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Influence of co-culture on osteogenesis and angiogenesis of bone marrow mesenchymal stem cells and aortic endothelial cells

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ABSTRACT

Co-culture of bone forming cells and endothelial cells to induce pre-vascularization is one of the strategies used to solve the insufficient vascularization problem in bone tissue engineering attempts. In the study, primary cells isolated from 2 different tissues of the same animal, rat bone marrow stem cells (RBMSCs) and rat aortic endothelial cells (RAECs) were co-cultured to study the effects of co-culturing on both osteogenesis and angiogenesis. The formation of tube like structure in 2D culture was observed for the first time in the literature by the co-culture of primary cells from the same animal and also osteogenesis and angiogenesis were investigated at the same time by using this co-culture system. Co-cultured cells mineralized and formed microvasculature beginning from 14 days of incubation. After 28 days of incubation in the osteogenic medium, expression of osteogenic genes in co-cultures was significantly upregulated compared to RBMSCs cultured alone. These results suggest that the co-culture of endothelial cells with mesenchymal stem cells induces both osteogenesis and angiogenesis.
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1. Introduction

Although bone heals itself, bone defects which are caused by infection, trauma, cancer, or diseases such as osteoporosis usually result in fracture non-unions and require tissue grafts. On the other hand, allo or xenografts have a high complication rate including the risks of infection and immune rejection (Salgado et al., 2004). Bone tissue engineering strategies address the problem of healing critical size bone defects by combining cells to regenerate the tissue, growth factors to guide cell behavior and scaffolds to provide a support for the cells to form the ultimate 3D shape of the targeted tissue (Yarlagadda et al., 2005; Chan and Leong, 2008; Lee and Atala, 2014). The main problem in these traditional bone tissue engineering attempts is the insufficient vascularization of newly formed bone tissue (Hofmann et al., 2008; Santos et al., 2009; Amaral et al., 2009; Ghanaati et al., 2011; Liu et al., 2013). Without proper vascularization and blood supply, cells in the tissue engineered construct suffer from hypoxia, depletion of nutrients and accumulation of waste products. In addition, biochemical signaling is disrupted, affecting tissue homeostasis and eventually making tissue regeneration difficult (Ghanaati et al., 2011; Aguirre et al., 2010).

In bone, a highly vascularized tissue, blood vessels play especially important roles in fracture healing by satisfying the oxygen and nutrient requirements, delivering hormones and directing inflammatory signals and cells to the wound site (Bai et al., 2013).

In most of the tissues, cells typically can survive up to a distance of 200 μm from the nearest capillary network and those further away from the capillaries suffer because they depend on simple diffusion for the transport of the nutrients and oxygen, and for waste removal (Lovett et al., 2009). Diffusion, however, is not adequate for thick and dense tissues like bone without proper vessel network in regeneration process and tissue engineering constructs can only rely on the ingrowth of host vessels which might not be early enough (Rouwkema et al., 2006). On the other hand, the contribution of infiltration of the local blood vessels is quite limited since it is very slow and only possible up to a depth of several hundred micrometers from the implant surface (Jabbarzadeh et al., 2008; Wang et al., 2010) and it is even harder in mineralized tissues such as bone. Thus, for the successful integration of tissue engineered constructs formation of a stable and functional vascular network is essential (McFadden et al., 2013). In order to obtain a fully functional, vascularized bone construct, bone tissue engineering researchers began including pre-vascularization of the construct as a

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step before implantation. One of the strategies to achieve pre-vascularization involves seeding the scaffolds with endothelial cells (ECs) that can spontaneously form vessel-like networks before seeding the bone forming cells (Levengood et al., 2011).

Another strategy is to use a co-culture of endothelial cells with bone forming cells to achieve vascularization simultaneously with osteogenesis. Since bone is a tissue formed by several cell types, co-cultures of heterogeneous cells can better mimic the *in vivo* microenvironment than monotypic cell cultures (Goubko and Cao, 2009). Besides, co-culture of ECs with bone forming cells allow researchers to study how the cellular crosstalk between these cell types affect functionality of the others (Kirkpatrick et al., 2011).

Interactions between heterogeneous cells provide the cues essential for differentiation, organization, and homeostasis as soluble signals and *via* cell-to-cell interactions. In bone, ECs in addition to being a component of angiogenesis, secrete regulatory molecules such as bone morphogenic proteins (BMPs), endothelins and prostaglandins that control the differentiation and activity of osteoblasts (Bai et al., 2013). Osteoblasts, in turn, influence EC activity by secreting angiogenic factors such as VEGF and bFGF (Santos et al., 2009). In direct co-cultures, cell-to-cell interactions between two cell types have also been shown to constitute the niche that influences cell fate including osteogenic differentiation (Villars et al., 2002; Saleh et al., 2011) and angiogenesis (Loibl et al., 2014).

In this study, mesenchymal stem cells isolated from rat bone marrow (BMSC) were directly co-cultured with endothelial cells isolated from rat aorta to study the effect of this co-habitation on angiogenesis and bone formation. Cell proliferation was studied to assess the compatibility of these cells. Differentiation to osteogenic lineages was assessed by ALP activity as an early osteoblast marker, von Kossa staining to observe mineralization and quantitative PCR to detect the expression of osteogenic genes by the BMSC. In addition, angiogenic tube formation was studied by confocal microscopy. This study involves co-culturing of two primary cell types of the same animal and the effects of this co-culture strategy on both osteogenesis and angiogenesis were investigated since there is a need for the literature to look the events not only from osteogenesis or angiogenesis point of view but also from both sides. It was also aimed to obtain an appropriate protocol for the successful primary endothelial and mesenchymal stem cells co-culture through seeding techniques and media compositions for a better osteogenesis and vascular structure formation.

2. Materials and methods

2.1. Isolation and growth of cells

This study was conducted after approval by Yeditepe University Animal Research Local Ethics Committee (YÜDHEK). Rat aortic endothelial cells (RAECs) were isolated from inside lumen of abdominal aorta of rats according to Kobayashi et al. (2005). Briefly, blood perfusion was applied to anesthetized 4-week-old Sprague Dawley male rats. When the perfusion was ended, aorta was exposed and dissected from the aortic arch to abdominal aorta. Connective and adipose tissue around the aorta was removed with a fine forceps and scissors under the stereomicroscope. Aorta was incubated in a collagenase Type II (Gibco, Invitrogen, USA) (2 mg/mL) solution at 37 °C for 45 min. RAECs were removed from aorta by flushing the lumen of the aorta with DMEM (Gibco, Invitrogen, USA) containing 20% fetal bovine serum (FBS) (Gibco, Invitrogen, USA) and plated in collagen Type I (Roche, Germany) coated tissue culture flasks. In order to prevent any cross contamination by smooth muscle cells or fibroblasts, medium was removed after 1.5 h incubation at 37 °C. Attached cells were washed with warm PBS (Gibco, Invitrogen, USA) and rat aortic endothelial cell growth medium was added (Cell Applications, USA). Medium was changed twice a week.

RBMSCs were isolated from the same rats by flushing the bone marrow of the femur and the tibia. RBMSCs were cultured in DMEM supplemented with 10% FBS (Gibco, Invitrogen, USA) and 100 units/mL penicillin-streptomycin-fungicide mixture (Pan Biotech, Germany). Medium was changed twice a week.

Cell passages of 3–5 were used for all of the experiments performed.

2.2. Characterization of cells

Specific cell surface antigens of passage 3 RBMSCs were analyzed by flow cytometry (FACSCalibur – BD Pharmingen, USA). Cell surface markers analyzed for RBMSCs were: a) rat specific hematopoietic lineage markers, CD 45 (BD Pharmingen, USA) and CD 11a (BD Pharmingen, USA), and b) rat specific MSC markers, CD 90 (BD Pharmingen, USA) and CD 29 (BD Pharmingen, USA). Briefly, 5×10^5 RBMSCs were incubated for 1 h at 4 °C with conjugated antibodies and then washed with PBS (Gibco, Invitrogen, USA) twice to remove excess antibodies. Cells were then resuspended in 400 μL PBS, assayed using FACSCalibur (Becton Dickinson, USA) and the data was analyzed using Cell Quest software (Becton Dickinson, USA).

RAECs were incubated with CD31 (PECAM-1) primary antibody (LSBio, USA) for 1 h at 4 °C followed by incubation with FITC conjugated secondary antibody (Santa Cruz Biotechnology, USA) for 1 h at 4 °C and observed by fluorescence microscope (Nikon, Eclipse TE200, USA). To further characterize RAECs, Angiogenesis Assay (Cell Biolabs, USA) was performed according to the company's instructions in order to test the ability of the isolated endothelial cells to form angiogenic tubes on extracellular matrix (ECM) gel. Formed tubes were observed by both bright field and fluorescence microscopes after being stained with Calcein AM in the Angiogenesis Assay kit (Cell Biolabs, USA).

2.3. Co-culture and preparation of co-culture media

RBMSCs and RAECs were mixed in 5:1 ratio prior to seeding. Cell suspensions containing 5×10^4 RBMSCs and 1×10^4 RAECs were transferred to 6-well plates and then medium was added into each well. Only RBMSC and only RAEC containing wells were included as controls. Since the media for the co-culture groups had to support both cell types, growth media of RBMSCs and RAECs were mixed in 1:1 ratio. In the differentiation studies, supplements required for the differentiation of RBMSCs, such as ascorbic acid (Sigma-Aldrich, USA) (50 μM) dexamethasone (Sigma-Aldrich, USA) (100 nM) and β-glycerophosphate (Sigma-Aldrich, USA) (10 mM), were added to each respective medium. Media and supplements added are listed in Table 1.

2.4. Assessment of cell proliferation

MTS test (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, USA) was used to determine cell proliferation (Kose et al., 2003). Briefly, MTS reagent (200 μL) was added to each well of the 6-well plate and incubated for 2 h at 37 °C in a CO_2 incubator. Absorbance was determined at 490 nm using an Elisa Plate Reader (BIO-TEK, ELx800, USA).

2.5. Determination of alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was measured spectroscopically using ALP kit (RANDOX Laboratories, Ireland). Cells were first lysed with Tris (Sigma-Aldrich, USA) buffer (0.1 M, pH 9.0) containing 0.01% Triton® X-100 (Sigma-Aldrich, USA). Cell lysates were subjected to 3 successive freeze-thaw cycles by freezing at −20 °C for 10 min and thawing at 37 °C for 10 min. Then, samples were sonicated for 10 min on ice with 30 s breaks every minute. Each sample (100 μL) was mixed with 20 μL of *p*-nitrophenyl phosphate solution supplied by ALP kit (RANDOX Laboratories, Ireland). Absorbance was measured every minute at 405 nm for 10 min using Elisa Plate Reader (BIO-TEK, ELx800, USA). ALP activity was calculated using a calibration curve

Table 1
Growth and differentiation medium used for cell culture studies and their ingredients (Dex: dexamethasone, Asc. acid: ascorbic acid, β-GP: β-glycerophosphate, RECGM: rat endothelial cell growth medium).

Samples	Medium name	Medium type	Medium supplements		
			Dex	Asc. acid	β-GP
Diff (+) co-culture	Osteogenic differentiation medium for co-cultures	DMEM (4.5 g/L glucose): RECGM (1:1)	0.1 μM	50 μg/mL	10 mM
Diff (+) RBMSC	Osteogenic differentiation medium for RBMSC	DMEM (4.5 g/L glucose)	0.1 μM	50 μg/mL	10 mM
Diff (−) co-culture	Growth medium for co-cultures	DMEM (4.5 g/L glucose): RECGM (1:1)	–	–	–
Diff (−) RBMSC	Growth medium for RBMSC	DMEM (4.5 g/L glucose)	–	–	–
Diff (−) RAEC	Growth medium for RAEC	RECGM	–	–	–

constructed with known concentrations of ALP (Millipore, USA) in nmol/min units. Values were normalized using DNA concentration.

2.6. Determination of mineralization

Mineralized nodules in cultures were assessed with von Kossa staining (American Master Tech Scientific, USA) after fixation with 2% (*w*/*v*) paraformaldehyde (Sigma-Aldrich, USA). Briefly, 1% silver nitrate solution was added on the cells after fixation and cells were exposed to UV for 20 min. Reaction was stopped by the addition of 5% sodium thiosulfate solution. Light microscopy images of the mineralized bodies were obtained through inverted microscope (Nikon, Eclipse TC100, USA).

2.7. Assessment of microvascularization

Cells were fixed with 3.7% (*w*/*v*) formaldehyde (Sigma-Aldrich, USA) containing 0.001% (*v*/*v*) Tween® 20 (AppliChem, Germany) for 30 min and then stained with Alexa Fluor® 546 Phalloidin (Molecular Probes, Invitrogen, USA) for 50 min to observe the morphology of the cells. Fluorescently tagged cells were then observed using confocal microscope (Leica, TCS SP2, Germany). Three images corresponding to each group were analyzed by uploading the images *via* the Wimasis Web platform (https://mywim.wimasis.com) to the automated analysis tool WimTube. The resulting data were calculated and graphed by Excel.

2.8. Real-time PCR

Total RNA was isolated using Roche High Pure RNA isolation kit (Roche, Germany). Then, mRNA was converted to single strand cDNA using oligo(dT) primers with Sensiscript Reverse Transcription Kit (Qiagen, Netherlands). Real-time PCR experiments were performed using Maxima SYBR Green Master Mix (Thermo Scientific, USA) and reaction was carried out in CFX96 Touch™ Real Time PCR Detection System (Bio-Rad, USA). Primer sequences for mRNAs of housekeeping gene GAPDH, and osteogenic genes Runx2, Collagen Type I (Col I), Osteocalcin (OC), and Osteonectin (ON) are shown in Table 2.

2.9. Statistical analysis

Data is presented as the mean ± standard deviation resulting from independent experiments. Two-tailed *t*-test was applied to compare the mean values between groups. Differences were considered significant when $p < 0.05$. * indicates significant difference with $p < 0.05$. * * indicates significant difference with $p < 0.01$.

Table 2
Sequences of primers specific for housekeeping and osteogenic mRNAs.

Genes	Primer sequences (5′ → 3′)	
	Forward	Reverse
GAPDH	CGATCCCGCTAACATCAAAT	GGATGCAGGGATGATGTTCT
Runx2	TCACTACCAGCCACCGAGAC	ACGCCATAGTCCCTCCTTTT
Col I	CAGGCTGGTGTGATGGGATT	AAACCTCTCTCGCCTCTTGC
OC	AAGTCCCACACAGCAACTCG	GTCCTGGAAGCCAATGTGGT
ON	CACTGGCTGTGTTGGAAACG	GTGGAGGAGACAGCAAGGTC

3. Results

3.1. Characterization of RBMSCs

RBMSCs were tagged with conjugated antibodies against rat CD45, CD11a, CD29, and CD90 (BD Pharmingen, USA). The flow cytometry of RBMSCs indicated that the cells were negative for the hematopoietic stem cell markers CD45 and CD11a (Fig. 1a and b), and positive for mesenchymal stem cell markers CD29 and CD90 (Fig. 1c and d) showing that after several medium changes and passages hematopoietic cells were eliminated and the cell source used in the study was pure mesenchymal stem cells.

3.2. Characterization of RAECs

Isolated RAECs had polygonal morphology (Fig. 2a) before confluency and showed characteristic cobblestone like morphology when they reached confluency. They were positive for CD31 (PECAM-1) (Fig. 2b). RAECs formed angiogenic tubes 4 h after seeding on ECM gel (Fig. 2c, d, e, and f).

The positive staining by CD31 (PECAM-1) antibody and tube formation confirmed that the cells were of endothelial origin and not contaminated by smooth muscle cells or fibroblasts.

3.3. Assessment of cell proliferation

An increase was observed in the cell numbers of all the samples during the 14 days of incubation (Fig. 3). When cultured alone, RAECs proliferated more compared to the co-cultured cells indicating that in the presence of RBMSCs their high proliferation rate was repressed.

It was also observed that addition of the differentiation medium decreased RAEC proliferation both when alone and in the co-culture. Differentiation medium decreased the proliferation rate of the other cells, the RBMSCs too, because the cells were directed towards differentiation. Lowest cell proliferation was observed with single RBMSC cultured owing to their longer doubling times (46 h), twice as long as that of the endothelial cells (26 h) (data not shown). Thus, endothelial cells showed higher proliferation rates in the co-cultures.

The most important results obtained from cell proliferation assay were that RBMSCs and RAECs were compatible when co-cultured directly on tissue culture plates. According to our preliminary studies, RAECs dominated the culture covering the space reserved for RBMSCs too when they were used in ratios of 1:1, 2:1, and 4:1 (RBMSC:RAEC) (data not shown). However, RAECs did not dominate the RBMSCs when they were co-cultured in a ratio of 5:1. This showed that both types of cells managed to proliferate under a set of conditions which is

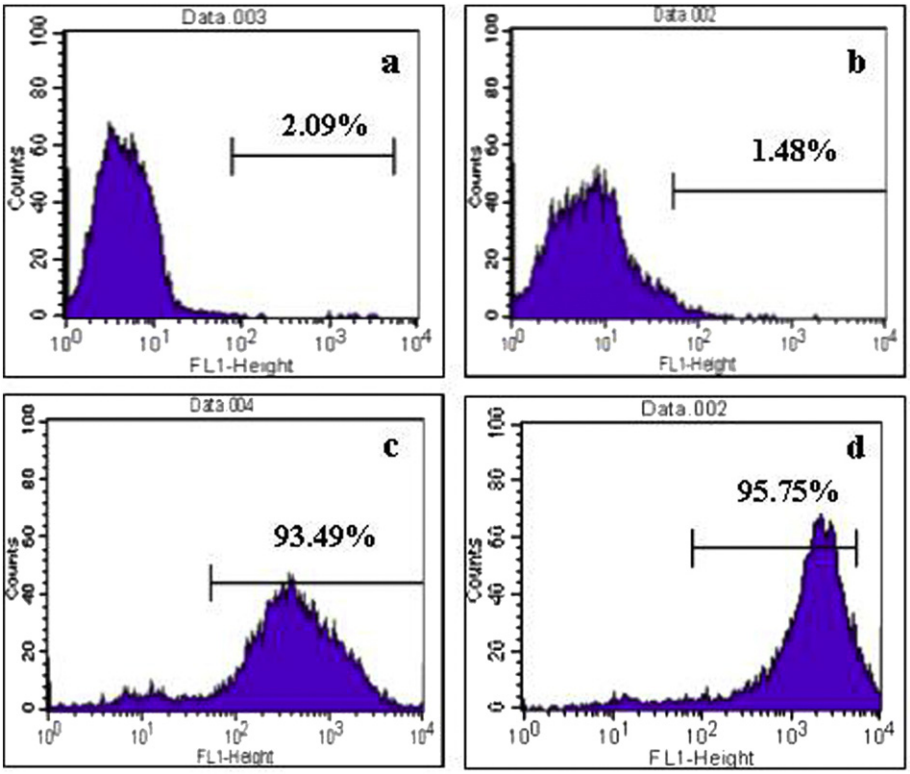

Fig. 1. Flow cytometry histogram of a) CD45, b) CD11a, c) CD29, and d) CD90 labeled RBMSCs (obtained by FACSCalibur). Fractions of positive gated cells were indicated on histograms.

appropriate for both types of cells without leading to the domination of one cell type over the other.

3.4. ALP activity

ALP activity assay was performed for RBMSCs, RAECs and for their co-cultures when they were cultured in differentiation or growth media listed in Table 1. ALP activity was measured using *p*-nitrophenylphosphate as the substrate. As it was expected, no ALP activity was observed in RAEC samples whether in differentiation medium or not (+Diff, −Diff) because these cells are of endothelial origin and no ALP activity can be expected of them (Fig. 4). On the other hand, with RBMSC, ALP activity was shown even in the absence of the osteogenic supplements. RBMSC –Diff group showed ALP activity possibly due to the presence of cells already committed to osteoblastic phenotype. When RBMSC –Diff and Co-culture –Diff are compared a distinct enhancement due co-culturing of MSCs with ECs is observed indicating the importance of the cross talk between the two cell types.

Our results showed that both Co-culture samples (with and without differentiation medium) exhibited more ALP activity than both RBMSC (with and without differentiation medium) samples. The co-cultures expressed more ALP on Day 1 than RBMSCs showing that co-culturing with endothelial cells encourages earlier commitment of MSCs to osteoblasts. ALP activity declined during the 14 days of incubation.

3.5. Assessment of microvascularization

When the morphologies of the cells and the structures formed by them were examined, multiple tube-like structures were observed in co-cultures after 14 (Fig. 5a and b) and 21 days of incubation (Fig. 5c and d) possibly formed by RAECs. As expected, in the absence of the EC no tube-like structures were observed. However, it was interesting to observe significantly less amount of tube-like structures for 21 days (Fig. 5g and h) when RAECs were incubated alone indicating a distinct positive role of RBMSCs in microvessel formation.

3.6. Assessment of mineralization

Mineralization is a late marker and the final stage of osteogenesis. A positive staining (brown) of mineralized nodules by von Kossa was observed in the co-cultures after 7 days of incubation (Fig. 6a) whereas a positive staining could be detected only after 14 days when RBMSCs were incubated alone (Fig. 6e). Since mineralization is a late osteoblastic differentiation marker, positive staining after 14 days of incubation is an appropriate time for the initiation of mineralization (Aronow et al., 1990). After 21 days both co-culture samples and RBMSC alone samples were largely mineralized (Fig. 6g and h). Only the RAECs samples showed no sign of mineralization throughout the 21 days of incubation (Fig. 6c, f, and i). Thus it can be concluded that co-culturing accelerates the initiation of mineral deposition.

3.7. Assessment of gene expression

In order to understand the molecular background of osteogenesis in samples, quantitative PCR was performed using primers for mRNAs of bone specific genes. mRNA expression levels of osteogenic genes were normalized using the expression level of housekeeping gene GAPDH (Fig. 7). On Day 14, both the only RBMSC and co-culture samples showed a similar gene expression profile except for osteonectin (ON) being significantly higher in the co-culture samples. Runx2 gene, which controls the commitment of mesenchymal cells to the osteoblastic lineage (Zhang et al., 2009) seemed to be expressed ubiquitously in all samples with similar amounts. Collagen Type I (Col I), major component of the bone organic matrix (Luz and Mano, 2010), was expressed in both the RMBSC and the co-culture samples after 14 days of incubation in the osteogenic medium, and it was significantly up regulated in the RBMSC and the co-culture samples after 28 days in the osteogenic medium. In the co-culture samples, expression of Col I was significantly higher than the RBMSC samples indicating a higher level of bone ECM synthesis. Osteocalcin was also expressed only in detectable amounts in the osteogenic medium after

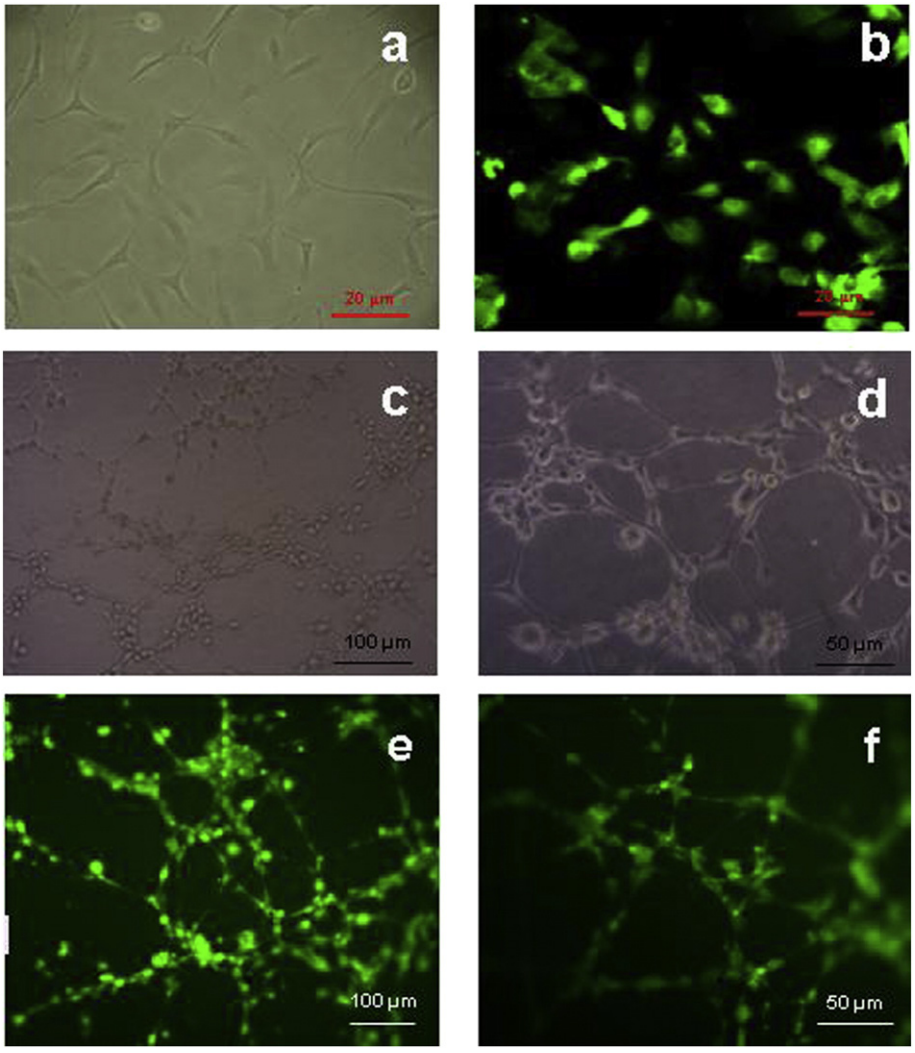

Fig. 2. Characterization of endothelial cells. a) Brightfield microscopy of polygonal morphology of endothelial cells before they reach confluency. b) Fluorescence microscopy image of RAECs that are positive for PECAM-1. c and d) Brightfield microscopy of endothelial cells that form angiogenic tubes on ECM gel. e and f) Fluorescence microscopy of endothelial cells that form angiogenic tubes on ECM gel. Scale bars: a and b: 20 μm, c and e: 100 μm, d and f: 50 μm.

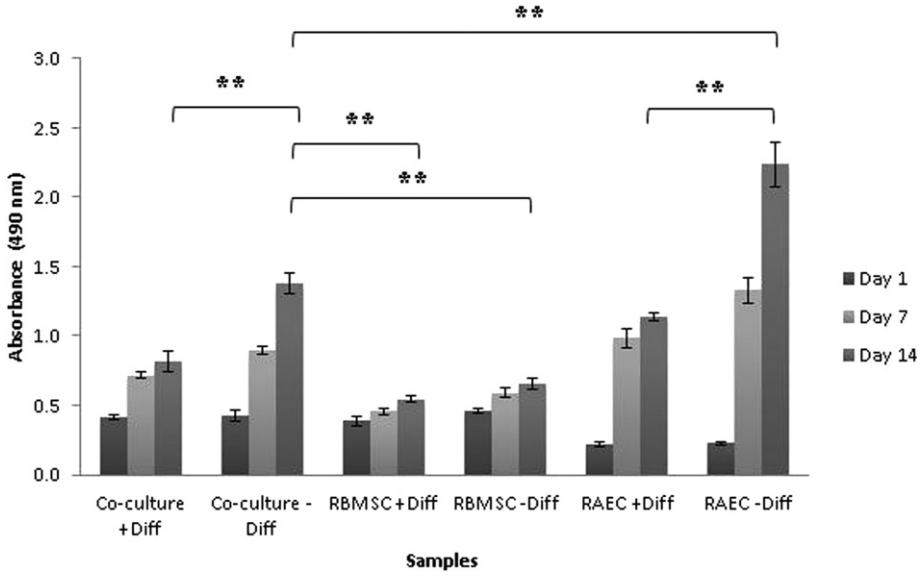

Fig. 3. Cell Proliferation by MTS assay throughout 14 days of incubation of co-culture, RBMSCs and RAECs cultured with growth (−Diff) or differentiation (+Diff) medium. ** indicates a significant difference with a $p < 0.01$.

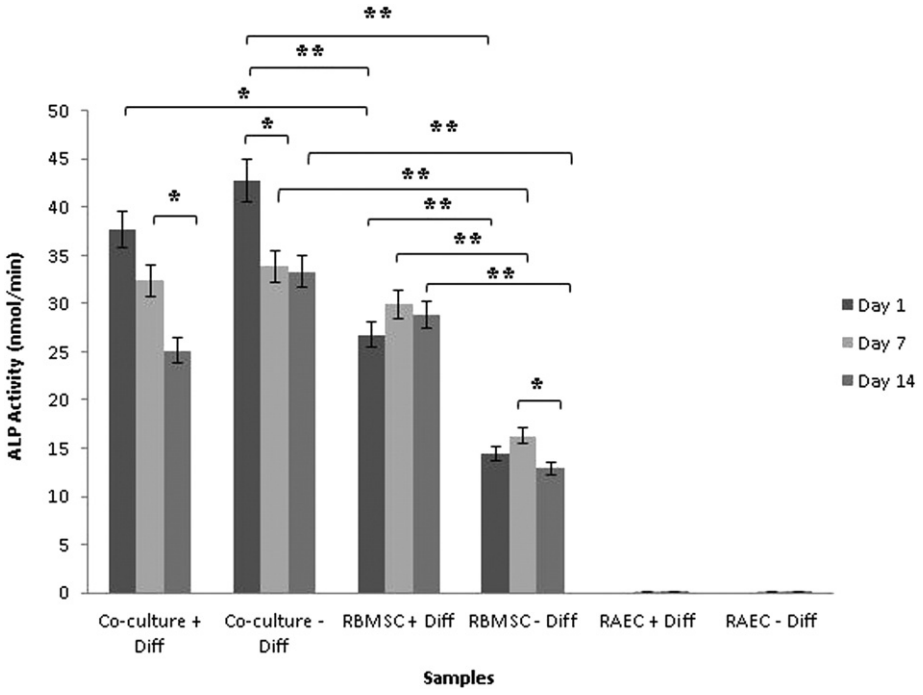

Fig. 4. ALP activity throughout 14 days of incubation in growth or differentiation mediums. * indicates a significant difference with a $p < 0.05$. ** indicates a significant difference with $p < 0.01$.

14 days of incubation in both RBMSC and co-culture samples, and was up regulated only in the co-culture samples after 28 days. High expression of bone specific genes also showed that endothelial cells did not dominate the culture during the 28 days of incubation allowing the survival, and eventually, the differentiation of RBMSCs towards osteogenic lineages.

4. Discussion

Today, it is considered a must to study the crosstalk between different cell types involved in the tissue regeneration in order to understand the underlying mechanism (Kirkpatrick et al., 2011). Most of the co-culture studies related to bone tissue engineering until now, however, involve only co-culture of mesenchymal stem cells with cell lines (Hofmann et al., 2008; McFadden et al., 2013; Saleh et al., 2011; Dahlin et al., 2014; Kim et al., 2013; Kang et al., 2013) which probably does not reflect the actual interactions between cells. Besides, most of the studies previously done in this specific field investigate the effects of culturing of endothelial cells with bone forming cells in 2D or in 3D on osteogenesis or angiogenesis alone. In this study, co-culture of primary cells from two cell sources (rat bone marrow derived

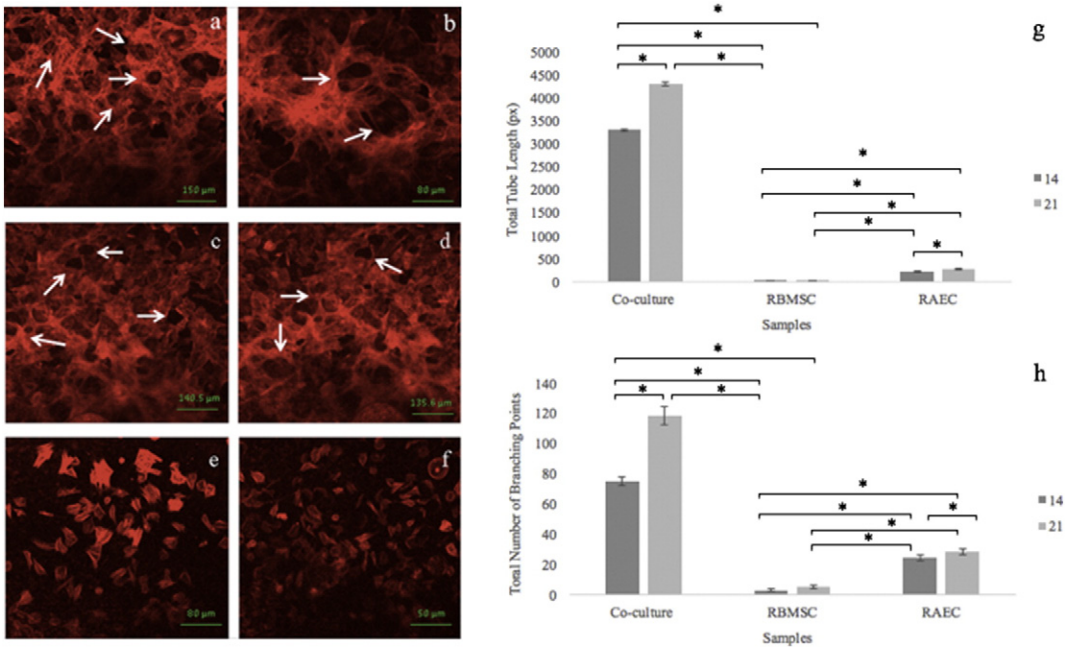

Fig. 5. Confocal microscopy of co-cultures after (a, b) 14 days and (c, d) 21 days of incubation (10× objective). As controls (e) Only RBMSCs and (f) only RAECs after 21 days of incubations were included. Arrows in a, b, c, and d show the regions that the endothelial cells have formed angiogenic tubes. g) Total tube length (px) and h) Number of branching points analyzed by Wimasis WimTube tool. The scale bars are: a) 150 μm, b) 80 μm, c) 140.5 μm, d) 135.6 μm, e) 80 μm and f) 50 μm. * indicates a significant difference with a $p < 0.05$.

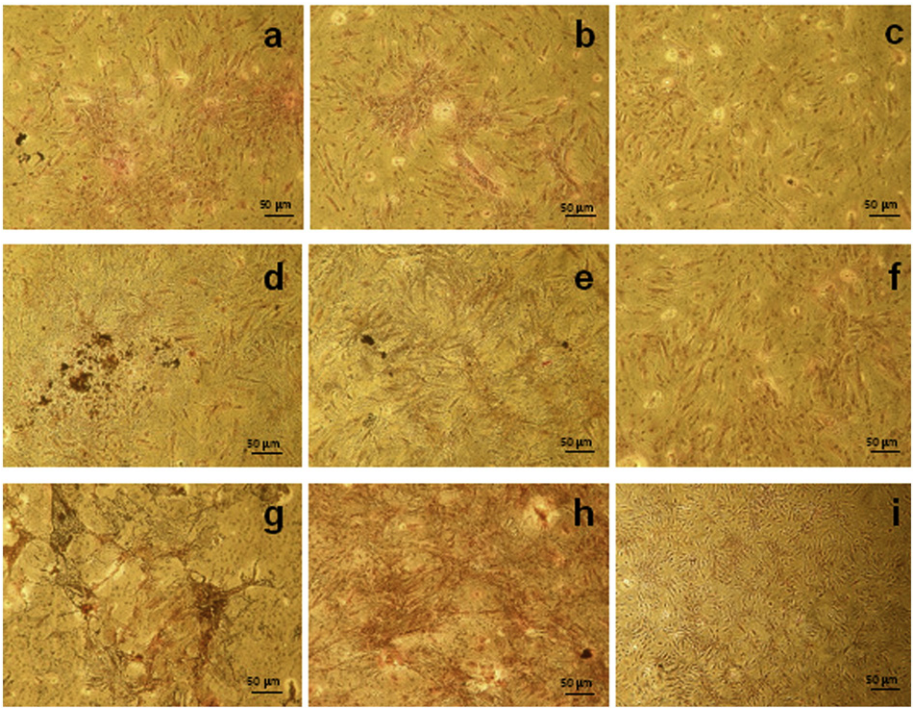

Fig. 6. von Kossa staining of (a, d, g) Co-culture samples; (b, e, h) only RBMSC samples; (c, f, i) only RAEC samples after 7 days (first row), 14 days (second row) and 21 days (third row) of incubation (10× objective). The scale bars are 50 µm.

mesenchymal stem cells and rat aortic endothelial cells) were used to study the effect of co-culturing on both osteogenesis and on vessel formation.

In the present work, it was possible to achieve proliferation of these two cell types without the domination of one cell type over the other. This was achieved by co-culturing RAECs and RBMSCs in a ratio of 5-to-1; when higher ratios were used the RAECs dominated the culture. It was also stated by Kirkpatrick et al. (2011) that a lower proportion of the more proliferative and less fastidious cell type will usually be necessary, otherwise the more proliferative one would dominate the culture if seeded in higher proportions.

In the co-culture groups the high proliferation rate of the RAECs was repressed regardless of using growth or differentiation medium. This can possibly due to the halving of angiogenic factors' concentrations present in the endothelial growth medium when mixed with RBMSC growth or differentiation medium (Table 1). Similar results were also obtained by Gershovich et al. (2013). On the other hand, results contrary to ours were also reported by other researchers, such as Jones et al. (1995) who reported that osteoblasts increased the proliferation rate of endothelial cells *in vitro* when they were co-cultured. Also addition of differentiation medium decreased RAEC proliferation when they are cultured alone or in the co-culture due to the effect of differentiation medium supplements such as dexamethasone, ascorbic acid, and β-glycerophosphate.

In addition to proliferation of both cell types in culture, RBMSCs showed significantly higher ALP activity starting from day 1 of culture

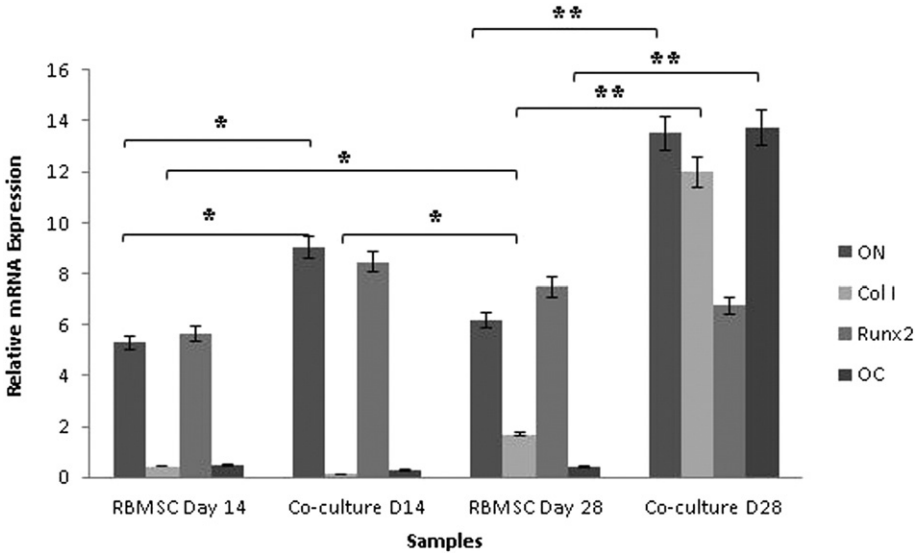

Fig. 7. Relative mRNA expression of osteogenic genes in RBMSC and co-culture samples after 14 and 28 days of incubation. ON: Osteonectin, Col I: Collagen Type I, OC: Osteocalcin Activity. * indicates a significant difference with a $p < 0.05$. ** indicates a significant difference with a $p < 0.01$.

when they were co-cultured with endothelial cells. Alkaline phosphatase (ALP) is an important early osteoblastic differentiation marker, showing the commitment of stem cells to become an osteoblast. It reduces phosphate-containing substances to produce free phosphate for bone mineralization and hydrolyzes pyrophosphate (PPi), a known inhibitor of hydroxyapatite formation, regulating the mineralization process (Sun et al., 2009). ALP activity declined throughout the 14 days of incubation in this study since ALP activity decreases when mineralization is initiated as was reported by (Yokose et al. (2000).

Several studies so far reported that culture of bone forming cells with endothelial cells, or with microvessel cell-conditioned media, led to decreased ALP activity (Meury et al., 2006; Sun et al., 2007).

However, while other studies are in agreement with ours, contrary results were also reported. It was stated that co-cultures of Human Mesenchymal Stem Cells (HMSC) and Human Umbilical Vein Endothelial Cells (HUVEC) showed higher ALP activity compared to HMSCs monoculture (Ma et al., 2011). Xue et al. (2009) also demonstrated a 5-fold increase in ALP expression when MSCs were co-cultured with ECs < 20% of the total cell population. Several others demonstrated that endothelial cells are capable of inducing osteoblast differentiation of rat and human osteoprogenitor cells *in vitro* as well as osteogenesis *in vivo* (Ma et al., 2011; Guillotin et al., 2004). Gershovich et al. (2013) also observed a decrease in cell number and increase in ALP activity with the co-culture. This is possibly due to the presence of soluble factors such as VEGF expressed by RAECs, an agent essential for angiogenesis, fracture repair and mineralization in response to bone injury (Street et al., 2002). Literature states that inhibition of VEGF blocks FGF-2 or BMP-2 induced angiogenesis, BMP-7 induced of primary osteoblast differentiation, and BMP-4-induced bone formation (Richard et al., 2003).

One other issue is the type of contact between the cells. Direct contact between two cell types (one angiogenic and the other osteogenic) is said to be necessary for the induction of osteogenic differentiation because some researchers found that increased ALP activity could only be observed when there is direct contact (Villars et al., 2002, 2000). During direct contact of RBMSC and RAEC, transmembrane proteins and gap junctions may be involved in increasing the differentiation capacity. Endothelial cells might induce mineralization by driving the mesenchymal stem cells into an osteoblastic phenotype. This was also suggested by other co-culture studies (Sun et al., 2007; Kaigler et al., 2005, 2006) and implies that endothelial cells are “osteoinductive”. One such study, which explores the injectable capsules that co-encapsulate adipose derived stem cells and endothelial cells to stimulate the formation of vascularized new bone tissue upon implantation, found that osteogenesis is enhanced by the co-encapsulation even in the absence of differentiation factors dexamethasone and ascorbic acid (Correia et al., 2016).

Gene expression profile of osteogenic genes in RBMSCs also supported our findings of differentiation of RBMSCs towards osteogenic lineage since cells expressed bone specific transcription factor Runx2 and ECM components Col I, OC and ON. Synthesis of bone ECM is an important part in the bone regeneration process since it provides structural support and physical environment for cells to attach, grow, migrate and respond to signals as well as giving the tissue its mechanical properties such as rigidity and elasticity that is intrinsic to bone. Expression of bone ECM components Col I, OC and ON were significantly up-regulated in co-cultures after 28 days of incubation compared to only RBMSCs indicating a contribution of endothelial cells to differentiation of RBMSCs' towards osteoblasts. Higher expression of Col I, OC and ON in co-culture samples not only showed higher synthesis of bone organic matrix but this up regulation of expression of those genes were also correlated with higher mineralization observed in co-cultures since both OC and ON have multiple Ca^{2+} binding sites for the nucleation of hydroxyapatite as the component of bone inorganic matrix.

In co-cultures not only endothelial cells influenced the RBMSCs, and therefore, osteogenesis but in return RBMSCs influenced the ECs and therefore contributed to angiogenesis as microvessel formation could only be observed in the co-culture samples. Hofmann et al. (2008) also noticed tube-like structures in co-cultures of primary human osteoblasts and HUVECs on polyurethane scaffolds. However, it was surprising in this study to observe significantly less tube-like structures or complex structures for up to 21 days with the RAECs samples, and we interpret this as a positive role played by the RBMSCs in microvessel formation. This role might again be due to direct contact between heterotypic cells or to paracrine signaling. It is also a novel finding of our study to observe tube-like structures in 2D co-culture.

The role of MSCs in the co-cultures in the formation of tube-like structures can be direct or indirect. It was previously demonstrated that MSCs can contribute to vessel formation directly by differentiating into ECs (Oswald et al., 2004; Silva et al., 2005; Song et al., 2007) or indirectly, by secreting VEGF to induce ECs for angiogenesis (Boomsma and Geenen, 2012). Aguirre et al. (2010) reported that MSCs also participated in the formation of tube-like structures along with the endothelial progenitor cells suggesting the possibility of some MSC differentiating into endothelial-like cells or vessel supporting cells.

5. Conclusion

In this study we demonstrated the positive influence of co-culturing mesenchymal stem cells and endothelial cells in both osteogenesis and angiogenesis processes. Co-culture of these two cell types might be a solution for the insufficient vascularization problem of bone tissue engineering attempts since vascularization is very important for successful and complete regeneration of the tissue through osteogenesis and mineralization processes. This study also proposes a protocol for the successful co-culture of primary endothelial cells and mesenchymal stem cells through original seeding techniques and media compositions.

Although the outcome of this research is valuable from the practical point of view, the mechanism behind endothelial cell induced angiogenesis and the role of MSCs in microvessel formation require further investigation. We plan to apply mesenchymal stem cells and endothelial cells to 3D scaffolds because their co-culture appears to be promising for use in the treatment of critical-sized bone defects.

Financial & competing interests/Disclosure

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