

Cyclic stretch promotes the ossification of ligamentum flavum by modulating the Indian hedgehog signaling pathway

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Abstract. The Indian hedgehog (IHH) signaling pathway is an important pathway for bone growth and development. The aim of the present study was to examine the role of the IHH signaling pathway in the development of the ossification of ligamentum flavum (OLF) at the cellular and tissue levels. The expression levels and localization of the osteogenic genes Runt-related transcription factor 2 (*RUNX2*), *Osterix*, alkaline phosphatase (*ALP*), osteocalcin (*OCN*) and *IHH* were evaluated in OLF tissues by reverse transcription-quantitative PCR (RT-qPCR) and immunohistochemistry. Non-ossified ligamentum flavum (LF) sections were used as control samples. The tissue explant method was used to obtain cultured LF cells. In addition, OLF cells were subjected to cyclic stretch application for 0, 6, 12 or 24 h. The expression levels of osteogenic genes, and the IHH signaling pathway genes *IHH*, Smoothed (*SMO*), GLI family zinc finger 1 (*GLI1*), *GLI2* and *GLI3* were evaluated with RT-qPCR and western blotting. Osteogenic differentiation was further evaluated by assessing ALP activity and staining. Moreover, the effect of cyclopamine (Cpn), an IHH signaling inhibitor, on osteogenic differentiation was examined. The RT-qPCR

and immunohistochemical results indicated that the mRNA and protein expression levels of *RUNX2*, *Osterix*, *ALP*, *OCN* and *IHH* were significantly higher in the OLF group compared with the LF group. Furthermore, application of cyclic stretch to OLF cells resulted in greater ALP activity, and significant increases in mRNA and protein expression levels of *RUNX2*, *Osterix*, *ALP* and *OCN* in a time-dependent manner. Cyclic stretch application also led to significant increases in IHH signaling pathway genes, including *IHH*, *SMO*, *GLI1* and *GLI2*, while no significant effect was found on *GLI3* expression level. In addition, it was found that Cpn significantly reversed the effect of cyclic stretch on the ALP activity, and the expression levels of *RUNX2*, *Osterix*, *ALP*, *OCN*, *GLI1* and *GLI2*. Collectively, the present results suggested that the IHH signaling pathway may mediate the effect of cyclic stretch on the OLF cells.

Introduction

Ossification of the ligamentum flavum (OLF) is one of the degenerative changes to the ligamentum flavum (LF) that occur with aging (1). Since the first report of OLF in 1920, OLF has been investigated mainly in Asian countries (2). In China and Japan, the prevalence of OLF is 3.8% (3) and 6.2% (4), respectively. OLF arises from the lateral capsular portion and exhibits continuity with the bony laminae (5). Clinical manifestations of OLF include regional thoracic stiffness and pain, which usually progresses to myelopathy (6). The diagnosis of OLF is often missed or delayed due to its slow progression and the co-occurrence of other spinal diseases (5,6), which results in a heavy burden to both the patients and society.

OLF is a heterotopic ossification that occurs in the ligamentum (5,6). OLF usually starts from the degeneration and hypertrophy of the elastic fibrous tissues, which results in the production of fibrocartilage-like cells and the proliferation of chondrocytes (7). Subsequently, chondrocytes are calcified, and with the growth of small vessels into the LF tissues, mature bone structures are formed (7). However, the etiology of OLF is not fully understood. Multiple factors have been implicated in the development of OLF, including genetics (8), systematic hormones (9), inflammatory markers (10) and

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Abbreviations: *IHH*, Indian hedgehog; OLF, ossification of ligamentum flavum; *RUNX2*, Runt-related transcription factor 2; *ALP*, alkaline phosphatase; *OCN*, osteocalcin; LF, ligamentum flavum; Cpn, cyclopamine; *OPN*, osteopontin; *SOX9*, sex-determining region Y-box protein 9; *BCA*, bicinchoninic acid

Key words: ligamentum flavum, cyclic stretch, ossification, osteogenic differentiation, pathway, Indian hedgehog

mechanical stressors (11). Moreover, OLF is closely related to disturbance of the local mechanical microenvironment of the spine (12). The reduced stability of the middle and lower thoracic joints (T) due to long-term rotational motions has been indicated the anatomical basis of OLF (13). A previous study reported T9-T12 are the common segments affected by OLF, suggesting that increases in local stress caused by longitudinal axial instability of the middle and lower thoracic spine may be the biomechanical basis of OLF (14).

In rats, after periodic mechanical stress stimulation, LF of the caudal vertebrae shows similar pathology to that found in humans with OLF: An extensive proliferation of the chondrocyte-like cells and ectopic cartilage formation near the insertion point of the LF (15). In addition, Cai *et al* (16) found that the expression levels of the ossification markers osteopontin (*OPN*) and sex-determining region Y-box protein 9 (*SOX9*) are significantly upregulated after stress stimulation. In contrast, normal ligamentum cells did not show this significant upregulation in expression levels (16). Therefore, periodic mechanical stress can facilitate the progression of OLF at the cellular level.

Indian hedgehog (*IHH*), a member of the secretory morphogen signaling family, is a secreted molecule that regulates cells via concentration gradients (17). The *IHH* signaling pathway is key for bone growth and development (18). *IHH* is one of the essential signaling pathways mediating heterotopic ossification of the extremities (19). Furthermore, the *IHH* pathway can directly or indirectly promote the proliferation and hypertrophy of the chondrocytes, induce the expression of parathyroid hormone-related peptide in cells surrounding the chondrocytes, facilitate the differentiation of resting chondrocytes into proliferating ones and induce differentiation of deep perichondrocytes into osteoblasts (20,21). In an *IHH*^{-/-} mouse model, no deep perichondrocytes differentiation into osteoblasts is observed (22). Regard *et al* (23), found that the *IHH* pathway independently causes heterotopic ossification of the extremities; furthermore, inhibition of the *IHH* pathway significantly reduced the degree of heterotopic ossification. Thus, previous studies have indicated that the *IHH* signaling pathway may play a role in the process of bone development and heterotopic ossification. However, whether the *IHH* signaling pathway mediates osteogenic differentiation during ossification of LF requires further investigation.

Currently, there is no effective medication to prevent or delay the progress of OLF (5). Once ossification causes oppression to the spinal cord, the only treatment solution for OLF is surgery (5). However, OLF progression does not decrease or stop, despite this surgical intervention (24-26). Therefore, it is necessary to investigate the signaling pathways underlying the progression of OLF in order to understand the pathogenesis of OLF. The aim of the present study was to examine the involvement of the *IHH* signaling pathway in the development of OLF at the cellular and tissue levels, by simulating the *in vivo* stress environment of the LF. The present results will help in the understanding of the mechanisms underlying the development of OLF, and provide evidence for potential targets in novel therapeutic strategies.

Materials and methods

Patient specimens. The Ethics Committee from The Second Military Medical University approved the present study.

Participants provided written informed consent prior to specimen collection. The diagnosis of OLF was confirmed by clinical symptoms and radiological examinations. Patients were included if they received posterior open decompressive laminectomy between January 2016 and January 2019 at Changzheng Hospital, Second Military Medical University. A total of 18 LF tissue samples (male patients, 10; female patients, 8; mean age, 61.2 years; age range, 52-73 years) from patients with OLF were obtained, of which 10 samples were harvested for cell culture. The remaining eight samples were used for histology. The non-ossified LF samples from 12 patients were used as controls (male patients, 7; female patients, 5; mean age, 56.2 years; age range, 42-68 years), of which four samples were harvested for cell culture. The remaining eight samples were used for histology. Patients in the control group underwent posterior surgical procedures for disc herniation (n=7) and fractures (n=5). Thus, eight samples from the OLF group and eight samples from the control group were used for the tissue experiments. Whole pieces of ligaments were isolated and harvested after removing the lamina during the surgery. Patients who had congenital bone diseases or musculoligamentous tissue abnormalities were excluded.

Cell culture. A tissue explant method (16) was used to obtain the cultured LF cells. The LF and OLF tissues were obtained aseptically during surgery. For OLF tissues, the ossified areas were separated and removed under a microscope to avoid contamination with osteogenic cells. The LF and OLF ligaments were digested in 0.25% trypsin, followed by 250 U/ml type I collagenase (Gibco; Thermo Fisher Scientific, Inc.). Subsequently, the fragments were washed with PBS and cultured at 37°C with 5% CO₂ in a 10-cm dish with DMEM: Nutrient Mixture F-12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 µg/ml penicillin/streptomycin. The cultures were left undisturbed for 2 days and then replaced with fresh medium. The cells, which were obtained from the explants, were treated with 0.25% trypsin containing 0.02% EDTA for 1-2 min at 37°C, re-suspended and cultured for further passages. Cells at passage three were used for subsequent experiments.

Procedures of cyclic stretch application. A Flexcell FX-5000 strain unit (Flexcell International Corporation) was used in this study, with procedures similar to a previous study (16). Cells were cultured (5x10⁵ cells/well) in a flexible bottomed polystyrene plate (6 wells) with type I collagen (0.15 mg/ml) coated at the bottom (Flex I, Bioflex Plates; Flexcell International Corporation). After cell attachment, cyclic stretch was applied to the cells at 37°C for different durations (0, 6, 12 or 24 h). Based on a previous study (16), the stretch parameter was set at a maximum 15% elongation. In the present study, the cultured cells were subjected to 15% elongation for 10 sec and then relaxation for the next 10 sec. In pathway inhibition tests, subconfluent cells were pre-treated with 0.5 µM Cyclopamine (Cpn; Sigma-Aldrich; Merck KGaA) for 2 h at 37°C and subsequently subjected to cyclic stretch for 24 h at 37°C.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from OLF and LF samples or cultured OLF cells using TRIzol[®] reagent (Invitrogen; Thermo Fisher

Table I. Primers used for reverse transcription-quantitative PCR.

Target	Primer sequence	Genbank accession no.
<i>GAPDH</i>	F: 5'-AGGTCGGTGTGAACGGATTTG-3' R: 5'-TGTAGACCATGTAGTTGAGGTCA-3'	NM_008084
<i>RUNX2</i>	F: 5'-ATGCTTCATTCGCCTCACAAA-3' R: 5'-GCACTCACTGACTCGGTTGG-3'	NM_001146038
<i>Osterix</i>	F: 5'-ATGGCGTCCTCTCTGCTTG-3' R: 5'-TGAAAGGTCAGCGTATGGCTT-3'	AF477981.1
<i>ALP</i>	F: 5'-CCAACCTCTTTTGTGCCAGAGA-3' R: 5'-GGCTACATTGGTGTGAGCTTTT-3'	NM_007431
<i>OCN</i>	F: 5'-CTGACCTCACAGATCCCAAGC-3' R: 5'-TGGTCTGATAGCTCGTCACAAG-3'	X04142
<i>IHH</i>	F: 5'-CGGCTTTGACTGGGTGTATT-3' R: 5'-AAAATGAGCACATCGCTGAA-3'	KR710697.1
<i>SMO</i>	F: 5'-CTGTCCTGCGTCATCATCTTT-3' R: 5'-CCACAGCAAGGATTGCCAC-3'	NM_005631.5
<i>GLI1</i>	F: 5'-AAGCGTGAGCCTGAATCTGT-3' R: 5'-CAGCATGTACTGGGCTTTGA-3'	BC013000.2
<i>GLI2</i>	F: 5'-CGACACCAGGAAGGAAGGTA-3' R: 5'-TGCACAGAACGGAGGTAGTG-3'	BC172434.1
<i>GLI3</i>	F: 5'-CTTTGCAAGCCAGGAGAAAC-3' R: 5'-TTGTTGACTGTGTGCCATT-3'	BC113616.1

F, forward; R, reverse; *ALP*, alkaline phosphatase; *RUNX2*, Runt-related transcription factor 2; *OCN*, osteocalcin; *IHH*, Indian hedgehog; *SMO*, Smoothened; *GLI*, GLI family zinc finger.

Scientific, Inc.), according to the manufacturer's instructions. RNA concentration was then measured and converted to cDNA using a PrimerScript RT kit (Takara Bio, Inc.), according to the manufacturer's instructions. The mRNA expression levels of the osteogenic genes Runt-related transcription factor 2 (*RUNX2*), *Osterix*, alkaline phosphatase (*ALP*) and osteocalcin (*OCN*), and the IHH signaling pathway genes *IHH*, Smoothened (*SMO*), GLI family zinc finger 1 (*GLI1*), *GLI2* and *GLI3* were quantified using SYBR Premix Ex Taq™ II (Takara Bio, Inc.). *GAPDH* was used as an endogenous control gene. The following qPCR conditions were used: Initial denaturation at 95°C for 10 min; followed by 40 cycles of 95°C for 30 sec; and final extension at 60°C for 1 min. The relative expression levels of each gene were calculated by the $2^{-\Delta\Delta C_q}$ method (27). Primer sequences used for qPCR are presented in Table I.

Western blot analysis. Protein samples were extracted from the OLF cells using RIPA lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Detailed procedures were described previously (28). The bicinchoninic acid protein assay (Pierce; Thermo Fisher Scientific, Inc.) was used to measure the protein concentration. The protein samples (~60 µg/lane) were separated by electrophoresis using 10% separating gel and 5% stacking gel, and then transferred onto PVDF membranes. After blocking with skimmed milk in 5% Tris-buffered saline with 0.05% Tween-20 for 1 h at room temperature, the membranes

were subsequently incubated overnight at 4°C with primary antibodies targeted against: *RUNX2* (cat. no. ab23981; Abcam; 1:1,000), *Osterix* (cat. no. ab22552; Abcam; 1:500), *ALP* (cat. no. ab67228; Abcam; 1:500), *OCN* (cat. no. ab93876; Abcam; 1:500), *IHH* (cat. no. ab39634; Abcam; 1:1,000), *SMO* (cat. no. ab113438; Abcam; 1:500) and *GAPDH* (cat. no. ab181602; Abcam; 1:5,000). The next day, membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. ab205718; Abcam; 1:2,000) for 1 h at room temperature and detected with electrochemiluminescence (GE Healthcare). Protein expression was quantified using Image J software (v1.51; National Institutes of Health) with *GAPDH* as the loading control.

Immunohistochemical analysis. Immunohistochemistry was performed in OLF and LF tissues following previously described (28). OLF and LF samples were fixed with 4% formaldehyde for 24 h at room temperature, embedded in paraffin and cut at 4-µm thickness. The sections underwent antigen retrieval at 120°C for 10 min in a citrate solution (10 mmol/l; pH 6.0). After blocking with 1% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) for 37°C at 15 min, the sections were subsequently incubated overnight at 4°C with the following primary antibodies: Rabbit polyclonal antibody against *RUNX2* (cat. no. ab23981; Abcam; 1:500), *Osterix* (cat. no. ab22552; Abcam; 1:200), *ALP* (cat. no. ab224335; Abcam; 1:400), *OCN* (cat. no. ab93876; Abcam; 1:200), *IHH*

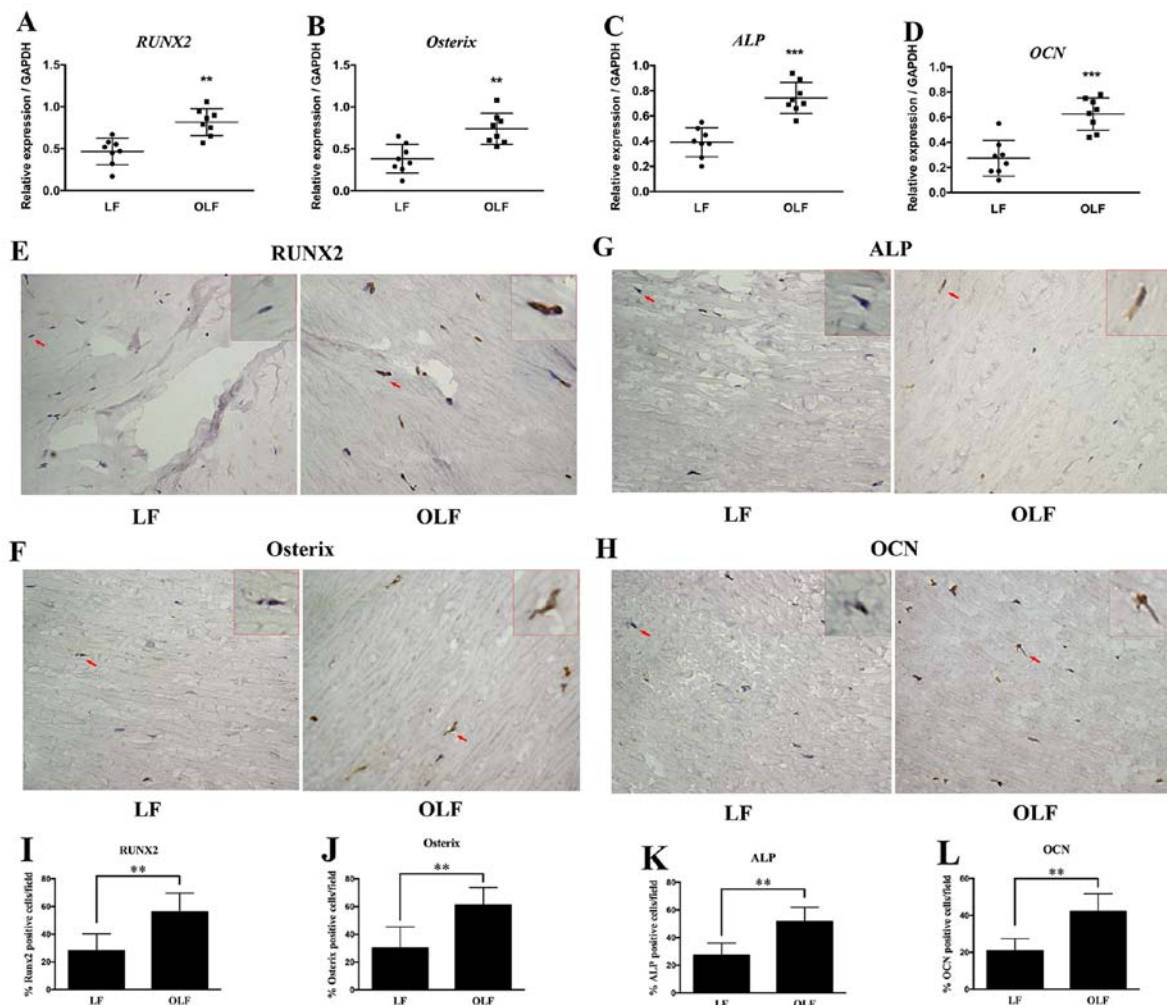


Figure 1. Expression of *RUNX2*, *Osterix*, *ALP* and *OCN* in LF and OLF tissues. Reverse transcription-quantitative PCR results for the mRNA expression levels of (A) *RUNX2*, (B) *Osterix*, (C) *ALP* and (D) *OCN* in the LF and OLF groups. Immunohistochemistry images of (E) *RUNX2*, (F) *Osterix*, (G) *ALP* and (H) *OCN*. Quantification of the protein expression levels of (I) *RUNX2*, (J) *Osterix*, (K) *ALP* and (L) *OCN* in the LF group and OLF group. Magnification, x400. The red arrows represent the typical cells, which were magnified and shown in the upper right-hand corner (magnification, x 1,600). N=8. **P<0.01, ***P<0.001 vs. LF samples. OLF, ossification of ligamentum flavum; LF, ligamentum flavum; *RUNX2*, Runt-related transcription factor 2; *ALP*, alkaline phosphatase; *OCN*, osteocalcin.

(cat. no. ab39634; Abcam; 1:200) and SMO (cat. no. ab113438; Abcam; 1:100). The next day, the sections were incubated with the HRP-conjugated secondary antibody (cat. no. ab205718; Abcam; 1:2,000) for 1 h at room temperature, followed by incubation with 3,3'-diaminobenzidine solution for 2-5 min at room temperature and counterstaining with hematoxylin at room temperature for 30 sec-1 min. Stained cells were identified using a ZEISS light microscope (magnification, x400; ZEISS Axio Imager A2; Carl Zeiss AG). Within each section, 200 cells were counted at random and the proportion of immunopositive cells was calculated (29). For quantitative analysis, images from three different sections of each sample were analyzed using Image J software (v1.51; National Institutes of Health).

Measurement of ALP activity and histochemical staining. ALP activity was quantitatively measured using an LabAssay ALP kit (cat. no. 291-58601; Wako Pure Chemical Industries, Ltd.) and histochemical staining was performed using a ALP color development kit (cat. no. C3206; Beyotime Institute of

Biotechnology) as previously described (30). Cell pellets were obtained by centrifugation at 3,000 x g for 5 min at 4°C. The total cellular protein was collected from the cultured cells by lysing cell pellets with RIPA lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.). The concentration of the protein was measured using the BCA method (Pierce; Thermo Fisher Scientific, Inc.). The protein solution was then analyzed for ALP activity using the ALP kit. The absorbance at 450 nm was measured using Spectra Max 250 spectrophotometer. The ALP-specific activity was determined using a standard curve and normalized to the protein concentration (U/mg). For histochemical staining, the cultured cells were washed with PBS and then fixed in 4% formaldehyde for 10 min at room temperature. The cells were stained with ALP reagent at 37°C for 30 min, and ALP-positive cells were stained blue. Stained cells were identified using a ZEISS light microscope (magnification, x400; ZEISS Axio Imager A2; Carl Zeiss AG). In total, five random fields in each well were used for the analysis. ALP staining intensities were applied to evaluate the ability of osteogenic differentiation in OLF cells.

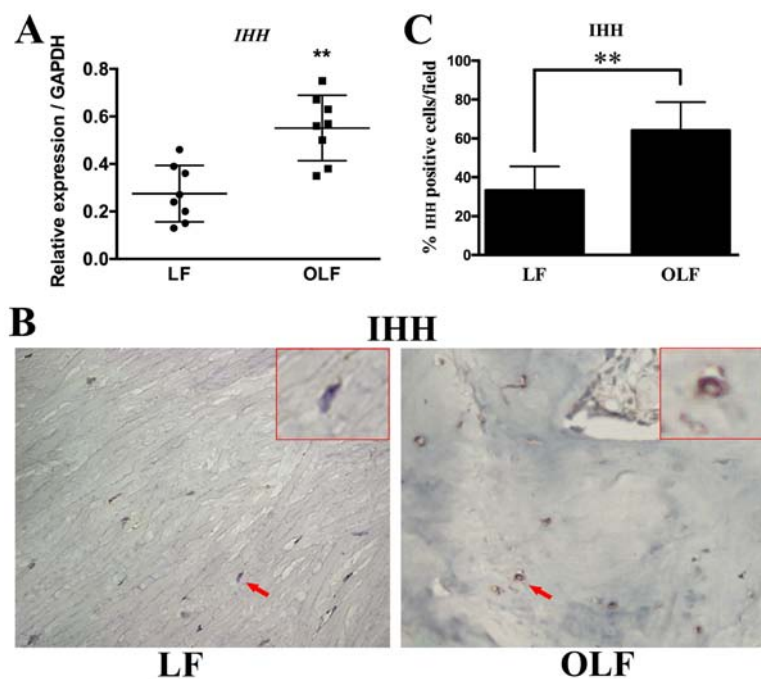


Figure 2. Expression level of *IHH* in LF and OLF tissues. (A) Reverse transcription-quantitative PCR results showed the mRNA expression levels of *IHH* between the LF group and OLF group. (B) Representative images of immunohistochemistry staining from each group. Magnification, x400. The red arrows represent the typical cells, which were magnified and shown in the upper right-hand corner (magnification, x1,600). (C) Immunohistochemistry results of the protein expression levels of *IHH* in the LF group and OLF group. N=8. **P<0.01 vs. LF samples. *IHH*, Indian hedgehog; OLF, ossification of ligamentum flavum; LF, ligamentum flavum.

Statistical analysis. Statistical analyses were performed using SPSS v19.0 (SPSS, Inc.). The experiments were repeated three times. Data are presented as the mean \pm SD. The results were analyzed with a Student's *t*-test, or one-way ANOVA followed by Tukey's post hoc test or Dunnett's test where appropriate. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of osteogenic genes in OLF. *RUNX2*, *ALP*, *Osterix* and *OCN* are important genes that define the osteogenesis process (31,32). In the present study, the mRNA and protein expression levels of *RUNX2*, *Osterix*, *ALP* and *OCN* were compared between the LF and OLF groups (Fig. 1). RT-qPCR and immunohistochemical analyses indicated that the OLF group had higher mRNA and protein expression levels of *RUNX2* compared with the LF group. Moreover, the OLF group had significantly higher mRNA and protein expression levels of *Osterix*, *ALP* and *OCN* compared with the LF group (P<0.01). Therefore, the present results suggested that the differentiation of fibroblasts into osteoblasts plays a role in OLF development.

Expression of *IHH* signaling genes in OLF. The mRNA and protein expression levels of *IHH* in both groups are shown in Fig. 2. The RT-qPCR and immunohistochemical results revealed that the OLF group had significantly higher mRNA and protein expression levels of *IHH* compared with the LF group (P<0.01). Thus, *IHH* may be involved in the osteogenesis of OLF.

Effects of cyclic stretch on osteogenic differentiation of LF and OLF cells. The mRNA expression levels of osteogenic

genes and *IHH* signaling genes in OLF and LF cells are shown in Fig. 3. It was demonstrated that application of cyclic stretch to OLF cells resulted in significant increases in mRNA expression levels of *RUNX2*, *Osterix*, *ALP* and *OCN*, and the *IHH* signaling genes *IHH* and *SMO* at 24 h. Moreover, in LF cells the application of cyclic tensile strain significantly increased the mRNA expression level of *ALP*, but not of *RUNX2*, *Osterix*, *OCN*, *IHH* and *SMO* (Fig. 3).

To further examine the effect of mechanical stress on the osteogenesis of OLF cells, the expression levels of osteogenic genes were compared after OLF cells were treated with or without cyclic stretch for 6, 12 and 24 h. It was found that cyclic stretch caused a significant elevation in the mRNA expression levels of *RUNX2*, *Osterix*, *ALP* and *OCN* at all time points (Fig. 4A-D). Furthermore, cyclic stretch was determined to significantly increase the protein expression levels of *RUNX2*, *Osterix* and *ALP* at 6, 12 and 24 h (Fig. 4E-G). In addition, it was demonstrated that cyclic stretch caused a significant increase in the protein expression level of *OCN* at 12 and 24 h (Fig. 4H). ALP activity was also significantly enhanced at 12 and 24 h, with the greatest increase observed at 24 h (Fig. 4I and J). Collectively, the present results indicated that mechanical stress induced the osteogenesis process during the development of OLF.

Effects of cyclic stretch on the *IHH* signaling pathway. The *IHH* signaling pathway, which includes *IHH*, *SMO* and *GLI*, is an important pathway in the osteogenic differentiation (33). To further examine the effect of local stress on the *IHH* signaling pathway and osteogenesis in OLF, cyclic stretch was applied to OLF cells for 6, 12 and 24 h. The relative ratios of *IHH*, *SMO* and *GLI* expression levels in OLF cells

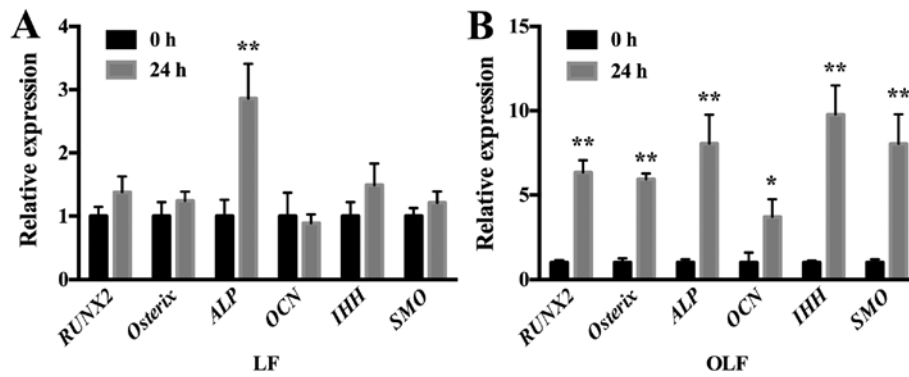


Figure 3. Expression level of osteogenic genes and *IHH* signaling genes in LF and OLF cells after cyclic stretch. (A) Reverse transcription-quantitative PCR results showed that in LF cells, cyclic stretch increased the ALP expression level, while other gene expression levels did not change. (B) Relative mRNA expression levels of *RUNX2*, *Osterix*, ALP, *OCN*, *IHH* and *SMO* significantly increased after the application of cyclic stretch in OLF cells. * $P < 0.05$, ** $P < 0.01$ vs. 0 h. Data are presented as the mean \pm SD. N=3. OLF, ossification of ligamentum flavum; LF, ligamentum flavum; *RUNX2*, Runt-related transcription factor 2; ALP, alkaline phosphatase; *OCN*, osteocalcin; *IHH*, Indian hedgehog.

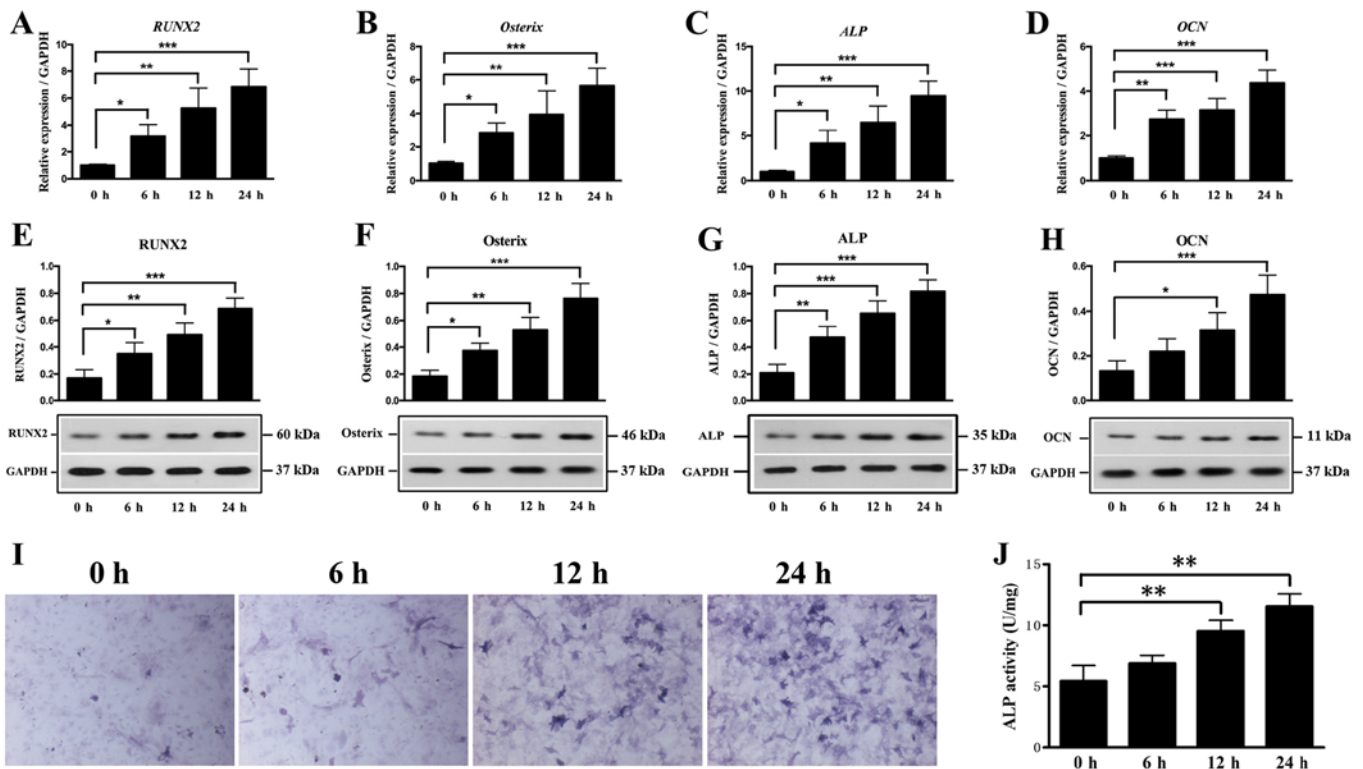


Figure 4. Effect of cyclic stretch on osteogenic genes and ALP activity. Expression levels of (A) *RUNX2*, (B) *Osterix*, (C) ALP and (D) *OCN* in OLF cells were detected after cyclic stretch treatment for 0, 6, 12 and 24 h. Protein expression levels of (E) *RUNX2*, (F) *Osterix*, (G) ALP and (H) *OCN* after cyclic stretch. (I) Representative images of ALP staining. Magnification, x400. (J) Cyclic stretch increased ALP activity in OLF cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are presented as the mean \pm SD. N=3. OLF, ossification of ligamentum flavum; *RUNX2*, Runt-related transcription factor 2; ALP, alkaline phosphatase; *OCN*, osteocalcin.

are shown in Fig. 5. It was found that cyclic stretch significantly increased the mRNA and protein expression level of *IHH* at 6, 12 and 24 h (Fig. 5A and B). Moreover, the mRNA and protein expression levels of *SMO* were significantly increased at all time points (Fig. 5C and D). Cyclic stretch also significantly upregulated the expression level of *GLI1* at 6, 12 and 24 h, and the mRNA expression level of *GLI2* at 12 and 24 h (Fig. 5E and F); however, no significant effect on *GLI3* expression level was detected (Fig. 5G). Therefore, the present results suggested that local stress could promote

the expression of osteogenic markers by regulating the *IHH* signaling pathway in OLF cells.

IHH signaling is involved in osteogenesis induced by cyclic stretch. Cyclopamine (Cpn) can downregulate the *IHH* signaling pathway by binding to *SMO* directly, which is a key component in *IHH* signaling (34). To examine whether cyclic stretch can directly affect the expression levels of *RUNX2*, *Osterix*, ALP and *OCN* via the *IHH* signaling pathway, OLF cells were treated with cyclic stretch alone or both cyclic stretch and Cpn. Fig. 6

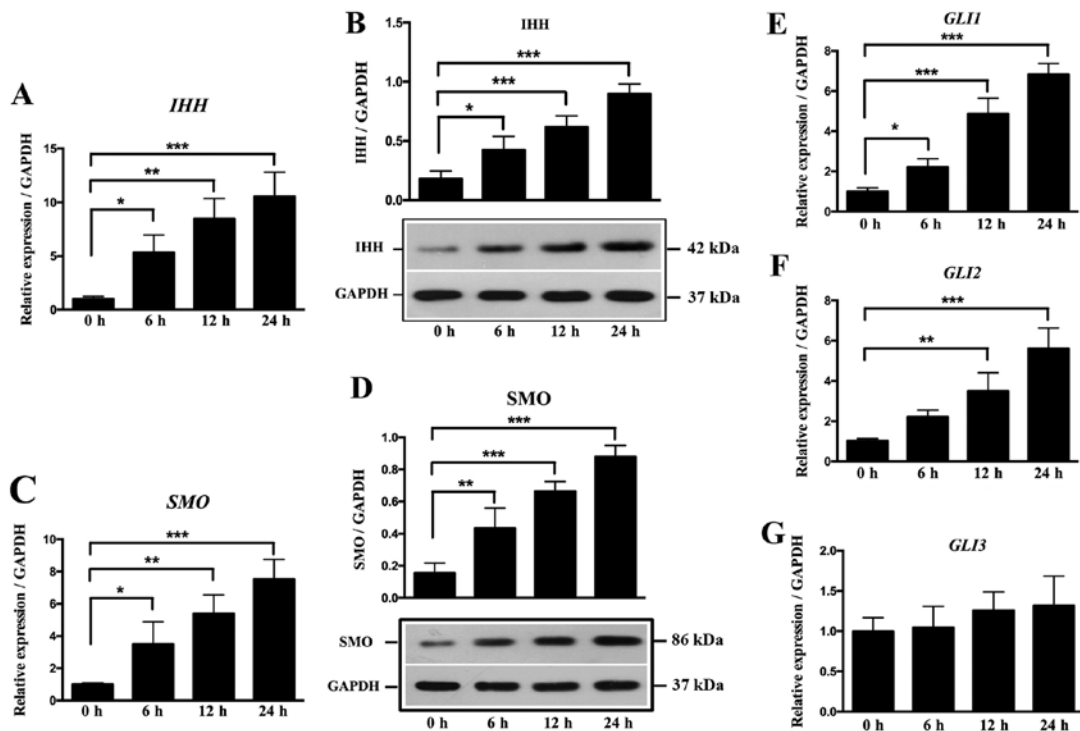


Figure 5. Effect of cyclic stretch on the expression of molecules involved in the *IHH* signaling pathway in OLF cells. Cyclic stretch of OLF cells resulted in significant increases in the (A) mRNA and (B) protein expression levels of *IHH*. Cyclic stretch of OLF cells resulted in significant increases in the (C) mRNA and (D) protein expression levels of *SMO* at 6, 12 and 24 h. mRNA expression levels of (E) *GLI1* and (F) *GLI2* were significantly increased after cyclic stretch. (G) No significant effect was observed on the expression level of *GLI3*. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are presented as the mean \pm SD. N=3. OLF, ossification of ligamentum flavum; *RUNX2*, Runt-related transcription factor 2; *ALP*, alkaline phosphatase; *OCN*, osteocalcin; *IHH*, Indian hedgehog; *SMO*, Smoothed; GLI, GLI family zinc finger.

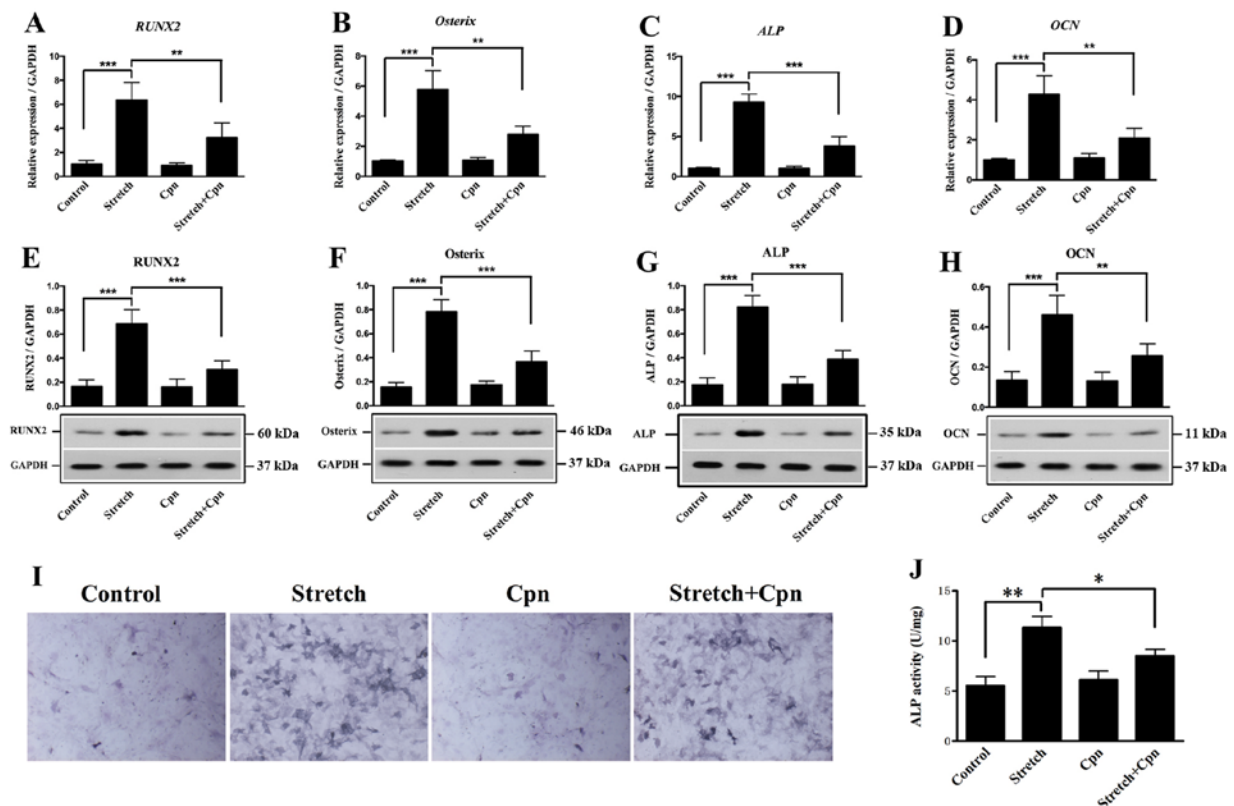


Figure 6. Effect of the Indian hedgehog signaling pathway inhibitor Cpn on the expression levels of *RUNX2*, *Osterix*, *ALP* and *OCN* in OLF cells. mRNA expression levels of (A) *RUNX2*, (B) *Osterix*, (C) *ALP* and (D) *OCN*. Protein expression levels of (E) *RUNX2*, (F) *Osterix*, (G) *ALP* (H) and *OCN* were significantly decreased in the stretch + Cpn group compared with cyclic stretch alone. (I) Representative images of *ALP* staining. Magnification, x400. (J) *ALP* activity was significantly reduced in the Cpn + stretch group compared with cyclic stretch alone. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are presented as the mean \pm SD. N=3. OLF, ossification of ligamentum flavum; *RUNX2*, Runt-related transcription factor 2; *ALP*, alkaline phosphatase; *OCN*, osteocalcin; Cpn, cyclopamine.

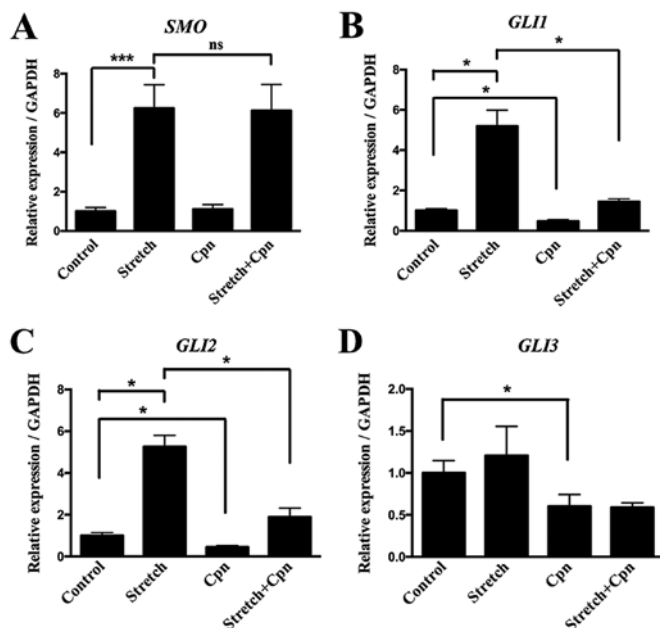


Figure 7. Effect of Cpn on the expression levels of Indian hedgehog signaling-related genes in OLF cells. mRNA expression levels of (A) *SMO*, (B) *GLI1*, (C) *GLI2* and (D) *GLI3*. * $P < 0.05$, *** $P < 0.001$. Data are presented as the mean \pm SD. $N = 3$. OLF, ossification of ligamentum flavum; Cpn, cyclopamine; ns, not significant; *SMO*, Smoothed; *GLI*, GLI family zinc finger.

showed the expression levels of *RUNX2*, *Osterix*, *ALP* and *OCN* after Cpn treatment. It was found that the expression levels of all genes were significantly decreased in the Cpn + cyclic stretch group compared with the cyclic stretch alone group (Fig. 6A-H). Furthermore, ALP activity was reduced with Cpn treatment compared with cyclic stretch alone (Fig. 6I and J). It was demonstrated that cells treated with cyclic stretch showed a significant increase in the expression levels of the downstream IHH signaling molecules *SMO*, *GLI1* and *GLI2*. However, cyclic stretch did not significantly alter the expression of *GLI3* compared with the control group. Moreover, Cpn alone decreased the expression of *GLI1*, *GLI2* and *GLI3* compared with the control group. The effect of cyclic stretch on the expression of *GLI1* and *GLI2* was decreased by the addition of Cpn (Fig. 7). Thus, the present results provided evidence of the role of the IHH signaling pathway in the pathogenesis of OLF.

Discussion

OLF in East Asian populations is more common compared with individuals from other regions, and is characterized by pathological heterotopic ossification in the LF (5,6). Various genetic, mechanical and inflammatory factors are involved in the development OLF (35), but the molecular mechanistic pathways remain to be elucidated. IHH signaling is an essential signaling pathway mediating heterotopic ossification of the extremities (19). To the best of our knowledge, the present study is one of the first to examine whether IHH signaling mediates osteogenic differentiation during OLF. It was found that the expression levels of osteoblastic markers and *IHH* were higher in OLF tissues compared with in LF tissues. Moreover, cyclic stretch elevated the expression levels of osteogenic differentiation markers in the LF cells of patients with OLF.

Furthermore, it was demonstrated that osteogenic induction was attenuated by inhibiting IHH signaling. Collectively, the present results suggested that the IHH signaling pathway may play a role in the pathogenesis of OLF.

The development of OLF involves chondrogenic and osteogenic differentiation of the fibroblasts into osteoblasts (7). Therefore, examining osteogenic differentiation of the LF may help to understand the pathogenesis of OLF. Previous studies have identified higher expression levels of osteogenesis-related genes in the OLF cells and tissues (16,36). Using RNA sequencing, Yang *et al* (36), examined gene expression profiles of LF cells in both immature and mature ossification groups. Moreover, Yang *et al* (36) found that 42 osteogenesis-related factors were differentially expressed, of which the expression levels of *SOX11*, *RUNX2*, *Osterix* and secreted phosphoprotein 1 were increased. Cai *et al* (16), performed immunohistochemistry in the decalcified paraffin OLF sections, and found that β -catenin and *SOX9* were immunopositive in premature chondrocytes. In addition, Cai *et al* (16) identified that the expression of *RUNX2* and *OPN* was significantly increased in hypertrophic chondrocytes surrounding the calcification front. According to a previous *in vitro* study (37), cultured OLF cells have higher mRNA expression levels of β -catenin, *RUNX2*, *SOX9* and *OPN* compared with non-OLF cells. In line with these previous results, the present study found that the expression levels of the osteoblastic markers *RUNX2*, *Osterix*, *ALP* and *OCN* were higher in OLF tissues compared with LF tissues. Thus, the process of fibroblasts differentiating into osteoblasts may be an important physiopathological mechanism in OLF.

Mechanical and genetic factors may facilitate the development of OLF (5). Previous studies have demonstrated that the cyclic stretch could initiate and promote the ossification process by modulating the osteogenic differentiation of the spinal ligament cells (15,38). The present study applied cyclic stretch to OLF cells, and found that both ALP activity and the expression levels of *RUNX2*, *Osterix*, *ALP* and *OCN* were elevated as the duration of the stretch force was increased; these results were in line with previous results (11,39). Ning *et al* (39), showed that cyclic mechanical application produces higher ALP activity and expression levels of *ALP*, *BMP2*, *OPN* and *Osterix* in the multiple-level OLF group compared with the single-level group. Furthermore, a previous *in vitro* study revealed that mechanical stress significantly increases the mRNA expression levels of *RUNX2*, *ALP* and *OCN* in OLF cells compared with the control group (11).

The present results indicated that cyclic stretch increased the expression levels of *IHH*, *SMO*, *GLI1* and *GLI2* genes, which are involved in the IHH signaling pathway, at different time points. Furthermore, the present study blocked the IHH signaling pathway by treating OLF cells with Cpn, which is a potent inhibitor of the IHH signaling pathway (40). It was found that the impact of cyclic stretch on the expression levels of osteogenic genes was significantly attenuated after Cpn treatment. Thus, the present results suggested that cyclic stretch may affect osteogenic differentiation of OLF cells via the IHH signaling pathway. IHH signaling plays a critical role in prenatal endochondral bone formation and limb development, as well as postnatal bone formation (18,41). Therefore, the complete ablation of IHH signaling can lead to the closure of the growth

plate and immature bone growth (18,41). IHH signaling may also participate in the bone repair process after a fracture (42). Furthermore, abnormal bone development and ectopic bone formation due to overexpression of the IHH signaling have been previously reported (22,43). In the present study, the expression of *IHH*, *SMO*, *GLI1* and *GLI2* was upregulated by cyclic stretch, suggesting the IHH signaling pathway was involved in the pathogenesis of OLF. By contrast, it has been shown that the upregulation of patched 1 (*PTCI*) can decrease IHH signaling function (44). Moreover, it has been suggested that several IHH molecules can interact with the increased *PTCI*, resulting in reduced *SMO* release (45). However, the present study did not identify any changes in the expression level of *GLI3* under cyclic stretch conditions. Thus, *GLI3* may not play a critical role in the regulation of osteogenic differentiation of OLF cells.

There are certain limitations to the present study. First, only the IHH signaling pathway was investigated, and other signaling pathways are likely involved in the differentiation of OLF cells. A previous study (16) demonstrated that cyclic stretch significantly elevated the expression levels of β -catenin, *RUNX2*, *SOX9* and *OPN*, which indicates that the β -catenin signaling pathway may be involved in the pathogenesis of OLF. Second, the present study was conducted *in vitro*. Therefore, *in vivo* OLF models are required to assess the therapeutic effect of inhibiting IHH signaling on the progression of OLF. In addition, only the effects of mechanical stress on osteogenic differentiation were investigated, and other types of stress, such as compression and shear force, were not examined. Thus, further studies are required to investigate the specific mechanisms and therapeutic targets for OLF.

In conclusion, the present results suggested that the expression levels of osteogenic markers and IHH signaling genes were higher in OLF cells. Moreover, inhibition of the IHH signaling pathway reduced the expression levels of osteogenic markers. Thus, the IHH signaling pathway may be involved in the progression of OLF. If cell differentiation can be regulated by modulating this pathway, the homeostatic balance of cell differentiation could be maintained and the ossification of spinal ligaments may be prevented..

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RG and CS performed experiments, analyzed data and prepared the manuscript. CY and YZ analyzed and interpreted

the data, and prepared the manuscript. XC and XZ designed experiments, analyzed data and finalized the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Second Military Medical University, and the experimental procedures were performed in accordance with the standard guidelines. Informed consent was obtained from the patients or relatives for the collection of LF during surgery, and the use of these samples for scientific research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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