Uniaxial Cyclic Stretch Enhances Osteogenic Differentiation of OPLL Derived Primary Cells via YAP-Wnt/β-catenin Axis

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Uniaxial Cyclic Stretch Enhances Osteogenic Differentiation of OPLL Derived Primary Cells via YAP-Wnt/β-catenin Axis

Running title: YAP-Wnt/β-catenin axis promotes OPLL progression

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Abstract

The pathogenesis of ossification of the posterior longitudinal ligament (OPLL) remains inadequately understood. Mechanical stimulation is one of the important pathogenic factors of OPLL. As one of the mechanical stimulation transduction signals, the yes associated protein (YAP) interacts with the Wnt/β-catenin signaling pathway, which plays an important role in osteogenic differentiation. This study aimed to demonstrate the role of YAP-Wnt/β-catenin axis in cell differentiation induced by mechanical stress. Primary cells extracted from posterior longitudinal ligament tissues from OPLL or non-OPLL patients were subjected to sinusoidal uniaxial cyclic stretch (5%, 0.5 Hz, 3 days). The expression of RUNX2, Collagen I, Osterix, OCN and ALP were
compared between the static group and the experimental group. In addition, the cytoskeleton was observed using phalloidin staining, and the phosphorylation states and nuclear location of YAP were identified by immunofluorescence. The results showed that mechanical stretching loading increased the expression of osteogenic genes and proteins in the OPLL group, while had no significant effect on the control group. When OPLL cells were stretched, the YAP exhibited obvious nuclear translocation and the Wnt/β-catenin pathway was activated. Knocking down YAP or β-catenin can weaken the impact on osteogenic differentiation induced by mechanical stimulation. YAP-mediated mechanical stimulation promotes osteogenic differentiation of OPLL cells through Wnt/β-catenin pathway, and this progress is independent of Hippo pathway.

**Key words:** Ossification of the posterior longitudinal ligament (OPLL); Mechanotransduction; Heterotopic ossification; Yes-associated protein (YAP); Cytoskeleton remodeling; Wnt/β-catenin signaling

**List of Abbreviations:**

- OPLL: Ossification of the posterior longitudinal ligament
- YAP: Yes-associated protein
- RUNX2: Runx-related transcription factor 2
- COL1: Collagen 1
- OCN: Osteocalcin
- ALP: Alkaline phosphatase
- FOP: Fibrodysplasia ossificans progressive
- FBS: Fetal bovine serum
- PDMS: Polydimethylsiloxane
- GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
- HRP: Horseradish peroxidase
- BSA: Bovine serum albumin
- DAPI: 6-diamidino-2-phenylindole
- RhoA: Ras homolog family member A
Introduction

Ossification of the posterior longitudinal ligament (OPLL) is a pathological condition of heterotopic bone formation of the spinal ligament, leading to spinal cord compression and neurological symptoms (Zhang et al., 2021). Previous studies have investigated the osteogenic characteristics of spinal ligament fibroblasts in patients with OPLL, yet its pathogenesis is still not fully understood (Ohara, 2018; Yan et al., 2017). Clinical reports showed that in comparison with stand-alone laminoplasty, cervical laminoplasty combined instrumented fusion suppressed the progression of OPLL (Kang et al., 2019; Katsumi et al., 2016; Lee et al., 2017). Vivo experiments in animal models obtained a similar conclusion, when rat tail or thoracic vertebra was subjected to repeated excessive traction stimulation, heterotopic ossification of local ligaments occurred (Tsukamoto et al., 2006; Zhao et al., 2021). Although these clinical features have been described in detail, the specific molecular mechanism remains to be elucidated. The underlying mechanism may be related to the high expression of osteogenesis related genes (OPN, COL1, BMP-2) promoted by mechanical stimulation. In addition, several vitro experiments have confirmed that cytokines and transcriptional factors may have been proposed to play a key role in this process (Chen et al., 2018a; Nakajima et al., 2020; Shi et al., 2019a; Shi et al., 2019b; Sugita et al., 2020). When the physical conditions that can be sensed by cells change, such as changes in matrix stiffness or geometry, or there are fluid shear forces or tensile stresses loading on the cells, the mechanical signals may be converted into recognizable biological signals, which will affect the biological behaviors of cells (Czeisler et al., 2016; Guo et al., 2015; Kim and Asthagiri, 2011).

Yes-associated protein (YAP) is a transcriptional co-activator protein that shuttles between the nucleus and cytoplasm, and regulates gene expression through nuclear translocation (Moya and Halder, 2019). The activity of YAP can be regulated by cell morphology, cell density, extracellular matrix stiffness, and various mechanical stresses (tensile force, fluid shear force, etc) (Aragona et al., 2020; Cai et al., 2021; Gao et al., 2020). When the active YAP enters nucleus, it will participate in the regulation of other signal transduction pathways, such as Wnt/β-catenin pathway, Hippo pathway and TGF-β pathway, so as to play a biological role of each pathway. Previous studies have shown that the YAP-Wnt/β-catenin axis plays an important role in the process of cell osteogenic differentiation. When cells sense changes in the external physical cues, YAP removes the phosphate
group to form active-YAP and activates the Wnt/β-catenin pathway, thereby promoting cell osteogenic differentiation (Huang et al., 2020; Zhao et al., 2022).

In addition, YAP can directly bind to RUNX2 after entering the nucleus and assist RUNX2 in initiating osteogenic transcriptional regulation (Zhou et al., 2022). Yang et al. showed that YAP-Shh self-amplifying loop was an important cause of heterotopic ossification of tendon tissues in fibrodysplasia ossificans progressive (FOP) (Cong et al., 2021). In addition, the high activation state of YAP/RhoA pathway stimulated by mechanical stress will cause the abnormality of BMP pathway that lead to promote osteogenic differentiation of FOP mesenchymal progenitors (Stanley et al., 2019). Given that both OPLL and FOP are endochondral heterotopic ossification, the specific mechanism of YAP in the occurrence and development of OPLL has not been reported. In this study, we explored the possible mechanism of YAP in accelerating OPLL progression induced by mechanical stimulation, in order to provide new insights into the pathogenesis of OPLL.

2 Materials and Methods

2.1 Clinical diagnosis and spinal ligament samples

Ten patients presenting with cervical OPLL and ten non-OPLL patients as control groups were selected for this study (Table 1). The diagnosis of OPLL or non-OPLL was confirmed by clinical signs and symptoms and cervical spine computed tomography. All patients underwent cervical anterior decompression surgery, and the posterior longitudinal ligaments (PLL) were collected intraoperatively. All patients enrolled in this study provided written informed consent for the research study protocol. The ethics approvals were provided by the institutional review board of the Seventh Affiliated Hospital of Sun Yat-sen University (2020SYSUSH-055).

2.2 Primary ligament fibroblasts culture

Posterior longitudinal ligament specimens were harvested during the anterior cervical decompression surgery. The ligament tissues were carefully dissected from a non-ossified site to avoid any possible contamination with osteogenic cells. Enzymatic digestion method was applied to PLL cell extraction according to protocol (Chen et al., 2018a). In short, PLL tissues were entirely minced and digested by 0.2% type II collagenase (Gibco, Life Technologies, America) for 4 h at 37°C. The digested tissues were then transferred to DMEM/F12 (Gibco, Life Technologies, America) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, America) and 1%
penicillin/streptomycin (Gibco, Gibco, Life Technologies, America) in an incubator at 5% CO₂ and 37°C. When confluent, the cells should be passaged after digesting with 0.25% Trypsin-EDTA (Gibco, Gibco, Life Technologies, America). Cells transferred to the third passage were used in the following experiments. According to previous reports, inverted phase-contrast microscopy and vimentin staining were used for cell type identification. Vimentin positive rates of more than 90% were considered qualified for cell extraction.

2.3 Stretch apparatus

To mimic the tensile force exerted on cells during neck flexion and extension, a custom-made uniaxial dynamic apparatus was applied for dynamic stretching culture. The apparatus is characterized by uniaxial sinusoidal stretch stimulation with multiple chambers, making it possible to keep the same frequency between each unit. Polydimethylsiloxane (PDMS, SLYGARD, America), with excellent cytocompatibility and high translucency, was used to fabricate stretchable cell culture dishes according to a previous protocol (Rodriguez et al., 2019).

2.4 Application of cyclic stretch to cells.

Before cells were seeded at a density of 1×10⁴/cm², the flexible-bottomed plates were coated with type I collagen with 5µg/cm² (Gibco, America). After reaching 80% confluence, the medium was refreshed and cyclic tensile force (5% amplitude) was applied to OPLL cells and non-OPLL cells at a frequency of 0.5 Hz for 9 h per day. Non-OPLL cells that were maintained in identical conditions without being stretched were used as control.

2.5 RNA extraction, cDNA synthesis, and quantitative real-time PCR analysis

The total RNA of cells was isolated by RNAeasy™ Animal RNA Isolation Kit (R0027, Beyotime, China) and 400ng of RNA was then converted to cDNA using a cDNA Synthesis Kit (Takara, Japan).

RT-qPCR was performed using Power Up SYBR Green Master Mix (Thermo Fisher Scientific, America) on a Real-Time System (Bio-Rad, America). As described in protocol, each reaction mixture consisted of 5µl of 2x Power Up SYBR Green Master Mix, 2µl of nuclease-free water, 0.5µl each of 10 µM forward and reverse primers and 2µl of cDNA. The applied cycle conditions are shown below: 50°C for 2 minutes and 95°C for 2 minutes followed by 44 cycles of 15s at 95°C and 1 minute at 60°C. Primer sequence design, primer construction and purification were provided by Sangon Inc. The sequences are provided in Table 2. Results were normalized to housekeeping gene
glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression using the $2^{\Delta\Delta Ct}$ algorithm.

### 2.6 Total protein extraction and western blot analysis

Total protein was extracted from with RIPA buffer (Thermo Fisher Scientific, America) and the concentrations of total proteins were determined by a BCA kit (Boster, China). Proteins were electrophoresed in premade polyacrylamide gels containing SDS, and transferred to polyvinylidene fluoride (PVDF) membranes from gels. After blocking with 5% non-fat milk (Solarbio, China) for 1 h, the PVDF membranes were incubated with primary antibody overnight at 4°C and then incubated with secondary antibody (coupled with horseradish peroxidase) for 1 h at room temperature. The protein signal was visualized by an ECL chemiluminescence kit (EpiZyme, China), and the grayscale value of band was quantified using Image J software.

### 2.7 Alkaline phosphatase staining

The presence of ALP in the cell layers was assessed according to the manufacturer’s instructions (Beyotime, China). The OPLL cells and non-OPLL cells from the control and stretch treatment groups were rinsed with PBS three times and fixed with 4% paraformaldehyde for 20 min. The fixed cells were rinsed with PBS three times again and then BCIP/NBT liquid substrate was added to each stretchable dished for 1 h at 37°C. Finally, cells were washed with ddH2O and then captured with a digital camera and observed with optical phase contrast microscope.

### 2.8 Immunofluorescence analysis

After culturing and being stretched in the flexible culture dishes, OPLL cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature and then permeabilized with 0.3% Triton X-100 (sigma, America) as well as blocked with 5% bovine serum albumin (BSA, BioFroxx, Germany) for 1 hour. The permeabilized cells were incubated with a YAP antibody (Abcam, 1:1000, Britain) diluted in PBS at 4 °C overnight. Washed with PBS for 5 minutes 3 times, the cells were probed with goat anti-rabbit IgG in darkness for 1 h. What’s more, cells were counterstained with phalloidin (Meilunbio, China) in order to better observe the changes of the cytoskeleton according to the kit instructions. At last, nuclei were marked with 6-diamidino-2-phenylindole (DAPI, Abcam, Britain). The fluorescent images were acquired using a fluorescence microscope (Leica, Germany).

### 2.9 Lentivirus packaging and cell transfection

ShRNA sequences targeting YAP or CTNNB1 (β-catenin) as well as scrambled control
sequences were cloned into a GV248 or GV493 vector for lentivirus packaging (Genechem, China). Primer details (forward F and reverse R) are listed in Table 2. To produce lentivirus for exogenous YAP and CTNNB1 knock-down, the core plasmid was co-transfected with the packaging plasmids pMD2.G and psPAX2 into 293T cells by lipofectamine 3000 transfection kit (Invitrogen, America). The medium was refreshed 6 h post-transfection. Supernatants containing virus were collected at 24 and 48 hours. When target cells were grown to 60% confluence in 6-well plates, they will be transfected with virus with 8 μg/ml polybrene (Beyotime, China). And when cells fusion rate reached to 90%, they were transferred to 10 cm² dishes. Fluorescence microscopy were used to observe whether the cells have been successfully transfected, and the transfected cells were labeled green. To obtain stable strains, all cells are screened with 2 μg/ml puromycin. The knockout efficiency of the stably transfected cells was tested by qPCR and western blot. Compared with the control group, the cells with the highest transfection efficiency were selected for further experiments.

2.10 Statistical analysis

Statistical analyses were performed using SPSS version 20 (SPSS, Chicago, USA). Quantitative data are presented as mean ± standard deviation. Differences between two samples were conducted using an unpaired student’s t-test when the data met a normal distribution, otherwise the Mann-Whitney U test will be used. For comparisons of more than two groups, one-way analysis of variance followed by a Turkey test was performed. Statistical significance was set at p<0.05.

3 Results

3.1 Identification of primary cell types extracted from posterior longitudinal ligament

The primary cells were transferred to P3 and then used in subsequent experiments. Vimentin staining was required to determine the cell type. Given that vimentin is a type of intermediate filament that mainly resides in fibroblast, here we took vimentin as a fibroblast marker, and performed the immunofluorescence assay (Fig. 1A). The results showed that the cells derived from the cervical PLL were mostly fibroblast-like cells, with red fluorescence in the cytoplasm.

3.2 Biocompatibility of the stretchable culture plate and evaluation of cell status after dynamic culture

The morphology of cells grown on the plate is an important indicator for assessing cell-material interactions. The effect of mechanical stretch on cell viability was assessed by live/dead staining
after stretching immediately. The results showed no significant differences in cell viability and proliferation between mechanically loaded cells and static cells (Fig.1 B1-B2). The SEM images showed that cells on the surface of the material appear good adhesion with pseudopodia birth (Fig. 1 C1). These results proved that the composite exhibits good cell-material interaction and good biocompatibility. In addition, when the cells received mechanical stretching stimulation, they gradually changed from the spread state to the bar shape observed by scanning electron microscopy (Fig. 1 C1-C2).

3.3 Stretching stimulation promotes the osteogenic genes expression in OPLL cells

RUNX2, COL1, Osterix, OCN and ALP are considered important markers of osteogenic differentiation. The qPCR results showed that the OPLL group demonstrated enhanced ossification as reflected by the induction of osteogenic markers, namely, ALP, COL1, Osterix, OCN and ALP, at the mRNA levels (Fig. 2A) and protein levels (Fig. 2B). In parallel, there was no significant difference in the elevation of these markers in non-OPLL group. Alkaline phosphatase staining showed the same results to what has been confirmed with qPCR. As shown in figure 2C, the ALP activity was significantly increased in the stretched OPLL-derived cells but not the control cells. In summary, these results demonstrated that cyclic stretch significantly promoted the osteogenic differentiation of OPLL-derived cells while the control cells were less affected by mechanical stimulation.

3.4 Stretching stimulation induces actin cytoskeleton reorganization and YAP nuclear translocation

In order to investigate how the cytoskeleton changes to respond to mechanical stretching, OPLL-derived primary cells were subjected to cyclic mechanical stretching for 9 h per day at a 5% amplitude. Subsequently, the F-actin was stained by phalloidin. We observed that cells displayed a markedly different cell morphology after dynamic culture. As shown in Figure 3A, stretching loading caused cellular elongation, while the static cells showed a flattened, pancake-like shape, indicating that stretching induced actin cytoskeleton reorganization in these cells. The remodeling of the cytoskeleton leads to a change in the location of YAP protein expression. The immunofluorescence results showed that YAP was distributed primarily in the cytoplasm in the static group, while it began to migrate progressively to the nucleus when cells received tensile stress stimulation (Fig. 3C). When the stretching force lasted for 9 hours, the distribution of YAP in the
nucleus and cytoplasm was approximately equal. When the cells were subjected to continuous 3d
(9h/d) dynamic stretch culture, YAP was predominantly distributed in the nucleus. In order to
measure the shape change of cells, we introduced a concept of cell aspect ratio. This concept could
be defined as the length of the longest axis divided by the length of the short axis across the cell
nucleus. The measurement results demonstrated that the cells that were cultured dynamically may
exhibit a higher aspect ratio compared with the static group (Fig. 3B).

This observation was further confirmed by RT-qPCR and western blot analysis. Cell elongation
increased the expression of gene as well as dephosphorylated protein of YAP. At the meantime, we
found that the expression of ras homolog family member A (RhoA), a cytoskeleton regulation gene,
was up-regulated with the increase of cell stretch (Fig. 3 D-E). This indicated that cytoskeletal
changes activate the expression of RhoA, which in turn arouse the downstream YAP expression.

Taken together, the results indicated that OPLL-cells could respond to mechanical stretch by
adjusting cytoskeleton organization. Driven by such cytoskeletal morphological changes, YAP is
translocated from the cytoplasm to the nucleus to participate in downstream transcriptional
regulation.

3.5 Activation status of Hippo and Wnt pathways after stretch stimulation

YAP, as a key gene of crosstalk between Hippo pathway and Wnt/β-catenin pathway, has been
shown to dephosphorylate and enter the nucleus to regulate the expression of osteogenic genes after
mechanical stimulation. Here, we detected the expression of key genes in these two pathways. The
results showed that the expression of LATS, MST and TEAD in OPLL cells did not change
significantly after mechanical stimulation (Fig.4 A-B), while the expression of β-catenin
significantly increased (Fig.4 C-D). WB detection reached a similar result with qPCR, and there
was no significant change in the expression of LATS and TEAD, that is, there was no significant
change in the Hippo pathway. However, the expression of phosphorylated β-catenin was
significantly decreased, while the non-phospho (active) β-catenin was significantly increased,
suggesting that the Wnt/β-catenin pathway was activated abnormally, and β-catenin was involved
in the regulation of downstream biological behavior.

3.6 Knockdown of YAP or β-catenin reduced osteogenic gene changes in OPLL cells induced
by tensile mechanical stimulation

OPLL cells with YAP or β-catenin knockdown were generated using an shRNA lentiviral
vector (Fig. 5A). Immunofluorescence results showed that both lentiviruses obtained good transfection effects in cells (Figure 5B). According to the results of qPCR and WB, the best transfection sequence was selected for subsequent experimental study (Figure 5 C-D). After successful transfection, cells were subjected to stretch stimulation with described parameters to evaluate the effect of tensile mechanical stimulus on osteogenic gene variation. Compared with scramble groups, the YAP or β-catenin shRNA-transduced OPLL cells expressed significantly lower levels of RUNX2, COL1, Osterix, OCN, and ALP. Meanwhile, OPLL cells that have been knocked down with both YAP and β-catenin decreased responses to mechanical stimulation. Compared with one gene alone, knockdown of two genes at the same time had lower expression of osteogenic markers (Fig. 5 E-F). Our findings suggested that the downregulation of YAP or β-catenin expression relieved the osteogenic differentiation of OPLL cells driven by stretch mechanical stimulation.

Discussion

OPLL, a disease characterized by progressive heterotopic ossification of the PLL (Xu et al., 2022), is a common disease in Asia with an incidence of 1.9% - 4.3% (Yan et al., 2017). OPLL is a multifactorial disease that involves genetic and environmental factors (Boody et al., 2019). Among all environmental factors, cyclic tensile strain plays an important role in the progression of ossification (Nishida et al., 2011). The anatomical features of the PLL determine its susceptibility after mechanical stimulus loading. PLL is a two-layer structure that is subjected to distraction stress longitudinally and regulates spinal instability. The superficial layer is in close contact with the dura mater and bridges 3 or 4 vertebra, whereas the deep layer is posterior to the vertebral body and connects two adjacent vertebrae. Due to these anatomical features, the PLL has distraction tension along its longitudinal axis and a large mechanical overload. Given these anatomical features when the tensile stress is oppressive or the frequency is high, the ligament tissue may undergo pathological changes, such as inflammation or local heterotopic ossification (Ranganathan et al., 2015). Previous studies have confirmed that when OPLL-derived cells were subjected to mechanical stretching stimulation, the expression of osteogenic genes in cells was up-regulated, while there was no significant change in non-OPLL cells (Bhatt et al., 2007; Yang et al., 2011). They believed that tensile stress may increase of expression of connexin43 and abnormally activate multiple signaling
pathways in OPLL-derived primary cells (Chen et al., 2017; Chen et al., 2016; Sugita et al., 2020).

Furthermore, endoplasmic reticulum stress response, the disorder of ATP, prostaglandin I2 and endothelin metabolism were also reported to play a role in this pathological process (Iwasawa et al., 2006; Ohishi et al., 2003; Sawada et al., 2008; Shi et al., 2019a). Although a few studies have elucidated the molecular mechanism for the increase of osteogenic genes caused by mechanical stimulation from multiple perspectives, there are still many issues that have not been fully understood, such as how OPLL cells perceive mechanical stimulation and how mechanical signals are converted into biological signals that can be recognized by cells. Based on these questions, we conducted this study.

In this study, we not only revealed the possible mechanism of osteogenic differentiation of OPLL cells after tensile mechanical stimulation but also elucidated how cells sense mechanomechanical signals. Mechanotransduction starts with the ability of cells to probe the physical features of the microenvironment through integrins and other adhesive proteins and to counterbalance extracellular forces by adjusting their own tensional state through actomyosin contractility and organization of the F-actin cytoskeleton (Totaro et al., 2018).

Our results suggested that when cells receive tensile mechanical stimulation, the cytoskeleton will remodel and YAP will dephosphorylate thus letting it transfer from cytoplasm to nucleus. After entering the nucleus, YAP well directly bind to RUNX2 or activate Wnt/β-catenin pathway to promote osteogenic differentiation of cells, while these biological effects are independent of the Hippo pathway.

YAP, as one of the essential mechanosensitive transcriptional activator (Aragona et al., 2020; Zhong et al., 2013a; Zhong et al., 2013b), has been reported to play an important role in the osteogenic differentiation of ligament cells induced by stretch stimulation (Jia et al., 2019; Yang et al., 2018). Phosphorylated YAP is located in the cytoplasm and interacts with the cytoskeleton, preventing its entry into the nucleus and gradually degrading in the cytoplasm (Lin et al., 2017; Mo et al., 2012). Previous studies have shown that different stretching amplitudes have different effects on cell differentiation, and the stretching amplitude of 5%-10% promotes the differentiation of cells towards osteogenic direction. In addition, the influence of stretch regulation on YAP is also related to stretch amplitude. The stretching amplitude of 5% can significantly promote YAP activation and motivate it to participate the downstream transcriptional regulation (Chen et al., 2018b; Cui et al.,...
Cui et al. have confirmed that YAP began to transfer from cytoplasm to nucleus when 5% tensile stress was lasted for 3 hours in human umbilical vein endothelial cells. When the dynamic culture time was extended to 6 hours, YAP was almost completely expressed in the nucleus (Cui et al., 2015). These results are consist with ours, 9h stretching stimulation could promote cytoskeleton remodeling and increase YAP expression in the nucleus. When the cell senses that the physical environment has changed, the mechanical signals will be transmitted to integrin receptors on the cell membrane, which will further trigger the change in the expression of focal adhesion. Changes in cell surface proteins induce the remodeling of F-actin and the alteration of cytoskeletal structure that triggers the activation of the Rho pathway. The activated RhoA will contact the non-phospho YAP, prompting it to enter the nuclear triggering the expression of a series of transcription factors, and ultimately affecting cell differentiation (Saidova and Vorobjev, 2020; Xue et al., 2017). In this process, the shift of F-actin is necessary for mechanical signals transduction, and YAP will lose its activity when F-actin is blocked or the Rho pathway is inhibited (Mammoto and Ingber, 2009). Similar results were observed in OPLL-derived cells, when the cell shape changes from flat to long spindle, the position of YAP protein expression will change.

In addition, changes in the cytoskeleton structure alter the mechanical connection between the cell membrane and the nuclear envelope. Alberto et al. showed that force applied to the nucleus directly drives YAP nuclear translocation by decreasing the mechanical restriction of nuclear pores to molecular transport (Elosegui-Artola et al., 2017). When cells exposed to a high matrix stiffness environment, there will establish a mechanical connection between the nucleus and the cytoskeleton, that allows forces exerted through focal adhesions to reach the nucleus. Therefore, the stretched nuclear pores reduce their mechanical resistance to molecular transport and increase YAP nuclear import. The junction of cells and the open state of membrane proteins also affected the nuclear expression of YAP. Super-resolution imaging showed that different cell density will affect the location of YAP expression in cells, and a high cell density will inhibit YAP entry into the nucleus (Gao et al., 2020). What's more, the open state of PIEZO1, a mechanically sensitive protein, could also affect the activation of YAP. Pathak et al. showed that knocking out the PIEZO1 gene will inhibit the stretched-stimulation-induced nuclear transfer of YAP in the neural stem cells (Pathak et al., 2014).

Similar to previous research, we found that the dephosphorylation of YAP induced by
stretching stimulation was involved in downstream regulation, and this progress was dependent of Wnt/β-catenin pathway rather than Hippo pathway (Elosegui-Artola et al., 2017; Li et al., 2019). YAP activity is essential for maintaining β-catenin nuclear expression and the YAP-Wnt/β-catenin axis is one of the important links in maintaining bone homeostasis and inhibiting adipogenic differentiation of cells (Jia et al., 2019; Pan et al., 2018). Phosphorylated YAP (inactive form) is an integral component of the β-catenin destruction complex (Azzolin et al., 2014), which traps phosphorylated β-catenin and makes it degraded progressively in the cytoplasm. The regulation of β-catenin through a cytoplasmic destruction complex forms the crux of the Wnt signaling cascade (Schaefer et al., 2018). When active YAP dissociated from the destruction complex, the Wnt pathway is activated by promoting β-catenin detachment from the complex to form activated β-catenin (Azzolin et al., 2014; Schaefer et al., 2018) (Fig. 6).

There were several limitations of our study. Firstly, all PLL tissues we obtained were too fragmented to distinguish the specific anatomical structures, so it is hard for us to conduct HE or immunofluorescence staining to verify our experimental results at the tissue level. Secondly, limited by the function of the custom-made uniaxial dynamic apparatus, only sinusoidal stretching mode can be carried on, that is, the cells did not have any rest during the stretching process, which may not consistent with normal cervical physiological activity. Thirdly, the diagnosis of disease is based on imaging examination and clinical symptoms, which may not be accurate for some people who carry the susceptibility gene but do not develop the disease were classified as the control group. Finally, due to the difficulty in constructing an animal model of local heterotopic ossification of the posterior longitudinal ligament caused by mechanical stimulation, our experiment was conducted in vitro only.

**Conclusion**

Primary cells derived from patients with OPLL exhibit a highly responsive state to tensile stress and uniaxial stretching culture could promote the expression of osteogenic genes in these cells. Mechanical stretching loading induces YAP activation and nuclear translocation by cytoskeleton remodeling, and subsequently facilitates the osteogenesis of OPLL cells. In addition, dephosphorylated YAP activated Wnt/β-catenin pathway to synergistically regulate cell osteogenic differentiation, which is independent of Hippo pathway. More in vivo studies are needed to confirm
the results of the in vitro experiments.

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**Conflicts of Interest:** The authors declare no conflict of interest.
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strain facilitates ossification of the cervical posterior longitudinal ligament via increased Indian


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**Figure legends**

**Fig. 1** (A) Vimentin staining showed that more than 90% of the cells extracted from the tissues of the posterior longitudinal ligament were fibroblasts (scale bar=50 μm). (B) AM/PI staining showed that stretching culture did not increase cell mortality (scale bar=50 μm); (C) Cells form before and after stretching were scanned by SEM images.

**Fig. 2** (A) mRNA expression levels of ossification markers (RUNX2, COLA1, Osterix, OCN and ALP) were compared in non-OPLL and OPLL cells. (B) Protein expression levels of ossification markers were examined between non-OPLL and OPLL cells. (C) ALP staining was used to evaluate alkaline phosphatase activity in different culture conditions.

**Fig. 3** (A) The expression of YAP protein in OPLL-derived primary cells of the posterior longitudinal ligament gradually shifted from cytoplasm to nucleus after receiving stretching stimulation (scale bar=50 μm); (B) Ratio of long axis to short axis after stretching; (C) The proportion of YAP nuclear expression during cell stretching; (D-E) Gene and protein expression levels of YAP and RhoA.

**Fig. 4** (A-D) Dephosphorylated YAP activated Wnt/β-catenin pathway to synergistically regulate cell osteogenic differentiation, which is independent of Hippo pathway.

**Fig. 5** (A) Plasmid construction diagram; (B) The transfection efficiency was determined by immunofluorescence after lentivirus transfection (scale bar=50 μm); (C-D) Knockdown efficiency of transfected cells was detected at gene and protein levels. (E-F) Changes in gene and protein levels of osteogenic markers after transfected cells were subjected to mechanical stretch stimulation.

**Fig. 6** Mechanism of osteogenic differentiation induced by stretching stimulation in OPLL-derived primary cells.

**Table 1** Demographic data for patients in the study. CSM: cervical spondylotic myelopathy.

**Table 2** Primer sequences used in this study.
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793x1411mm (72 x 72 DPI)
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190x338mm (300 x 300 DPI)
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1799x1195mm (72 x 72 DPI)
Table 1: Demographic data for patients in the study.

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F: female; M: male; CSM: cervical spondylotic myelopathy; CDH: cervical disc herniation.
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