Comparison of the osteogenic differentiation of orofacial bone marrow stromal cells prior to and following marsupialization in patients with odontogenic cyst

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Received May 28, 2017; Accepted October 18, 2017

DOI: 10.3892/mmr.2017.7949

Abstract. Decompression has been considered a valuable tool for odontogenic cystic lesions to minimize cyst size with low morbidity and recurrence. However, whether decompression has a role in regulating stem cell properties of orofacial bone marrow stromal cells (BMSCs) around the cysts has not been fully investigated. The present study compared the stem cell marker profile and osteogenic differentiation potential of orofacial BMSCs prior to and following marsupialization (pre-BMSCs vs. post-BMSCs) in the same individuals. The results demonstrated that post-BMSCs proliferated significantly faster, displayed higher colony-forming unit-fibroblast capacity and demonstrated higher expression of octamer binding protein 4, Nanog and SRY-related HMG box 2 when compared with the pre-BMSCs. Notably, the osteogenic potential was greater in the post-BMSCs compared with in pre-BMSCs, by demonstrating that the protein and mRNA expression levels of osteopontin, runt-related transcription factor 2, osteocalcin, alkaline phosphatase and osterix were upregulated in pre-BMSCs. Furthermore, the phosphorylated levels of extracellular signal-regulated kinase and c-Jun N-terminal kinase were enhanced in post-BMSCs. In conclusion, the study indicated that decompression influences the stem cell properties of orofacial BMSCs, and further studies are needed to verify the findings.

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Key words: marsupialization, odontogenic cyst, bone marrow stromal cells, osteogenesis

Introduction

Odontogenic cysts are the most common lesions in the jaw, which usually grow large along with resorbing the bone and expanding into surrounding tissues, leading some vital structures (i.e., inferior alveolar neurovascular bundle, and maxillary sinus) damage and causing facial asymmetry, displacement of teeth, and pathologic fractures (1-3). Removal of the cysts is necessary, but enucleation of these lesions can bring forth a risk of complications such as infection, fracture of the jaw, or nerve injury. Recently, decompression with marsupialization has been recommended as a more conservative treatment for large odontogenic cysts. Decompression can mitigate the intramural pressure and reduce the cytokines production, in favor of maintenance of pulp vitality, preservation of the inferior alveolar nerve or maxillary sinus, prevention of fracture of the jaw, and minimization of the recurrence risk (4-7).

Human bone marrow stromal cells (BMSCs) have been widely studied and used as a therapeutic tool in numerous studies and clinical trials due to their unique characteristics, including ease of isolation, amplification *in vitro*, immunological tolerance and multipotent capacity (8,9). Previous studies have showed that BMSCs from human alveolar/jaw bone have osteogenic potential to promote regeneration of alveolar bone (10). Moreover, differentiation of BMSCs into osteoblasts is regulated by many factors including growth factors, cytokines, hormones and mechanical stress. Whilst decompression causes a reduction in the cyst volume with new bone formation, it is unclear if BMSCs respond to these changes. In this study, we examined the effects of decompression on the 'stemness' and osteogenic potential of alveolar BMSCs around the cysts.

Materials and methods

Samples and cell culture. Trabecular bone isolated with a rongeur from five patients with odontogenic cysts (three women and two men at the age ranging from 29 to 37 years), who provided informed consent at the Department of stomatology of the First Hospital of Jiaxing, Zhejiang, China.

The study was carried out under approved guidelines of the Ethical Committee. Trabecular bone around cysts was separately collected during the marsupialization surgery and the second-stage procedure of cysts excision in same individuals. Nucleated cells isolated from each sample were cultured to establish primary BMSC using α-modified Eagle's medium (α-MEM; Gibco-BRL, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (FBS) (Equitech-Bio, Kerrville, TX, USA), 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamine (Sangon Biotech Co., Ltd., Shanghai, China), as previously described (11,12). Cultures were maintained at 37˚C in a humidified atmosphere of 5% CO_2 and the culture medium changed thrice a week. Primary BMSCs from before (pre-BMSCs) and after (post-BMSCs) marsupialization were further expanded and then used at passage 2-4 for *in vitro* experiment.

Cell proliferation assay. BMSCs were seeded into 96-well plates at a density of $5x10^3$ cells/well and incubated overnight in α-MEM medium. Then Cell Counting Kit-8 (CCK8; KeyGen Biotech Co., Ltd., Nanjing, China) solution was added to each well and incubated at 37˚C for 1 h at room temperature. Absorbance at 450 nm was measured spectrophotometrically with a MRX II absorbance reader (Dynex Technologies, Inc., Chantilly, VA, USA). The proliferation assay was performed each day for 18 days consecutively.

Colony forming efficiency assay. For colony forming unit-fibrolast (CFU-F) assays, BMSCs were cultured in triplicate 25 cm^2 plastic culture flasks at 10^3, 10^4 and 10^5 cells/flask with 6 mls of non-osteogenic growth medium (13). After incubation for 10 days without medium change, cultures were washed with PBS, fixed with 100% methanol and dyed bv an aqueous solution of saturated methyl violet (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Aggregates of 50 or more cells were counted as colonies.

Flow cytometric analysis. BMSCs were trypsinized with 0.25% trypsin-EDTA, centrifuged at 1,500 g for 5 min, resuspended and aliquoted at PBS containing 3% FBS into a fluorescence-activated cell sorting (FACS) tube. Each aliquot containing $1x10^5$ cells were incubated with saturating concentrations of primary antibodies or control IgG at room temperature for 30 min. The cells were washed twice by PBS and incubated with a fluorescent conjugated secondary antibody for 30 min at room temperature in dark. The following monoclonal antibodies (mAbs) were used: CD34-PE, CD90-PE, CD44-FITC, and CD45-FITC (Becton-Dickinson, Franklin Lakes, NJ, USA). Events were collected with FACScan (BD Bioscience), and the data were analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

Immunofluorescence staining. BMSCs were were fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilised with 0.1% Triton X-100 in PBS for 5 min. After rinsing with phosphate-buffered solution (PBS), the cells were blocked with 5% horse serum in PBS for 1 h at room temperature and then were incubated with fluorescein isothiocyanate (FITC)-conjugated STRO-1 (sc-47,733 FITC; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and with phycoerythrin (PE)-conjugated CD105 (Santa Cruz, sc-18,838 PE) antibodies antibody (1:100 dilution) for 2 h at room temperature. After washing three times with PBS, cells were stained with DAPI (100 ng/ml) for 3 min. All of the images were captured with a fluorescence microscope (BX50 Olympus, Tokyo, Japan).

Osteogenic differentiation. BMSCs were trypsinized, resuspended and seeded in 96-well culture plates in a density of $1x10^4$ cells/well. The next day, the growth medium was replaced by osteogenic medium to induce osteogenic differentiation (14). The cells were grown for 28 days and the medium was refreshed twice a week. Alkaline phosphatase (ALP) staining to detect osteoblasts was performed with the ALP kit and protocol (Sigma) on cultures grown in osteogenic medium for 7 days. All images were captured on a Olympus IX70 microscope (Olympus, Tokyo, Japan) at the same magnification and light intensity and analyzed by ImageJ software (NIH, Bethesda, MD, USA). Image segmentation was used to generate percent stained values for each field of view. At the 28th day of differentiation, the BMSCs were washed once with PBS and fixed with 4% paraformaldehyde (Sigma) for 15 min at room temperature. At the end of osteogenic stimulation, cells were fixed with 4% paraformaldehyde (Sigma) for 15 min, washed with dH_2O, and stained with 1% Alizarin Red S for 30 min to assess the formation of the mineralized matrix. The level of calcium deposition was quantified by elution of AR-S following incubation in 10% cetylpyridinium chloride (Sigma-Aldrich) for 1 h at room temperature. Samples of the resulting solution were distributed on a 96-well plate and absorbance was read at 570 nm. All experiments were performed in triplicate with independent samples.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from BMSCs using Trizol reagenl (Invitrogen, Carlsbad, CA, USA) and then reverse-transcribed with PrimeScript RT Master mix Kit (Takara, Otsu, Japan) according to manufacturer's recommended protocol. Quantitative real-time PCR was performed using an Eppendorf Mastercycler ep realplex machine (Eppendorf, Germany) and using SYBR Premix Ex Taq™ II Kit (Takara) according to the manufacturer's instructions. The primers were present in Table I. Real-time PCR reactions were carried out with the following conditions: Initial denaturation step at 95˚C for 10 min, followed by 40 cycles of 5 sec at 95˚C and 34 sec at 60˚C. The specificity of PCR products was checked by melting curve analysis and gel electrophoresis. Relative mRNA expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method after by normalizing to GAPDH as an internal control (15).

Western blotting. The BMSCs were collected and lysed in cell lysis buffer containing protease inhibitors at 15 min (Sangon Biotech Co., Ltd.). Then, total protein concentration in every lysate was calculated by a bicinchoninic acid (BCA) protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equivalent amounts of protein samples (100 μg) were electrophoresed through 10-15% gels by SDS-PAGE and subsequently transferred to PVDF membranes. The membranes were then blocked for 1 h in 10% skimmed milk in Tris-buffered saline Tween

Table I. The primer sequences for PCR.

Gene	Forward primers (5'-3')	Reverse primers (3'-5')	Product size (bp)
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC	196
ALP	GGACCATTCCCACGTCTTCAC	CCTTGTAGCCAGGCCCATTG	237
RUNX2	CCCGTGGCCTTCAAGGT	CGTTACCCGCCATGACAGTA	190
OCN	CCCAGGCGCTACCTGTATCAA	GGTCAGCCAACTCGTCACAGTC	224
OPN	CAGTTGTCCCCACAGTAGACAC	GTGATGTCCTCGTCTGTAGCATC	230
Osterix	GCAGCTAGAAGGGAGTGGTG	GCAGGCAGGTGAACTCTTC	218
OCT4	GTATTCAGCCAAACGACCATC	CTGGTTCGCTTTCTCTTTCG	326
Nanog	ATTCAGGACAGCCCTGATTCTTC	TTTTTGCGACACTCTTCTCTGC	360
SOX2	GACTTCACATGTCCCAGCACTA	CTCTTTTGCACCCCTCCCATT	298
c-myc	GCTGCTTAGACGCTGGATTT	TAACGTTGAGGGGCATCG	252

OPN, osteopontin; *RUNX2*, runt-related transcription factor 2; *OCN*, osteocalcin; *ALP*, alkaline phosphatase; *OCT4*, octamer binding protein 4; *SOX2*, SRY-related HMG box 2.

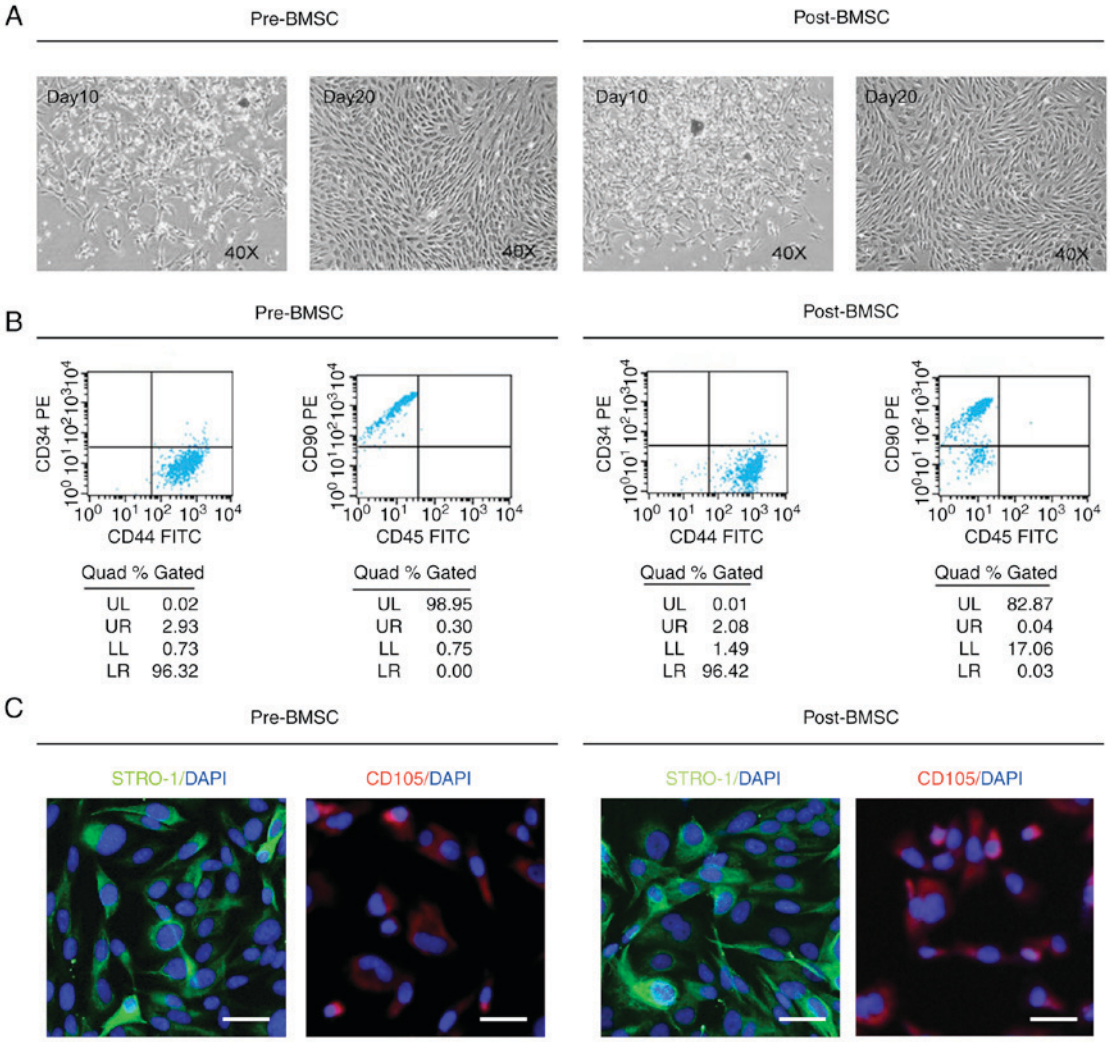

Figure 1. Isolation of pre-BMSCs and post-BMSCs from orofacial bone. (A) Morphological aspects of the pre-BMSCs and post-BMSCs. (B) Flow cytometry analysis to detect the cell surface markers expression (CD34, CD44, CD45 and CD90) of the pre-BMSCs and post-BMSCs. (C) Immunofluorescence analysis of STRO-1 and CD105 expressions in pre-BMSCs and post-BMSCs (Scale bar, 100 μm).

(TBST) at room temperature and then incubated overnight at 4˚C with primary antibodies: Anti-osteopontin (OPN; ab8448, 1:1,000), anti-runt-related transcription factor 2 (Runx2; ab54868, 1:1,000), anti-osterix (ab94744, 1:500), anti-ALP

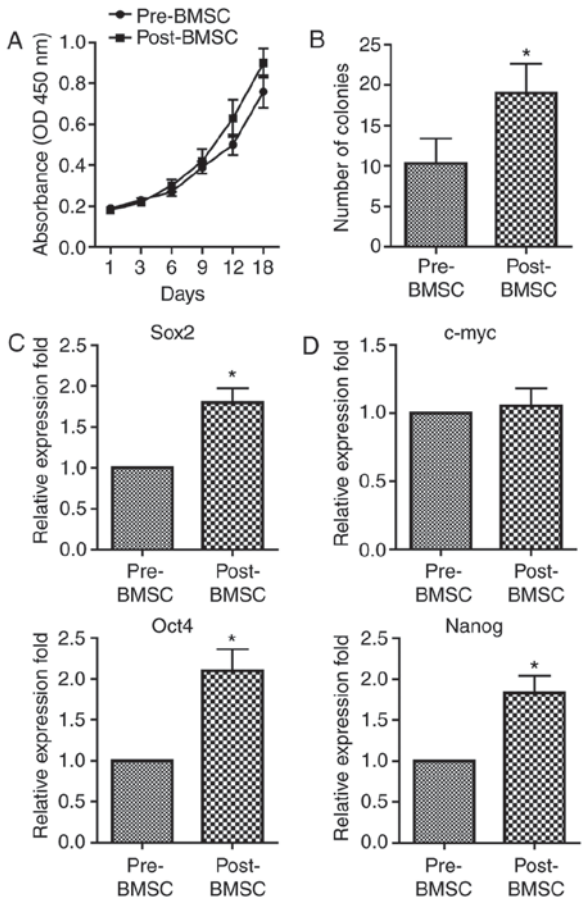

Figure 2. Growth and self-renewal capacity of pre-BMSCs and post-BMSCs. (A) The proliferation of pre-BMSCs and post-BMSCs from day 2 to 16 days. (B) Colony forming unit fibroblast (CFU-F) in pre-BMSCs and post-BMSCs. (C and D) mRNA expression of stemness genes in pre-BMSCs and post-BMSCs evaluated by qRT-PCR. Results are mean ± SD. *P<0.05 compared with pre-BMSCs.

(ab83259, 1:500), and anti-osteocalcin (OCN; ab76690, 1:2,000) (all from Abcam, Cambridge, MA, USA), extracellular signal-regulated kinase1/2 (ERK1/2; no. 9102, 1;1,000), phosphorylated ERK1/2 (p-ERK1/2; no. 4370, 1;1,000), c-Jun N-terminal kinase (JNK; no. 9,252, 1:1,000), phosphorylated JNK (p-JNK; no. 9,251, 1:1,000), anti-p38 mitogen-activated protein kinase (p38 MAPK; no. 9,212, 1:1,000), phosphorylated-p38 MAPK (p-p38 MAPK; no. 9,215, 1:1,000), and anti-glyceraldehyde-phosphate dehydrogenase (GAPDH) (all from Cell Signaling Technology, Inc., Danvers, MA, USA), used at dilutions recommended by the manufacturer. Following three times washing with TBST, membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Relative protein bands intensities were quantified using RapidStep™ ECL reagent (EMD Millipore, Billerica, MA, USA).

Statistical analysis. Results are showed as the mean ± standard deviation (SD) and all experiments were separately repeated at least three times. Statistical analysis was performed using the GraphPad Prism statistical package (version 5.0) and the Student's t-test was performed to assess differences between experimental groups. Differences were considered statistically significant at P<0.05.

Results

Isolation and identification of BMSCs in culture. BMSCs grew as adherent monolayers with a tendency to grow in clusters under microscope, and showed fibroblast-like morphology (Fig. 1A). The surface markers of mesenchymal stem cells (MSCs) were identified by flow cytometry and the results revealed positive staining for MSC markers (CD44 and CD90), and negative for hemacyte antigen markers (CD34 and CD45). Moreover, two early MSC markers STRO-1 and CD105, were also present on both pre- and post-BMSCs by confocal microscopy (Fig. 1C). All the results indicated that the isolated BMSCs were of mesenchymal origin and of high purity (Fig. 1B).

Post-BMSCs proliferate faster and have an increased number of CFU-F and higher expression of stemness genes than pre-BMSCs. We first analysed the proliferation rate of pre-BMSCs and post-BMSC populations. The results showed that the capacity of proliferation was significantly higher in the post-BMSCs with respect to pre-BMSCs (Fig. 2A). Then, primary cultures of BMSCs were established at clonal density in order to obtain discrete colonies. The CFU-F of post-BMSCs was significant increased when compared with the pre-BMSCs, indicating that the self-renewal capability is significantly higher in post-BMSCs (Fig. 2B).

Next, we questioned if the decompression operation could influence the stemness genes expression in BMSCs. We observed that both pre-BMSCs and post-BMSCs expressed the transcripts of the embryonic stem cells including octamer binding protein 4 (OCT4), Nanog, SRY-related HMG box 2 (SOX2) and c-myc. Importantly, the mRNA expression of OCT4, Nanog and SOX2 was significantly higher in the post-BMSCs, whereas no significant difference in c-myc was detected between the two population (Fig. 3C). Collectively, these results clearly indicate that decompression surgery can influence several stem cell properties of BMSCs.

Osteogenic potential is higher in the post-BMSCs than in pre-BMSCs. Last, to test whether the pre-BMSCs and post-BMSCs exhibited differences in osteogenic capacity Alizarin Red S staining was employed to observe the calcium deposition in the osteogenic differentiation of BMSCs *in vitro*. The results showed that decompression operation could enhance the osteogenic differentiation potential of the BMSCs (Fig. 3A). These results were further confirmed by spectrophotometric quantification, with significant more absorbance noted in the post-BMSCs (Fig. 3B). Moreover, both pre- and post-BMSCs displayed a positive color signal of the ALP staining, the post-BMSCs showed a more intensive signal compared with the pre-BMSCs (Fig. 3C).

To further investigate the effects of decompression operation on osteogenic differentiation of BMSCs, the gene and protein expression levels of OPN, RUNX2, OCN, ALP and osterix were determined by RT-PCR and Western blotting, respectively. Post-BMSCs cultured in osteogenic medium showed a significant upregulation of protein levels in OPN, RUNX2, OCN, ALP and osterix than pre-BMSCs (Fig. 3A). RT-PCR analyses were consistent with the protein expression, demonstrating an increase in the gene levels of OPN,

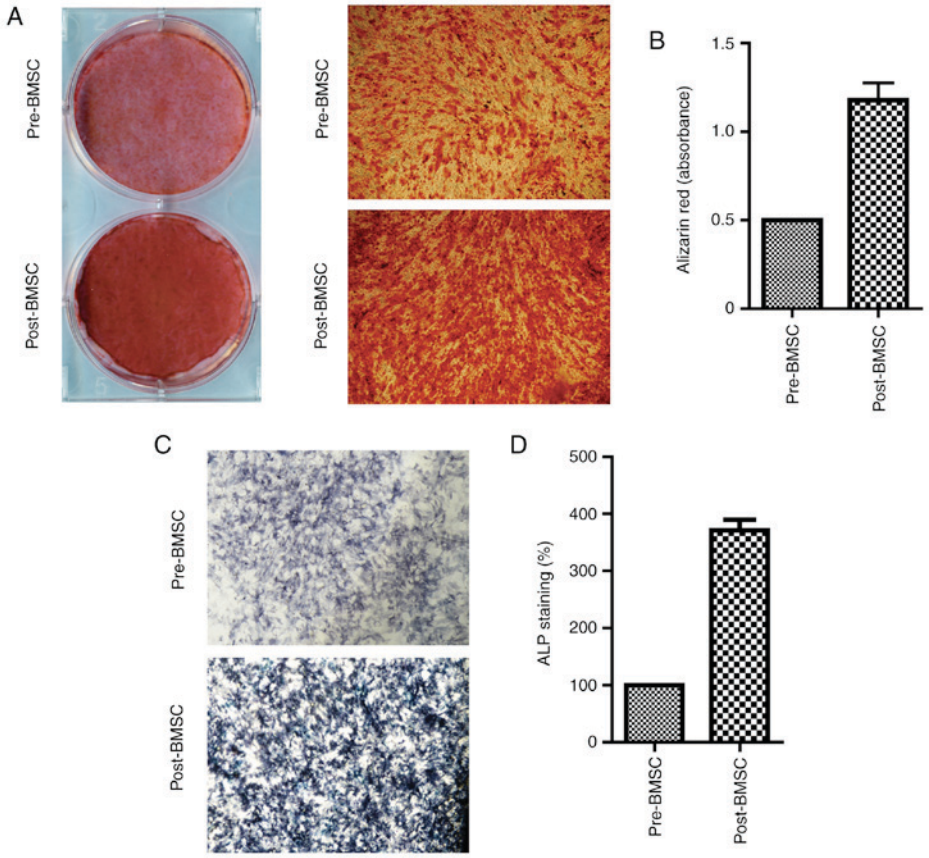

Figure 3. Osteogenic differentiation potential of pre-BMSCs and post-BMSCs. (A) Osteogenic differentiation of pre-BMSCs and post-BMSCs stained with alizarin red. (B) Alizarin red quantification of mineralized deposits in sorted fractions of pre-BMSCs compared to post-BMSCs. (C) Osteogenic differentiation of pre-BMSCs and post-BMSCs stained with alkaline phosphatase. (D) Quantitation of alkaline phosphatase staining in (C) using the ImageJ software.

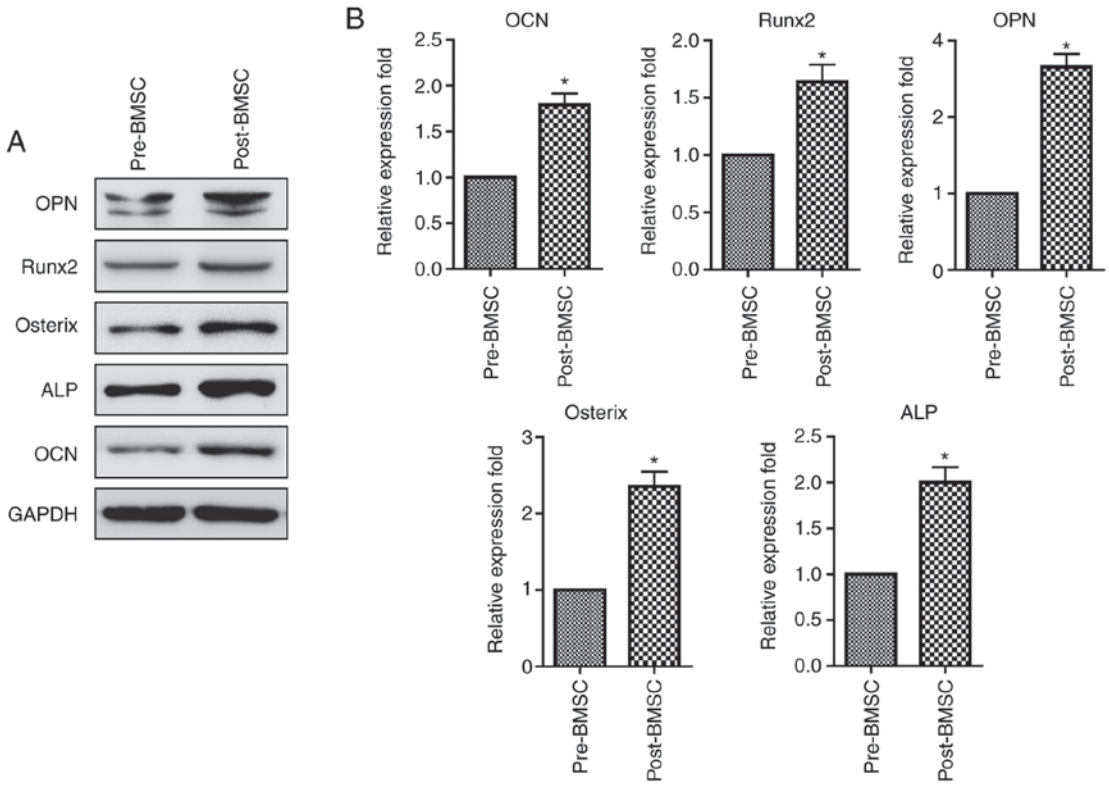

Figure 4. The expression of osteoblast-associated genes in pre-BMSCs and post-BMSCs. (A) Protein expression and (B) mRNA expression of osteoblast marker genes in pre-BMSCs compared to post-BMSCs. *P<0.05 compared with pre-BMSCs.

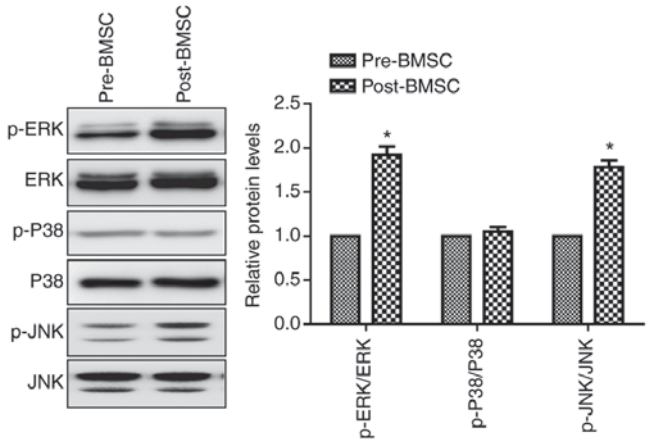

Figure 5. The activation of ERK, p38 MAPK and JNK signaling pathway in pre-BMSCs and post-BMSCs. Phos-phorylation of key kinases involved in ERK, p38 MAPK and JNK pathways were assessed by western blot analysis. *P<0.05 compared with pre-BMSCs.

RUNX2, OCN, ALP and osterix in post-BMSCs with respect to the pre-BMSCs (Fig. 4B).

Decompression operation activates ERK/JNK signaling pathway. To reveal the underlying mechanisms of decompression operation on proliferation, stemness and osteogenic differentiation of BMSCs, the phosphorylation of MAPK signaling pathway was evaluated in BMSCs. Western blot analysis illustrated phosphorylated levels of ERK and JNK were obviously enhanced in post-BMSCs, while no difference in phosphorylated level of p38 MAPK (Fig. 5), suggesting the activation of ERK/JNK signaling pathway was an important mechanisms for decompression operation.

Discussion

BMSCs have the characteristics of self-proliferation and multi-differentiation (e.g., osteogenic, chondrogenic, and adipogenic) potential, but marrow aspiration from iliac crest is an invasive procedure. It has been previously confirmed that orofacial (maxilla and mandible) BMSCs had the same fibroblastic shape as that isolated from the iliac crest, and their proliferative and osteogenic potentials were similar to those of iliac crest derived BMSCs (10,12). Therefore, orofacial BMSCs may be a cell source for promoting regeneration and remodeling of orofacial bone in patients with periodontal disease. However, many factors, such as growth factors, cytokines, hormones and mechanical stress, can influence osteogenic differentiation capability of BMSCs. In the present study, we aimed to explore the role of decompression in regulating the osteogenic differentiation of the orofacial BMSCs around the odontogenic cystic lesions.

In this study, we successfully isolated and characterised orofacial BMSCs from same individuals before and after marsupialization surgery. We observed that both pre-BMSCs and post-BMSCs highly expressed the BMSC markers such as CD44 and CD90. However, the proliferation and CFU-F capacity were significantly higher in post-BMSCs compared to pre-BMSCs. Importantly, post-BMSCs expressed a high level of OCT4, Nanog and SOX2, indicating that marsupialization surgery plays a key role in regulating the pluripotency of BMSCs around cysts tissues. Furthermore, a significant difference between the osteogenic potential of pre-BMSCs and post-BMSCs was detected in our current study. In fact, the osteogenic differentiation capacity of the post-BMSCs was significantly higher with respect to the pre-BMSCs. Collectively, all of these findings suggested that decompression of odontogenic cysts could promote the stemness and osteogenic potential of orofacial BMSCs.

There are several reasons may be explained for the effect of marsupialization on orofacial BMSCs. One reason was that the intracystic pressure decreased after marsupialization could promote osteogenesis in BMSCs. It has been shown that the intracystic fluid pressure in odontogenic jaw cyst could reach up to 38-47 mmHg (16), and reduced to 0 mmHg after marsupialization. Yang *et al* (17) demonstrated that BMSCs showed a typical appearance of osteoblast after two weeks of induction by intermittent negative pressure, and the activity of ALP and expression of OPN increased significantly. Wiesmann *et al* (18) also confirmed that mechanical stimulation could promote the expression of collagen type I and osteonectin in BMSCs. Another reason was that the expression of inflammatory factors such as interleukin-1α (IL-1α) and prostaglandin E2 (PGE2) was decreased by marsupialization could inhibit osteoclastogenesis in BMSCs (19). It has been showed that IL-1α and PGE2 evoked an increase in receptor activator of nuclear factor-κB ligand (RANKL) mRNA, and a decrease in osteoprotegerin (OPG) mRNA in BMSCs (20,21). All these factors may enhance the differentiated function of osteoblasts and bone formation. Further studies by using an *in vitro* model were needed to confirm all these potential mechanisms, which might help the application of marsupialization in patients with odontogenic cyst.

MAPKs, comprised of ERK, P38 MAPK and JNK, are serine-threonine protein kinases and participate in a mass of cellular activities, such as proliferation, inflammation, migration and differentiation (22,23). To elucidate the possible underlying mechanism, we finally evaluated the phosphorylation of ERK, p38 MAPK and JNK pathways in pre- and post-BMSCs. Results showed that the phosphorylated levels of ERK and JNK, rather than p38 pathway, were obviously enhanced in post-BMSCs compared to pre-BMSCs, suggesting that decompression operation might participate in the modulation of proliferation, stemness and differentiation through regulating ERK and JNK pathways in orofacial BMSCs.

In summary, our results show that decompression has a crucial role in regulating stem cell properties of orofacial BMSCs. Further understanding of the relation between intracystic pressure and cytokines changes and osteogenic differentiation potential of orofacial BMSCs are needed.

Acknowledgements

This study was supported by grants from the Science and Technology Plan Project of Jiaxing Zhejiang Province, China (no. 2016BY28004) and Medical and Health Science and Technology Project of Zhejiang Province, China (no. 2017KY650).

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