Capillary network formation by endothelial cells differentiated from human bone marrow mesenchymal stem cells

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Abstract
Human bone marrow derived mesenchymal stem cells (HBMSCs) have the potential to differentiate into cells such as adipocyte, osteocyte, hepatocyte and endothelial cells. In this study, the differentiation of hBMSCs into endothelial like-cells was induced in presence of vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF-1). The differentiated endothelial cells were examined for their ability to express VEGF receptor-2 (VEGFR2) and von willebrand factor (vWF). Then the cells were adopted to grow and develop capillary network in a semisolid gel matrix in vitro. The capillary network formation in a well of 24-well plate was found to be 85% in presence of VEGF (50ng/ml) and IGF-1 (20ng/ml) of the culture media. These data may suggest that the expression of endothelial markers in endothelial like-cells derived from hBMSCs is associated with their ability to form capillaries.
Keywords: Mesenchymal stem cell; Endothelial cell; Angiogenesis; Differentiation; *In vitro*.

INTRODUCTION

Angiogenesis is an essential biological process for embryonic development, organogenesis, wound healing, reproductive tissue cycles, and tumorgenesis (Shibuya, 2001). Bone marrow derived stem cells (BMSCs) are a mixed population of cells derived from the bone marrow. These marrow cells have been broadly categorized into haematopoietic and mesenchymal lineages based on defined cell surface markers such as CD34 and CD45 (Prockop *et al*., 1997). Despite their plasticity and great potential for proliferation and differentiation, there is a wide variation in the characteristics, expandability and multipotentiality of the cells isolated from different bone marrow aspirates (Digirolamo *et al*., 1999; Chiu, 2003; Dominici *et al*., 2001; Prockop *et al*., 2003). Also, the bone marrow derived haematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs), mononuclear cells (MNCs) and mesenchymal stem cells (MSCs) have been demonstrated to transdifferentiate into cardiomyocyte and endothelial cells following engraftment into the myocardium (Tomita *et al*., 1999; Orlic *et al*,. 2001; Bittira *et al*., 2002; Jackson *et al*., 2001; Toma *et al*., 2002; Tomita *et al*., 2002; Davani *et al*., 2003). Bone marrow derived stem cells (BMSCs) that contribute to angiogenesis have been identified by surface markers such as VEGFR2, Tie-2, endothelial cadherin, CD34, CD146 and E-selectin (Asahara *et al*., 1999a; 1999b). Endothelial cells (ECs) can be differentiated from angioblast in the embryo (Mikkola *et al*., 2002) and endothelial progenitor cells (EPCs), mesoangioblast and multipotent adult progenitor cells (MAPCs), or side population in the adult bone marrow (Luttun *et al*., 2002; Reyes, 2002). EPCs can also contribute to vessel growth by releasing angiogenic growth factors (Rehman *et al*., 2003).

In this study, we could show the differentiation

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process of HBMSCs to endothelial cells by adding insulin-like growth factor-1 (IGF-1) in addition to VEGF and the effects of IGF-1 on the differentiated endothelial cells migration were investigated by evaluation of CXCR4 protein expression. CXCR4 as a chemokine receptor together with its ligand SDF-1 plays an important role in vasculogenesis (Orlic *et al*., 2002). The expression of VEGFR2, CD31 and vWF were determined at protein and mRNA levels using immunocytochemical (ICC) and RT-PCR methods, followed by development of capillary network formation in an extracellular matrix (ECM) gel. The functionality of the cells was confirmed by showing the ability of the cells to form capillary network formation.

MATERIALS AND METHODS

Reagents and antibodies: Ficoll-Hypac, penicillin, streptomycin, and Trypsin-EDTA were purchased from Biochrom (Berlin, Germany). Dulbecco's-modified Eagle's Medium-High Glucose (DMEM-HG), GlutaMAX-I™, fetal bovine serum (FBS) and α-Minimal Essential Medium (α-MEM) were from the Gibco Invitrogen (Carlsbad, CA, USA). Oil red-O-staining, alkaline phosphatase staining (ALP) kits, Medium Complete with Trace Elements 131 (MCDB131), ECM gel solution, phycoerythrin (PE) - conjugated anti-mouse IgG and 4′, 6-diamidino-2-phenylindole (DAPI) were from Sigma (St. Louis, USA). Anti-CD44, anti-CD166, anti-CD105, anti-CD34, anti CXCR4, anti-CD31, anti-von Willebrand factor (vWF) and anti-VEGFR2 were from eBioscience (USA). Vascular endothelial growth factor (VEGF) and insulin like growth factor-1 (IGF-1) were from Peprotech (NJ, USA). Human umbilical vein endothelial cell line (HUVEC) was obtained from the National Cell Bank, Pasteur Institute of Iran**.**

Isolation of HBMSCs: Bone marrow aspirations were collected from healthy donors (age 20-49 years) at the Bone Marrow Transplantation Center, Shariati Hospital, Tehran, Iran, after obtaining informed consent. MSCs were isolated and cultured based on previously reported methods (Majumdar *et al*., 1998; Pittenger *et al*., 1999; Phinney *et al*., 2002). The method was modified as described below:
Bone marrow aspirate (7-10 ml) was layered over a Ficoll-Hypac (d=1.077 g/ml) and centrifuged at 2200 rpm for 20 min at room temperature. Mononuclear cells at the interface were recovered and washed twice in phosphate buffered saline (PBS). The cells were seeded into 25 cm^2 flasks containing DMEM-HG, supplemented with 2mM GlutaMAX-I™ (L-alanyl-L-glutamine), 10 U/ml penicillin, 100 µg/ml streptomycin, and 10% FBS. MSC cultures grew at 37°C in 5% CO_2. Non-adherent cells were removed, after 24 h by washing with PBS. The medium was changed subsequently every 3 days. Two weeks later the culture reached 90% confluency. MSCs were recovered using 0.25% trypsin-EDTA and replated at a density of 5,000-6,000 cells per cm^2 of surface area as passage 1 (P_1) cells (Phinney *et al*., 2002).

Osteogenic and adipogenic transdifferentiation of HBMSCs: In order to confirm the source (MSCs) and the process of differentiation, we carried out a transdifferentiation MSCs to osteocytes and adipocytes. To induce adipocyte differentiation, HBMSCs were cultured in DMEM supplemented with 10% FBS, 1 µM dexamethasone, 200 µM indomethacin, 1.7 µM insulin, 500 µM isobutyl-methylxanthine, 0.05 U/ml penicillin, and 0.05 µg/ml streptomycin for two weeks. For adipocyte identification, intracellular lipid accumulation was visualized using Oil red-O-staining. Cells were fixed in 10% solution of formaldehyde in aqueous phosphate buffer for one h, washed with 60% isopropanol, and stained with Oil red-O-solution (in 60% isopropanol) for 10 min, followed by repeated washings with distilled water before being de-stained in 100% isopropanol for 15 min (Janderova *et al*., 2002; Ramriez *et al*., 1992). For the osteogenic differentiation, HBMSCs were induced for two weeks in α-MEM supplemented containing 10% FBS, 0.1 µM dexamethasone, 10 µM β-glycerophosphate, and 50 µM ascorbate-phosphate (Covas *et al*., 2003; Kim *et al*., 2004). Control cultures without the differentiation stimuli were maintained concurrently with the differentiation experiments and stained in the same manner. The differentiated cells were identified by specific histochemical staining for ALP using a commercial kit.

Immunophenotyping of HBMSCs and the differentiated cells: Expanded HBMSCs and the differentiated cells were detached from the culture flasks using PBS (pH 7.4) containing trypsin (0.05%) and EDTA (0.02%), washed once with DMEM and once with

PBS. The cells (10^6/ml) were then suspended in 50 µl PBS and incubated either with fluorescein isothiocyanate *(*FITC) or PE-conjugated antibodies in dark for 45 min at 4°C. The following antibodies were used for immunophenotyping: anti-CD44-FITC, anti-CD166-FITC, anti-CD105-PE, anti-CD34-FITC and anti-CXCR4-FITC. Simultaneously, the cells were incubated with an isotype antibody as a negative control to exclude non-specifically labeled cells from the calculation. At the end of the incubation period, the cells were washed twice with PBS supplemented with 2% bovine serum albumin (BSA) and fixed in 1% paraformaldehyde solution in PBS. Analysis was performed using a flow cytometer (Partech, Germany). Before each test, the cell viability that was more than 95% was determined with trypan-blue staining in a Neubauer slide.

Induction of HBMSCs differentiation to endothelial-like cells *in vitro*: Confluent HBMSCs were cultured in MCDB131 media containing 5% FBS, 50 ng/ml VEGF and 20 ng/ml IGF-1 and incubated at 37°C for 5 days. The medium was changed every 3 days.

Immunocytochemical (ICC) analysis: The differentiated endothelial cells and undifferentiated HBMSCs were fixed in 4% paraformaldehyde in PBS containing 0.1% Triton X-100 for 15 min at room temperature. Non-specific binding was avoided by several washings with PBS containing 1% BSA. The cells were then incubated overnight at 4°C separately with human primary antibodies prepared against vWF (1:1000) and VEGFR2 (1:500) and CD31 (1:1000). At the end of the incubation period, for VEGFR2 assay the cells were washed three times with PBS, and incubated with the PE-conjugated anti-mouse IgG (1:100) for 1h at 37°C. Finally, the cells were observed under a fluorescence microscope (Nikon, TE 2000, Japan).

Reverse transcriptase-polymerase chain recation (RT-PCR): First-strand cDNA was synthesized from total RNA (200ng) isolated from the differentiated endothelial cells using guanidine thiocyanate then cDNA was amplified by *Taq* DNA polymerase dissolved in PCR buffer (Qiagen, USA) in a 50 µl reaction mixture containing 0.2 mmol/l dNTPs and 40 pmol of VEGFR2 specific primers (Sense; 5′-CTGGCATGGTCTTCTGTGAAG-3′. Antisense; 5′-AATACCAGTGGATGTGATGCG-3′), vWF primers: (Sense: 5′-AATGTTGTGGGAGATGTTTGC-3′. Antisense: 5′-GTGGATATCCACCTCTACTTCA-GAC-3′). The PCR protocol consisted of 5 min of initial denaturation at 94°C, followed by 25 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 64°C, 2 min of extension at 72°C, and a final extension step of 10 min. The PCR product (20 µl) was separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Similarly, mRNA was isolated from HUVEC line and processed for RT-PCR and considered as positive control sample. β-actin (a house keeping gene) was also used as internal control.

Capillary network formation by the differentiated endothelial cells: Following induction of differentiation, the cells were allowed to develop capillary-like network formation in ECM gel in vitro. 200 µl ECM gel solution was added to each well of a 24-well microplate and incubated for 2h at 37°C. Then induced MSCs (4×10^4) were plated onto ECM gel and incubated for 1h. The induced cells were cultivated in MCDB131 medium containing 5% FBS, 50 ng/ml VEGF and 20 ng/ml IGF-1 for 7 days by changing the culture medium every 3 days. The cells were then examined under a phase contrast microscope (Nikon, Japan) for the formation of capillary structures.

RESULTS

Cell culture and characterization of HBMSCs by flow cytometry: MSCs were separated according to the standard methods for the isolation of mononuclear cells from bone marrow using Ficoll-Hypac. The cells were subjected to flow cytometry for the presence or absence of hematopoietic and endothelial markers. MSCs typically expressed the surface antigens CD105, CD44 and CD166 (Fig. 1), but were negative for CD34, which is the early hematopoietic marker.

Differentiation of HBMSCs into adipocytes and osteoblasts: HBMSC cultures in adipogenic differentiation medium after 14 days led to the appearance of larger round cells presenting numerous fat vacuoles in the cytoplasm. These lipid droplets were more than 80% as judged by Oil red-O-staining (Fig. 2A), while untreated control cultures did not show such lipid droplets in their cytoplasm (Fig. 2B). HBMSCs under

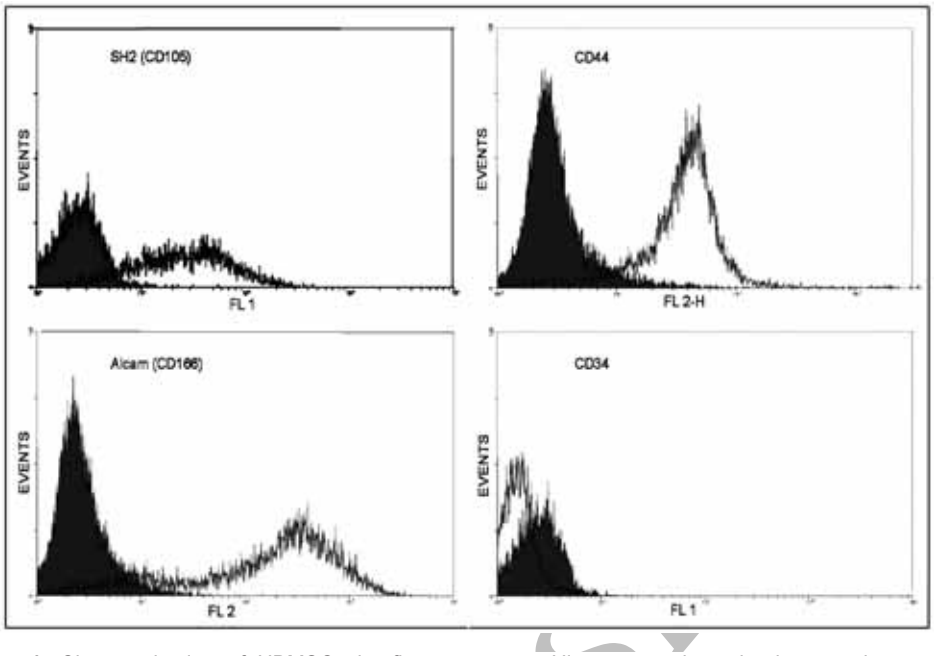

Figure 1. Characterization of HBMSCs by flow cytometry. Histograms show the immunophenotype of HBMSCs isolated from the human bone marrow. HBMSCs were positive for CD44, CD105, and CD166. These cells were negative for CD34. The shaded area shows the profile of the negative control. The data shown are representative of those obtained in six different experiments.

osteogenic inductive medium led to the appearance of refringent crystals in the cells after 2 weeks. By the end of the second week of stimulation, more than 90% of the HBMSCs also became ALP positive (Fig. 2C). Untreated control cultures growing in regular medium in absence of the osteogenic differentiation stimuli did not show spontaneous osteoblast formation even after 3 weeks of cultivation (Fig. 2D).

IGF-1 increases CXCR4 expression: The expression of CXCR4 was evaluated on the differentiated cells by flow cytometry analysis 3 days after treating with IGF-1. The results showed that more than 50% of the cells expressed CXCR4 by IGF-1 stimulation compared to untreated cells, which expressed CXCR4 in less than 25% of cell population (Fig. 3).

Ability of endothelial cells to form capillaries: *In vitro* differentiation of HBMSCs on ECM gel containing the inducing factors (VEGF and IGF-1) resulted in formation of tubes resembling angiogenesis (Fig. 4). Capillary network formation was started two days (day 7 of the differentiation process) after transferring the induced HBMSCs to ECM gel. The network formation was completed on day 12 of differentiation on ECM. Examination of 4X images for each plate (24 wells) showed that capillary network form more than 80% of the plate (Table 1). The area of tubulogenesis was assessed by counting the number of tubes in each well under phase contrast microscope. It is worth monitoring that continuation of the process resulted in granulation of the cells (day 14 onwards) on ECM.

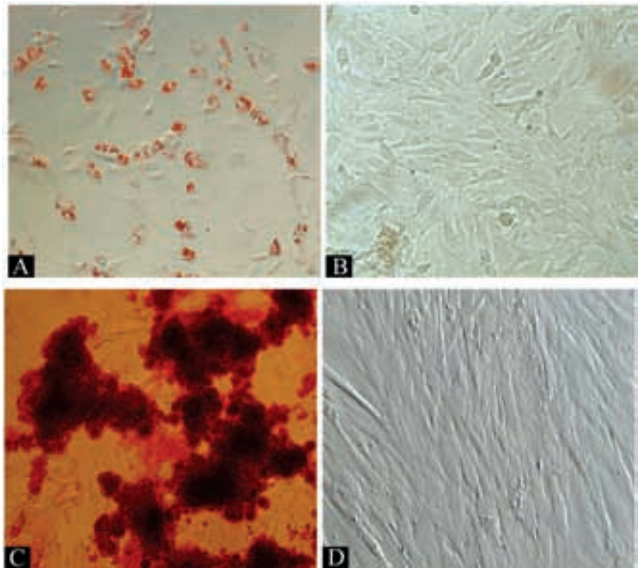

Figure 2. Isolation and differentiation characteristics of human bone marrow-derived mesenchymal stem cells. **(A)** The results of Oil red-O-staining in differentiated adipogenic cells from HBMSCs. **(B)** Negative control for adipogenic cell . **(C)** Results of osteogenic differentiation from HBMSCs. **(D)** Negative control for osteogenic cell. (All figures with a 10X magnification)

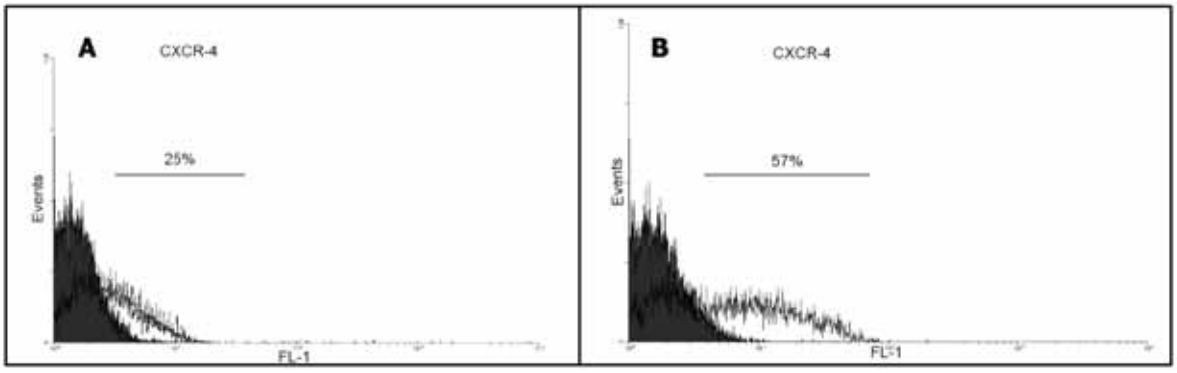

Figure 3. Flow cytometry analysis of the differentiated cells for CXCR4. **(A)** CXCR4 expression in the absence of IGF1. **(B)** CXCR4 expression in the presence of IGF-1. The shaded area shows the profile of negative control.

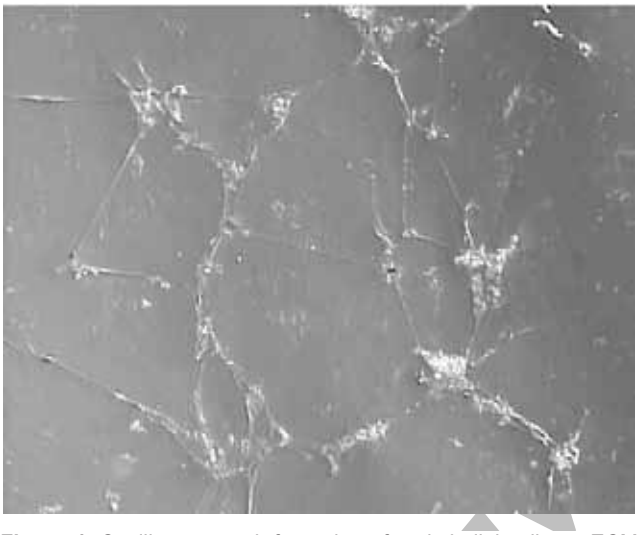

Figure 4. Capillary network formation of endothelial cells on ECM gel. Capillaries were observed on day 12 of differentiation using light microscope (10X magnification).

Concurrently, plates were seeded with HBMSCs in absence of growth factors and considered as control (undifferentiated). The results of tubulogenesis were fairly different in cells before and after differentiation. Relatively small fraction of the undifferentiated cells (~30%) participated in formation of a non-specific aggregates (Table 1).

Characterization of the differentiated endothelial cells: ICC assays revealed that unlike the undifferentiated MSCs, the endothelial-like cells (~70%) were positive for VEGFR2, vWF and CD31 (Fig. 5). The endothelial specific markers in the differentiated endothelial cells were assessed by RT-PCR. VEGFR2 and vWF specific mRNA gene products were visualized as two bands of 795 and 329 bp respectively. A 198 bp PCR product belongs to β-actin-specific mRNA which was used as internal control equally expressed in the cells before and after differentiation. Expression of endothelial specific genes (VEGFR2 and vWF) was positive in samples obtained from the differentiated endothelial cells (Fig. 6). Whereas, in case of hBMSCs both VEGFR2 and vWF related mRNA expression was negative.

DISCUSSION

Differentiation of the endothelial cells (ECs) from different sources including, the angioblast in the embryo (Mikkola *et al*., 2002), the EPCs, mesoangioblast and multipotent adult progenitor cells (MAPCs) or side population in the adult bone marrow (Luttun *et al*., 2002; Reyes, 2002) has been reported. According to Rehman *et al*., (2003), EPCs contribute to vessel growth by releasing angiogenic growth factors. The relative ease of isolating MSCs from bone marrow and the great plasticity of the cells make them ideal tools for an autologous or allogeneic cell therapy (Horwitz *et al*., 1999; Koc *et al*, 2002). Serial analysis of gene expression (SAGE) revealed that single cell derived colonies of MSCs expressed mRNAs of multiple cell lineages, including characteristic epithelial and

Table 1. Differences in capillary like network formation during differentiation.

HBMSCs	Capillary network formation (%)
Undifferentiated	30 ± 1
Differentiated	85 ± 1

Analysis of capillary network formation was performed based on in vitro angiogenesis kit (BD, Biosciences). Tube formation was observed under a phase contrast microscope on day 12. Tube areas were calculated from 4X images for each plate (24 wells).

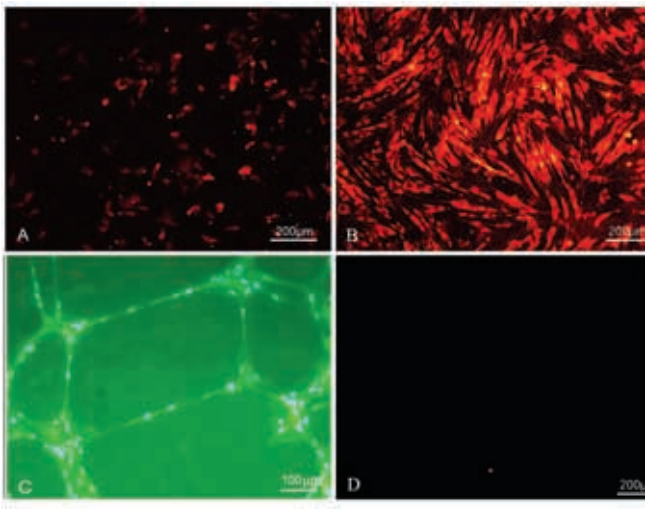

Figure 5. Immunocytochemistry analysis for the detection of the specific endothelial markers. **(A)** Immunostaining of differentiated HBMSCs toward endothelial cells for VEGFR2. **(B)** CD31. **(C)** vWF. **(D)** CD31, VEGFR2 and vWF were negative for undifferentiated cells.

endothelial molecules like Epican and Keratins (Tremain *et al*., 2001). Endothelial cells are specialized cells capable of building up the blood capillaries. Because of their unique morphological and molecular properties, they are readily distinguished from the other cell types. Endothelial cells are generally identified with their surface antigenic markers, particularly VEGFR2, FLT-1 and vWF are additional markers for functional endothelial cells (Asahara *et al*., 1997). Our study shows that HBMSCs are capable of differentiating into endothelial cells *in vitro*, which make them attractive candidates for the development of autologous tissue grafts. The culture medium used for differentiation of human BMSCs to endothelial cells was modified by adding IGF-1, which increases the expression of CXCR4 protein and enhances SDF-1 induced MSC migration *in vitro* (Li *et al*., 2007). Also, IGF-1 prevents cell death in the differentiated cells (Tögel *et al*., 2007). Preliminary results obtained from the treatment of HBMSCs with IGF-1 indicate that IGF-1 enhances CXCR4 expression on the differentiated cells compared to untreated cells. Also, ICC was used to distinguish endothelial-like cells from the undifferentiated stem cells. Moreover, it was demonstrated that the differentiated cells posses high levels of specific protein markers i.e., CD31, VEGFR2 and vWF. The expression of VEGFR2 and vWF were determined at mRNA levels (using RT-PCR), which support the results of protein accumulation as shown by ICC. Expression of the surface markers, together with CD31, VEGFR2 and vWF are probably common to all the vascular endothelial cells regardless the source and origin. The progressive formation of capillaries was associated with the increased expression of endothelial markers.

Following induction of HBMSCs to the differentiated endothelial cells, the formation of capillary-like structures in semisolid medium (ECM gel) was markedly enhanced (Fig. 4). We also found that the induced HBMSCs form tube-like structures when cultivated in semisolid medium and in the presence of VEGF markedly enhanced this behavior. Therefore, MSCs may be an alternative source of differentiation into endothelial cells for clinical therapies such as tissue replacement or vascularization of artificial organs. Overall results indicate that the endothelial like cells derived from hBMSCs expressing endothelial markers are capable of developing capillary networks *in vitro*.

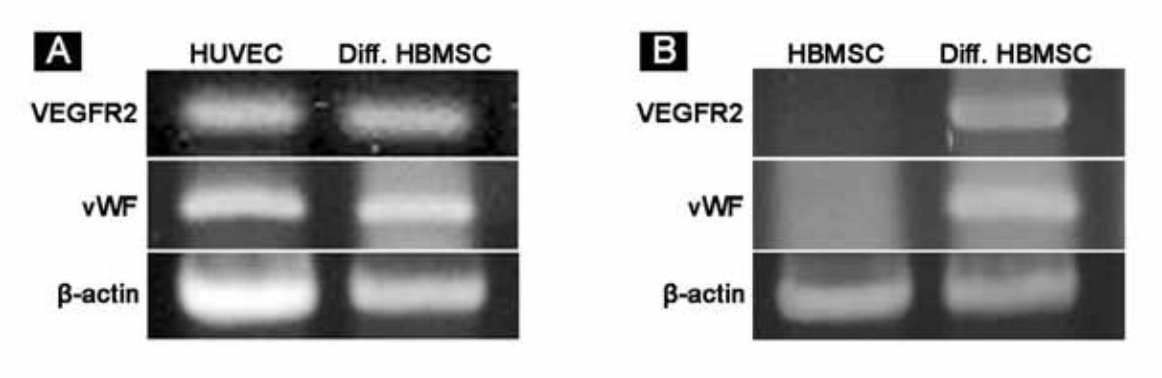

Figure 6. RT-PCR analysis for the determination of the specific endothelial gene expression. **(A)** RT–PCR results of specific endothelial genes for HUVEC as a positive control and the differentiated endothelial cells (Diff. HBMSC) are the same. **(B)** There was not any gene expression for VEGFR2 and vWF in HBMSC. The size of PCR products for VEGFR2 and vWF were 795 bp and329 bp, respectively.

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