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ORIGINAL ARTICLE

TERM

Derivation and Differentiation of Bone Marrow Mesenchymal Stem Cells from Osteoarthritis Patients

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Osteoarthritis (OA) of the knee is a degenerative joint disease caused by the progressive reduction of the articular cartilage surface that leads to reduced joint function. Cartilage degeneration occurs through gradual loss in extracellular matrix components including type II collagen and proteoglycan. Due to limited inherent self repair capacity of the cartilage, the use of cell-based therapies for articular cartilage regeneration is considered promising. Bone marrow mesenchymal stem cells (BM-MSCs) are multipotent cells and are highly capable of multilineage differentiation which render them valuable for regenerative medicine. In this study, BM-MSCs were isolated from OA patients and were characterized for MSC specific CD surface marker antigens using flowcytometry and their differentiation potential into adipocytes, osteocytes and chondrocytes were evaluated using histological and gene expression studies. BM-MSCs isolated from OA patients showed short spindle shaped morphology in culture and expressed positive MSC related CD markers. They also demonstrated positive staining with oil red O, alizarin red and alcian blue following differentiation into adipocytes, osteocytes and chondrocytes, respectively. In addition, chodrogenic related genes such as collagen type II alpha1, cartilage oligomeric matrix protein, fibromodulin, and SOX9 as well as osteocytic related genes such as alkaline phosphatase, core-binding factor alpha 1, osteopontin and RUNX2 runt-related transcription factor 2 were upregulated following chondrogenic and osteogenic differentiation respectively. We have successfully isolated and characterized BM-MSCs from OA patients. Although BM-MSCs has been widely studied and their potential in regenerative medicine is reported, the present study is the first report in our series of experiments on the BMSCs isolated from OA patients at King Abdulaziz University Hospital, Jeddah, Saudi Arabia.
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INTRODUCTION

Osteoarthritis (OA) of the knee is a degenerative joint disease, associated with progressive reduction of articular cartilage within the synovium, subchondral bone sclerosis and hypertrophy [1]. It is characterized by loss of extracellular matrix (ECM) components in the cartilage and bone. OA is associated with gradual degradation of type II collagen and proteoglycan that ultimately leads to diminished joint function [2-4]. OA is an age-related disease affecting individuals above the age of 65 [5]. Causative factors involved in the development of OA include genetic predisposition, overweight, inflammation, autoimmune arthritis, metabolic arthritis and joint trauma [5-7]. Due to the limited self-renewal and regeneration capability of chondrocytes self restoration and repair of the articular cartilage is difficult. As a consequence, the severity of OA tends to increase with time and the currently available treatment does not offer complete cure [8-9].

The use of cell-based therapies for articular cartilage repair has shown promise for over two decades [10-12]. Autologous chondrocyte implantation (ACI) is used to restore degenerated cartilage; however, the implanted chondrocytes are associated

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with several limitations including insignificant regeneration potential, availability of lesser number of chondrocytes and their native integration which in turn lead to poor therapeutic outcome [13-15]. ACI involves surgical procedure to obtain cartilage from the healthy joints and transplantion to the defect area [10-16].

Adult mesenchymal stem cells (MSCs) can be used to regenerate defective cartilage as an alternative therapeutic approach to OA [17]. MSCs are multipotent progenitors that have the potential and capability to differentiate into several cell types including skeletal muscle, bone, cartilage, fat, as well as neural precursors [18,19]. MSCs can be easily isolated from multiple sources such as bone marrow (BM), synovium, adipose tissue and umbilical cord [20-22]. Several important characteristics of BM-MSCs make them an attractive population of cells for cartilage repair. Autologous BM-MSCs are preferable than allogeneic MSCs as they have low immunogenicity apart from their self-renewal, proliferation, expansion and chondrogenic differentiation potential [23-25].

BM-MSCs has been widely studied for the multilineage differentiation capacity compared to other MSCs sources, which justify their use for both research and translational studies [17,18,26]. In this study, bone marrow stem cells were isolated form osteoarthritic patients to evaluate their differentiation potential into chondrocytes, osteocytes and adipocytes *in vitro*.

MATERIALS AND METHODS

This study was approved (no. 11-557) by the Institutional Ethical Committee, King Abdulaziz University (KAU). BM aspirates (~4−6 mL, n=7) was obtained following informed patient consent from the iliac crest of osteoarthritic patients who underwent total knee replacement at the Orthopedics Department, KAU, Jeddah, Saudi Arabia.

Isolation and culture of human BM-MSCs

The BM aspirate (~2 mL/T-175 cm^2/tissue culture flask) were plated in complete BM-MSCs culture medium to aid attachment and proliferation as previously described [3]. Briefly, the samples were cultured in a low glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA), supplemented with 200 mM GlutaMax™ (Invitrogen, Carlsbad, CA, USA), in combination with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), and 5 ng/mL basic fibroblast growth factor-2 (Invitrogen, Carlsbad, CA, USA). The samples were plated in T-175 cm^2 tissue culture flasks (Greiner Bio-One, Kremsmünster, Austria) and incubated in a humidified incubator (37°C, 5% CO_2). The first mediachange was done on day 5, and thereafter the media was changed every 3−4 days. After reaching 80−95% confluency, cells were subpassaged and cells from early passages were used in experiments.

Flow cytometry analysis

The expression of MSC-specific CD surface antigen markers was analyzed in BM-MCs cultured at passages 1−6 using FACSAria III flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, the cultured cells were trypsinized using 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) centrifuged at 3000 rpm×10 min and pelleted. The cell pellet was resuspended in in PBS containing 3% FBS and counted. Aliquots of cell suspension containing 1×10^5 cells were stained with the following antibodies CD105 and CD90 (FITC), CD73 (PerCP-Cy5.5), CD44 (PE-Cy7), CD29 (APC), CD34 (DAPI), and CD45 (PE) (BD Biosciences, Franklin Lakes, NJ, USA). Expression data were evaluated using the FACSDIVA software (BD Biosciences, Franklin Lakes, NJ, USA).

Multilineage differentiation assay

Chondrogenic differentiation

BM-MSCs from early passasges (P3−P5) were about seeded at a density of 2×10^4 cells/well in 24- well plates and incubated in complete culture medium until it reached 80% confluency. Culture medium was then replaced with chondrogenic induction medium consisting of high glucose DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 1% insulin transferrin and selenium (Sigma, St. Louis, MO, USA), 100 nM dexamethasone (Sigma, St. Louis, MO, USA), 50 μg/mL ascorbic acid-2-phosphate (Sigma, St. Louis, MO, USA), 10 ng/mL transforming growth factor (TGF-β3) (Sigma, St. Louis, MO, USA), 2 mM of L-glutamine (Invitrogen, Carlsbad, CA, USA), 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) and 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA, USA). The control group was cultured in DMEM-LG (Invitrogen, Carlsbad, CA, USA) supplemented with 2% FBS (Invitrogen, Carlsbad, CA, USA). Respective culture medium was changed for every 3 days and culture continued for 21 days. At the end of differentiation period the cells were fixed with 10% formalin for 30 minutes and stained with Toluidine Blue (Sigma, St. Louis, MO, USA) to analyze collagen and proteoglycan depositions indicative of chondrogenic fate.

Osteogenic differentiation

BM-MSCs were plated at a seeding denisity of 2×10^4 cells/well in 24- well plates and grown to 70−80% confluency in complete culture medium. The cells were then continued to be cultured in osteogenic differentiation medium for 21 days. The os-

teogenic medium comprised of DMEM-LG (Invitrogen, Carlsbad, CA, USA), 10% FBS (Invitrogen, Carlsbad, CA, USA) and supplemented by 100 nM dexamethasone (Sigma, St. Louis, MO, USA), 50 μg/mL ascorbic acid-2-phosphate (Sigma, St. Louis, MO, USA), 10 mM sodium β-glycerophosphate (β-GP) (Sigma, St. Louis, MO, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). The control cells were cultured in DMEM-LG (Invitrogen, Carlsbad, CA, USA) supplemented with 2% FBS (Invitrogen, Carlsbad, CA, USA). Respective culture medium was changed for every 3 days and culture continued for 21 days. After 21 days, the cells were fixed with cold 70% ethanol for 1 hour and stained with Alizarin Red stain (Sigma, St. Louis, MO, USA) for detection of bone mineralization indicative of osteogenic differentiation.

Adipogenic differentiation

BM-MSCs from early passages were seeded at a density of 2×10^4 cells/well in 24- well plates and incubated in complete culture medium until it reaches 80% confluency. The cells were then incubated in adipogenic induction medium for 21 days using StemPro Adipogenesis Differentiation Kit (Invitrogen, Carlsbad, CA, USA). The control cells were cultured in DMEM-LG (Invitrogen, Carlsbad, CA, USA) supplemented with 2% FBS (Invitrogen, Carlsbad, CA, USA). Respective medium was replaced every 3 days. Following the differentiation period of 21 daysthecells were fixed in 10% formalin for 20 minutes and stained with Oil Red O (Sigma, St. Louis, MO, USA) to detect fat vacuolations indicative of adipocytic differentiation.

Gene expression analysis by quantitative real time PCR

BM-MSCs (1×10^5) from early passages (P3−P4) (were cultured in T-25 cm^2 tissue culture flask in complete culture medium until the cells were 80% confluent. The cells were then switched to either chondrogenic or osteogenic real time polymerase chain reaction (RT-PCR).

Induction medium as previously described. Control cells were cultured in basal medium containing 2% FBS. Total RNA was extracted using RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. cDNA was synthesized using reverse transcriptase system kit (Promega, Madison, WI, USA). RT-PCR was performed using SYBRGreen master mix (Life technologies, Carlsbad, CA, USA). Cartilage-related genes in-

Table 1. Oligonucleotides for cartilage and bone

Gene	Primer sequence	Product size
GAPDH	Forward: 5′-GCACCGTCAAGGCTGAGAAC-3′	73 bp
	Reverse: 5′-GGATCTCGCTCCTGGAAGATG-3′	
COL2A1	Forward: 5′-GTGACAAAGGAGAGGCTGGA-3′	145 bp
	Reverse: 5′-CCTCTAGGGCCAGAAGGAC-3′	
ACAN	Forward: 5′- AGACTTGGTGGGGTCAG-3′	468 bp
	Reverse: 5′-GATGTTTCCCCACTAGTG-3′	
COMP	Forward: 5′-GGAGATCGTGCAGACAATGA-3′	147 bp
	Reverse: 5′-GAGCTGTCCTGGTAGCCAAA-3′	
FMOD	Forward: 5′-CAACCAGCTGCAGAAGATCC-3′	96 bp
	Reverse: 5′-CAGAAGCTGCTGATGGAGAA-3′	
SOX9	Forward: 5′-GTACCCGCACTTGCACAAC-3′	96 bp
	Reverse: 5′-GTAATCCGGGTGGTCCTTCT-3′	
ALP	Forward: 5′-GGACCATTCCCACGTCTTCAC-3′	96 bp
	Reverse: 5′-CCTTGTAGCCAGGCCCATTG-3′	
CBFA1	Forward: 5′-CACTGGCGCTGCAACAAGA-3′	127 bp
	Reverse: 5′-CATTCCGGAGCTCAGCAGAATAA-3′	
OPN	Forward: 5′-ACAGCCACAAGCAGTCCAGATT-3′	58 bp
	Reverse: 5′-TGCTCATTGCTCTCATCATTGG-3′	
RUNX2	Forward: 5′-GAGGTACCAGATGGGACTGTG-3′	113 bp
	Reverse: 5′-TCGTTGAACCTTGCTACTTGG-3′	

GAPDH: glyceraldehyde 3-phosphate dehydrogenase, COL2A1: collagen type II alpha 1, ACAN: aggrecan, COMP: cartilage oligomeric matrix protein, FMOD: fibromodulin, ALP: alkaline phosphatase, CBFA1: core-binding factor alpha 1, OPN: osteopontin, RUNX2: runt-related transcription factor 2, *SOX9* = (Sex Determining Region Y)-Box 9

cluding collagen type II alpha 1 (COL2A1), aggrecan (ACAN), cartilage oligomeric matrix protein (COMP), fibromodulin (FMOD), (Sex Determining Region Y)-Box 9 (SOX9) and osteogenic-related genes namely, alkaline phosphatase (ALP), core-binding factor alpha 1 (CBFA1), osteopontin (OPN) and runt-related transcription factor 2 (RUNX2) expressions were analyzed. The primers were taken from earlier published studies and the details are provided in Table 1. Glyceraldehyde 3-phosphate dehydrogenase was used as the internal control. qRT-PCR was performed at following cycling conditions; initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 58°C for 1 min.

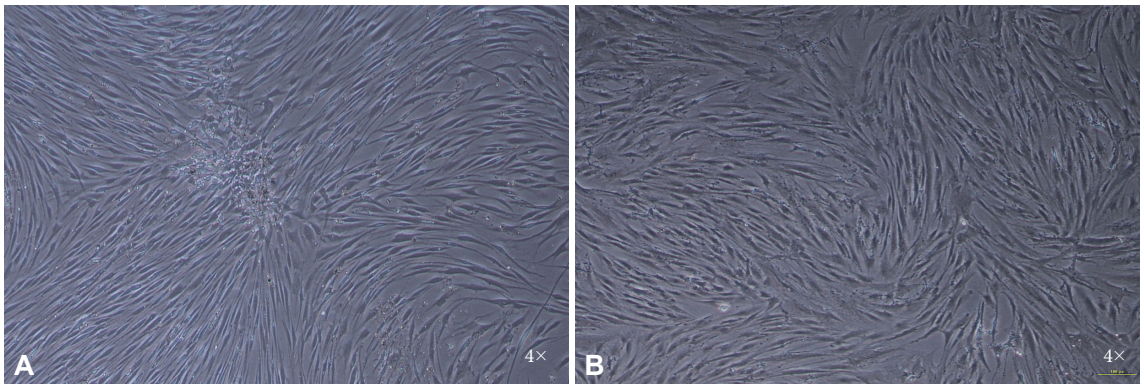

Figure 1. Phase contrast images of bone marrow derived mesenchymal stem cells (BM-MSCs) showing the characteristic fibroblastic morphology. (A) BM-MSCs on day 5 primary culture (magnification, 4×). (B) Expanded BM-MSCs at third passage (P3) (magnification, 4×).

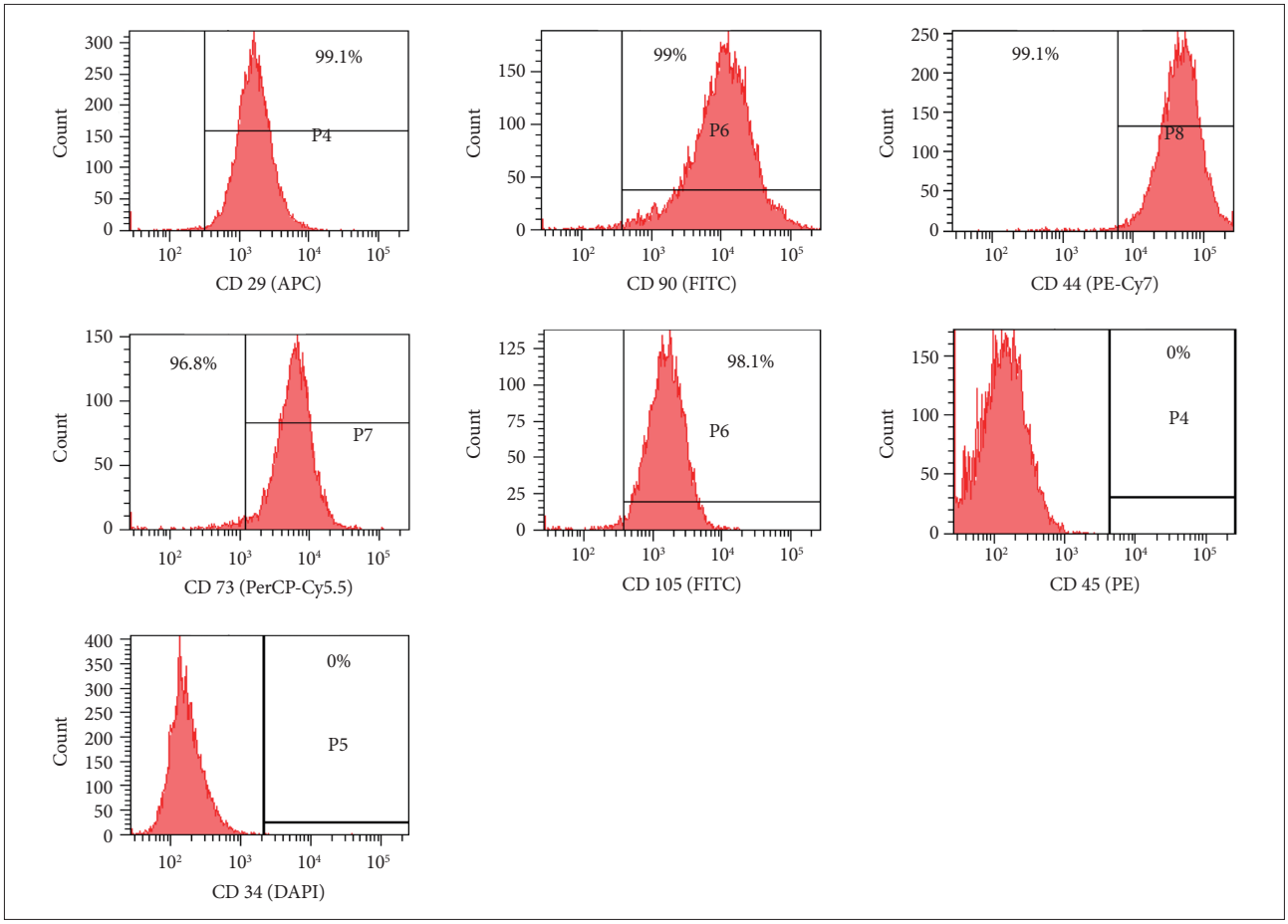

Figure 2. Hisotgram of flow cytometry analysis of BM-MSCs revealed high expression of CD29, CD90, CD44, CD73, and CD105 surface markers. The BM-MSCs were negative for CD45 and CD34 expression. BM-MSCs: bone marrow mesenchymal stem cells.

RESULTS

Phenotypic characterization and surface antigen expression of human BM-MSCs

The isolated cells from human BM formed colonies of cells starting from day 5 of culture. These cells grew to 80–95% confluency within 3 weeks of culture exhibiting fibroblastic spindle morphology (Fig. 1A). Adherent cells were subpassaged and expanded in new T-75 cm^2 tissue culture flasks. The subpassaged cells grew faster unlike initial passage and they reached 95% confluence within 3–7 days (Fig. 1B). FACS analysis demonstrated that 95% of cells showed strong expression of CD29, CD90, CD44, CD73, and CD105. These cells were negative for both CD34 and CD45 (Fig. 2).

Differentiation of human BM-MSCs

Human BM-MSCs that were cultured for 21 days in chondrogenic medium, demonstrated chondrocyte-like cells stained positively with Toluidine blue, while the control cells were devoid of any chondrocyte-like cells and also showed negative staining (Fig. 3A and B). BM-MSCs cultured in osteogenic induction medium formed clusters of calcium depositions as shown by positive Alizarin red staining, while the controls did not show any mineralization nodules and were also negative for the alizarin stain. (Fig. 3C). Also the BM-MSCs cultured in adipogenic induction medium formed lipid vacuolations that positively stained with Oil Red O (Fig. 3D). The controls did not show any lipid vaculoations and were also negative for histology.

Gene expression

All of the chondrogenesis related genes analyzed namely COL2A1, COMP, FMOD, SOX9, and ACAN were upregualted in the differentiated cells compared to the controls. Col2A1 was highly upregulated compared to rest of the genes, and showed 25 fold increase at day 21 in the differentiated cells. ACAN showed moderate upregualtion and had 8 fold increase compared to the control. COMP, FMOD, and SOX9 showed mild upregualtion and the fold increases were 1.7, 2.2, and 2.3, respectively compared to the control (Fig. 4A).

Similarly, ostoegenic related genes namely ALP, CBFA1, OPN, and RUNX2 were upregualted compared to control. ALP showed the highest expression with a fold increase of 17.2 compared to the control. Rest of the genes showed only mild upregulation and their fold increases were 2.0, 1.7, and 1.5 fold for

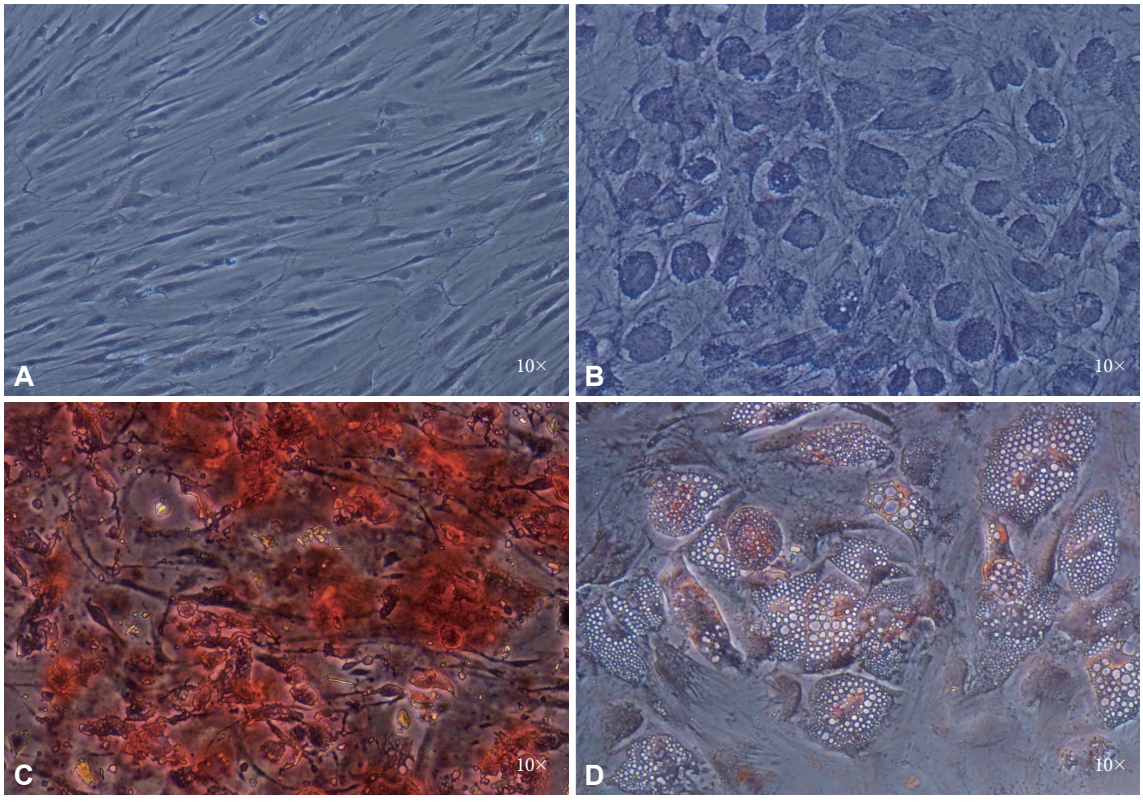

Figure 3. Multilineage differentiation of BM-MSCs. (A) Undifferentiated BM-MSCs (magnification, 10×). (B) Toluidine blue staining showed chondrocyte like cells following 21 days of differentiation using cartilage differentiation medium (magnification, 10×). (C) Alizarin red staining indicated clusters of calcium depositions (magnification, 10×). (D) Lipid vacuolations stained with Oil Red O following adipocytic differentiation (magnification, 10×). BM-MSCs: bone marrow mesenchymal stem cells.

OPN, CBFA1, and RUNX2, respectively (Fig. 4B).

DISCUSSION

MSCs are multipotent progenitor cells capable of self-regeneration and have potential to differentiate into multiple cell types [27,28]. These cells provide promising opportunities for cellular therapies and tissue engineering applications to repair variety of damaged tissues or diseased organs [5-29]. The anti-inflammatory and immunosuppressive properties of MSCs are major advantages for these cells to be used in the context of transplantation and clinical applications [9,30]. These cells secrete several biological mediators such as growth factors, cytokines, and chemokines that are crucial in regulating regeneration and other cellular functions including anti-apoptotic, anti-fibrotic, mitogenic, angiogenic and wound-healing [5,31].

In the present study, BM-MSCs were isolated from OA patients and cultured *in vitro* to evaluate their capacity of differentiation into multiple cell types, especially their chondrogenic potential. Several studies have compared the ability of MSCs isolated from different sources with regard to their proliferation and innate differentiation potential into chondrocytes and osteocytes [32]. The results of chondrogenic and osteogenic differentiation of BM-MSCs to those of adipose derived stem cells (ADSCs), as well as MSCs derived from umbilical cord (UBM-SCs) showed that BM-MSCs were the most suitable and beneficial [33-35]. An earlier study compared efficiency of chondrogenic differentiation between human BM-MSCs and ADSCs were obtained from the same donors [33]. BM-MSCs were reported to be more efficient than ADSCs following both quantitative and qualitative analysis of cartilage related gene and protein expression [33,36]. BM-MSCs may thus be the ideal cell type for cell-based therapies for cartilage regeneration as they have demonstrated acceptable clinical results [5,32].

Recently, The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapies (ISCT) proposed minimum criteria to identify MSCs, which includes adherence to plastic during expansion in culture, multipotent differentiation, and expression MSCs specific cell surface antigen markers [37,38]. The MSCs are characterized by positive expression of STRO-1, CD73, CD105, CD106, CD145, and CD166 as well negative expression for CD11b, CD31, CD34, CD45, and

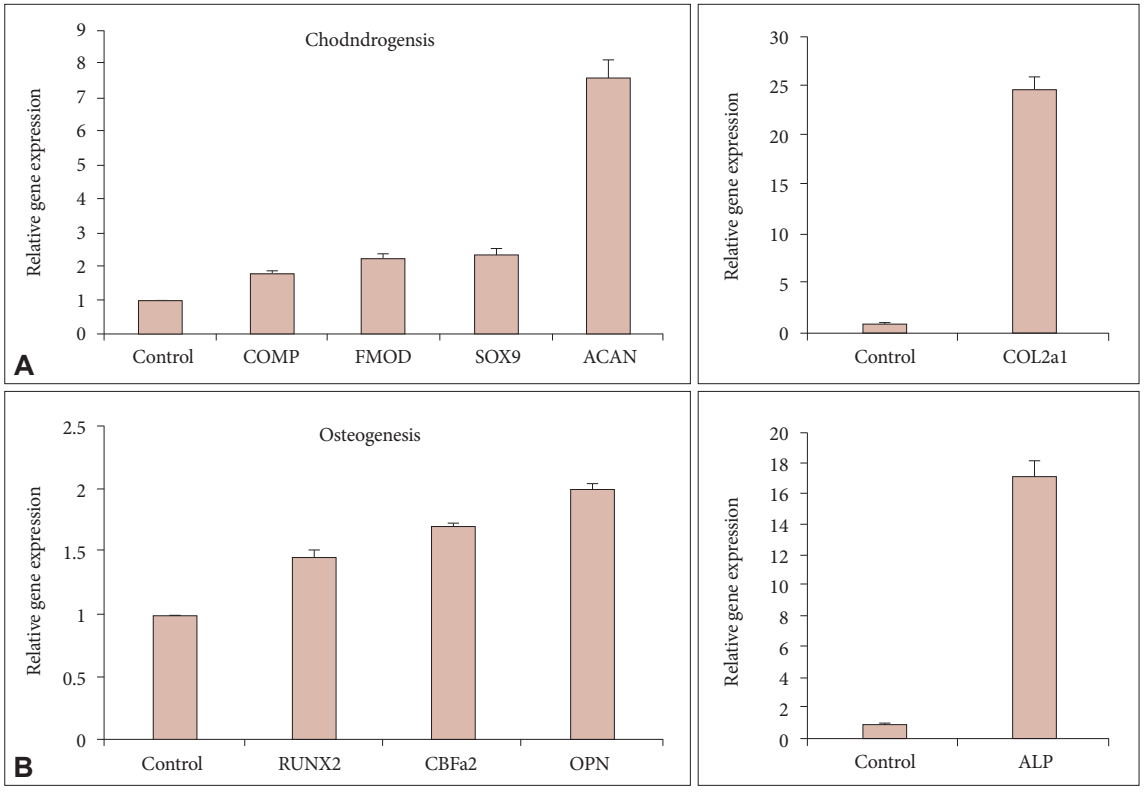

Figure 4. Gene expression analysis by qRT-PCR following 21 days of differentiation showing chondrogenic and osteogenic related genes. (A) Chondrogenic gene expression (COMP, FMOD, SOX9, ACAN, and COL2a1). (B) Osteogenic gene expression (RUNX2, CBFa2, OPN, and ALP). Values are expressed as mean SEM (n=9). RT-PCR: real time polymerase chain reaction, COMP: cartilage oligomeric matrix protein, FMOD: fibromodulin, SOX9: (Sex Determining Region Y)-Box 9, ACAN: aggrecan, COL2a1: collagen type II alpha 1, RUNX2: runt-related transcription factor 2, CBFa2: core-binding factor alpha 1, OPN: osteopontin, ALP: alkaline phosphatase, SEM: standard error of the mean.

CD117 [37].

In our present study, the phenotypic characteristics of BM-MSCs fulfill the stipulated minimal criteria of the ISCT. Isolated BM-MSCs formed adherent fibroblastic colonies in primary culture. Morphologically, cells from OA patients resembled the same characteristics as reported from the normal donors. Furthermore, the analysis of selective MSCs surface antigen markers showed strong positive expression for CD29, CD90, CD44, CD73, and CD105. In addition, these cells demonstrated completely negative expression of the hematopoietic stem cells cell surface antigens CD34 and CD45.

MSCs are highly capable of multilineage differentiation potential [13,17]. In this study we successfully stimulated BM-MSCs to yield chondroblasts, osteocytes and adipocytes. TGF- family of proteins including TGF-β1, TGF-β2, and TGF-β3 are mainly used to promote chondrogenic differentiation of MCSs [39]. In fact, TGF-β2 and TGF-β3 produce more accumulation of proteoglycans and collagen II [40]. We used TGF-β3, and this provided the much needed cell signaling for BM-MSCs to undergo chondrogenic transformation. In addition, the observed positive staining with toluidine blue is indicative of the presence of proteoglycans in the ECM which confirms efficient differentiation into chondrocytes.

Furthermore, gene expression analysis showed high expression of some of the chondrogenesis related genes, especially the COL2A and ACAN. Immature cartilage has low COL2A expression, whereas they become highly expressed in mature cartilage [40]. Moreover, the cartilage ECM contains large amount of type II collagen and ACAN in addition to several other components including COMP, FMOD and SOX9 [40]. Our results also showed minimal expression of both COMP and FMOD at day 21. Interestingly, these two are the earliest ECM components expressed during chondrogenesis [40]. Therefore, an analysis at an earlier time point would have possibly shown higher expression of both COMP and FMOD. In addition, the transcription factor SOX9 that plays an important role in chondrogenesis also showed low expression levels. The results of this study was found to be similar to earlier studies, suggesting that SOX9 is most likely expressed at low level and is adequate for matrix production [32,41].

Dexamethasone, β-GP and ascorbic acid, are identified to be essential to induce differentiation of MSCs into osteocytes [42,43].Others have reported that dexamethasone alone may promote MSCs differentiation to osteocytes and is associated with osteocalcin and OPN gene expression [44]. We also used dexamethasone, β-GP and ascorbic acid to aid osteogenic differentiation and we observed formation of mineralized ECM with calcium deposition as detected by Alizarin red staining. RT-PCR studies also demonstrated increased expression of some of the osteogenic related genes namely ALP, OPN, RUNX2, and CBFa1 compared to the control cells. Similar to other studies ALP gene expression levels were highly increased compared to the undifferentiated cells. However, OPN, RUNX2, and CBFa1 were found to be expressed at low levels in the present study. Earlier studies have reported increased CBFa1, ALP, and OPN gene expression levels within 2−4 weeks following similar differentiation protocols [32,45].

Conclusion

In this present study, human BM-MSCs were isolated and characterized from OA patients. Their differentiation potential into chondrocytes, osteocytes and adipocytes were demonstrated using both histological and gene expression analysis. Although it is an established fact that MSC harvested from BM are capable of multilineage differentiation *in vitro*, this is our first report in our series of *in vitro* and *in vivo* experiments on the BM-MSCs from OA patients.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

This study was approved (no. 11-557) by the Institutional Ethical Committee, King Abdulaziz University (KAU).

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