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Original article

BMP-2 can promote the osteogenic differentiation of human endometrial stem cells

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Background: Human endometrial-derived stem cells (hEnSCs) as multipotent accessible source of cells are known as useful cell candidates in the field of bone tissue engineering. However, the effect of bone morphogenic protein-2 (BMP-2) as an osteoinductive growth factor has not been clearly ascertained.

Objective: To evaluate the effect of the remarkable osteoinductive growth factor BMP-2, on promotion of osteogenic differentiation in hEnSCs.

Methods: Endometrial biopsies were obtained from healthy women referred to the hospital for infertility treatment. After tissue digestion in collagenase, the isolated endometrial cells were expanded in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS. The propagated cells were characterized based on the expression of endometrial (CD90, CD105), endothelial (CD31), and hematopoietic (CD34, CD133) stem cell markers. Cells were differentiated in osteogenic medium containing DMEM supplemented with 10% FBS, 10 nM dexamethasone, 50 μg/ml Ascorbic acid, and 10 mM β-glycerophosphate in the presence or absence of BMP-2 for 21 days. Alizarin red staining was performed to verify the matrix mineralization. Immunocytochemical staining was conducted to detect the expression of OCT-4, CD133, and osteopontin as well as osteocalcin. The expression of osteoblast transcripts, including osteopontin, osteonectin, and alkaline phosphatase (ALP) were analyzed by semi quantitative PCR.

Results: The expanded EnSCs were spindle shaped. They were positive for the expression of Oct-4, CD90, and CD105, while they were negative for endothelial and hematopoietic markers. The matrix mineralization was confirmed by Alizarin red in both groups at day 21. Although the expression of osteopontin and osteocalcin was detected in both groups by immunological staining, the expression of osteocalcin was more intense in the presence of BMP-2. ALP, Osteonectin and osteopontin transcripts were expressed in all groups; however, the expression of ALP and osteopontin was upregulated in the presence of BMP-2.

Conclusion: BMP-2 as an osteoinductive growth factor, could promote the osteogenic differentiation of EnSCs in vitro.

***Keywords*:** Bone morphogenetic protein-2, endometrial stem cells, osteogenic differentiation

Abbreviations

ALP = Alkaline phosphatase
BMP = Bone morphogenic protein
ESCs = Embryonic stem cells
hEnSCs = Human endometrial-derived stem cells
HSCs = Hematopoietic stem cells
MAPCs = Multipotent adult progenitor stem cells
MSCs = Mesenchymal stem cells
UCBSCs = Umbilical cord blood stem cells

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Bone is responsible for the shape, mechanical support and protection of the body. Additionally, bone contributes to the mineral homeostasis of the body and can participate in endocrine regulation of energy metabolism [1]. Because the sources of bone autologous grafts for bone regenerative medicine are scarce [2], the application of stem cells is a promising approach. These undifferentiated cells are characterized based on their capacity to proliferate (self-renewal) and produce tissue specific committed progenitor and differentiated cells [3-5]. So far, various attempts have been made in vitro and in vivo to

induce osteogenesis in different types of cells; including mesenchymal stem cells (MSCs) [1, 2, 6-9] hematopoietic stem cells (HSCs), multipotent adult progenitor cells (MAPCs), and umbilical cord blood (UCBSCs), and embryonic stem cells (ESCs) [10-13]. Beside bone marrow and blood, the focus of interest in the field of tissue engineering currently lies on other ethically accessible cell reservoirs such as human endometrium. As a dynamic tissue, human endometrium undergoes cycles of growth and regression during each menstrual cycle. It is known that human endometrium contains low numbers of endometrial stem cells (EnSCs), which are responsible for monthly reconstruction and regeneration of the human endometrium [14, 15, 17-19]. Interestingly, not only in young women, endometrial regeneration also happens in postmenopausal women taking estrogen replacement therapy. Easy to access and high clonogenicity, and relatively fewer ethical and technical problems, nominate these types of cells as unique potential cells [18-23].

Being committed to an osteo lineage is a complex process in which various environmental factors including hormones and growth factors are involved [24-26]. Among different osteoinductive growth factors, our focus of interest in this study is on bone morphogenic protein 2 (BMP2). This glycoprotein is found in high amounts in bone tissues and is responsible for regenerative abilities of demineralized bone grafts used in periodontal therapy [27]. As a protein, this growth factor accounts for the recruitment of osteoprogenitor cells to the site of bone formation [24, 25]. The ability of BMP2 to promote osteogenesis led us to evaluate the effect of this protein on promotion of osteogenesis in EnSCs. The levels of mineralization and the expression of osteoblast- specific transcripts and proteins were our criteria to evaluate the process of osteogenesis in this in vitro study.

Methods

Biopsies from endometrium

Endometrial biopsies were obtained from women referred to the hospital for infertility treatment. Endometrial abnormality, GnRH agonist therapy, and previous administration of any hormones within the last 3 months, were our exclusion criteria in this study. To provide endometrial biopsies, written informed consent was obtained from each donor in compliance with the regulations of the Tehran University of medical sciences ethical committee. Biopsies were obtained from 10 normal women on cycle days of 19–24 based on routinely used protocols [23].

Isolation of human endometrial stem cells (EnSCs)

The biopsies were washed twice in Dulbecco's phosphate buffered saline (DPBS). After cutting the tissue into small pieces, the samples were incubated in Hank's balanced salt solution (HBSS), supplemented with 25 mM 4-(2 hydroxyethyl)-1 piperazineethanesulfonic acid (HEPES), and 1 mg/ml collagenase (all from Gibco, Eggenstein, Germany) at 37 °C for 30–45 minutes. The cell suspension was then passed through a 70 μM sieve (BD Biosciences, Bedford, MA, USA) to remove glandular epithelial particles. After centrifugation at 1200 rpm for 5 minutes, the cells were loaded on Ficoll (1.77 g/cm; Sigma, St. Louis, MO, USA) and centrifuged at 1000*g* for 30 minutes to separate mononuclear cells. The isolated mononuclear cells were cultured in DMEM/F12 medium containing 10% FBS (Fetal bovine serum; Gibco), 1% penicillin/streptomycin and 1% and incubated at 37 °C in an atmosphere of 5% CO_2 [14, 23].

Characterization of EnSCs

To characterize the phenotype of cultured EnSCs, passaged-3 cells were detached using 0.25% trypsin-EDTA (Gibco) and suspended in 100 μl of HBSS buffer supplemented with 2% bovine serum albumin (BSA) (Sigma). Each cell sample was separately incubated with the following antibodies in the dark at 4 °C for 20 minutes. CD44, CD90, and CD34, as well as CD133 were all conjugated with fluorescein isothiocyanate, while CD105 and CD31 were conjugated with phycoerythrin (all from BD Biosciences-Pharmingen, San Diego, CA, USA). Before cell analysis by using flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), cells were washed twice with HBSS buffer. Positive expression was defined as the level of fluorescence greater than 95%. For intracellular staining, cells were washed twice in Hank's solution with 2% BSA and fixed with 4% paraformaldehyde (PFA) for 1 hour. Then the cells were permeabilized by using 0.5% Tween 20 and 0.1% Triton X-100 in PBS (T-PBS). Primary antibodies (Abcam, Cambridgeshire, UK) were added to PBS and the cells were incubated for 20 minutes with the corresponding primary antibodies (all from Abcam) at the concentrations recommended by the manufacturer. Cells were then washed twice in PBS. Corresponding

secondary antibodies with fluorescent conjugates were subsequently diluted in PBS at the concentrations suggested by the manufacturer. After Incubation for 20 min, cells were washed twice by HBSS buffer and then analyzed by using the flow cytometer (Becton Dickinson).

Induction of osteogenesis

Cells (2×10^4 cells/ml) were seeded in each well of a 24- well plates containing DMEM supplemented with 10% FBS. Twenty-four hours later, the medium was exchanged with osteogenic medium containing DMEM supplemented with 10% FBS, 10^{-7} M dexamethasone, 50 μg/ml L-ascorbic acid-2-phosphate, and 10 mM β-glycerophosphate. We considered this culture as the osteogenic medium (OM) group. The other cell culture was treated with osteogenic medium plus BMP2 at 10ng/ml. The cells were kept in humidified chamber at 37 °C and 5% CO_2 for 21 days. The medium was changed every 3 days.

Alizarin red staining

Alizarin red staining was used to verify the mineralization of the extracellular matrix. Briefly, at third week post- induction, the cultures were fixed in 4% paraformaldehyde for 30 min and then stained with 2% alizarin red staining solution (Sigma). The results were analyzed qualitatively based on the intensity of the alizarin red-stained areas.

Immunocytochemical staining

At the end of the third week, the cells were fixed with 4% paraformaldehyde (Sigma) for 30 minutes at room temperature. After permeabilization with 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes, the cells were blocked with goat serum for 1 hour and then incubated at 4 °C with primary antibodies; including anti-Oct4 (1:200; Abcam), anti-CD133 (1:200; Abcam), anti-osteopontin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-osteocalcin (1:200; Santa Cruz) overnight. Secondary rabbit anti-mouse IgG-FITC (Santa Cruz), at a dilution of 1:200 was used as the secondary antibody in this experiment. Between each step, slides were washed with PBS and nuclei staining were performed using 4′,6-diamidino-2-phenylindole (DAPI, Sigma). Cells were examined using a fluorescence microscope (Olympus BX51, Tokyo, Japan).

RNA extraction and semiquantitative RT-PCR

To evaluate the expression of osteoblast- specific genes during osteogenic differentiation of EnSCs, we performed a semiquantitative reverse transcription-polymerase chain reaction at 21 days postinduction. Total RNA was extracted using a Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequently, 5 μg of total RNA was converted into cDNA by using Moloney-murine leukemia virus (M-MLV) superscript II reverse transcriptase (Promega, Madison, WI, USA) and random hexamer primer (Oligo dT18; Promega).The intensity of each band was quantified by using ImageJ software. The respective primer sequences are listed in **Table 1**.

Table 1. The primer sequences applied in RT-PCR

Gene		Primer sequence (5′–3′)	Accession number
β-actin	F	CGTGACATTAAGGAGAAG	NM_001101.3
	R	TGATGGAGTTGAAGGTAG	
Osteonectin	F	GGAAGAAACTGTGGCAGAGG	NM_003118.3
	R	TGCTGCACACCTTCTCAAAC	
Osteopontin	F	CTACAGACGAGGACATCAC	NM_001101.3
	R	CTCATTGCTCTCATCATTGG	
Alkaline phosphatase (ALP)	F	CCTCGTTGACACCTGGAAG	NM_003118.3
	R	CTGGTAGTTGTTGTGAGCATAG	

Results

Cell expansion and characterization

Human endometrial cell primary cultures contained cells that predominantly had a fibroblastic shape. This morphology was maintained throughout the subcultures (**Figure 1**). Flow cytometric analysis indicated that the majority of passaged-3 cells tended to express OCT-4, CD90, and CD105. Endothelial and hematopoietic markers that included CD31 and CD34 were expressed at very low frequencies (**Figure 2**). Immunocytochemistry analysis for OCT4 and CD133 showed that EnSCs were positive for OCT4 and negative for CD133 (**Figure 3**).

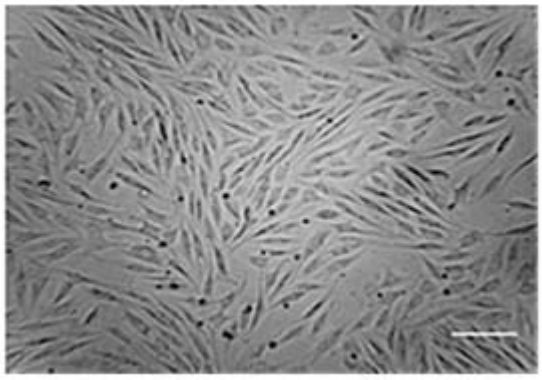

Figure 1. Human endometrial stem cells at passage 3 (scale bar: 100 μm)

Assessment of mineralization

Based on our observation, at third week of induction, the amount of deposited matrix was increased in the BMP2-treated cultures in comparison with the group without BMP2 (**Figure 4**).

Immunocytochemical staining

Immunocytochemical staining revealed that osteopontin and osteocalcin were expressed in both groups; however, the expression of osteocalcin was more intense in the presence of BMP-2 when compared to the OM group (**Figure 5**).

RT-PCR analysis

RT-PCR was conducted to investigate the expression of osteoblast markers at the level of mRNA. Our results showed that in the presence of BMP2, the expression of osteopontin and alkaline phosphatase transcripts increased in comparison with our control group, which was only treated with osteogenic medium. The level of expression of osteopontin increased 3.02-fold and the amount of Alp increased 2.16-fold in comparison with control group as shown in **Figure 6**.

Discussion

It is well documented that the human endometrium contains a low number of endometrial stem cells, which seem to belong to the family of MSCs [18, 28]. These cells evolve monthly, remodel the human endometrium [18, 28-31] and have the capacity to differentiate into chondrogenic, adipogenic, osteogenic, neurogenic, and cardiogenic cell lineages [19, 21-23, 32-34]. Based on this evidence, the endometrium may be an alternative source of MSC-like cells for bone tissue engineering purposes, obtainable with no extra-morbidity than that required for other sources of stem cells [14, 35]. Herein we evaluated the osteogenic differentiation capacity of human endometrial stem cells, as an ethically accessible source of cells, in the presence or absence of BMP-2 in vitro. We evaluated the differentiation of human endometrial stem cells into osteoblast-like cells using morphological and molecular criteria. We observed the mineralized extracellular in both groups; however, the intensity of the alizarin red-stained positive areas was qualitatively more remarkable in BMP-2 treated group. Immunochemical staining revealed that osteopontin and osteocalcin, as dominant proteins expressed during osteogenesis, were expressed in both cultures, but the expression of osteocalcin, as a mature osteoblast specific protein [36] was more intense in the presence of BMP-2 in comparison with cultures without this growth factor. On the other hand, RT-PCR analysis showed the expression of osteoblast markers including osteopontin, osteonectin, and alkaline phosphatase at 21 days post-treatment. Interestingly, treatment with BMP-2 increased the expression of osteopontin and alkaline phosphatase in these cells. Based on previous work, BMP-2 increases the proliferation and induces a change in cell shape and expression of alkaline phosphatase [37, 38]. Upregulation of this enzyme leads to deposition of inorganic phosphate into the matrix and increases the amount of mineralized areas in the extracellular matrix [39]. On the other hand, osteopontin as another osteoblast marker is expressed in osteoblast differentiating cells. Expression of this transcript subsequently ends to the increase of osteocalcin as a mature osteoblast cell marker [40]. The potency of osteogenic media alone and in combination with BMP-2 in induction of differentiation is well supported

[41-43]. Essentially, BMPs play an important role during prenatal development and bone regeneration. They conduct the task of cytokines that fundamentally influences stem cell fate [44]. Although BMP signaling is relevant to osteoblast differentiation, how far it can drive differentiation towards osteogenesis is unclear; however, osteogenesis is the result of the effect of individual types of BMPs; such as BMP-2 [45] and the osteoinductive activity of bone morphogenetic proteins nominate them as important regulators in bone repair process and maintenance [46].

Conclusion

Self-renewal properties, beside the osteogenic potential, make endometrial stem cells a desirable source of cells in the field of bone tissue engineering. On the other hand, the osteopromotive effect of BMP-2 on this type of cells supports their application in production of tissue engineered bone constructs to regenerate tissue lost by large bone defects in the clinic.

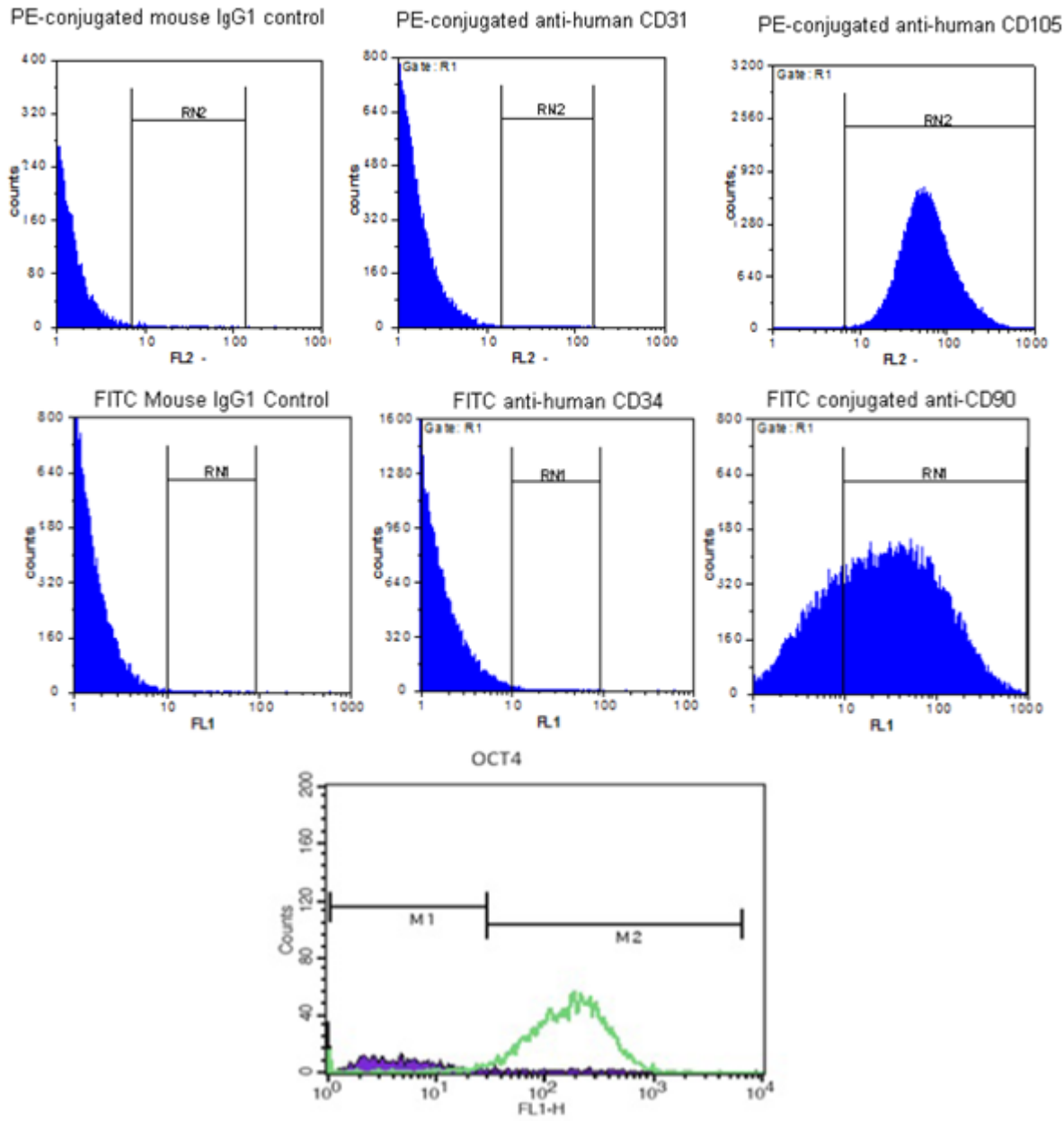

Figure 2. Flow cytometric analysis of isolated EnSCs for mesenchymal stem cell markers (CD90 and CD105), hematopoietic marker (CD34), endothelial marker (CD31), and embryonic stem cell marker (OCT4). The isolated cells are positive for CD90, CD105, and OCT4, but they are negative for CD31 and CD34.

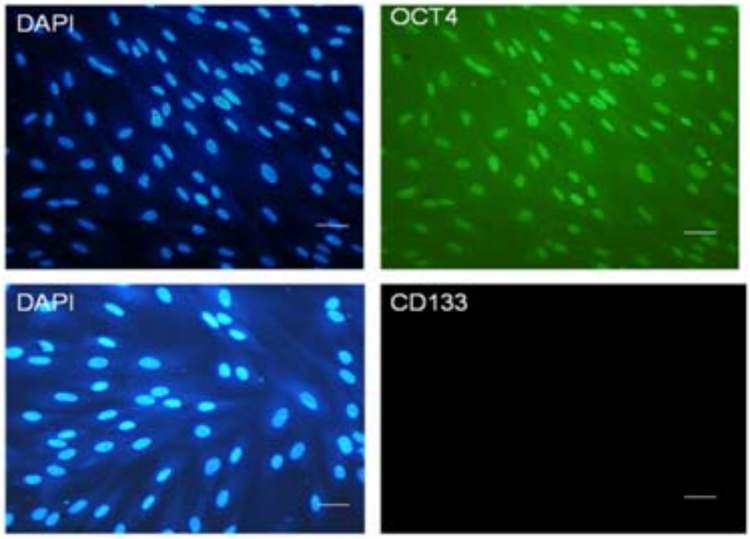

Figure 3. Human endometrial stem cells are positive for OCT4 and negative for CD133 (a hematopoietic marker). Nuclei were stained with DAPI (scale bar: 100 μm).

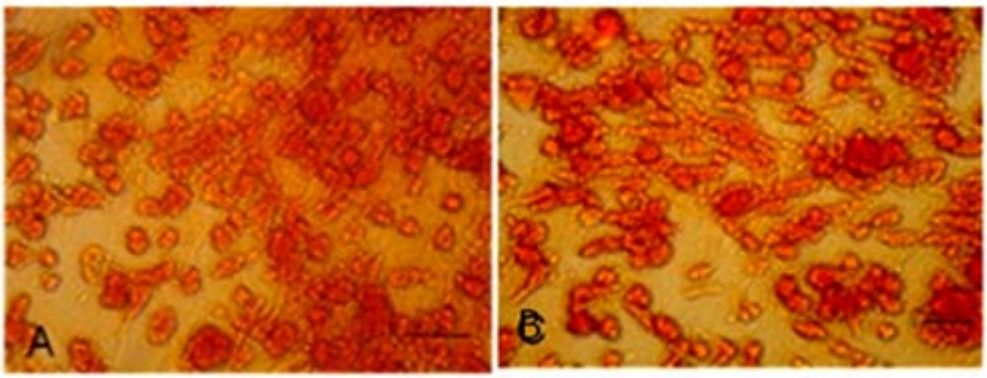

Figure 4. Alizarin red staining at day 21. Cells were incubated with (**A**) or without BMP-2 (**B**) for 21 days (magnification 10×).

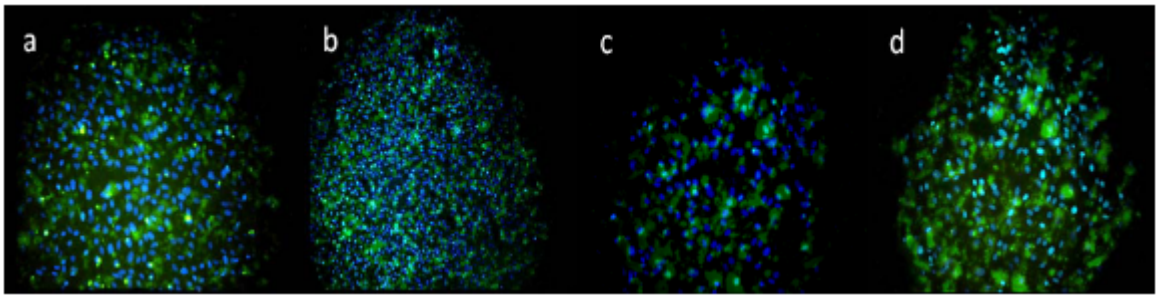

Figure 5. Immunostaining of EnSCs to detect the expression of osteoblast markers, osteopontin and osteocalcin, at day 21 in the presence or absence of BMP-2. Cells were stained for the expression of osteopontin in the absence (a) and presence (b) of BMP-2. Cells were stained for the expression of osteocalcin in the absence (c) and presence (d) of BMP-2.

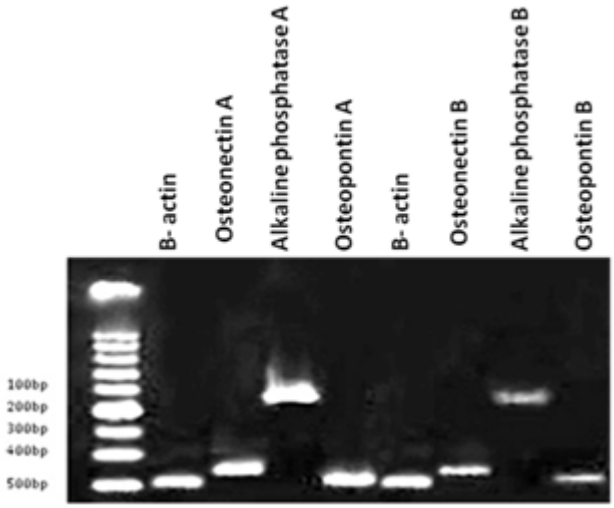

Figure 6. Representative semiquantitative RT-PCR for the expression of osteoblast transcripts in the presence (**A**) and absence (**B**) of BMP-2 after 21 days. β-actin was used as an internal standard.

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