and EPCs to assess how the different combinations affected osteogenesis.

The current results demonstrated that co-cultures of MSCs/HSCs and MSCs/EPCs/HSCs had higher expression levels of the genes encoding COL1A, OPN, ALP, and OCN compared with the control group, suggesting higher osteogenic activity. These findings demonstrated that HSCs promoted the osteogenic properties of MSCs, and that this promotion was further enhanced by co-culture with EPCs. This is consistent with a synergistic effect on osteogenesis mediated by interactions among MSCs, EPCs, and HSCs; however, further studies are required to uncover the exact molecular details.

Mineralization was evaluated by measuring ALP activity and calcium content. Although ALP activity was slightly higher in the MSC/EPC/HSC and MSC/HSC groups compared with the MSC/EPC group, there were no significant differences in ALP activity among the three groups.

However, ALP levels were previously reported to increase mainly at the beginning of differentiation from MSCs (fewer than 7 days),²³ whereas we measured ALP activity after 21 days of co-culture, and this longer culture period may have led to the small observed differences among the groups. We also evaluated mineralization by measuring calcium content. Although calcium content did not vary significantly among the groups, it was lower in the MSC/EPC and MSC/EPC/HSC groups. However, mineralization in the MSC/HSC/EPC group at day 21 was lower than that in the MSC/HSC group, which was in contrast to the results for ALP activity and the osteogenic gene expression patterns. This indicated the existence of a more complex mechanism in the interaction of MSC/HSC/EPC. HSCs can differentiate into osteoclasts to reduce mineralization, but this was not observed in the co-culture system, indicating that HSCs did not weaken mineralization. We further explored this result by examining the expression levels of several osteogenesis-related genes (*ALPL*, *COL1A1*, *BGLAP*, and *OSTP*) in each co-culture group. Generally, genes associated with osteogenesis had higher expression levels in the MSC/HSC and MSC/EPC/HSC groups. We detected the gene expression levels at 28 days post-treatment, during the later stage of osteogenesis, when *OSTP* and *BGLAP* had higher expression levels than *COL1A1* and *ALPL*, which mainly function at the early stage of osteogenesis.

MSCs are negatively affected by ischemia. It was reported¹⁵ that 50% of MSCs undergo apoptosis within 48 hours after injection into patients or mice. However, recent studies^{21,22} demonstrated that co-culturing MSCs and EPCs enhanced both osteogenesis and angiogenesis. Here, we co-cultured MSCs and EPCs using two concentrations of VEGF and serum concentrations to optimize angiogenesis.

Stem cells developed into tube-like structures within 24 hours, which is important for MSC survival and osteogenesis. We also showed that tube formation depended on serum and on the interaction between MSCs and EPCs, but was unaffected by VEGF concentration.

This study had some limitations. Although we showed the upregulation of genes involved in osteogenesis, more studies are needed to investigate the process of osteogenesis.

Collectively, our study provides evidence that co-culturing MSCs with either HSCs or EPCs/HSCs upregulates the expression of genes involved in osteogenesis. However, despite the changes in gene expression, the tested co-culture conditions did not significantly affect the process of osteogenesis.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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