Mechanical stress affects the osteogenic differentiation of human *ligamentum flavum* cells via the BMP-Smad1 signaling pathway

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Abstract. The aim of the present study was to investigate the effects of mechanical stress on the osteogenic differentiation of human *ligamentum flavum* cells via the bone morphogenetic protein (BMP)-Smad1 signaling pathway. Mechanical stress increased cell proliferation and induced osteogenic differentiation of human cells derived from the ossification of the *ligamentum flavum* (OLF). In addition, mechanical stress activated osteocalcin (OC), alkaline phosphatase (ALP) and runt-related transcription factor 2 (RUNX-2) mRNA expression, and suppressed Ets proto-oncogene 1 (Ets-1) and sex determining region Y-box 2 (SOX-2) mRNA expression in OLF cells. Src protein expression was suppressed by mechanical stress in human OLF cells. In addition, the protein expression levels of BMP, phosphorylated (p)-mothers against decapentaplegic homolog-1 (Smad1) and p-p38-mitogen-activated protein kinases (p38MAPK) were increased by mechanical stress. These results demonstrate that mechanical stress effectively increases cell proliferation, promotes the osteogenic differentiation rate of OLF cells, activates OC, ALP and RUNX-2, and suppresses Ets-1 and SOX-2 potentially via the BMP-Smad1 and Src-p38MAPK signaling pathways.

Introduction

Ossification of the *ligamentum flavum* (OLF) is the most common etiology of thoracic spinal stenosis (1). Although the causes and pathogenesises are unclear, OLF is thought to

occur due to genetic and environmental factors, including diet, humidity, metabolic disturbance and athletic injury (2). A number of surgical procedures are used to treat OLF in the clinic, including lamina eroding decompression, double-door lamina resection decompression, the whole vertebral plate decompression peel method, and lamina decompression and implant surgery (3). The lamina eroding decompression method is a relatively simple operation; however, the double-door and vertebral plate decompression methods require high-speed drills and osteotomes (1). The loss of the spinous process and vertebral plate weaken the stability of the posterior column and, after a significant period of time, may produce unstable regions of reduced pressure (4). To maintain the intact structure of the *canalis vertebralis*, lamina decompression and implant surgery are performed (5). Implanted vertebral plates ensure that the structure of the *canalis spinalis* is maintained and prevents scar tissue from invading the *canalis spinalis* (6). In addition, vertebral plates provide attachment points for paravertebral muscles and prevent amyotrophy. The fixation of miniature titanium plates maintains the integrity of the spinal cord. However, when compared with laminectomies, vertebral plate implant surgery is more complex, with longer operating times and increased levels of bleeding (7).

Mechanical stress stimulation serves an important regulatory function in bone formation and remodeling (8). Mechanical stimuli target bone tissues and are transmitted as one of three local signals, which include fluid shear stress, cytomorphosis or stress-generated potentials (9). Cells sensitive to transformation stress transduce these local signals into primary signals via the following three pathways: The integrin-cytoskeleton system, the calcium channel pathway and the primary cilium pathway (10). These primary signals are subsequently transmitted as downstream signals via signal transduction pathways (11). These signals induce a number of alterations, including those associated with genetic expression, energy metabolism and material synthesis.

OLF is a common disease, which is characterized by heterotopic osteogenesis of the posterior longitudinal ligament of the cervical vertebra in China (12). Unregulated growth of an ossific mass in the *canalis spinalis* may lead to severe spinal cord and nerve root entrapment; however, the pathogenesis of OLF is currently unclear (13). The aim of the present study was to determine the effect of mechanical stress on the rate of

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osteogenic differentiation of human *ligamentum flavum* cells, and to elucidate the underlying mechanisms involved.

Materials and methods

Cell culture and generation of the mechanical stress model. Human OLF tissues (3–5 g) were obtained during surgery from a patient admitted to the Shanghai Tenth People's Hospital, Shanghai, China (aged 53 years; male; stage IV lumbar degenerative disease, during March 2015) that was diagnosed with lumbar degenerative disease. Written informed consent was provided by the patient for the use of their tissue samples in the present study. The tissues were cut into 1–2-mm³ sections and digested with 2% type-I collagenase (Sunshine Biotechnology Co., Ltd., Nanjing, China) at 37°C for 30 min. Nucleated cells (10,000 cells/cm²) were recovered and plated as mononuclear cells to obtain a higher yield. Cells were cultured in α -minimum essential medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 15% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C in 5% CO₂; the medium was refreshed every 2 days. Following 1 week of culture, the cells were transferred to culture flasks coated with fibronectin (5 μ g/cm², Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). A specially-designed four-point bending apparatus with flexible silicon-bottomed chambers was generated as described previously (14), and used to induce the mechanical stress model. The cells were then subjected to uniaxial tensile strain (0.5 Hz, 2,000 min, three times/day).

Cell viability. Cells were distributed into control, unstretched and stretched groups.

In the control group, cells were treated with PBS; in unstretched group, cells were subjected to uniaxial tensile strain without voltage; in stretched group, cells were subjected to uniaxial tensile strain (0.5 Hz, 2,000 min, three times/day).

Following 3 weeks of mechanical stress, cells (1–2x10³) were cultured in a 96-well plate for 24 h. A total of 20 μ l MTT assay reagent (5 mg/ml; Beyotime Institute of Biotechnology, Haimen, China) was then added to each well, and the plates were incubated at 37°C and 5% CO₂ for 4 h. Following incubation, the culture medium was removed and 150 μ l dimethyl sulfoxide (DMSO) was added to each well and allowed to dissolve the formazan crystals for 20 min at 37°C. Cell viability was measured using a microplate reader (Omega Bio-Tek, Inc., Norcross, GA, USA) at a wavelength of 490 nm.

Osteogenic differentiation. Cells (1x10⁵) from the different groups were fixed in 95% ethanol overnight at 4°C, and stained with Oil Red O (0.25%) for 15 min at room temperature. The cells were then washed three times with 70% ethanol for 5 min. Osteogenic differentiation was observed using a microscope in three field of view (Axio Imager 2; Zeiss GmbH, Jena, Germany).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). After 3 weeks mechanical stress, cells (1x10⁶) from the different groups were cultured on a 6-well plate for 24 h. Total RNA was isolated using TRIzol reagent (Invitrogen;

Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. A total of 2 ng RNA was reverse-transcribed into cDNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. For qPCR analysis, 1 ng cDNA template was used for each reaction and sequences were amplified using a StepOne™ Real-Time PCR system and Power SYBR® Green PCR Master Mix (both from Applied Biosystems; Thermo Fisher Scientific, Inc.). Amplification reactions were conducted with an initial step at 95°C for 1 min, followed by 40 cycles of 55°C for 30 sec and 60°C for 30 sec. The primers used for qPCR analysis are listed in Table I and gene expression was normalized to the levels of β -actin and analyzed using the 2^{- $\Delta\Delta C_q$} method (15).

Western blot analysis. Cells (1x10⁶) after 3 weeks mechanical stress from the different groups were lysed with ice-cold lysis buffer containing a 10 μ g/ml of protein inhibitor mixture (1:100; Roche Diagnostics, Indianapolis, IN, USA). Protein concentrations were then determined using a DC Protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A total of 50 μ g protein was separated by 8–12% SDS-PAGE and electrophoretically transferred onto 0.22 μ m polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were then washed with 5% non-fat dried milk in Tris-buffered solution plus 0.05% Tween-20 (TBST) for 2 h, prior to incubation with anti-Src (catalog no. sc-8995, dilution, 1:200), anti-bone morphogenetic protein (catalog no. sc-9003, BMP; dilution, 1:200) (both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-phosphorylated (p)-mothers against decapentaplegic homolog-1 (catalog no. 13820, p-Smad-1; dilution, 1:400; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-p-p38-mitogen-activated protein kinases (catalog no. sc-7975-R, p38MAPK; dilution, 1:300) and anti- β -actin (catalog no. sc-7210, dilution, 1:400) (both from Santa Cruz Biotechnology, Inc.) at 4°C overnight. The membranes were washed with TBST for 5 min three times and were subsequently incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (catalog no. A0208, dilution, 1:4,000; Beyotime Institute of Biotechnology) at room temperature for 1 h, and detected with electrochemiluminescence western blot detection reagents (Beyotime Institute of Biotechnology). Protein expression was analyzed using Quantity One software version 3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All data are expressed as the mean $\pm$ standard deviation, and represent the mean values of at least three independent experiments using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). Comparisons among groups were analyzed using one-way analysis of variance, followed by the Tukey Kramer post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cell morphology. OLF cells were extracted using a fibronectin differential-adhesion assay, and OLF cell morphology was observed following 1 week of culture. As shown in Fig. 1, OLF cells exhibited a uniform morphology and were well-distributed

Table I. Primer sequences used to analyze target gene expression levels by reverse transcription-quantitative polymerase chain reaction.

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')
OC	ATGAGAGCCCTCACACTCCTC	GCCGTAGAAGCGCCGATAGGC
ALP	TGGAGCTTCAGAAGCTCAACACCA	ATCTCGTTGTCTGAGTACCAGTCC
RUNX-2	ATCTCGTTGTCTGAGTACCAGTCC	ATCTCGTTGTCTGAGTACCAGTCC
SOX-2	CCCCCTGTGGTTACCTCTTC	CCCCCTGTGGTTACCTCTTC
Ets-1	GAGTTCAGCCTGAAGGGTGT	CACATCCTCTTTCTGCAGGATCT
β -actin	GTGGGGCGCCCCAGGCACCA	CTTCCTTAATGTCACGCACGATTTC

OC, osteocalcin; ALP, alkaline phosphatase; RUNX-2, runt-related transcription factor 2; SOX-2, sex determining region Y-box 2; Ets-1, Ets proto-oncogene 1.

Figure 1. Cell morphology. Cell morphology of OLF cell. Magnification, x20. The cells remained shuttle shape until the end of week 1. OLF cell morphology was observed after 3 weeks mechanical stress.

in the culture flasks. In addition, the cells exhibited a shuttle shape until the end of week 1 (Fig. 1).

Cell viability. When comparing the viability of OLF cells among the different experimental groups, the control and unstretched groups demonstrated similar levels of cell viability at 1, 2 and 3 weeks (Fig. 2). However, mechanical stress significantly increased OLF cell viability following 2 ($P<0.05$) and 3 weeks ($P<0.01$) of mechanical stimulation when compared with the controls (Fig. 2).

Osteogenic differentiation. Oil Red O staining was performed to analyze the effect of 3 weeks of mechanical stress on the osteogenic differentiation of OLF cells. The osteogenic differentiation rate of the control group appeared to be similar to that of the unstretched group (Fig. 3). By contrast, mechanical stress effectively promoted the osteogenic differentiation rate and increased the size of OLF cells, when compared with the control group (Fig. 3).

Expression of osteocalcin (OC), alkaline phosphatase (ALP) and runt-related transcription factor 2 (RUNX-2) mRNA. RT-qPCR analysis was performed to investigate OC, ALP and RUNX-2 mRNA expression levels following mechanical

Figure 2. The percentage viability of cells derived from patients with ossification of the *ligamentum flavum* in the control, unstretched and stretched groups following 1, 2 and 3 weeks of mechanical stress. # $P<0.05$ and ## $P<0.01$ vs. the control group. Control, untreated control group; unstretched, non-mechanical stress group; stretched, mechanical stress group.

stress stimulation at 3 weeks. As shown in Fig. 4, the mRNA expression levels of OC, ALP and RUNX-2 were comparable when comparing the control group and unstretched groups. By contrast, mechanical stress significantly increased the expression of OC, ALP and RUNX-2 mRNA in OLF cells when compared with the control group ($P<0.01$; Fig. 4).

Expression of Ets proto-oncogene 1 (Ets-1) and sex determining region Y-box 2 (SOX-2) mRNA. In order to investigate the mechanisms underlying the effects of mechanical stress on osteogenic OLF cell differentiation, Ets-1 and SOX-2 mRNA expression was determined by RT-qPCR analysis. Following quantitative analysis, no significant difference in Ets-1 and SOX-2 mRNA expression levels were observed between the control and unstretched groups (Fig. 5). However, mechanical stress significantly inhibited Ets-1 and SOX-2 mRNA expression in OLF cells when compared with the control cells ($P<0.01$; Fig. 5).

Expression of Src protein. Western blot analysis revealed that the control and unstretched groups exhibited similar levels of Src protein expression (Fig. 6). By contrast, mechanical stress significantly inhibited Src protein expression in OLF cells when compared with the control group ($P<0.01$; Fig. 6).

Expression of p-p38MAPK protein. To further elucidate the underlying mechanisms of mechanical stress on osteogenic OLF cell differentiation, p-p38MAPK protein was detected

using western blot analysis. No significant differences in p38MAPK protein expression were observed between the control and unstretched groups (Fig. 7). However, mechanical stress significantly increased p-p38MAPK protein expression, when compared with the control group ($P<0.01$; Fig. 7).

Expression of BMP protein. BMP protein expression was determined by western blot analysis. As shown in Fig. 8, no significant difference in BMP protein expression was observed between the control and unstretched groups. However, mechanical stress significantly increased BMP protein expression in OLF cells when compared with the control group ($P<0.01$; Fig. 8).

Expression of Smad-1 protein. p-Smad-1 protein expression in OLF cells was analyzed using western blot analysis. As shown in Fig. 9, no significant difference in the protein expression of p-Smad-1 was observed between the control and unstretched groups. However, mechanical stress significantly increased p-Smad-1 protein expression in OLF cells, when compared with the control group ($P<0.01$; Fig. 9).

Discussion

Following the action of mechanical stress stimuli, bone tissues transform mechanical stress signals into local signals, which target stress-sensitive cells via the integrating integrin-cytoskeleton system, the calcium channel pathway and the primary cilium pathways (16). Stress-sensitive cells transform these primary biochemical signals into downstream signals through a signal transduction channel (6). These signals subsequently induce alterations in gene expression, energy metabolism and material synthesis. In the present study, mechanical stress effectively increased cell viability and promoted the osteogenic differentiation rate of OLF cells. Mechanical stimuli induce alterations in bone microstructure and produce a number of local signals, which are recognized by mechanical stress-sensitive cells (17). Li *et al* (18) demonstrated that mechanical strain regulates osteogenic differentiation in bone marrow stromal cells (BMSCs).

An ongoing study showed the complex underlying mechanisms of signal transduction are currently unclear and are therefore the focus of a study (19). The process by which cells produce extracellular chemical energy from mechanical energy is of particular interest (19,20). Previous studies have demonstrated that cyclical mechanical stress is regulated by kinases to promote the proliferation of chondrocytes by functioning as extracellular signaling molecules (19,21). In the present study, mechanical stress significantly increased OC, ALP and RUNX-2 mRNA expression levels, while significantly inhibiting Ets-1 and SOX-2 miRNA expression in OLF cells. Li *et al* (18) demonstrated that mechanical strain regulates osteogenic differentiation in BMSCs via Runx2, Osterix, and collagen-I.

Src, a member of the Src family, possesses tyrosine protein kinase activity. Phosphorylation of the Tyr residue on the activation loop activates specific enzymes and the delivery of extracellular signals to the cells, thereby facilitating cell proliferation and migration (22). A previous study demonstrated that the migration activity of murine fibroblast

Figure 3. Osteogenic differentiation. Representative images of Oil Red O-stained ossification of the *ligamentum flavum* cells in the control, unstretched, and stretched groups (magnification x20). Control, untreated control group; unstretched, non-mechanical stress group; stretched, mechanical stress group.

Figure 4. Expression of (A) OC, (B) ALP and (C) RUNX-2 mRNA in ossification of *ligamentum flavum* cells following mechanical stress, as determined by reverse transcription-quantitative polymerase chain reaction analysis. ^{##} $P<0.01$ vs. control group. OC, osteocalcin; ALP, alkaline phosphatase; RUNX-2, runt-related transcription factor 2; control, untreated control group; unstretched, non-mechanical stress group; stretched, mechanical stress group.

cells in Src-knockout rats is reduced, which further supports the role of Src in cell migration (22). The interactions between Src, the extracellular matrix and integrin are essential for the induction of ras homolog gene family, member A (RhoA), and in turn, the activation of RhoA is closely associated with cell migration (23). The present study revealed that mechanical stress significantly suppressed Src protein expression in OLF cells. Paravicini *et al* (24) demonstrated that mechanical stretch influences vascular fibrosis during hypertension, which appeared to be independent of c-Src and p38MAPK.

p38MAPK is a core component of the MAPK cascade. Phosphorylation of p38MAPK induces enzyme activation and serves a fundamental role in cell proliferation, differentiation and migration (25). p38MAPK at different cellular locations possess different functions. p38MAPK on the cytomembrane

Figure 5. Expression of (A) Ets-1 and (B) SOX-2 mRNA in ossification of *ligamentum flavum* cells following mechanical stress as determined by reverse transcription-quantitative polymerase chain reaction analysis. ^{##}P<0.01 vs. control group. Ets-1, Ets proto-oncogene 1; SOX-2, sex determining region Y-box 2; control, untreated control group; unstretched, non-mechanical stress group; stretched, mechanical stress group.

Figure 6. Expression of Src protein in ossification of the *ligamentum flavum* cells following mechanical stress. (A) Western blotting analysis of Src protein expression and (B) quantification of Src protein expression levels following normalization to β-actin expression. ^{##}P<0.01 vs. control group. Control, untreated control group; unstretched, non-mechanical stress group; stretched, mechanical stress group.

Figure 8. Expression of BMP protein in ossification of the *ligamentum flavum* cells. The expression of BMP protein by (A) western blotting analysis and (B) quantification of BMP protein expression following normalization to β-actin expression. ^{##}P<0.01 vs. control group. BMP, bone morphogenetic protein; control, untreated control group; unstretched, non-mechanical stress group; stretched, mechanical stress group.

Figure 7. Expression of p-p38MAPK protein by (A) western blotting analysis, and (B) quantification of p-p38MAPK protein expression following normalization to β-actin expression. ^{##}P<0.01 vs. control group. p38MAPK, p38-mitogen-activated protein kinase; p-p38MAPK phosphorylated p38MAPK; control, untreated control group; unstretched, non-mechanical stress group; stretched, mechanical stress group.

Figure 9. Expression of p-Smad-1 protein in ossification of the *ligamentum flavum* cells by (A) western blotting analysis and (B) quantification of p-Smad-1 protein expression following normalization to β-actin expression. ^{##}P<0.01 vs. control group. p-SMAD-1, phosphorylated-mothers against decapentaplegic homolog-1; control, untreated control group; unstretched, non-mechanical stress group; stretched, mechanical stress group.

participates in cellular effects, and following mechanical trauma, induces the migration and differentiation of stomach

smooth muscle cells (26). This function does not depend on the intranuclear transcription. The present study demonstrated

that mechanical stress significantly increased p-p38MAPK protein expression in OLF cells.

A study confirmed that the BMP-Smad-1 signaling pathway regulates specific aspects of the osteoblast life cycle, including differentiation from mesenchymal stem cells to osteoblasts, osteogenic cell proliferation, the vitality of osteoblast mineralization and osteoclast coupling (27). However, abnormal differentiation is triggered by downregulated Runx2 and Osterix expression (28). Empirical data collected from a recent study indicated that the loss of BMP-Smad signaling significantly increased Osterix and Runx2 protein expression (29,30). The results of the present study demonstrated that mechanical stress significantly increased BMP and p-Smad1 protein expression in OLF cells. Kido *et al* (31) revealed that mechanical stress activates the Smad pathway in osteoblasts. In addition, Grottkau *et al* (32) demonstrated that mechanical stretching induced osteogenesis through BMP-2 mRNA expression in adipose-derived stem cells and BMSCs.

In conclusion, the present study demonstrated that mechanical stress effectively increased cell viability and promoted the osteogenic differentiation rate of OLF cells potentially via the activation of OC, ALP and RUNX-2, and the suppression of Ets-1 and SOX-2 signaling. These results suggest that the BMP-Smad-1 signaling pathway may serve an important role in mediating the intracellular signaling effects of mechanical stress on osteogenic differentiation via the Src and p38MAPK signaling pathways in OLF cells.

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