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Examination of Vascular Capacity of Scaffold Free Human Microtissue During Osteogenesis

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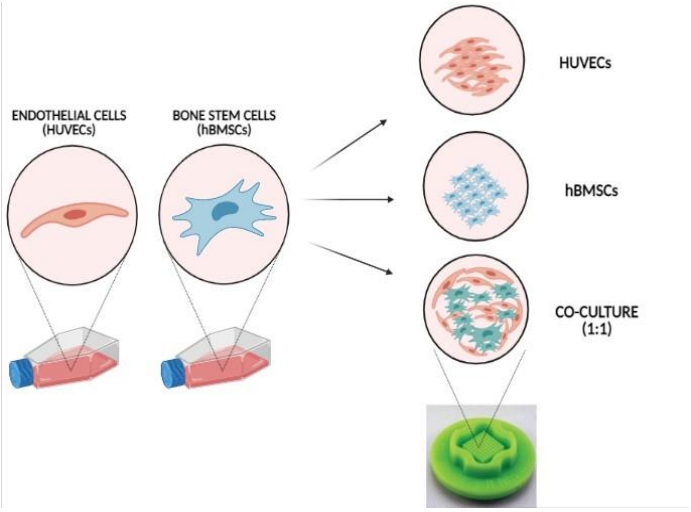
HIGHLIGHTS

- Endothelial cells support proliferation and viability of stem cells
- The coculture of endothelial and stem cell promoted to vascular capacity
- VEGF is one of the key protein for regulation of vascularization in coculture

Abstract: Vascularization is crucial strategy for bone tissue constructs. To mimic natural bone structure, researchers benefit from co-culture strategy by using stem cell and endothelial cells. The correlation of them supports proliferation of both cells. Moreover, the combination of them contributes vascularization marker expression such as Vascular Endothelial (Ve)-cadherin, Platelet Endothelial Cell Adhesion Molecule (PECAM) and Vascular Endothelial Growth Factor (VEGF). Detection of expression rate of markers may be limited in monolayer. Three dimensional scaffold free microtissues (3D SFMs) are used to understand natural expression rate of vascular markers and to form in vivo mimetic environments. To analyze proliferation and vitality of SFMs, diameter measurements and live and dead assay were performed. For vascular analysis, relative Ve-cadherin, PECAM and VEGF mRNA fold changes were calculated by qPCR. Moreover, VEGF immunofluorescence staining was performed to analyze expression of VEGF. Diameters and vitalities of SFMs coculture increased compared with only Human Umbilical Vein Endothelial Cells (HUVEC) and only Human Bone Mesenchymal Stem Cell (hBMSC) groups. mRNA expression of vascular marker was higher than only HUVEC and only hBMSC groups. The highest VEGF protein expression rate was observed in coculture rather than the other groups. hBMSC:HUVEC coculture showed that association of both cell contributes proliferation of cell viability. Moreover, the coculture showed upregulation of vascular marker and high VEGF protein expression. Co-culture system may be preferred to understand more straight responses in terms of vascularization during osteogenic differentiation.

Keywords: Vascularization; bone tissue engineering; Co-culture; Scaffold Free Microtissues.

GRAPHICAL ABSTRACT



INTRODUCTION

Bone tissue engineering (BTE) aims to develop bone regeneration strategies for crucial levels of bone fracture or damage [1, 2]. Recovery of bone damage depends on bone cell activity containing attachment, proliferation, migration and stem cell differentiation. Their environments conduct the activation of bone cells because the niche of bone cells regulates especially stem cell differentiation [1, 3]. Stem cells and endothelial cells contact each other in natural tissue. The relation supports the vascularization of bone [4]. The vascularization phenomenon is necessary to continue the viability of stem cells [5]. Vascularization supports vitality of cells by supplying oxygen and nutrients into tissue. Additionally, vascular structure removes wastes of cell metabolism.

Vascularization is regulated by endothelial cells [5-7]. One of the most commonly used endothelial cell type is Human Umbilical Vein Endothelial Cells (HUVECs) because of easy to obtain. HUVECs are located in the inner layer of vein and closely contact with stem cells to initiate vascularization. Previous studies have shown that the combination of HUVECs and Bone Marrow Stem Cells (BMSCs) increased viability of human BMSC and even vasculogenesis of bone. Another study, conducted by Nguyen LH and coauthors reported that HUVECs contribute to osteogenesis by supporting vascularization [8]. As HUVECs promote vascularization, they express some critical important markers such as Platelet Endothelial Cell Adhesion Molecule (PECAM), Ve-cadherin (Ve-cad), and Vascular Endothelial Growth Factor (VEGF). The molecular cues maintain viability and differentiation of stem cells. PECAM is an early vascular molecule that adhesion of endothelial cells. The adhesion of endothelial cells affects stem cells. PECAM may also trigger the vascularization of HUVEC and BMSCs coculture during osteogenesis. Ve-cadherin is another vascular marker that plays a fundamental role in adhesions of endothelial junctions [9, 10]. The molecule regulated endothelial contacts to control proliferation, apoptosis and growth [9, 11]. Signaling through VE-cadherin affects endothelial cell conduct by balancing action of growth factor receptors, intracellular messengers, and proteins that control quality record [11]. Actually, VEGF, is also another vasculogenic marker, is synthesized by endothelial cells and it is needed for the growth and viability of HUVECs during vasculogenesis [8, 12]. In addition, VEGF also supports stem cell viability in natural bone ECM. Furthermore, VEGF is an initiator for vascular growth during bone healing [8, 13]. In natural tissue, the relationship between osteoblasts and endothelial cells is controlled by VEGF releasing of osteoblasts, which forms vascular structure [6, 8]. Actually, the combination improves osteogenic markers such as alkaline phosphatase [8]. VEGF gets in close contact not only PECAM [14, 15] but also Ve-cadherin [11, 15] for vasculogenesis.

The investigation of VEGF molecule is critically important for the vasculogenesis of bone. There are many literature reports related to VEGF molecule expression fate of HUVECs in 2 dimensional (2D) monolayer culture

system [5, 6, 8, 16, 17]. However, 2D system is limited in terms of transmission of signal molecules. 2D system is restricted genes and protein expression rate. The reason for limitations may depend on devoid of ECM. ECM is the primary network for cell behaviors. ECM is more presentable by 3D scaffold free microtissues (SFMs) rather than 2D system. This is because 3D SFMs can produce self ECM just as in vivo. The natural ECM production in vitro mimics natural tissue from the point of high cellular connection. Gene and protein expression profile in 3D SFMs shows similarity to in vivo system.

In light of these information, the study aims to investigate vasculogenic potential of hBMSC:HUVEC co-culture and examine interactions of vascular markers such as PECAM, Ve-cadherin and VEGF in 3D SFMs.

MATERIAL AND METHODS

Materials

All materials were purchased from Sigma-Aldrich, St. Louis, Missouri, USA such as DMEM: F12, L-Glutamine, Penicillin Streptomycin, Triton X-100, Paraformaldehyde, DAPI (D9542), Fetal Bovine Serum (FBS), Phosphate Buffer Saline (PBS), Bovine Serum Albumin (BSA). Endothelial Cell Growth Medium SingleQuots (EGM) media was purchased from Lonza, Basel, Switzerland. Live and Dead Kit (Cellstain) was taken by Dojindo, Munich, Germany. For qPCR, all materials were taken from invitrogen, Thermo, Waltham, Massachusetts, USA. All antibodies were purchased by Santa Cruz Biotechnology Inc., Santa Cruz, California, USA. HUVECs were taken from Animal Cell Culture Laboratory of Bioengineering Department of Ege University, Turkiye. hBMSCs (300665-102) were taken from CLS Company, Eppelheim, Germany.

Cultivation of hBMSC and HUVEC

Both HUVECs and hBMSCs were cultured with DMEM: F12 containing 10% FBS, 10 mM L-Glutamine, 1% penicillin/streptomycin [18]. For both cells, culture was humidified incubator at 37 °C and 5% CO_2. The media of cells was refreshed every 2 days. After cells reached nearly 90 % confluency, they prepared to construct SFMs.

Fabrication of SFMs with only hBMSC, only HUVEC and hBMSC:HUVEC coculture

To form SFMs, agarose molds were prepared by 3D petri dish. Then, agarose incubated with media to ready for cell seeding [19]. SFMs of only hBMSC or only HUVEC were created with 100.000 cells. Co-cultured SFMs were also created total 100.000 cells (1:1 hBMSC:HUVEC). The SFMs were incubated with vascular EGM media (containing hydrocortisone, fibroblast growth factor (hFGF-B), insulin-like growth factor (R3-IGF-1), ascorbic acid, epidermal growth factor (hEGF), GA-1000 (gentamicin, amphotericin-B), heparin) supplemented with osteogenic compounds (100 nM dexamethasone, 50 µg/mL ascorbic acid, 10 mM ß-glycerophosphate).

Micrographs and Diameter Measurement of SFMs

Images of SFMs were taken to control under light microscope (Olympus, CKX41, Waltham, MA, USA) on day 1, 4 and 7. The diameter measurements of SFMs were analyzed on days 1, 4 and 7 by Image J (NIH, USA). The average diameter was calculated triple repeated SFMs.

Viability of SFMs

To understand vital effects of hBMSC and HUVEC coculture, live and dead assay was performed by Double Staining Kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) [20]. While the vital cells were stained bv Calcein-AM/DMSO (green), dead cells were stained by propidium iodide/purified water (red). After 15 min incubation time, cells were observed under fluorescence microscope. Micrographs of SFMs were also captured and merged. Then, color intensity was calculated for each experimental group. Green color intensity of HUVEC was measured and estimated as 100 % on day 1. The green intensities of only hBMSC and coculture were evaluated by comparing the HUVEC green intensity. The average color intensities were calculated.

Relative mRNA expressions of Ve-cadherin, PECAM and VEGF on SFMs

The fold differences of Ve-cadherin, PECAM and VEGF were determined at each time point described in previous studies [20, 21]. The sequences of the genes have been given in Table 1 [20]. Firstly, total RNA was

obtained from SFMs [20]. Then, extracted RNA was turned double stranded cDNA. 1 µg of cDNA was used to analyze fluorescence dye which is SYBR Green for qPCR (Step One Plus, Applied Biosystems, CA, USA). The fold differences of PECAM, Ve-cadherin and VEGF were calculated as $2^{(-\Delta\Delta CT)}$ formula.

Table 1. The forward and reverse primer sequences of vasculogenic markers [20].

GAPDH	F: GAAATCCCATCACCATCTTCC
	R: CCAGCATCGCCCCACTT
Ve-cadherin	F: TCACCTGGTCGCCAATCC
	R: AGGCCACATCTTGGGTTCCT
PECAM	F: GCTGACCCTTCTGCTCTGTT
	R: TGAGAGGTGGTGCTGACATC
VEGF	F: ATCTTCAAGCCATCCTGTGTGC
	R: GCTCACCGCCTCGGCTTGT

Immunofluorescence Staining of VEGF

To identify VEGF protein expression on SFMs, immunoflurescence staining was examined. For immunofluorescence staining, hBMSC:HUVEC SFMs were washed two times with PBS and fixed with 4% paraformaldehyde [22-24]. After 30 min. incubation at the room temperature, SFMs were lysed by 1% Triton X-100 in PBS for an hour. Then, they were blocked by 1.5% BSA for 2 hours. Primary mouse monoclonal anti-VEGF antibody (red) added into SFMs. Then, DAPI (blue) was added into SFMs solution. After 20 min incubation time at room temperature, all SFMs were washed two or three times with PBS. Fluorescence dye was observed under the microscopy. All images were taken and merged by CellSense Entry software.

RESULTS

To analyze formation and enlargements of the SFMs, SFMs images were taken on days 1, 4 and 7 as shown in Figure 1 during vasculogenesis.

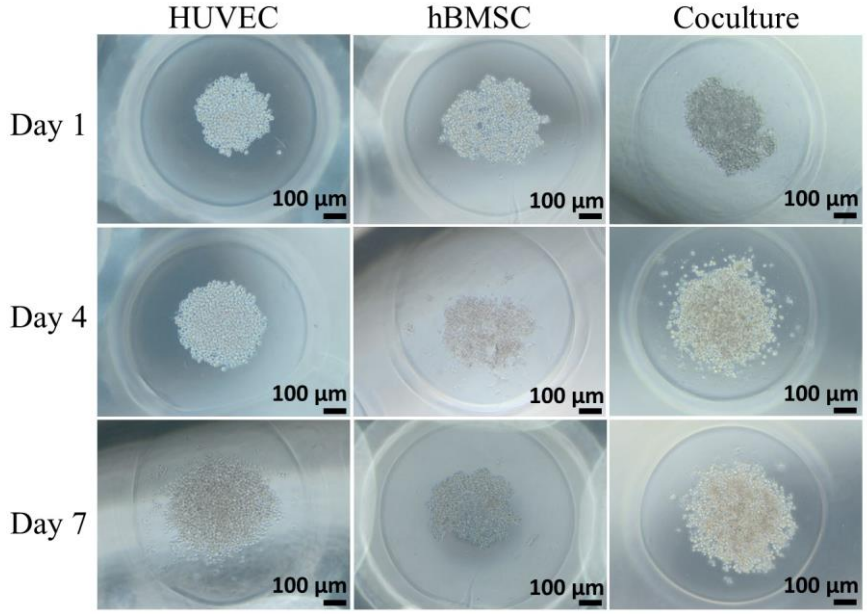


Figure 1. Images of HUVEC, hBMSC and coculture SFMs on days 1, 4 and 7 (Scale:100 µm).

Changes in the morphology of SFMs were well observed. All SFMs were greatly constructed until day 1. Additionally, the forms of all SFMs remained intact until the 7th day. The averages of SFMs were calculated from taken micrographs and given in Figure 2.

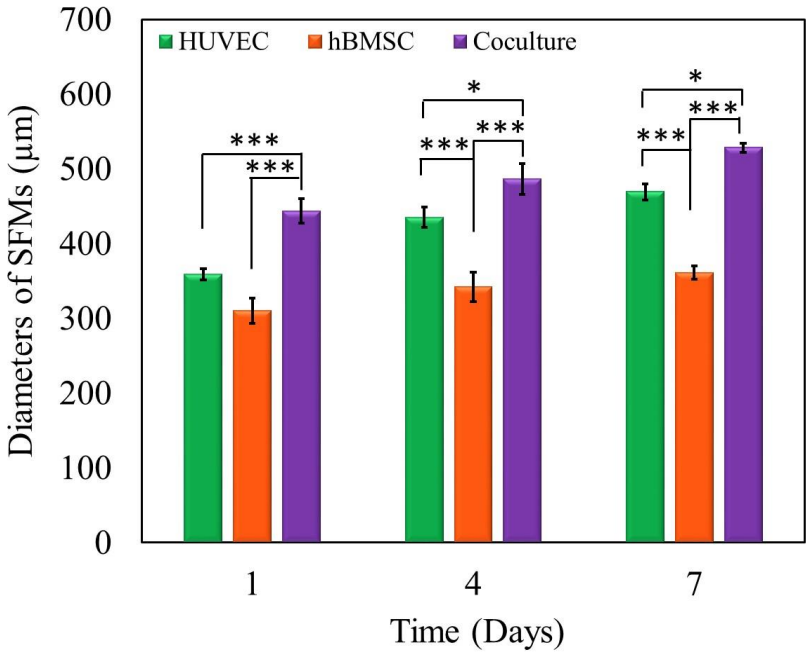


Figure 2. Diameter measurements of SFMs on days 1, 4 and 7. Results were analyzed by two way ANOVA and followed Tukey posthoc test (p*<0,05, p**<0,01 and p***<0,001).

Diameters of SFMs of only hBMSC, only HUVEC and coculture were measured as 310±17, 359±8,and 444±17 µm, respectively on day 1. For day 4, diameters were calculated as 342±20, 435±14, and 486±20 µm, respectively. At the end of the 7th day, diameters were 361±9, 469±11, 528±6 µm, respectively. The coculture SFMs showed statistically significant enlargement in each time point compared with only hBMSC and only HUVEC groups. The viability assay was also performed on days 1, 4, and 7 to compare experimental groups as shown in Figure 3.

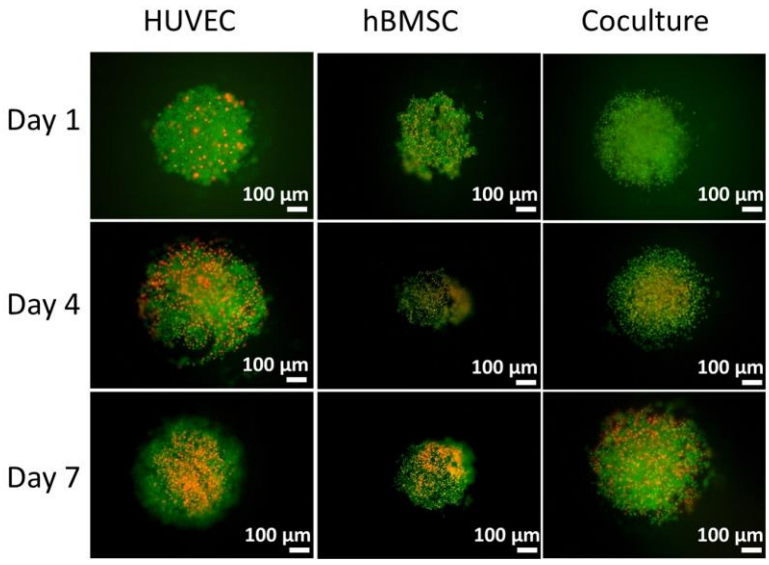


Figure 3. Viability analysis of SFMs on days 1, 4 and 7 (Scale:100 µm). While green color represents live cells, red color represents dead cells.

The viabilities of all SFMs were increased day by day. End of the 7th day, the highest viable cell was observed in coculture. Green color intensities of SFMs were measured and given in Figure 4.

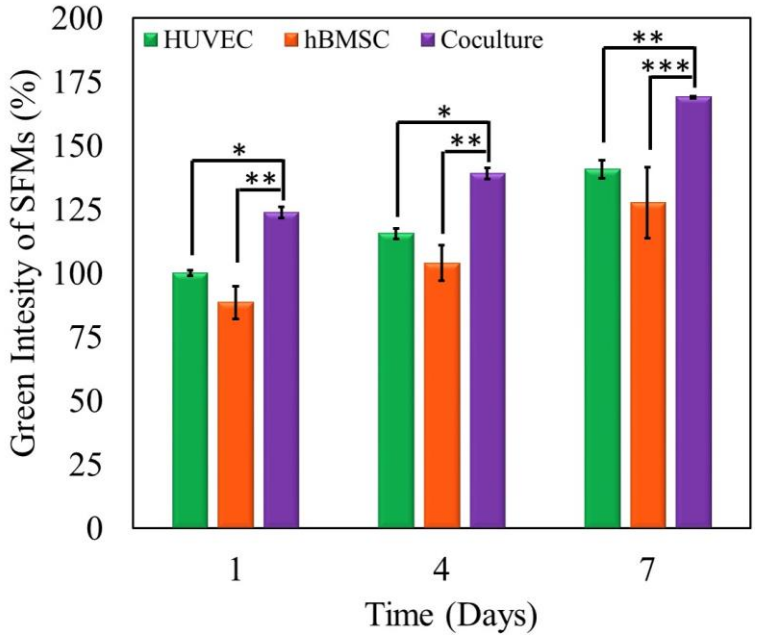


Figure 4. Green intensity measurement of SFMs. Results were analyzed by two way ANOVA and followed Tukey posthoc test (p*<0,05, p**<0,01 and p***<0,001).

The green intensity of only HUVEC was estimated as 100%. Only hBMSC and coculture groups were compared with HUVEC intensity. The viable cell intensities of only hBMSC and co-culture were calculated as 89±6 and 124±2, respectively on day 1. For day 4, intensities of only HUVEC, only hBMSC and co-culture were 116±2, 104±7 and 139±2, respectively. Finally, viability ratios were 141±4, 128±14 and 169±0,4, respectively on day 7. Fold differences of vascular genes such as Ve-cadherin, PECAM and VEGF were analyzed as shown in Figure 5 (A-C).

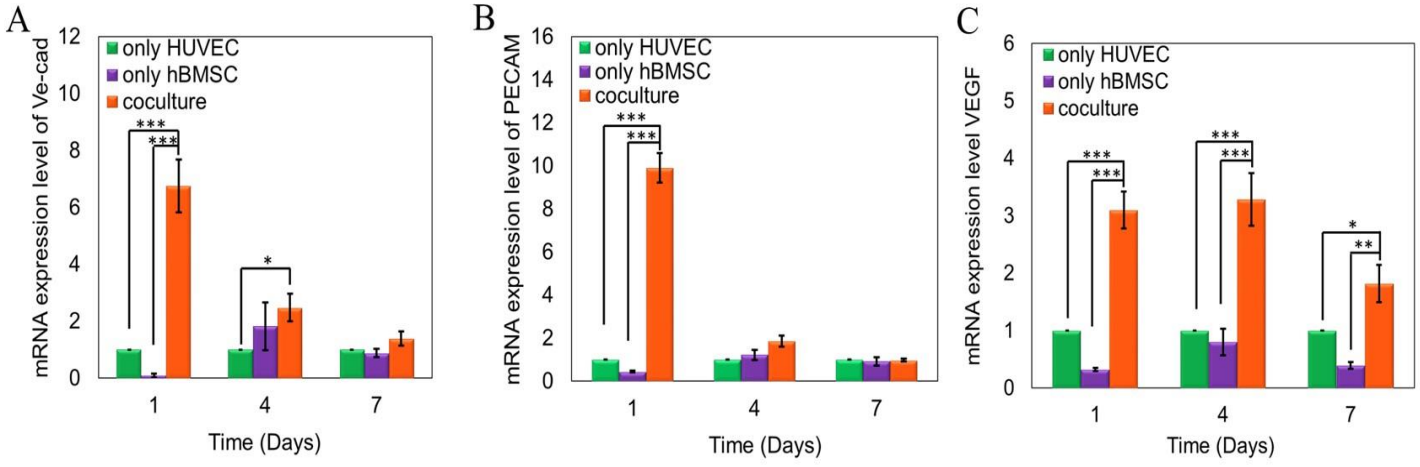


Figure 5 (A-C). Vascular mRNA expression of SFMs on days 1, 4 and 7. A) Ve-cadherin B) PECAM C) VEGF. Only HUVEC gene expression was estimated as 1. Relative gene expressions of only hBMSC and coculture were calculated by $2^{-(\Delta\Delta CT)}$ method and compared with only HUVEC. Results were analyzed by two way ANOVA and followed Tukey posthoc test (p*<0,05, p**<0,01 and p***<0,001).

Fold differences were calculated according to expression profile of only HUVEC SFMs which was estimated as 1. The expression rate of SFMs of coculture was significantly increased. On the first day, SFMs of the Ve-cadherin relative mRNA fold difference of only hBMSC and coculture were measured as 0,10±0,15 and 6,76±0,93, respectively. On day 4, Ve-cadherin gene expression rate was tested as 1,83±0,84 and 2,48±0,24, respectively. For day 7, the rates were 0,88±0,15 and 1,39±0,24, respectively. PECAM mRNA fold changes of only hBMSC and coculture were 0,44±0,04 and 9,91±0,68, respectively on day 1. Then, expression rates were 1,22±0,24 and 1,87±0,25, respectively on day 4. Finally, PECAM fold differences were calculated as 0,92±0,20 and 0,98±0,07, respectively. The expression rates of VEGF of only hBMSC and coculture were 0,32±0,03 and 3,10±0,32, respectively on day 1. Additionally, the VEGF changes were measured as 0,81±0,23 and 3,28±0,46, respectively on day 4. On the last day of vasculogenesis, VEGF fold differences were calculated as 0,40±0,06 and 1,82±0,33, respectively.

The confirmation of VEGF mRNA expression of coculture SFMs, it was performed through immunofluorescence staining as given in Figure 6 (A-C).

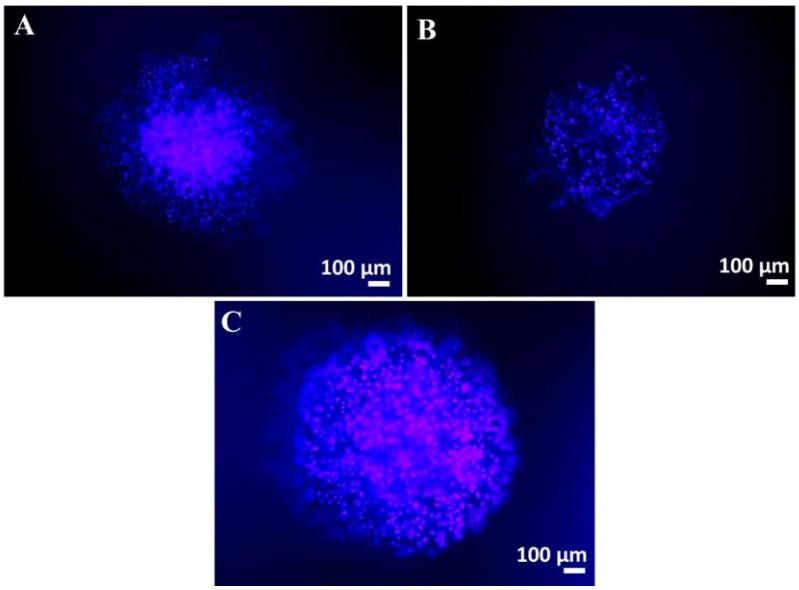


Figure 6. Images of VEGF immunofluorescence of HUVEC, hBMSC and coculture SFMs on days 1, 4 and 7 (Scale:100 µm). Nucleus on SFMs were dyed by DAPI (blue), and VEGF protein was dyed antibody (red).

According to DAPI staining, all SFMs showed nucleus forms but SFMs of coculture had more cell nuclei. VEGF the expression of vasculogenic maturation-related proteins was dramatically higher than only HUVEC and only hBMSC groups. Only HUVEC group showed much more VEGF expression than only hBMSC SFMs.

DISCUSSION

In the study, cell viability and vascular potential of hBMSC:HUVEC cocultivation were analyzed compared with only HUVEC and only hBMSC during osteogenesis. To give more natural cellular responses, it was fabricated SFMs and performed diameter measurement, viability assay, vascular gene expression and VEGF immunostaining.

All SFMs were greatly constructed in each group. Micrographs were followed day by day. Diameters of SFMs were measured on days 1, 4 and 7. Diameters of hBMSC:HUVEC SFMs were larger than only HUVEC and only hBMSC groups. The diameter enlargements of SFMs is linked with the proliferation rate of cell on SFMs [20, 25]. Thus, co-culture tends to enhance proliferation of cells on SFMs. On the other hand, a previous study showed that the combination of Adipose Derived MSC and endothelial cells promotes proliferation of endothelial cells [2]. Parallel to diameter results, the viability of co-culture was higher than only HUVEC and only hBMSC groups. The association of both cell lines were mutually increased viability. Endothelial cells are directly relevant in signal transduction with osteoprogenitor cells [26]. Previous studies also demonstrated that cells in coculture may enhance cell viability [2, 27].

Co-culture promotes not only endothelial cell proliferation but also the expression of vascular genes. mRNA fold differences of Ve-cadherin were shown as the highest in coculture group. In addition, bone like coculture may express osteogenic markers rather than only stem cell [26]. Ve-cadherin is a molecule that is found in endothelial junction point [11] and expressed in vascularization [9, 10]. The highest endothelial connection was observed in SFMs coculture compared with only HUVEC. Previous study clarified that following interaction with endothelial cells, MSCs aim to restore endothelium balance, perhaps via aiding the association of endothelial cell and VE-Cadherin [28]. Coculture, but not from endothelial or stem cells alone increased Ve-cadherin expression [29]. Similar to VE-cadherin, PECAM is another fundamental vascular probe that is an adhesion marker. PECAM may direct vascular fate of cells [30]. Thus, PECAM is closely related to VEGF in vascularization. In the study, coculture is more expressed PECAM vascular marker than only HUVEC group. Actually, only HUVEC showed less vascular marker expression rate than the control group. As it was expected, only hBMSC group didn't show any Ve-cadherin or PECAM expression. The connection of endothelial cells based on Ve-cadherin supports VEGF expression which is critically important factor for vasculogenesis [31]. VEGF is one of the major vascular markers which is regularly expressed during vascularization [23]. VEGF expression contributes to endothelial proliferation and maturation [8, 12]. Yu, J. and coauthors also showed that only Adipose Derived MSC (ADMSC) can even enhance VEGF expression [32]. A study which conducted by Nguyen and coauthors concluded that mRNA expressions of PECAM and VEGF in coculture were higher compared to the only HUVEC expression in collagen hydrogel-based scaffolds [33]. Moreover, VEGF mRNA fold expression of ADMSC spheroids was much more than monolayer ADMSC and ADMSC sheets [32] because spheroids support higher cell-to-cell interaction [34]. Additionally, ADMSC may trigger angiogenic potential of endothelial cells [23, 35]. Heidari-Moghadam A. and coauthors showed that coculture was a better mimicking platform than single cells [23]. In this study, it is observed that the highest VEGF fold change was in hBMSC:HUVEC coculture compared with only hBMSC and only HUVEC groups. Parallel to relative mRNA fold change, immunofluorescence results of coculture also showed the highest VEGF protein expression compared with only HUVEC group. Previous study concluded that VEGF promotes endothelial cell proliferation and viability [23]. Additionally, Pons J. and coauthors showed that producing VEGF from endothelial cells enhances mesenchymal stem cell proliferation and viability [36]. Moreover, low VEGF expression rate was limited regeneration capacity [7, 32]. Thus, the VEGF expression may induce both cell viabilities of stem cell and endothelial cells and also vascularization of bone like coculture in the study.

Consequently, the results of the study were consistent with the literature research concluded that co-culture of hBMSC:HUVEC could improve the proliferation and viability both only stem cell and only endothelial cells. It was clearly indicated that bone like coculture of hBMSC:HUVEC expressed highly vascular markers rather than only HUVEC. Moreover, VEGF is a key molecule not only endothelial cells but also stem cells. Thus, VEGF expression is critically important to form vascular networks between hBMSC and HUVEC. Thus, VEGF may contribute to the vascularization of bone-like forms.

CONCLUSION

The study fabricated SFMs to compare vascular capacities of bone-like co-culture and only endothelial cell. It is concluded that coculture pursued higher vascularization strategy than only HUVEC. To understand bone regeneration capacity in vitro monolayer culture, using coculture for further study may provide better vascular responses than only endothelial cell responses.

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Conflicts of Interest: The authors declare no conflict of interest.

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