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Original Article

Mechanical stimulation promotes osteogenic and chondrogenic differentiation of synovial mesenchymal stem cells through BMP-2

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Abstract: Mounting evidences have been shown that mesenchymal stem cells are present in various tissues including synovium. Given that environmental factors could have impacts on the differentiation of stem cells in vivo, we elucidated that whether mechanical stimulation may influence the process. In this study, we investigated the effect of cyclic mechanical stimulation on the osteogenic and chondrogenic differentiation of synovial mesenchymal stem cells (SMSCs) and its correlation with BMP-2. Cyclic mechanical stimulation was applied to SMSCs isolated from bilateral hip, knee joints and unilateral ankle joint of rabbits. SMSCs were grouped into two classifications: treated with mechanical stimulation (MS group) and without mechanical stimulation (no MS group). The mechanical stimulation was a cyclic tensile and compression: the amplitude and frequency of strain was 8%, 0.33 Hz and the peak of cyclic stress was 2.35 kilopascals (kPa) (1 kPa = 1.0 × 103 $^{N}/m^{2}$). The osteogenic and chondrogenic differentiation of SMSCs was determined by detecting expressions of osteogenesis markers (ALP and Cbfa1) and chondrogenesis markers (Sox9 and Col2a1). qRT-PCR and western blot showed that mechanical stimulation up-regulated expressions of osteogenesis markers (ALP and Cbfa1) and chondrogenesis markers (Sox9 and Col2a1) of SMSCs, respectively. In addition, BMP-2 was increased in SMSCs treated with mechanical stimulation during osteogenic and chondrogenic differentiation, and knockdown of BMP-2 leaded to the reversed result of the expression of osteogenesis markers and chondrogenesis markers. To conclude, our study confirm that repetitive mechanical stimulation increased the expression of BMP-2, and promoted osteogenic and chondrogenic differentiation of SMSCs.

Keywords: Mechanical stimulation, synovium mesenchymal stem cells, osteogenic differentiation, chondrogenic differentiation, BMP-2

Introduction

Articular cartilage is frequently damaged in different pathological situations such as sports injuries, accidents, trauma and osteoarthritis [1, 2]. Although a number of approaches based on tissue and cellular therapies have been explored, more studies and mechanism need to be investigated in the process.

Mesenchymal stem cells (MSCs) are multipotential non-hematopoietic progenitor cells that can differentiate into a variety of mesenchymal lineages such as osteoblasts, chondrocytes and adipocytes and have been found in many other adult tissues such as skeletal muscles, synovium, tendon and adipose tissues [3-5]. Synovial mesenchymal stem cells (SMSCs) are attractive cell sources for bone/cartilage regeneration because of their high expansion as well as osteogenic and chondrogenic potentials compared with other tissue-derived MSCs [6-8]. The clinical demands for bone-and cartilage-generating therapies increase along with our population of aging people [9].

The success of tissue engineering depends on bioactive factors, cells, their extracellular environment and a matrix or scaffold [10, 11]. Besides, mechanical stimulation is another important factor that regulates ostecytic and chondrocytic activities [12, 13]. It was found that mechanical stimulation modulated the

Table 1. The primers were used in study

Gene	Primers
BMP	5'-AAC AGA AACCTCATC ACC AA-3'
	3'-AGG TGC CTG TAG TGA GGT TT-5'
ColII	5'-CCC TGCCGGATC TGT GTC TG-3'
	3'-GCTCTA CCTCTCGGA CCC TG-5'
Cbfa1	5'-GGT CAA TCCTGGAGG ACC GC-3'
	3'-CTGTTA ACT TCT TAA GGT AA-5'
ALP	5'-GGG CAG ATG ACT GGC AAC CT-3'
	3'-CGA CCGTGGATG CGT GTG TG-5'
GAPDH	5'-TGAACG GAT TTG GCC GCA TT-3'
	3'-GGC CCC GAG TAA ACT TCC CG-5'

extracellular matrix (ECM) synthesis of the cartilage explants and the cultured chondrocytes in *vitro*. Though the alteration of chondrocyte aggrecan and type-II collagen gene expression has been proved to be modulated by mechanical stimulation [9], and sulphate uptake shows the same correlation with mechanical stimulation [10], the exact mechanism still remains unknown for a decrease in biosynthesis sho wed inconsistence with the mRNA expression [9]. And in that case, the BMP-2 functioned as a morphogen may play an important role in this process since up-regulation of bone molecular markers, such as BMP-2, Col I, ALP, has also been reported [14-16]. BMP-2 is a low-molecular-weight glycoprotein that functions as a morphogen and belongs to the transforming growth factor-β (TGF-β) super-family [17], and has been verified to be up-regulated in human MSCs (hMSCs) during osteogenic differentiation in response to chemical stimulation [18, 19]. However, till present no study has focused on the effect of mechanical stimulation on the expression of BMP-2 in the differentiation process of SMSCs or the correlation between them.

In this study, we investigated the effect of cyclic mechanical stimulation on the osteogenic and chondrogenic differentiation of SMSCs and especially revealed its correlation with BMP-2.

Materials and methods

Isolation and culture of SMSCs

SMSCs were generated from synovial membrane of bilateral hip, knee joints and unilateral ankle joint according to Suzuki's method [6]. Cells were cultured in the Rabbit Mesenchymal Stem Cell Medium (OriCell, USA) for 3 days. At the 3rd day, non-adherent cells were removed by PBS wash. Expansion of SMSCs lasted for 3 passages. The multi-differentiation potential of the cells were confirmed by osteogenic, adipogenic, and chondrogenic differentiation assays.

In vitro mechanical stimulation

SMSCs were 3D-embedded to small intestinal sub-mucosa scaffold (SIS) with the density of 1.0×10^6 cells/ml respectively. To avoid complex interaction between growth factors in serum and mechanical stimulation, SMSCs were cultured in serum-free medium at day 3 after 3D-embedding. Mechanical stimulation was applied using the Cell Stretcher System NS 500 (Scholar Tech, Osaka, Japan). The mechanical loading was repetitive. The amplitude and frequency of loading were set according to Ando, K.'s method and were adjusted for 8% uniaxial repeated tensile strain, 0.33 Hz; the 3D SIS was pressed with a cyclic pressure of 2.35 kilopascals (kPa) at its peak stress (1 kPa = 1.0×103 $^N/m^2$) (21). The mechanical stimulation lasted for 6 hours. Four hours after mechanical stimulation treatment, cells were harvested for further analysis.

Immuno blot

Cells were lysed with RIPA containing complete protease inhibitor cocktail (Roche, Indianapolis, IN) and proteins were separated by SDS-PAGE electrophoresis. Proteins were then transferred onto a polyvinylidene fluoride membrane and then blocked with BSA. Primary antibodies against ALP, Cbfa1, Sox9, Col2a1 and BMP-2 were obtained from Cell Signaling Technology. Proteins were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

RNA isolation, reverse-transcribed PCR and quantitative real-time PCR

SMSCs were harvested and total RNA was extracted using TRIzol (Ambion, Life technologies, U.S). Briefly, at least 10^6 cells were collected and washed by PBS, then added 1 mL TRIzol. The cell lysates and TRIzol were mixed thoroughly and left at room temperature for 5 min. 250 μL chloroform was added and shook vigorously for 15 sec. After 5 min, the mixture was centrifuged at 10000 rpm for 10 min. The

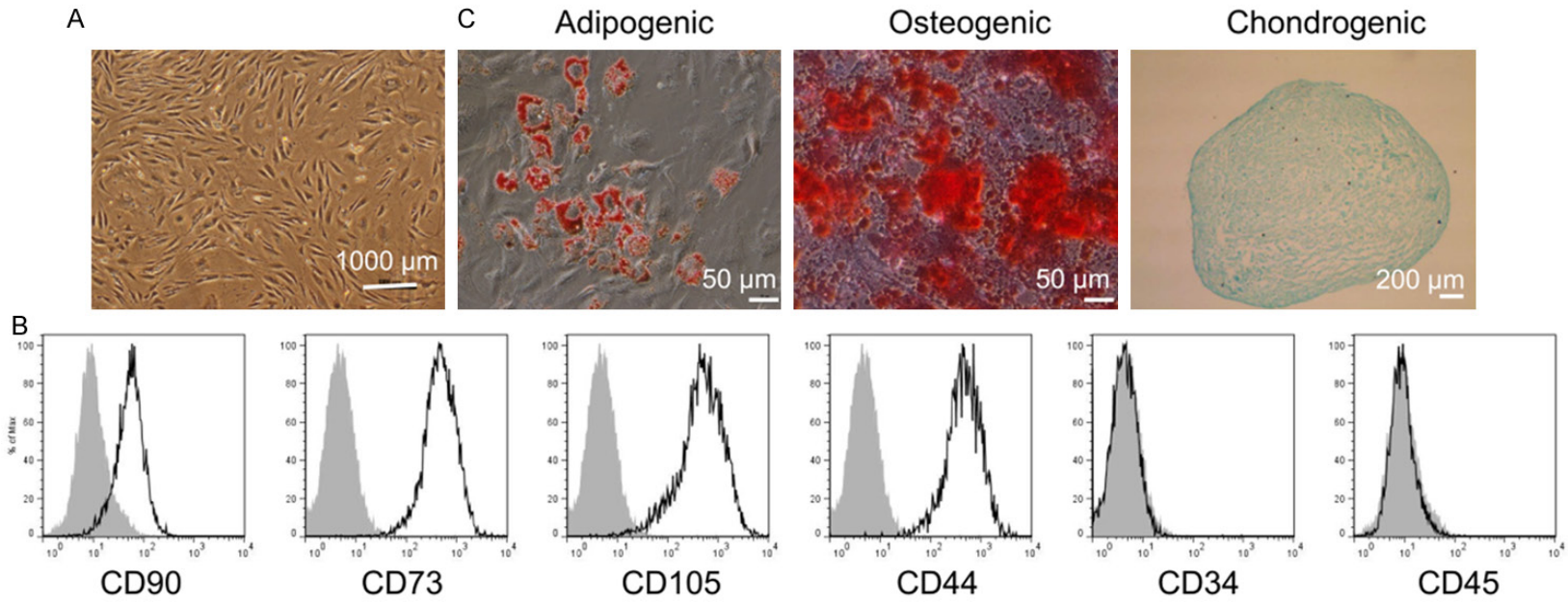

Figure 1. Culture and identification of SMSCs. A. Morphology of SMSCs. Size scale: 1000 μm. B. SMSCs were analyzed for the indicated markers by flow cytometry. Size scale (from left to right): 50 μm; 50 μm; 200 μm. C. SMSCs were able to differentiate into adipocytes, osteoblasts and chondrocytes under respective optimal conditions.

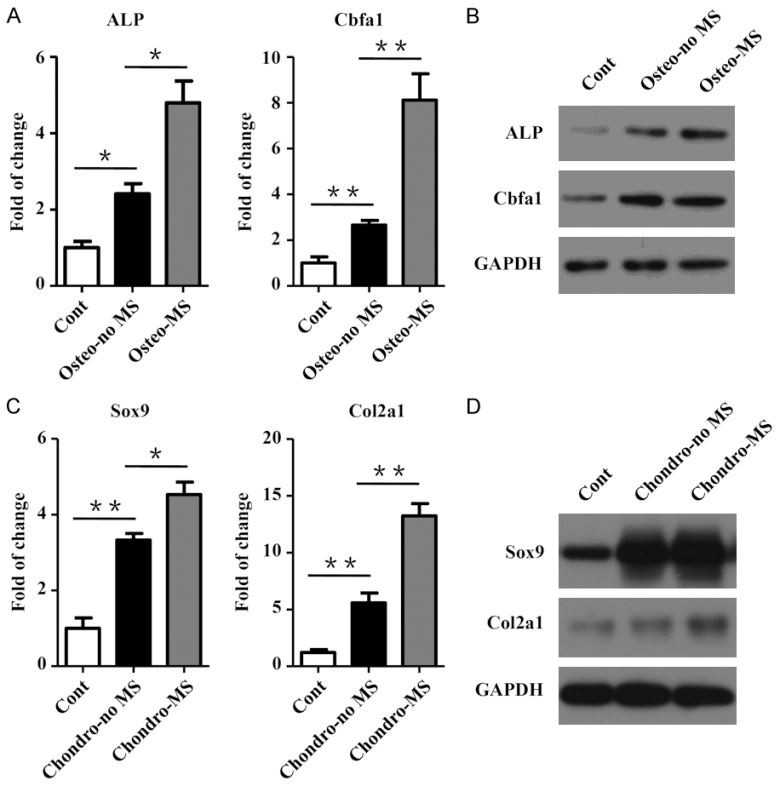

Figure 2. Effect of mechanical stimulation on osteogenesis and chongdrogenesis of SMSCs. SMSCs cultured in osteo-inductive medium or chondro-inductive medium were treated with/without mechanical stimulation. At indicated time points, cells were harvested for detection of markers of osteogenesis and chondrogenesis. A. mRNA levels of ALP and Cbfa1 in cells treated with/without mechanical stimulation under osteogenic condition were analyzed by qRT-PCR. B. Protein levels of ALP and Cbfa1 in cells treated with/without mechanical stimulation were analyzed by western blot. C. mRNA levels of Sox9 and Col2a1 in cells treated with/without mechanical stimulation under chondrogenic condition were analyzed by qRT-PCR. D. Protein levels of Sox9 and Col2a1 in cells treated with/without mechanical stimulation were analyzed by western blot. The data are represented as mean ± SD for triplicate samples. **P* < 0.05, ***P* < 0.01.

aqueous phase was carefully removed and added with 550 μL isopropanol, then centrifuged for 10 min at 12000 rpm. The pellet was air-dryed and dissolved in DEPC treated H_2O after washing by 75% ethanol. RNA was reverse-transcribed using SuperScript III First-Strand Synthesis System Kit as the protocol instructed (Invitrogen). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR Green I (BioRad, Hercules, CA). The PCR parameters were 95°C for 15 sec, 60°C for 1 min for 40 cycles. A melting curve analysis was collected. Ct was determined in the exponential amplification phase, and the amplification plots were analyzed using SDS software (Applied Biosystems). The relative expression level (defined as fold change) of the target gene was measured by the following equation: $2^{-\Delta\Delta Ct}$ ($\Delta Ct = \Delta Ct^{target} - \Delta Ct^{GAPDH}$; $\Delta\Delta Ct = \Delta Ct^{ms} - \Delta Ct^{no\ ms}$). The primers were listed in **Table 1**. GAPDH was used as an internal control to normalize for differences in the amount of total RNA in each sample.

Statistical analysis

All quantitative assays were calculated from at least 3 replicate samples. Data were presented as mean ± SD. All the data analysis was done using SPSS 18.0 (SPSS Inc, Chicago, IL). The quantitative PCR results were determined by Student's t-test (two-tailed). Differences of multiple treatment groups were compared within individual experiments by one-way ANOVA. *P < 0.05 was considered significant. **P < 0.01 was considered remarkably different.

Results

Isolation and identification of SMSCs

The lines of SMSCs were obtained from rabbits as described in Methods and Materials. SMSCs displayed spindle-like morphology (**Figure 1A**). Flow cytometric analysis demonstrated that SMSCs were $CD90^+CD73^+CD105^+CD44^+CD34^-CD45^-$ (**Figure 1B**). As expected, SMSCs were multipotent as demonstrated by their ability to differentiate into adipocytes, osteoblasts and chondrocytes under appropriate conditions (**Figure 1C**). Together, the data showed that the SMSCs isolated in this study were MSCs.

Mechanical stimulation promoted osteogenic and chondrogenic differentiation of SMSCs

To investigate the effect of mechanical stimulation on the differentiation of SMSCs, cells were cultured respectively and were treated with or

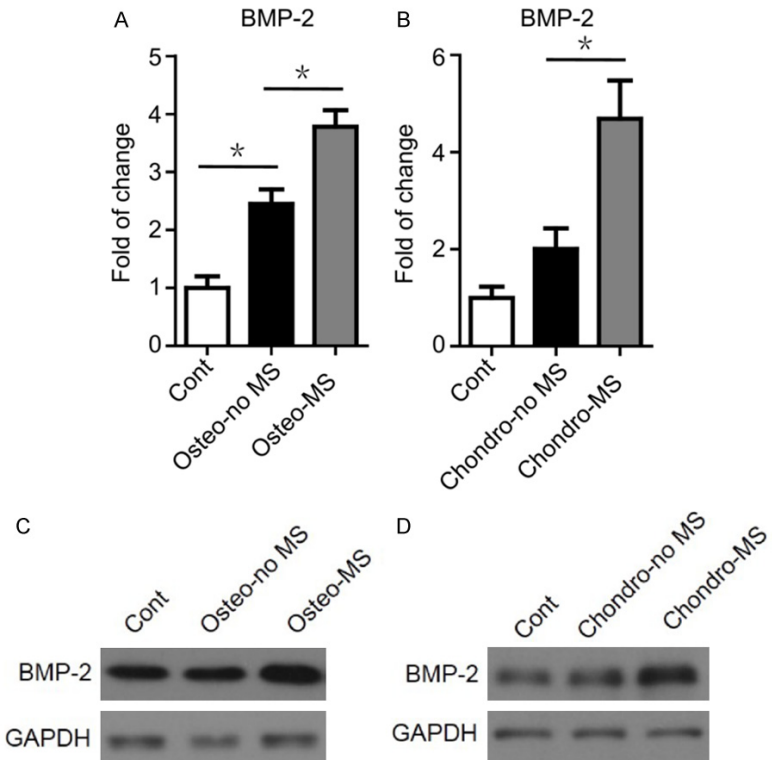

Figure 3. Expression of BMP-2 by SMSCs with/without mechanical stimulation treatment. During osteogenic or chondrogenic differentiation, SMSCs were treated with (MS group) or without mechanical stimulation (no MS group). And BMP-2 mRNA or protein in SMSCs were measured by qRT-PCR or western blot respectively. A. BMP-2 mRNA in SMSCs treated with/without mechanical stimulation during osteogenic differentiation. B. BMP-2 mRNA in SMSCs treated with/without mechanical stimulation during chondrogenic differentiation. C. BMP-2 protein in SMSCs treated with/without mechanical stimulation during osteogenic differentiation. D. BMP-2 protein in SMSCs treated with/without mechanical stimulation during chondrogenic differentiation. The data are represented as mean ± SD for triplicate samples. $*P < 0.05$, $**P < 0.01$.

without cyclic mechanical stimulation for 6 hours. At indicated time points, cells were harvested for detection of markers of osteogenesis and chondrogenesis. As detected by qRT-PCR, mRNA expression of ALP and Cbfa1, markers of osteogenesis, was significantly higher in cells treated with mechanical stimulation than that of without mechanical stimulation (**Figure 2A**). Consistently, the levels of ALP and Cbfa1 protein in cells treated with mechanical stimulation were significantly higher than those of without mechanical stimulation (**Figure 2B**).

Meanwhile, under chondrogenic conditions, mRNA expression of Sox9 and Col2a1, markers of chondrogenesis, was significantly higher in cells treated with mechanical stimulation than that of without mechanical stimulation (**Figure 2C**). The results were also confirmed by immuno blot (**Figure 2D**).

Mechanical stimulation promoted expression of BMP-2 in SMSCs during osteogenic and chondrogenic differentiation

Next, we investigated the possible correlation between specific genes and mechanical stimulation which promoted osteogenic and chondrogenic differentiation of SMSCs. As BMP-2 has been reported to be essential requirement for both osteogenic and chondrogenic differentiation of MSCs [20], we tested mRNA and protein levels of BMP-2 in SMSCs with mechanical stimulation. As determined by qRT-PCR, mRNA expression level of BMP-2 in SMSCs in MS groups during osteogenic or chondrogenic differentiation was significantly higher than that of control groups (**Figure 3A**, **3B**). Consistently, the trend could also be confirmed by protein level as determined using western blot (**Figure 3C**, **3D**).

Knockdown of BMP-2 inhibited the osteogenic and chondrogenic differentiation of SMSCs promoted by mechanical stimulation treatment

To identify whether the osteogenic and chondrogenic differentiation is mainly regulated by BMP-2, we utilized siRNA to inhibit the expression of BMP-2 and further evaluated different markers (**Figure 4A**, **4B**). We observed that the osteogenisis markers, ALP and Cbfa1 were down-regulated in SMSCs upon BMP-2 knockdown. The result of chondrogenisis markers was also proved (**Figure 4C**, **4D**). The alteration of different markers suggested that BMP-2 played a crucial role in the regulation of osteogenic and chondrogenic differentiation. Moreover, the mechanical stimulation which promoted the oteogenisis and chondrogenisis

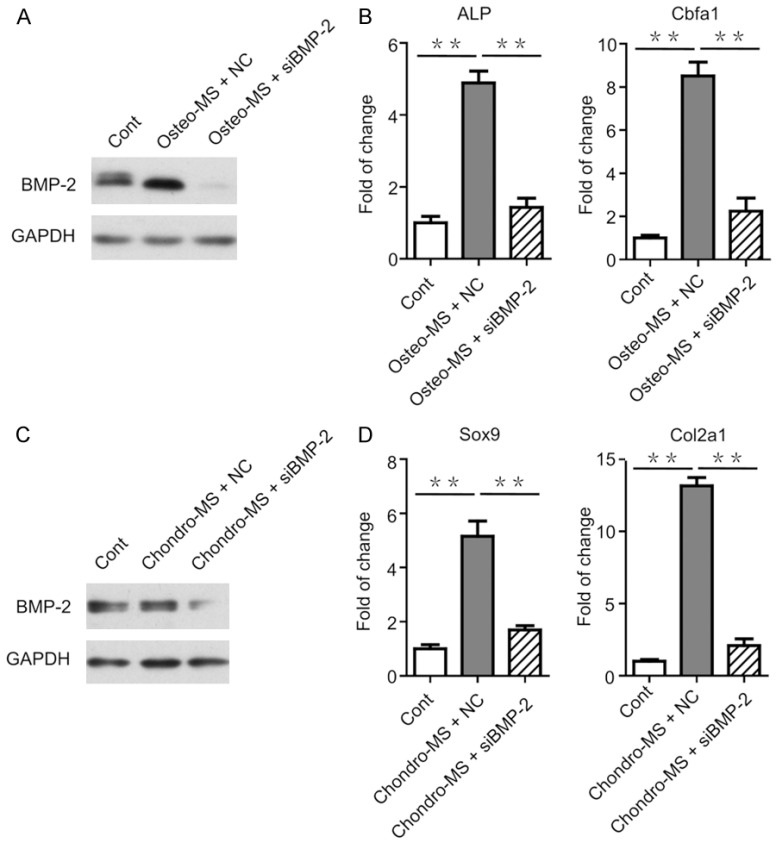

Figure 4. Knockdown of BMP-2 inhibited the osteogenic and chondrogenic differentiation of SMSCs promoted by mechanical stimulation treatment. siRNA was utilized to inhibit the expression of BMP-2 and further detected different markers. A. BMP-2 protein in SMSCs transfected with BMP-2 siRNA/negative control during osteogenic differentiation. B. mRNA levels of ALP and Cbfa1 in cells transfected with BMP-2 siRNA/negative control under osteogenic condition were analyzed by qRT-PCR. C. BMP-2 protein in SMSCs transfected with BMP-2 siRNA/negative control during chondrogenic differentiation. D. mRNA levels of Sox9 and Col2a1 in cells transfected with BMP-2 siRNA/negative control under chondrogenic condition were analyzed by qRT-PCR. The data are represented as mean ± SD for triplicate samples. $*P < 0.05$, $**P < 0.01$.

differentiation was mainly through BMP-2 pathway.

Discussion

Our study demonstrated that repetitive mechanical stimulation promoted the osteogenic and chondrogenic differentiation of SMSCs. Meanwhile, repetitive mechanical stimulation upregulated the expression of BMP-2 in SMSCs during osteogenic and chondrogenic differentiation. Differing from the previous single studies that only concentrated on the osteogenesis or chondrogenesis of SMSCs, our experiment indicated that both the osteogenesis and chondrogenesis capability of SMSCs could be strengthened by cyclic mechanical stimulation.

The success of tissue engineering depends on bioactive factors, cells, their extracellular environment and a matrix or scaffold [10]. Besides, mechanical stimulation is another important factor that regulates ostecytic and chondrocytic activities [12, 13]. Lohberger B et al proved that some osteogenesis genes' expression is a result of mechanical stimulation and determined genes like SPP1 and Col1A1. They also showed that the strain-induced bone remodeling may result from these mechanical stimulation and the expression changes of osteogenesis genes [26]. In that case, whether this phenomenon is suitable in synovial mesenchymal stem cells and the mechanism or correlation with specific gene is similar could help to correct the choice of applied force in the surgery. Moreover, Ando K et al [21] showed that mechanical loading rejuvenated the expression of the aggrecan and type II collagen genes at loading of 60 min/day compared to the other durations of loading, while BMP-2 and bFGF also increased aggrecan and type II collagen mRNA expression when used separately. Similarly, Guilak et al [22] indicated that compression of the tissue to physiological strain magnitudes acted as a signal which regulated chondrocyte biosynthetic and catabolic responses by the thickness of cartilage, while enhanced compression under higher strains may take responsibility for tissue and cell damage.

In the present study, the amplitude and frequency of strain were 8% and 0.33 Hz, respectively. The peak of cyclic stress was 2.35 kilopascals. It was reported that stretching

more than 10% and excessive time pressures were harmful to cells and tissues rather than beneficial [23-26]. However, it was suggested that the expression of BMP-2 could be enhanced even if the stretching was over 12% [27]. In our preliminary experiment we found that the viability of SMSCs was decreased when treated with stretching more than 10% and pressure time over 3 h. Thus we adjusted strain at 8%, 0.33 Hz and the peak stress at 2.35 kilopascals.

The mechanisms of mechanical stimulation increasing the capability of osteogenesis and chondrogenesis remain diverse. Though it was reported that ECM reconstruction was involved in this progress [21, 28], we found that BMP-2 might be one of the essential factors mediating that mechanical stimulation promoted osteogenesis and chondrogenesis. In agreement with our findings, research on tendon-derived stem cells and bone marrow-derived stem cells demonstrated that mechanical loading might promote osteogenic differentiation by increasing BMP-2 expression [27, 29]. Altogether, our research demonstrates that BMP-2 might play a pivotal role during mechanical stimulation-mediated osteogenesis and chondrogenesis.

Conclusions

Cyclic mechanical stimulation promoted the osteogenic and chondrogenic differentiation of synovial mesenchymal stem cells (SMSCs). The expression of osteogenesis markers (ALP and Cbfa1) and chondrogenesis markers (Sox9 and Col2a1) were utilized to determine the osteogenic and chondrogenic differentiation of SMSCs. Mechanical stimulation up-regulated expressions of osteogenesis markers (ALP and Cbfa1) and chondrogenesis markers (Sox9 and Col2a1) of SMSCs, respectively. BMP-2 was increased in SMSCs treated with mechanical stimulation during osteogenic and chondrogenic differentiation, and knockdown of BMP-2 leaded to the reversed result of the expression of osteogenesis markers and chondrogenesis markers. To conclude, our study demontrated that repetitive mechanical stimulation increased the expression of BMP-2, and promoted osteogenic and chondrogenic differentiation of SMSCs.

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Disclosure of conflict of interest

None.

Authors' contribution

PF, SC, ZD participated in the design of the study, carried out the animal experiments, analyzed the results and drafted the manuscript. RC participated in drafting the manuscript and revising the manuscript critically. JS and LZ participated in the evaluation of the results and data analysis. PF and QQ participated in the design of the study. All authors read and approved the final manuscript.

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