Expression of HIF-1α in cycling stretch-induced osteogenic differentiation of bone mesenchymal stem cells

HAIBO YU[1], WENYI YU[1], YING LIU[1], XIAO YUAN[2], RONGTAO YUAN[1] and QINGYUAN GUO[1,3]

[1]Department of Stomatology, The Affiliated Qingdao Municipal Hospital, Qingdao University; [2]Department of Orthodontics II, The Affiliated Hospital of Qingdao University, Qingdao, Shandong 266011; [3]Institute of Stomatology, Chinese PLA General Hospital, Beijing 100853, P.R. China

Received October 28, 2018; Accepted July 30, 2019

DOI: 10.3892/mmr.2019.10715

Abstract. During orthodontic treatment, mechanical force is applied to the teeth, and following a series of complex metabolism changes, the position of the teeth in the alveolar bone change. This process is closely associated with primitive bone mesenchymal stem cells (BMSCs), which may differentiate into osteoblasts precursor cell. A hypoxic microenvironment may be caused by orthodontic mechanical forces between the alveolar bone and the root. Hypoxia-inducible factor 1α (HIF-1α) is a specific receptor that adapts to a hypoxic environment. The present study was designed to investigate whether HIF-1α was involved in the osteoblastic differentiation of BMSCs induced by cyclic tensile stress. During this process, HIF-1α mRNA and protein expression were detected using a reverse transcription-quantitative polymerase chain reaction and western blotting. It was revealed that alkaline phosphatase activity increased in a time-dependent manner in three different stretching strength groups, which indicates that cyclic stretch promotes the osteogenic differentiation of BMSCs. The optimal force stage of osteogenesis was an unexpected discovery, which will provide theoretical guidance for selecting the most suitable orthodontic force for tooth movement in clinical orthodontic treatment. Most importantly, all experiments revealed that HIF-1α mRNA and protein were significantly increased following stretching treatment in BMSCs. It was therefore concluded that HIF-1α may be involved in BMSCs modulating osteogenic metabolism during exposure to cyclic stretch and a hypoxic microenvironment, which may prove useful for the reconstruction of a jaw during orthodontic treatment.

Correspondence to: Dr Qingyuan Guo, Department of Stomatology, The Affiliated Qingdao Municipal Hospital, Qingdao University, 5 Donghai Middle Road, Qingdao, Shandong 266011, P.R. China
E-mail: guoqingyuanqd@163.com

Key words: hypoxia-inducible factor 1α, cycling stretch, osteogenic differentiation, bone mesenchymal stem cell

Introduction

Classical histological change of tooth movement during orthodontic treatment is that the external force generates two different strains in the periodontal ligament; compression and tension (1). At the compression site, the periodontal ligament space narrows, the blood flow slows, collagen fibers and matrix degrade and absorb, osteoclastogenesis occurs and alveolar bone tissue is lost. At the tension site, the periodontal ligament space widens, the blood flow increases, collagen fibers and matrix proliferate, osteogenesis occurs and novel alveolar bone tissue is formed (2). Mechanical stress serves a vital function in bone metabolism during orthodontic tooth movement via the osteoblast and osteoclast processes. This type of tooth movement is considered a biological response to the physiologic equilibrium, when the dentofacial complex is interfered with by an externally applied force (3). Osteoblast activity serves a central function in the regulation of the bone remodeling process and orthodontic tooth movement (4). Notably, the formation of alveolar bone includes two aspects; first, that primitive bone mesenchymal stem cells (BMSCs) differentiate into osteoblast precursor cells, followed by the maturation of osteoblasts, including matrix formation and mineralization (5). However, the function of orthodontic force in the osteogenic differentiation of BMSCs still requires further study. In order to elucidate the association between orthodontic tooth movement and orthodontic-periodontal-bone tissue reconstruction, it is necessary to investigate the mechanism and regulation of osteogenic differentiation in BMSCs.

It is well recognized that the cellular microenvironment is necessary for regulating the phenotypic differentiation of BMSCs. Previous research has suggested that the differentiation of cells is regulated by complex interactions between genetic and biochemical factors (6,7). Subsequent studies have focused on biomechanical signals, which may serve a complementary or coordinating function to cytokines (8). Cells may develop corresponding biological changes in response to multifarious physiological stresses. Unfortunately, due to the complexity of the environment *in vivo*, revealing the impact of mechanical factors separately is often challenging. It is difficult to distinguish the effect of a single factor from the combined effect, particularly when studying the biological behavior of cells. Therefore, the effect on the cell stimulation

system mainly depends on the *in vitro* methods (9). Among the various mechanical stimulations, tensile stretch is a major factor in determining the strain and function of a cell (10). Under different tensile stress, the overall framework of the cytoskeleton is rearranged, resulting in the deformation of cells. Changing of the cell shape may initiate a signal, which in turn may be transmitted into the cell nucleus and produce various effects (11). Notably, the tension that is applied to *in vitro* culture cells may provide a greater physiological stimulation that better recapitulates the growth state of cells *in vivo* (12). In previous years, more studies have begun to focus on the osteogenic differentiation induced by tensile stress, as accumulating evidence suggests that tensile stress serves an important function in bone remodeling during orthodontic tooth movement (13,14).

A hypoxic microenvironment may be caused by orthodontic mechanical forces between the alveolar bone and the root, and a local hypoxic microenvironment is one of the main factors initiating bone remodeling (15). Interestingly, bone marrow was also demonstrated to have a hypoxic environment in nature (1-7% O_2), from which BMSCs are isolated (16). Hypoxia-inducible factor 1α (HIF-1α) is a specific regulator of hypoxia responses in all cells, and is also the common pathway of information transmission under hypoxia-inducible conditions (17), which has a vital influence in the adaption to hypoxia. However, it is unknown how HIF-1α regulates osteogenic differentiation induced by cycling stretch. One existing study demonstrates that regarding HIF-1α in osteogenic differentiation, being subjected to cycling stretch is still far from adequate, but it is helpful for clinical orthodontists using different methods to induce or regulate bone remodeling (18). As a consequence, elucidating the mechanism of osteogenic differentiation, particularly when mechanical stretch-induced, is essential to further understand the mechanism of bone remodeling during orthodontic tooth movement.

To investigate whether HIF-1α affects the osteogenic differentiation of BMSCs, a model of cyclic tensile stress loading on cells *in vitro* was established. Furthermore, different cycling stretch forces were used to determine the ideal condition and to investigate the association amongst force, HIF-1α expression and BMSC osteogenic differentiation. The present study demonstrated the necessity of mechanical forces in the osteogenic differentiation of BMSCs and investigated the potential intrinsic molecular mechanism of the expression of HIF-1α. Furthermore, the present study provided theoretical guidance for orthodontists applying different means and methods to induce or regulate bone remodeling during orthodontic tooth movement.

Materials and methods

Isolating and culture of BMSCs. BMSC primary cell culturing was performed with minor modifications, as previously described (19). In brief, the tibias and femurs were isolated from 10 4-week old male Sprague-Dawley rats (140±10 g). Rats were housed at 18-26˚C with ~55% humidity under a 12:12-h light:dark cycle with access to food and water *ad libitum*. The study design was submitted to and ethically approved by the Animal Ethical Committee of Shandong University (approval no. ECAESDUSM2013066; Shandong, China). Under aseptic circumstances, the bone marrow was flushed out and mixed with complete α-minimal essential medium (α-MEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 15% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. The harvests were plated into a culture flask and incubated for 3 days to allow for the attachment of adherent cells, in a humidified atmosphere containing 5% CO_2 at 37˚C. The culturing medium was changed every 3 days to remove the non-adherent cells and to provide the nutrition that the cells required.

MTT assay. BMSCs at third passage were cultured in α-MEM supplement with 10% FBS. Once 80% confluence had been reached, cells were seeded in 96-well plates at a density of $1x10^3$ cells/well and the culture medium was added up to 100 μl/well. The proliferation of cells was evaluated using a MTT assay. Cells were cultured for 1 week, and 6 wells/day were measured. Briefly, the culture medium was replaced with 200 μl MTT (5 mg/ml; Amresco, LLC, Solon, OH, USA) and the plates were incubated for 4 h at 37˚C. The supernatant was then removed and 150 μl of DMSO was added to each well. The absorbance at 490 nm was measured in a multiwall spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Osteogenic differentiation and alkaline phosphatase (ALP) assay. Cells on the 3rd passage were cultured in the growth medium for 24 h prior to testing the characteristics of osteogenic differentiation. The osteogenic differentiation medium included α-MEM containing 10% FBS, 10 mM β-glycerophosphate, 10 nM dexamethasone and 100 μM Vitamin C (all from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Osteogenesis analysis was performed at 1-week (ALP) and 4-week (ARS and calcium concentrations) time points. The extent of mineralization of *in vitro*-cultured BMSCs on different substrates was evaluated by using Alizarin red S (ARS) staining and an ALP activity assay.

For ARS, the samples were fixed in 70% ice-cold ethanol for 1 h at 4˚C, washed, and then were stained with 2% Alizarin red solution (pH 4.2; Sigma-Aldrich; Merck KGaA) for 10 min at room temperature and finally rewashed with distilled water.

After staining with Alizarin red, calcium was dissolved in 1 ml of 10% w/v cetylpyridinium chloride by gentle rocking for 30 min. The calcium concentration in the eluate (the solution was diluted at 1:10) was spectrophotometrically determined at 562 nm at 1-, 2-, 3- and 4-week time points. All values were normalized against the cultivation area.

ALP activity of each sample (n=3) was assayed using an ALP assay kit (Sigma-Aldrich; Merck KGaA). After incubation for 24 h, the medium was changed to mineralization medium for 7 days. Then, the BMSCs were rinsed twice with PBS, fixed with 10% formaldehyde for 30 min at room temperature, stained with 1 ml sodium nitrate, 1 ml FRV Alkaline and 1 ml Napthol As-BI ALK in 45 ml deuterium-depleted water (ddw) for 30 min at room temperature in the dark, and then washed with ddw and imaged using an optical microscope (magnification, x10). Red staining indicated an ALP activity positive rate of 80-90%.

Table I. Oligodeoxynucleotide primers used for the reverse transcription-quantitative polymerase chain reaction.

Gene	GenBank accession number	Primer sequence	Annealing (˚C)	Cycle (sec)	Length (base pairs)
GAPDH	NM 017008 8	F: 5'-AGGTCGGTGTGAACGGATTTG-3' R: 5'-TGTAGACCATGTAGTTGAGGTCA-3'	60	40	123
Hypoxia-inducible factor-1α	NM 024359	F: 5'AAGTCAGCAACGTGGAAGGT-3' R: 5'-ATCAGCACCAAGCACGTCAT-3	55	35	116

F, forward; R, reverse.

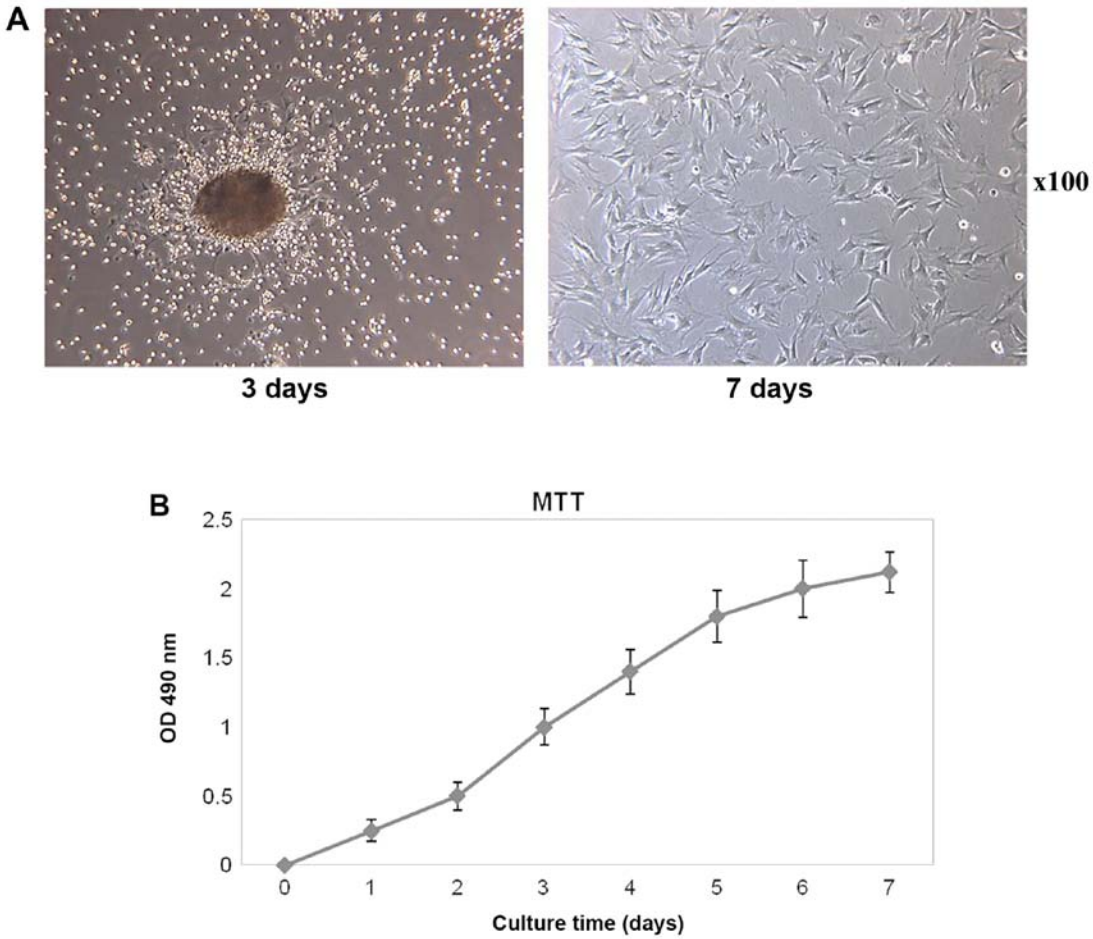

Figure 1. Culture and proliferation of BMSCs. (A) BMSCs were separately cultured for 3 and 7 days, and optical images revealed that the cells were confluent. Magnification, x100. (B) An MTT assay revealed that the cell proliferation of BMSCs increased steadily during one week. BMSC, bone mesenchymal stem cell; OD, optical density.

Colony-forming ability of BMSCs. Cells that had been passaged 3 times were seeded in 6-well culture plate at a gradient density of 0.01-1x10^5 cells/ml, in a humidified atmosphere containing 5% CO_2 at 37˚C. Culturing medium was changed every 3 days as described above. When formed, the colony was fixed using alcohol for 10 min at room temperature and washed using phosphate buffered saline. Then, 0.1% crystal violet dye was added into the plate for 5 min at room temperature and then washed away. Subsequent to being air-dried, the plate was observed using an optical microscope (magnifications, x10 and x100), and the colony forming units-fibroblastic (CFU-F) enrichment index was counted (one colony-forming unit was counted as >50 cells).

Surface antigen analysis of BMSCs. Flow cytometry was used to analyze the surface antigens of BMSCs. Following digestion for three times at room temperature and centrifugation at 150 x g for 3 min at room temperature, the density of the cells in the 3rd generation were adjusted to 1x10^6 ml. Cell-surface antigens were detected using FITC-conjugated [cluster of differentiation CD44 (1:100; cat. no. 203906), CD90 (1:100; cat. no. 206106)] or phycoerythrin-conjugated [CD45 (1:100;

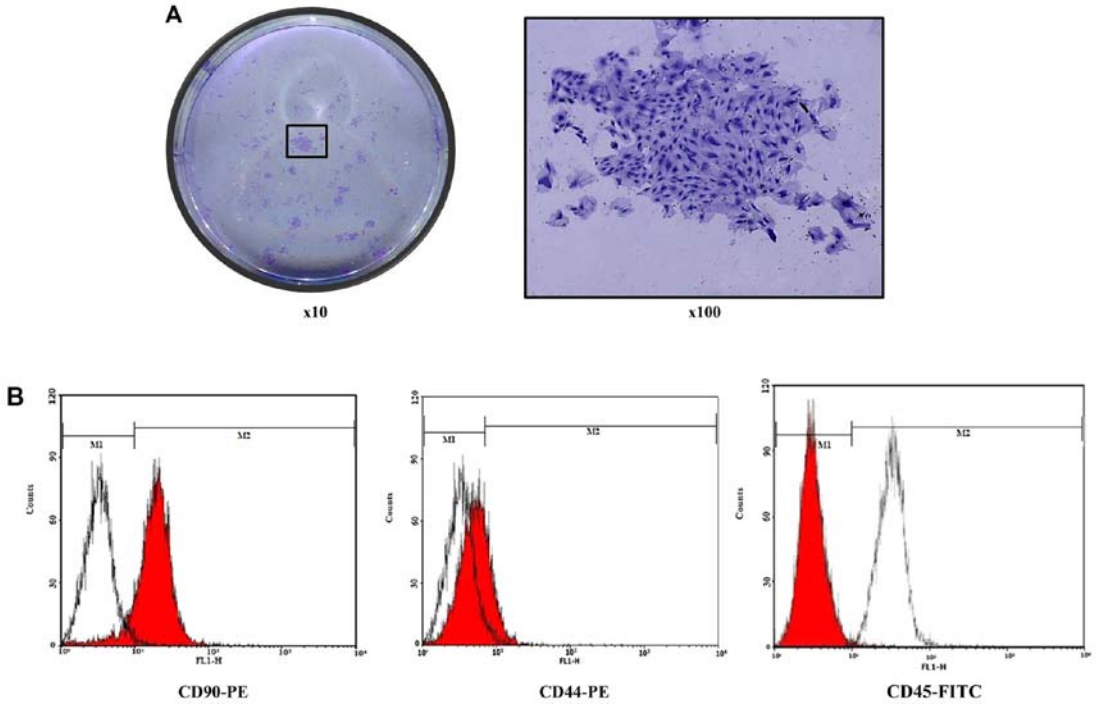

Figure 2. BMSC colony formation and phenotypes. (A) Colony formation was observed with using magnifications of x10 and x100. (B) In order to investigate the characterization of the BMSCs, the expression of the antigens on the cell surface were detected using flow cytometry. Results demonstrated that the harvested cells were positive for mesenchymal progenitor cell-like markers CD44 and CD90, but were negative for the hematopoietic marker CD45. BMSC, bone mesenchymal stem cell; CD, cluster of differentiation; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

cat. no. 202207)] antibodies (Biolegend, Inc.), and the results were analyzed using FlowJo 7.6 (FlowJo LLC).

Application of cyclic tensile strain. Cells were plated at a density of $1x10^5$ cells/ml in 2 ml serum-free medium on 6-well flexible silicone rubber BioFlex™ plates which were coated with rat tail collagen type I (Flexcell International Corp., Hillsborough, NC, USA). When the cells reached 80-90% confluence, the growth medium was replaced and mechanical strain was applied. The cyclic mechanical strain with a 1 HZ and sinusoidal curve set at 1, 5 and 15% elongation were applied for each treatment group, using a FX-5000T™ Flexcell Tension Plus™ unit (Flexcell International Corp.). The culture environment was the same as aforementioned. The BMSCs were collected after 0.5, 2, 6, 8 and 12 h of stretch stimulation.

Alkaline phosphatase (ALP) activity assay. The ALP activity of each sample (n=3) was assayed using an ALP assay kit (Sigma-Aldrich; Merck KGaA). Once the absorbance was measured at 405 nm, the optical density values were normalized to the amount of total proteins in each sample lysate.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated using RNAiso Plus (Takara Bio, Inc., Otsu, Japan). The concentration of the isolated RNA was determined at 260 nm using Gene Quant pro (GE Healthcare, Chicago, IL, USA) and reverse transcription was performed with the Prime Script® RT reagent kit with gDNA Eraser (Takara Bio, Inc.). RT-qPCR was performed using SYBR® Premix Ex TaqTM (Takara Bio, Inc.). The thermocycling conditions of the qPCR were as follows: Denaturation at 95˚C for 30 sec; 40 cycles at 95˚C for 50 sec and 60˚C for 20 sec; and a final dissociation stage (65˚C for 15 sec) was added at the end of the amplification procedure. GAPDH was used as an internal control. Each experiment for each sample was performed three times. The data were analyzed using the comparative quantitation cycle ($2^{-\Delta\Delta Cq}$) method and expressed as a fold change respective to the control (20). The primer sequences are listed in Table I.

Western blotting. Cell-aggregate lysates were extracted by lysing the cells in a western & immunoprecipitation protein extraction reagent (Beyotime Institute of Biotechnology, Haimen, China) with a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA) for 5 min on ice, and then the concentration of the total protein was determined using a BCA protein assay (Nanjing Keygen Biotech Co., Ltd., Nanjing, China). Subsequent to being heated for 5 min at 100˚C in a loading buffer, 20 μg of each detected protein sample was separated by 10% SDS-PAGE and then was electro-transferred into polyvinylidenedifluoride membranes (PVDF; EMD Millipore, Billerica, MA, USA). Then, the PVDF membranes were blocked for 0.5 h at room temperature with Tris-buffered saline (TBS) containing 5% nonfat dry milk powder and 0.05% Tween 20, and then incubated overnight at 4˚C with primary antibodies against HIF-1α (1:1,000; cat. no. ab216842) and GAPDH (1:5,000; cat. no. ab9485; both Abcam, Cambridge, MA, USA). The membranes were washed 3 times for 10 min each with TBS containing 0.05% Tween 20. Bound primary antibodies were detected by incubating for 1 h with horseradish peroxidase-conjugated goat anti-mouse (1:5,000; cat. no CW0102S) or anti-rabbit immunoglobulin G secondary antibodies (1:5,000; cat. no CW0103S; both CoWin Biosciences Co., Ltd., Beijing, China). The membranes

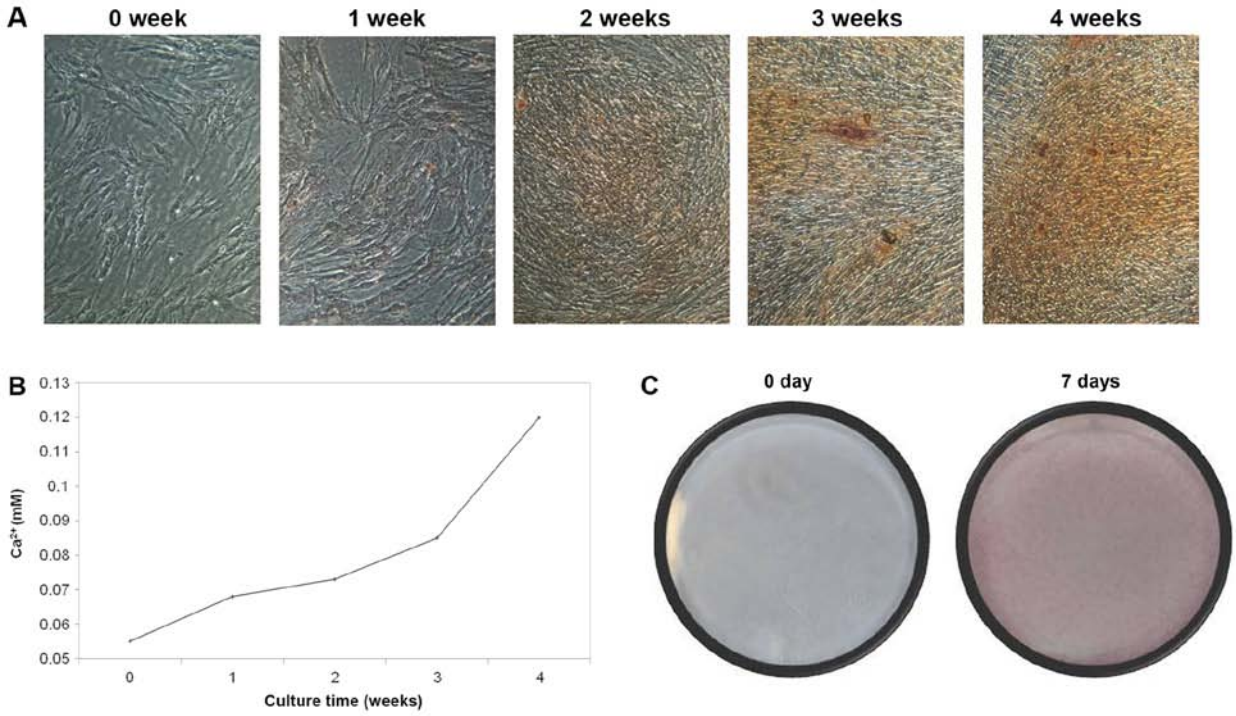

Figure 3. Mineral induction of BMSCs and evaluation of differentiation to an osteoblast phenotype. (A) Alizarin Red S staining revealed a time-dependent increase in the color intensity of osteogenic induction. (B) Quantification of the calcification was performed using Ca^{2+} measurement. (C) Differentiation of BMSCs to an osteoblast phenotype was evaluated using ALP staining. ALP staining for the mineral-induced BMSCs displayed substantially more red-colored staining compared with that of the control without the mineral induction. BMSC, bone mesenchymal stem cell; ALP, alkaline phosphatase.

were washed and developed using an ECL Reagent (EMD Millipore) according to the manufacturer's protocol, and were quantified using ImageQuant software (TL 8.1; GE Healthcare Life Sciences).

Statistical analysis. All data were presented as the mean ± the standard deviation of at least three independent experiments performed in duplicate. Statistical significance was evaluated using a one-way analysis of variance followed by multiple comparisons performed using a post-hoc Bonferroni's test using SPSS22.0 software (IBM Corp., Armonk, NY, USA). $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Culture of BMSCs. In the primary culture, the suspension of monocytes were round in shape. The suspension also contained other types of cells, including blood cells and other cells with the characteristics of adherent growth. BMSCs were isolated following adherence for 24-36 h. On the 3rd day, BMSCs grew at a rapid rate and other suspended cells were gradually discarded by exchanging the medium. Elongated spindle and polygon-shaped cells were observed via light microscopy, and cell clusters appeared (Fig. 1A). On the 7th day, cells were in typical spindle-shape and cell cluster was fused. Subsequent to the primary culture, the cells were passaged at a 1:2 ratio at 7 days when the 90% of the area of the bottom was covered by cells, suggesting the cells were in a good condition (Fig. 1A). As the cells in the 3rd generation still had decent cell viability, it suggested that the BMSCs possessed the capability to passage stably and may be used for further experimental study.

Proliferation capability of BMSCs. To assess the cell proliferation capability, an MTT test kit was used to detect the proliferative rates. The cell proliferation curve presented a slower rate initially (within the first 2 days), and followed by a logarithmic growth during days 3 to 6, which was similar to an S-shape curve. Subsequent to 7 days, the proliferation of the BMSCs entered a relative stable phase (Fig. 1B).

Clonal characterization of BMSCs. BMSCs were originally isolated from other cells via their ability to adhere to culture plastic and then form density-independent colonies termed CFU-F. To assess the clone formation characteristics of BMSCs, a limited dilution method was used by adding crystal violet dye into the plate. Following one week, it was observed through a light microscope that there was substantial purple colony formation, and a CFU-F was formed (Fig. 2A).

Flow cytometry. The 3rd passage of the BMSCs was used to investigate its phenotypes. The result revealed that 99.7% of the cells expressed CD44 and CD90. In contrast, expression of CD45, a pan-hematopoietic marker, was observed in a distinct population of 0.3% of the cells (Fig. 2B). The results indicated that the cells obtained through isolated culture were bone marrow stromal cells.

Osteogenic differentiation. Four weeks subsequent to the BMSCs being placed in osteogenic medium, mineralization nodules were observed following ARS staining. As presented

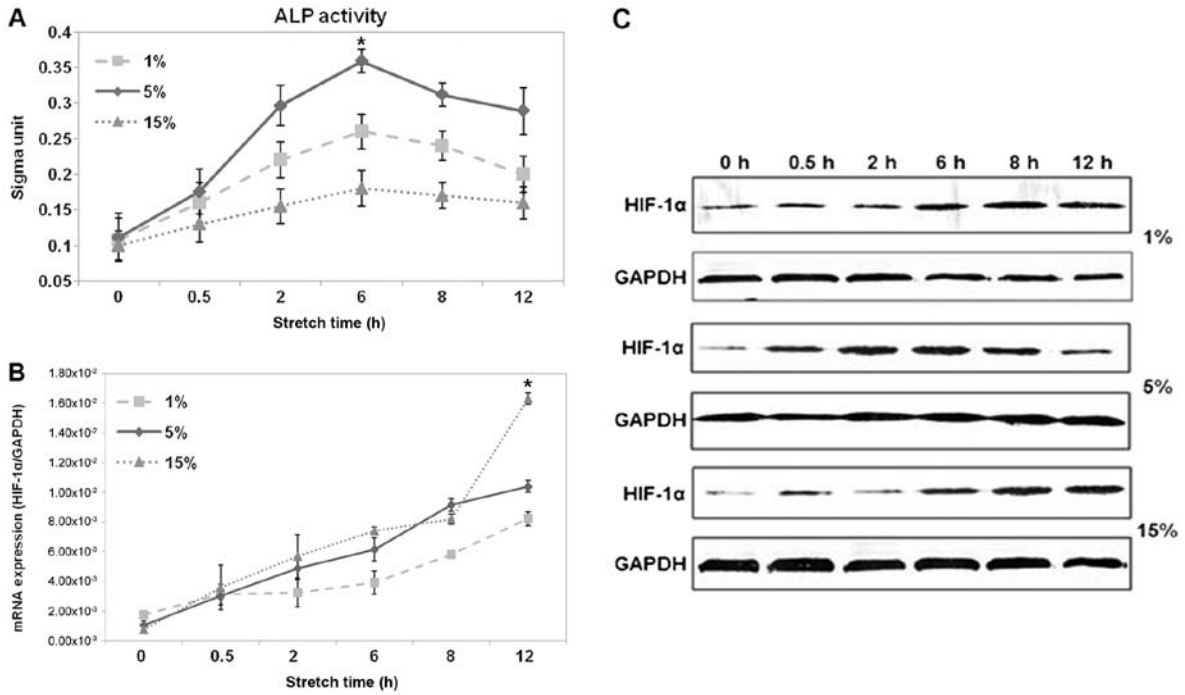

Figure 4. Cycling stretch stimulation was determined by measuring ALP and HIF-1α levels in BMSCs. (A) Cycling stretch stimulation was applied to BMSCs and the process of osteogenesis was assessed by measuring the presence of the osteoblast differentiation marker ALP. (B) Cycling stretch-induced increase in the expression of HIF-1α mRNA levels. (C) Western blot analysis results revealed a similar result. *P≤0.05 vs. 1%. ALP, alkaline phosphatase; BMSC, bone mesenchymal stem cell; HIF-1α, hypoxia-inducible factor 1α.

in Fig. 3A, with increased culture time, the proportion of mineralized nodules was increased. Following staining, the concentration of calcium ions was increased, which also exhibited a similar trend to ARS staining, reaching a peak at 4 weeks (Fig. 3B). In addition, ALP staining was used to detect the activity of ALP. The positive rate was as high as 80-90%, which indicates that the activity of ALP was elevated notably (Fig. 3C). The aforementioned results demonstrate that when in osteogenic medium, BMSCs may be induced to differentiate into osteoblasts *in vitro*.

Optimal force by ALP activity detection. In the present study, cycling stretch stimulation at 1 HZ was applied to BMSCs and whether the process of osteogenesis was switched on was assessed. The progression of osteogenesis was assessed using the presence of the osteoblast differentiation marker ALP. The results indicated that ALP activity increased with increasing culture time for all assessed groups (Fig. 4A). Additionally, the ALP activity of the medium force group (5%) was substantially higher compared with that of the weak force group (1%) and the strong force group (15%). Furthermore, at 6 h, the ALP activity of cells was substantially higher compared with any other time, which indicated that 6 h at 5% 1 HZ stretch is the ideal condition to stimulate osteogenic differentiation of BMSCs.

Effect of different cyclic stretch strengths on the expression of HIF-1α in BMSCs. To investigate whether HIF-1α participates in the stretch-induced osteogenic differentiation of BMSCs, the expression of HIF-1α at the mRNA and protein levels was determined. In the present study, the cells were exposed to different strengths of loading mechanical stimulation: A weak force group (1%), a medium force group (5%) and a strong force group (15%). Each group was stretched for 0, 0.5, 2, 6, 8 and 12 h, at 1 HZ. According to RT-qPCR analysis, it was revealed that there was a time-dependent increase in the expression of HIF-1α at the mRNA level, when BMSCs were exposed to 1 and 15% cycling stretch. Notably in 5% group, the HIF-1α mRNA level was slightly increased during the first 8 h, and peaked at 12 h, but then decreased a little (Fig. 4B). In accordance with the changes in mRNA levels, the protein expression of HIF-1α increased with increased stretch time in BMSCs, and reached a maximum at 12 h (Fig.4C). The above results suggest that mechanical stretch energized the activation of HIF-1α. Their changes were consistent with BMSC osteogenic differentiation. All results suggested that HIF-1α participated in stretch-induced BMSC osteogenic differentiation.

Discussion

The bone remodeling cycle may be altered by a number of different types of stimulation, including mechanobiological factors. Previous research has demonstrated that mechanical stress may participate in osteogenic differentiation (21). To date, numerous studies have focused on the mechanism of HIF-1α in different types of tumor (22,23). However, only a few reports so far have been able to shed light on the osteogenic differentiation of BMSCs (24). A clear molecular biological mechanism will help the application and generalization of HIF-1α in a clinical setting. Therefore, it is necessary to elucidate its mechanism in the osteogenic differentiation of BMSCs. In this context, the present study examined this topic, and concluded that a dynamic overexpression of osteogenic

genes and HIF-1α occurs in response to biomechanical cycling stretch. In the process of osteogenesis induced by mechanical stress, osteogenic differentiation is continuously enhanced under appropriate mechanical stretch, and HIF-1α is involved in this process.

BMSCs reside in bone marrow aspirated from tibias and femurs, which easily produce large quantities of BMSCs and which are available for transplantation (25). Furthermore, BMSCs are multipotent cells that favor the bone remodeling cycle and other mesenchymal tissues (25). Isolated expanded BMSC cultures differentiate, in a controlled manner, to multiple lineages (26). Therefore they are suitable 'volunteers' for studying osteogenic differentiation and bone formation, which was confirmed by the results of the present study. In the present study, Alizarin Red S staining was used to confirm that the isolated bone marrow monocytes exhibited multipotential differentiation. One previous study demonstrated that BMSCs emerged from marrow cell suspensions by selectively attaching to a culture dish and dividing to form colonies (25). In the present study, it was revealed that a large number of colonies were formed once the BMSCs grew against the wall of flask, whereas other cell types, such as blood cells, would not have formed colonies until several days of culture. This difference in the rate of colony formation is well documented for BMSCs in cultures derived from clonal and mass cell origin (27). Furthermore, to confirm the purity of the BMSCs, flow cytometry was performed. The positive expression of CD90 and CD44 markers indicated the presence of BMSCs in the pool of bone marrow cells. CD45, on the other hand, was negative, as a marker for hematopoietic cells (28). One previous study revealed that the third and fifth passage of the cells exhibited a uniform fiber-like shape similar to fibroblasts and the optimal cell purity was obtained (29). Due to this, cells in these passages are often used for experimental research. The results of the present study were consistent with the characteristics of BMSCs. Altogether, these results suggested that the cells that were isolated from rats were identified as BMSCs, which lay the foundation of the subsequent experiment.

Orthodontic tooth movement induced by mechanical stimuli depends on the remodeling capacity of local alveolar bone. Mechanical strain is known to be a fundamental physiological factor regulating bone formation and renewal (30). Numerous studies have examined the mechanism of mechanical stimuli. One focus in this area of research is regulating the osteogenic differentiation of stem cells (31). However, less attention has been dedicated to the consequences of cyclic equiaxial stretch (32). One prior study has suggested that equibiaxial cyclic strain stimulates human mesenchymal stem cells increase in matrix mineralization (33). Furthermore, another previous study demonstrated that cyclical stretching was a differentiation factor which was stronger compared with dexamethasone in the short term (34). However, another study reported that cyclic tensile stretch inhibits the osteogenic differentiation of human dental pulp stem cells (21). Therefore, the effect of cyclic tensile stress on stem cells remains controversial. In lieu of this, the present study established an external mechanical stimulation model using rat BMSCs and a mechanical loading device. Additionally, an ALP assay kit was used to examine the ALP activity of the BMSCs, as ALP is an important early stage osteogenic marker of stem cells (24). The results revealed that levels of the enzyme were low in the first half hour but increased substantially within 2 h, reaching maximal levels around 6 h and decreasing following that point (Fig. 3C), which indicated that ALP activity increased substantially and that cyclic tensile strain promotes the osteogenic differentiation of BMSCs under a certain conditions *in vitro*. One potential reason for the different results is that cells *in vivo* did not receive the same mechanical signal in the cell stretching loading system since the bone matrix filters the physical load. Furthermore, external biomechanical conditions are too complex to be simulated. It was reported that 20% of strain-activated apoptotic signaling pathways, and 25% of cyclic deformation, induced cell death directly (35). Therefore, the present study selected a 15% cyclic deformation at 1 HZ (60 cycles/min) stretch as a physiological limit of stress to induce the osteogenic differentiation of BMSCs. Three different loading mechanical stimulation groups were created: A weak force group (1%), a medium force group (5%) and a strong force group (15%), which mimicked the physiological conditions of occlusal force *in vivo* (36). In the present experiments, it was also revealed that the 5% elongation group exhibited the highest ALP activity, followed by 1% elongation. It was substantially increased at 6 h following loading and then decreased (Fig. 4A), which suggested that 6 h at 5% 1 HZ stretch is the ideal condition to stimulate the osteogenic differentiation of BMSCs. Similar optimal force was also identified in photothermal stress-induced osteogenesis (37). These results will form the theoretical guidance for application during orthodontic tooth movement.

Bone formation is initiated following the expansion of BMSCs, and ends with osteoblastic cells originating from the BMSCs (38). Numerous factors are involved in ensuring that BMSCs differentiate along osteoblastic cell lines, including growth factors, cytokines and the surrounding microenvironmental conditions (39). Interestingly, one previous study has demonstrated that a hypoxic microenvironment is a necessary condition during the bone regeneration process (40), whereas little is known on the mechanism underlying hypoxia in osteoblast differentiation and bone formation. Research has demonstrated that the transcription factor HIF-1α constitutes the principal mediator of cellular adaptation to hypoxia (41). HIF-1α is a transcriptional activator and participates in numerous pathophysiological processes under hypoxia (42). Previously, the classical theory was that HIF-1α activates the process of the epithelial-mesenchymal transition, which is fundamental for embryonic development and of particular importance to organ formation and differentiation (43). Additionally, De Luna *et al* (44) reported that hypoxia mediated HIF-1α accumulation results in reduced proinflammatory gene expression and ameliorates inflammation. These results indicate that hypoxia followed by HIF-1α production is closely associated with the proliferation, differentiation, invasion, metastasis and even prognosis of a tumor. Since stem cells and cancer cells share a lot of similarities in gene expression, cellular processes and signal transductions, HIF-1α may be involved in the BMSC osteogenesis process. In fact, others have reported the presence of a hypoxic micro-environment in the bone remodeling process and orthodontic tooth movement (15). Furthermore BMSCs originated from hypoxic stem cell niches, and survived under hypoxia, particularly in the bone marrow (45). Thus, it is reasonable to investigate whether HIF-1α was involved in the

cyclic stretch-induced osteogenic differentiation of BMSCs. In the present study, RT-qPCR and western blot analysis revealed that the mRNA and protein expression of HIF-1α increased uniformly under various conditions during osteogenic differentiation. The present results demonstrate that the *in vitro* mechanical stimulation model that was established contained a hypoxic microenvironment. Meanwhile extrinsic cyclic stretch may be used to realistically simulate the microenvironment of the cells *in vivo* (36). All these results indicate that the activation of HIF-1α was required for the cyclic stretch-induced osteogenic differentiation of BMSCs, which were in agreement with a previous report (46). These results reveal the mechanism of HIF-1α involvement in hypoxia-induced osteogenic differentiation.

The mechanism by which hypoxia regulates osteogenic differentiation has been investigated. Hsu *et al* (47) demonstrated that osteoblasts differentiated under hypoxia rely mainly on glycolysis for energy. They also confirmed that glycolysis was enhanced under hypoxia, and the metabolic switch to mitochondrial respiration during the osteogenic differentiation of human MSCs (hMSCs) was strongly compromised by hypoxia (47). Additionally, research suggests that vascular endothelial growth factor, a transcripional target of HIF-1α, serves an important function in the angiogenesis (48). It was indicated that osteogenesis is tightly coupled with angiogenesis during bone development and regeneration (40). These results are consistent with previous reports (49-51), which revealed HIF-1α is involved in hypoxia-induced osteogenic differentiation. However, the controversy remains. One previous study demonstrated that the overexpression of HIF-1α will decrease the expression of RUNX family transcription factor 2 (Runx2; a critical transcriptional regulator of osteoblast differentiation) and bone morphogenetic protein 2 (BMP2; another important osteogenic factor) in MSCs (52). Furthermore, a previous study reported that HIF-1α inhibits osteogenesis in hMSCs and suggested that hypoxia inhibits the expression of type 1 Runx2 and its downstream targets (53). Nevertheless, a number of studies have differing views on this issue. One study has suggested that hypoxia promotes the osteogenesis of hMSCs in a HIF-1α-dependent manner, which directly enhances the expression of Runx2 (54). Interestingly, Huang *et al* (40) hypothesized that hypoxia increases ALP activity and the production of type I/III collagen, which are all hallmarkers of MSC differentiation into osteogenic lineage cells. This discrepancy, however, may reflect the fact that different cells respond differently to hypoxia. Additionally, there is evidence confirming that at an early stage of osteogenesis, the hypoxic-mediated HIF-1α-Twist1 pathway promotes osteoblast differentiation, whereas at a late stage hypoxia substantially diminishes bone formation *in vivo* (55). This has provided a novel viewpoint defining the association between hypoxia and osteoblast differentiation. In other words, it may be stage-dependent. In brief, it was assumed that the potential reasons for these different results include a different cell species (mouse, rat and human), different stage of osteoblast differentiation (early, middle and late), differences in the experimental design (hypoxic state or mechanical stimuli) and different oxygen concentration (from 0.02 to 5% oxygen). One previous study has implicated Twist, a downstream target of HIF-1α, functioning as a transcription repressor of Runx2 through binding to the E-box located on the promoter of type 1 Runx2, resulting in the suppression of type 1 Runx2, followed by the suppression of BMP2, type 2 Runx2 and downstream targets of Runx2 in MSCs undergoing osteogenic differentiation (49). This has provided a neoteric molecular mechanism to explain the effect of hypoxia. Thus, the HIF-1α-Twist1 axis is involved in the MSC osteogenesis process, which may provide novel ideas to elucidate the association between HIF-1α and stretch-induced osteogenic differentiation.

In summary, the results of the present study suggest that cyclic tensile stretch promotes the osteogenic differentiation of BMSCs *in vitro*, as indicated by the upregulation of ALP activity. The present experimental results also revealed that the expression of HIF-1α increased specifically in response to certain mechanical tension with defined intensity and duration. Therefore, future research will investigate HIF-1α as a biological target of osteogenic differentiation induced by tensile stress. The function and mechanism of HIF-1α in bone remodeling are not well studied, nor is it clear whether HIF-1α functions as a promoter or an inhibitor for osteogenic differentiation.

Acknowledgements

The authors would like to thank Dr Qingyuan Guo (Department of Stomatology, The Affiliated Qingdao Municipal Hospital, Qingdao University) who provided guidance, and the technical support from the Director of Shandong Provincial Key Laboratory of Oral Tissue Regeneration.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 11602122) and the China Postdoctoral Science Foundation (grant no. 2017m623396).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HY and QG designed the study and edited the manuscript. WY and YL performed the experiments. XY and RY analyzed the data. All authors read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shandong Provincial Key Laboratory of Oral Tissue Regeneration.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Dibart S, Yee C, Surmenian J, Sebaoun JD, Baloul S, Goguet-Surmenian E and Kantarci A: Tissue response during Piezocision-assisted tooth movement: A histological study in rats. Eur J Orthod 36: 457-464, 2014.
2. Antoun JS, Mei L, Gibbs K and Farella M: Effect of orthodontic treatment on the periodontal tissues. Periodontol 74: 140-157, 2017.
3. Vlaikou AM, Kouroupis D, Sgourou A, Markopoulos GS, Bagli E, Markou M, Papadopoulou Z, Fotsis T, Nakos G, Lekka ME and Syrrou M: Mechanical stress affects methylation pattern of GNAS isoforms and osteogenic differentiation of hAT-MSCs. Biochim Biophys Acta Mol Cell Res 1864: 1371-1381, 2017.
4. Barbieri G, Solano P, Alarcón JA, Vernal R, Rios-Lugo J, Sanz M and Martín C: Biochemical markers of bone metabolism in gingival crevicular fluid during early orthodontic tooth movement. Angle Orthod 83: 63-69, 2013.
5. Ohgushi H: Osteogenically differentiated mesenchymal stem cells and ceramics for bone tissue engineering. Expert Opin Biol Ther 14: 197-208, 2014.
6. Connelly JT, García AJ and Levenston ME: Interactions between integrin ligand density and cytoskeletal integrity regulate BMSC chondrogenesis. J Cell Physiol 217: 145-154, 2008.
7. Moreau JE, Chen J, Horan RL, Kaplan DL and Altman GH: Sequential growth factor application in bone marrow stromal cell ligament engineering. Tissue Eng 11: 1887-1897, 2005.
8. Moreau JE, Bramono DS, Horan RL, Kaplan DL and Altman GH: Sequential biochemical and mechanical stimulation in the development of tissue-engineered ligaments. Tissue Eng Part A 14: 1161-1172, 2008.
9. Schaffer JL, Rizen M, L'Italien GJ, Benbrahim A, Megerman J, Gerstenfeld LC and Gray ML: Device for the application of a dynamic biaxially uniform and Isotropic strain to a flexible cell culture membrane. J Orthop Res 12:709-719, 1994.
10. Susilo ME, Bell BJ, Roeder BA, Voytik-Harbin SL, Kokini K and Nauman EA: Prediction of equibiaxial loading stress in collagen-based extracellular matrix using a three-dimensional unit cell model. Acta Biomater 9: 5544-5553, 2013.
11. Qi YX, Yao QP, Huang K, Shi Q, Zhang P, Wang GL, Han Y, Bao H, Wang L, Li HP, *et al*: Nuclear envelope proteins modulate proliferation of vascular smooth muscle cells during cyclic stretch application. Proc Natl Acad Sci USA 113: 5293-5298, 2016.
12. Ursekar CP, Teo SK, Hirata H, Harada I, Chiam KH and Sawada Y: Design and construction of an equibiaxial cell stretching system that is improved for biochemical analysis. PLoS One 9: e90665, 2014.
13. Lohberger B, Kaltenegger H, Stuendl N, Payer M, Rinner B and Leithner A: Effect of cyclic mechanical stimulation on the expression of osteogenesis genes in human intraoral mesenchymal stromal and progenitor cells. Biomed Res Int 2014: 189516, 2014.
14. Liu Y, Huang X, Yu H, Yang J, Li Y, Yuan X and Guo Q: HIF-1α-TWIST pathway restrains cyclic mechanical stretch-induced osteogenic differentiation of bone marrow mesenchymal stem cells. Connect Tissue Res 60: 544-554, 2019.
15. Niklas A, Proff P, Gosau M and Römer P: The role of hypoxia in orthodontic tooth movement. Int J Dent 2013: 841840, 2013.
16. Semenza GL and Wang GL: A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 12: 5447-5454, 1992.
17. Li Y, Padmanabha D, Gentile LB, Dumur CI, Beckstead RB and Baker KD: HIF- and non-HIF-regulated hypoxic responses require the estrogen-related receptor in Drosophila melanogaster. PLoS Genet 9: e1003230, 2013.
18. Zhou N, Hu N, Liao JY, Lin LB, Zhao C, Si WK, Yang Z, Yi SX, Fan TX, Bao W, *et al*: HIF-1a as a regulator of BMP2-induced chondrogenic differentiation, osteogenic differentiation, and endochondral ossification in stem cells. Cell Physiol Biochem 36: 44-60, 2015.
19. Nemeth K, Mayer B, Sworder BJ, Kuznetsov SA and Mezey E: A practical guide to culturing mouse and human bone marrow stromal cells. Curr Protoc Immunol 102: Unit 22F.12, 2013.
20. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
21. Yu HC, Wu TC, Chen MR, Liu SW, Chen JH and Lin KM: Mechanical stretching induces osteoprotegerin in differentiating C2C12 precursor cells through noncanonical Wnt pathways. J Bone Miner Res 25: 1128-1137, 2010.
22. Jang Y, Han J, Kim SJ, Kim J, Lee MJ, Jeong S, Ryu MJ, Seo KS, Choi SY, Shong M, *et al*: Suppression of mitochondrial respiration with auraptene inhibits the progression of renal cell carcinoma: Involvement of HIF-1a degradation. Oncotarget 6: 38127-38138, 2015.
23. Sung WW, Chu YC, Chen PR, Liao MH and Lee JW: Positive regulation of HIF-1A expression by EBV oncoprotein LMP1 in nasopharyngeal carcinoma cells. Cancer Lett 382: 21-31, 2016.
24. Sha Y, Lv Y, Xu Z, Yang L, Hao X and Afandi R: MGF E peptide pretreatment improves the proliferation and osteogenic differentiation of BMSCs via MEK-ERK1/2 and PI3K-Akt pathway under severe hypoxia. Life Sci 189: 52-62, 2017.
25. Bruder SP, Jaiswal N and Haynesworth SE: Growth kinetics, selfrenewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J Cell Biochem 64: 278-294, 1997.
26. Kusuyama J, Kamisono A, ChangHwan S, Amir MS, Bandow K, Eiraku N, Ohnishi T and Matsuguchi T: Spleen tyrosine kinase influences the early stages of multilineage differentiation of bone marrow stromal cell lines by regulating phospholipase C gamma activities. J Cell Physiol 233: 2549-2559, 2018.
27. Tang Y, Xu Y, Xiao Z, Zhao Y, Li J, Han S, Chen L, Dai B, Wang L, Chen B and Wang H: The combination of three-dimensional and rotary cell culture system promotes the proliferation and maintains the differentiation potential of rat BMSCs. Sci Rep 7: 192, 2017.
28. Xia CS, Zuo AJ, Wang CY and Wang YZ: Isolation of rabbit bone marrow mesenchymal stem cells using density gradient centrifugation and adherence screening methods. Minerva Med 104: 519-525, 2013.
29. Steinert AF, Kunz M, Prager P, Göbel S, Klein-Hitpass L, Ebert R, Nöth U, Jakob F and Gohlke F: Characterization of bursa subacromialis-derived mesenchymal stem cells. Stem Cell Res Ther 6: 114, 2015.
30. Jiang F, Xia Z, Li S, Eckert G and Chen J: Mechanical environment change in root, periodontal ligament, and alveolar bone in response to two canine retraction treatment strategies. Orthod Craniofac Res 18 (Suppl 1): S29-S38, 2015.
31. Stanley A, Heo SJ, Mauck RL, Mourkioti F and Shore EM: Elevated BMP and mechanical signaling through YAP1/RhoA poises FOP mesenchymal progenitors for osteogenesis. J Bone Miner Res: May 20, 2019 doi: 10.1002/jbmr.3760 (Epub ahead of print).
32. Estes BT, Gimble JM and Guilak F: Mechanical signals as regulators of stem cell fate. Curr Top Dev Biol 60: 91-126, 2004.
33. Simmons CA, Matlis S, Thornton AJ, Chen S, Wang CY and Mooney DJ: Cyclic strain enhances matrix mineralization by adult human mesenchymal stem cells via the extracellular signal-regulated kinase (ERK1/2) signaling pathway. J Biomech 36: 1087-1096, 2003.
34. Jagodzinski M, Drescher M, Zeichen J, Hankemeier S, Krettek C, Bosch U and van Griensven M: Effects of cyclic longitudinal mechanical strain and dexamethasone on osteogenic differentiation of human bone marrow stromal cells. Eur Cell Mater 7: 35-41, 2004.
35. Boccafoschi F, Sabbatini M, Bosetti M and Cannas M: Overstressed mechanical stretching activates survival and apoptotic signals in fibroblasts. Cells Tissues Organs 192: 167-176, 2010.
36. Parandakh A, Tafazzoli-Shadpour M and Khani MM: Stepwise morphological changes and cytoskeletal reorganization of human mesenchymal stem cells treated by short-time cyclic uniaxial stretch. In Vitro Cell Dev Biol Anim 53: 547-553, 2017.
37. Kajiya H, Katsumata Y, Sasaki M, Tsutsumi T, Kawaguchi M and Fukushima T: Photothermal stress triggered by near-infrared-irradiated carbon nanotubes up-regulates osteogenesis and mineral deposition in tooth-extracted sockets. Int J Hyperthermia 31: 635-642, 2015.
38. Friedenstein AJ: Precursor cells of mechanocytes. Int Rev Cytol 47: 327-359, 1976.
39. Han I, Kwon BS, Park HK and Kim KS: Differentiation potential of mesenchymal stem cells is related to their intrinsic mechanical properties. Int Neurourol J 21 (Suppl 1): S24-S31, 2017.
40. Huang J, Deng F, Wang L, Xiang XR, Zhou WW, Hu N and Xu L: Hypoxia induces osteogenesis-related activities and expression of core binding factor alpha1 in mesenchymal stem cells. Tohoku J Exp Med 224: 7-12, 2011.
41. Balamurugan K: HIF-1 at the crossroads of hypoxia, inflammation, and cancer. Int J Cancer 138: 1058-1066, 2016.
42. Shah YM: The role of hypoxia in intestinal inflammation. Mol Cell Pediatr 3: 1, 2016.

43. Zhu L and Zhao Q: Hypoxia-inducible factor 1a participates in hypoxia-induced epithelial-mesenchymal transition via response gene to complement 32. Exp Ther Med 14: 1825-1831, 2017.
44. De Luna N, Suárez-Calvet X, Lleixà C, Diaz-Manera J, Olivé M, Illa I and Gallardo E: Hypoxia triggers IFN-I production in muscle: Implications in dermatomyositis. Sci Rep 7: 8595, 2017.
45. Moirangthem RD, Singh S, Adsul A, Jalnapurkar S, Limaye L and Kale VP: Hypoxic niche-mediated regeneration of hematopoiesis in the engraftment window is dominantly affected by oxygen tension in the milieu. Stem Cells Dev 24: 2423-2436, 2015.
46. Jiang Y, Wang Y and Tang G: Cyclic tensile strain promotes the osteogenic differentiation of a bone marrow stromal cell and vascular endothelial cell co-culture system. Arch Biochem Biophys 607: 37-43, 2016.
47. Hsu SH, Chen CT and Wei YH: Inhibitory effects of hypoxia on metabolic switch and osteogenic differentiation of human mesenchymal stem cells. Stem Cells 31: 2779-2788, 2013.
48. Wei F, Yang S, Xu H, Guo Q, Li Q, Hu L, Liu D and Wang C: Expression and function of hypoxia inducible factor-1α and vascular endothelial growth factor in pulp tissue of teeth under orthodontic movement. Mediators Inflamm 2015: 215761, 2015.
49. Wang Y, Wan C, Gilbert SR and Clemens TL: Oxygen sensing and osteogenesis. Ann N Y Acad Sci 1117: 1-11, 2007.
50. Wan C, Shao J, Gilbert SR, Riddle RC, Long F, Johnson RS, Schipani E and Clemens TL: Role of HIF-1alpha in skeletal development. Ann N Y Acad Sci 1192: 322-326, 2010.
51. Menon A, Creo P, Piccoli M, Bergante S, Conforti E, Banfi G, Randelli P and Anastasia L: Chemical activation of the hypoxia-inducible factor reversibly reduces tendon stem cell proliferation, inhibits their differentiation, and maintains cell undifferentiation. Stem Cells Int 2018: 9468085, 2018.
52. Lampert FM, Kütscher C, Stark GB and Finkenzeller G: Overexpression of Hif-1a in mesenchymal stem cells affects cell-autonomous angiogenic and osteogenic parameters. J Cell Biochem 117: 760-768, 2016.
53. Yang DC, Yang MH, Tsai CC, Huang TF, Chen YH and Hung SC: Hypoxia inhibits osteogenesis in human mesenchymal stem cells through direct regulation of RUNX2 by TWIST. PLoS One 6: e23965, 2011.
54. Wagegg M, Gaber T, Lohanatha FL, Hahne M, Strehl C, Fangradt M, Tran CL, Schönbeck K, Hoff P, Ode A, *et al*: Hypoxia promotes osteogenesis but suppresses adipogenesis of human mesenchymal stromal cells in a hypoxia-inducible factor-1 dependent manner. PLoS One 7: e46483, 2012.
55. Chen X, Gu S, Chen BF, Shen WL, Yin Z, Xu GW, Hu JJ, Zhu T, Li G, Wan C, *et al*: Nanoparticle delivery of stable miR-199a-5p agomir improves the osteogenesis of human mesenchymal stem cells via the HIF1a pathway. Biomaterials 53: 239-250, 2015.