UNIAXIAL CYCLIC STRETCH ENHANCES OSTEOGENIC DIFFERENTIATION OF OPLL-DERIVED PRIMARY CELLS *VIA* THE YAP-WNT/B-CATENIN AXIS

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Abstract

The pathogenesis of posterior longitudinal ligament ossification (OPLL) remains inadequately understood. Mechanical stimulation is one of the important pathogenic factors in OPLL. As one of the mechanical stimulation transduction signals, the yes-associated protein (YAP) interacts with the Wnt/β-catenin signalling 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 d). The expression of runt-related transcription factor 2, collagen I, osterix, osteocalcin and alkaline phosphatase were compared between the static and the experimental groups. In addition, the cytoskeleton was detected using phalloidin staining while YAP phosphorylation states and nuclear location were identified using immunofluorescence. The results showed that mechanical stretching loading increased the expression of osteogenic genes and proteins in the OPLL group, while it had no significant effect on the control group. When OPLL cells were stretched, YAP exhibited an obvious nuclear translocation and the Wnt/β-catenin pathway was activated. Knocking down YAP or β-catenin could weaken the impact upon osteogenic differentiation induced by mechanical stimulation. YAP-mediated mechanical stimulation promoted osteogenic differentiation of OPLL cells through Wnt/β-catenin pathway and this progress was independent of the Hippo pathway.

Keywords: Ossification of the posterior longitudinal ligament (OPLL), mechanotransduction, heterotopic ossification, yes-associated protein (YAP), cytoskeleton remodelling, Wnt/β-catenin signalling.

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List of Abbreviations

ALP	alkaline phosphatase
AM	acetyl methoxy methyl ester
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BMP	bone morphogenetic protein
BSA	bovine serum albumin
CDH	cervical disc herniation
COL1	collagen type 1
CSM	cervical spondylotic myelopathy
CTNNB1	catenin beta 1
DAPI	6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
FBS	foetal bovine serum
FOP	fibrodysplasia ossificans progressive
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HE	haematoxylin-eosin
HRP	horseradish peroxidase
LATS	large tumor suppressor kinase
MST	macrophage stimulating
NBT	nitro blue tetrazolium
OCN	osteocalcin
OPLL	ossification of the posterior longitudinal ligament
OPN	osteopontin
PBS	phosphate-buffered saline
PDMS	polydimethylsiloxane
PI	propidium iodide
PLL	posterior longitudinal ligament
PVDF	polyvinylidene fluoride
RT-qPCR	real-time quantitative polymerase chain reaction
RIPA	radio-immunoprecipitation assay
RhoA	ras homologue family member A
RUNX2	runt-related transcription factor 2
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
Shh	Sonic hedgehog
TEAD	TEA domain family member
TGF-β	transforming growth factor-β
YAP	yes-associated protein

Introduction

OPLL is a pathological condition consisting of heterotopic bone formation within 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 suppresses the progression of OPLL (Kang *et al.*, 2019; Katsumi *et al.*, 2016; Lee *et al.*, 2017). *In vivo* experiments in animal models resulted in a similar conclusion: when rat tail or thoracic vertebra are subjected to repeated excessive traction stimulation, heterotopic ossification of local ligaments occurs (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 *in vitro* experiments have confirmed that cytokines and transcriptional factors may have 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 the 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 recognisable biological signals, which will affect the cell biological behaviours (Czeisler *et al.*, 2016; Guo *et al.*, 2015; Kim and Asthagiri, 2011).

YAP is a transcriptional co-activator protein that shuttles between the nucleus and the 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 the nucleus, it will participate in the regulation of other signal transduction pathways, such as Wnt/β-catenin, Hippo and TGF-β. 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 its own 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 it in initiating osteogenic transcriptional regulation (Zhou *et al.*, 2022). Cong *et al.* (2021) showed that YAP-Shh self-amplifying loop is an important cause of heterotopic ossification of tendon tissues in FOP. In addition, the high activation state of YAP/RhoA pathway stimulated by mechanical stress promotes osteogenic differentiation of FOP mesenchymal progenitors *via* the BMP pathway (Stanley *et al.*, 2019). Given that both OPLL and FOP are forms of endochondral heterotopic ossification, the specific mechanism of action of YAP in the occurrence and development of OPLL has not been reported. The present study explored the possible role of YAP in accelerating OPLL progression induced by mechanical stimulation in order to provide new insights into the pathogenesis of OPLL.

Materials and Methods

Clinical diagnosis and spinal ligament samples

10 patients presenting cervical OPLL and 10 non-OPLL patients as control groups were selected for the study (Table 1). The diagnosis of OPLL or non-OPLL was confirmed by clinical signs and symptoms as well as cervical-spine computed tomography. All patients underwent cervical anterior decompression surgery and the PLL were collected intraoperatively. All patients enrolled in the 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).

Primary ligament fibroblast culture

PLL 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 extraction of PLL cells was performed according to the protocol described by Chen *et al.* (2018a). In short, PLL tissues were entirely minced and digested for 4 h at 37 °C using 0.2 % type II collagenase (Gibco). Then, the digested tissues were plated into a T25 plate in DMEM/F12 (Gibco) supplemented with 10 % FBS (Gibco) and 1 % penicillin/streptomycin (Gibco) at 5 % CO_2 and 37 °C. When confluent, the cells were passaged after digesting using 0.25 % trypsin-EDTA (Gibco). Cells at the the third passage were used in the following experiments. In agreement with previous reports (Yang *et al*., 2011a), inverted phase-contrast microscopy and vimentin (abcam, ac8978, 1:1000) immunolabelling were used for cell type identification. Samples with a vimentin positive rate of more than 90 % were considered to be suitable for cell extraction.

Stretch apparatus

To mimic the tensile force exerted on cells during neck flexion and extension, a custom-made uniaxial dynamic apparatus was used for dynamic stretching culture. The apparatus is characterised by uniaxial sinusoidal stretch stimulation with multiple chambers, making it possible to keep the same frequency between each unit. PDMS (SLYGARD® 184, Merck), with excellent cytocompatibility and high translucency, was used to fabricate stretchable cell culture dishes, according to a previous protocol (Rodriguez *et al.*, 2019).

Application of cyclic stretch to cells

Before cells were seeded at a density of $1 \times 10^4/cm^2$, the flexible-bottomed plates were coated with 5 μg/cm^2 type I collagen (Gibco). After reaching 80 % confluence, the medium was changed and cyclic tensile force (5 % amplitude) was applied to OPLL and non-OPLL cells at a frequency of 0.5 Hz for 9 h/d. Non-OPLL cells that were maintained under identical conditions without being stretched were used as a control.

RNA extraction, cDNA synthesis and qRT-PCR analysis

Total RNA was isolated using the RNAeasy™ Animal RNA Isolation Kit (R0027, Beyotime, Shanghai, China) and 400 ng of RNA were converted to cDNA using a cDNA Synthesis Kit (Takara). RT-qPCR was performed using the PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) and a qRT-PCR system (Bio-Rad). Each reaction mixture consisted of 5 μL of 2× PowerUp™ SYBR™ Green Master Mix, 2 μL of nuclease-free water, 0.5 μL each of 10 μmol/L forward and reverse primer and 2 μL of cDNA. The applied cycle conditions were 2 min at 50 °C and 2 min at 95 °C followed by 44 cycles of 15 s at 95 °C and 1 min at 60 °C. Primer sequence design, primer construction and purification were provided by Sangon Inc. (Shanghai◎China). Primer sequences are provided in Table 2. Results were normalised to the housekeeping *GAPDH* gene expression using the $2^{-\Delta\Delta Ct}$ algorithm.

Total protein extraction and Western blot analysis

Total proteins were extracted using RIPA buffer (Thermo Fisher Scientific) and their concentrations

Table.1. Patients' demographic data. F: female. M: male.

OPLL	Case	Type	Age/sex	Non-OPLL	Case	Diagnosis	Age/sex
	1	mixed	56/M		1	CSM	65/F
	2	segmental	73/M		2	CSM	68/M
	3	continuous	37/M		3	CDH	57/F
	4	mixed	68/F		4	CSM	66/F
	5	continuous	52/M		5	CSM	56/F
	6	circumscribed	47F		6	CSM	72F
	7	continuous	66/F		7	CSM	70/M
	8	segmental	58/F		8	CSM	66/F
	9	mixed	50/F		9	CDH	55/M
	10	continuous	63/M		10	CSM	74/M

Table.2. Primer sequences used in the study.

Primer name	Sequence (5′—>3′)
ShYAP-1-F	CCGGCTGGTCAGAGATACTTCTTAACTCGAGTTAAGAAGTATCTCTGACCAGTTTTTG
ShYAP-1-R	AATTCAAAAACTGGTCAGAGATACTTCTTAACTCGAGTTAAGAAGTATCTCTGACCAG
ShYAP-2-F	CCGGAGTCAGAGTGCTCCAGTGAAACTCGAGTTTCACTGGAGCACTCTGACTTTTTTG
ShYAP-2-R	AATTCAAAAAAGTCAGAGTGCTCCAGTGAAACTCGAGTTTCACTGGAGCACTCT-GACT
ShYAP-3-F	CCGGGAGAGTACAGACAGTGGACTACTCGAGTAGTCCACTGTCTGTACTCTCTTTTTG
ShYAP-3-R	AATTCAAAAAGAGAGTACAGACAGTGGACTACTCGAGTAGTCCACTGTCTG-TACTCTC
ShCTNNB1-1-F	CCGGGCTTGGAATGAGACTGCTGATCTCGAGATCAGCAGTCTCATTCCAAGCTTTTTG
ShCTNNB1-1-R	AATTCAAAAAGCTTGGAATGAGACTGCTGATCTCGAGATCAGCAGTCTCATTC-CAAGC
ShCTNNB1-2-F	CCGGAGGTGCTATCTGTCTGCTCTACTCGAGTAGAGCAGACAGATAGCACCTTTTTTG
ShCTNNB1-2-R	AATTCAAAAAAGGTGCTATCTGTCTGCTCTACTCGAGTAGAGCAGACAGATAG-CACCT
ShCTNNB1-3-F	CCGGCGCATGGAAGAAATAGTTGAACTCGAGTTCAACTATTTCTTCCATGCGTTTTTG
ShCTNNB1-3-R	AATTCAAAAACGCATGGAAGAAATAGTTGAACTCGAGTTCAACTATTTCTTCCATGCG
ALP-F	GCTGTAAGGACATCGCCTACCA
ALP-R	CTCGTCACTCTCATACTCCACATCA
RUNX2-F	CACCACTCACTACCACACCTACCT
RUNX2-R	CTTCCATCAGCGTCAACACCATCA
OCN-F	AGGGCAGCGAGGTAGTGA
OCN-R	CCTGAAAGCCGATGTGGT
COL1-F	AGCAGACTGGCAACCTCAAGA
COL1-R	AGGGAGTTTACAGGAAGCAGACA
Osterix-F	GCAAGAGGTTCACTCGTTCGGATG
Osterix-R	TCAGGTGGTCGCTTCGGGTAAA
GAPDH-F	ACTTTGGTATCGTGGAAGGACTCA
GAPDH-R	CCAGTAGAGGCAGGGATGATGTT
MST1-F	CGCTCGCCATTGAATGACTTCC
MST1-R	GACCAGCACACTCTTCAGCATCT
MST2-F	TGATTCCCACAAATCCACCACCAA
MST2-R	CCTCTTCCAATTCTCGTTGCTGTTC
LATS1-F	TGCCCAAGGAAGATGAGAGTGAAA
LATS1-R	TTACGATGTAGACGCTGCTGATGA
LATS2-F	GGCTTCATCCACCGAGACATCAA
LATS2-R	CCACACCGACAGTTAGACACATCA
YAP-F	AACCGTTTCCCAGACTACCTTGA
YAP-R	GCTCCTCTCCTTCTATGTTCATTCC
TEAD1-F	GGTGGCTTAAAGGAACTGTTTGGA
TEAD-R	GAACTCTCGTACTGACTGGTTACAC
TEAD2-F	AGTGGTGGCTTCTACGGAGTGA
TEAD2-R	GTTCCTGGGTGTCTCTGTTTGTCA
RhoA-F	GGAAAGCAGGTAGAGTTGGCT
RhoA-R	GGCTGTCGATGGAAAAACACAT
β-catenin-F	ACAGGGTCTGGGACATTAGTCGTTA
β-catenin-R	TGTGAAGGCAGCAGCAAGTTCTT

were determined using the BCA kit (Boster, Wuhan, China). Proteins were electrophoresed in pre-made polyacrylamide gels containing SDS and transferred to PVDF membranes. After blocking with 5 % non-fat milk (Solarbio, Beijing, China) for 1 h, the PVDF membranes were incubated with primary antibody overnight at 4 °C and then with secondary antibody (coupled with HRP) for 1 h at room temperature. The protein signal was visualised using an ECL chemiluminescence kit (EpiZyme, China) and the grayscale value of the bands was quantified using Image J software.

ALP staining

The presence of ALP in the cell layers was assessed according to the manufacturer's instructions (Beyotime). OPLL and non-OPLL cells from the control and stretch treatment groups were rinsed with PBS 3 times and fixed using 4 % paraformaldehyde for 20 min. The fixed cells were rinsed with PBS 3 times and then BCIP/NBT liquid substrate was added to each stretchable dished for 1 h at 37 °C. Finally, cells were washed with ddH_2O and then imaged using a digital camera and observed using an optical phase contrast microscope (CKX53, Olympus, Germany).

Immunofluorescence analysis

After culturing and being stretched in the flexible culture dishes, OPLL cells were fixed using 4 % paraformaldehyde for 20 min at room temperature and then permeabilised using 0.3 % Triton X-100 (Sigma) as well as blocked using 5 % BSA (BioFroxx, Einhausen, Germany) for 1 h. The permeabilised cells were incubated at 4 °C overnight with a YAP antibody (ab205270, Abcam, 1:1000) diluted in PBS. After 3× washes in PBS for 5 min, cells were incubated for 1 h in the dark with goat anti-rabbit IgG (7074S, 1:1000, Cell Signaling Technology). Next, cells were counterstained with phalloidin according to the manufacturer's instructions (MB5940, 1:100, Meilunbio, Dalian, China) in order to better observe the changes to the cytoskeleton. At last, nuclei were stained with DAPI (ab104141, Abcam). Images were acquired using a fluorescence microscope (DM6B, Leica).

Lentivirus packaging and cell transduction

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, Shanghai, China). Primer details (forward and reverse) are listed in Table 2. To produce a lentivirus for exogenous YAP and CTNNB1 knockdown, the core plasmid was co-transduced with the packaging plasmids pMD2.G and psPAX2 into 293T cells using the Lipofectamine™ 3000 transfection kit (Invitrogen). The medium was changed 6 h post-transduction. Supernatants containing virus were collected at 24 and 48 h. Target cells were grown to 60 % confluence in 6-well plates and then transduced with the virus using 8 μg/mL polybrene (Beyotime). When cells fusion rate reached to 90 %, they were transferred to 10 cm^2 dishes. Fluorescence microscopy was used to observe whether the cells were successfully transduced since the transduced cells were labelled green. To obtain stable strains, all cells were screened using 2 μg/mL puromycin. The knockdown efficiency of the stably transduced cells was tested by RT-qPCR and Western blot. Compared with the control group, the cells with the highest transduction efficiency were selected for further experiments.

Statistical analysis

Statistical analyses were performed using SPSS version 20. Quantitative data are presented as mean ± standard deviation. Differences between two samples were measured using an unpaired student's *t*-test when the data displayed a normal distribution, otherwise the Mann-Whitney U-test was used. For comparisons among more than two groups, one-way analysis of variance followed by a Tukey test was performed. Statistical significance was set at $p < 0.05$.

Results

Identification of primary cell types extracted from PLL

Passage 3 primary cells were used in the experiments. Vimentin immunolabelling was required to determine the cell type. Given that vimentin is a type of intermediate filament that mainly resides in fibroblasts, it was considered to be a fibroblast marker in the immunofluorescence assay (Fig. 1**a**). Results showed that the cells derived from the cervical PLL were mostly fibroblast-like cells, with red fluorescence in the cytoplasm.

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 upon cell viability was assessed by live/dead staining immediately following stretching. Results showed no significant differences in cell viability and proliferation between mechanically-loaded and static cells (Fig.1**b**1,**b**2). SEM images showed that cells on the surface of the material presented a good adhesion, with development of pseudopodia (Fig. 1**c**1). These results proved that the composite exhibited 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 SEM (Fig. 1 **c**1,**c**2).

Stretching stimulation promoted osteogenic genes expression in OPLL cells

RUNX2, COL1, osterix, OCN and ALP are considered to be important markers of osteogenic differentiation.

RT-qPCR results showed that the OPLL group demonstrated enhanced ossification, as reflected by the induction of osteogenic markers, namely, RUNX2, COL1, osterix, OCN and ALP, at the mRNA levels (Fig. 2**a**) and protein levels (Fig. 2**b**). In parallel, there was no significant difference in the elevation of these markers in the non-OPLL group. ALP staining showed the same results as shown by RT-qPCR. As shown in Fig. 2**c**, ALP activity was significantly increased in the stretched OPLL-derived cells but not in the control cells. In summary, these results demonstrated that cyclic stretch significantly promoted the osteogenic differentiation of OPLL-derived cells while control cells were less affected by mechanical stimulation.

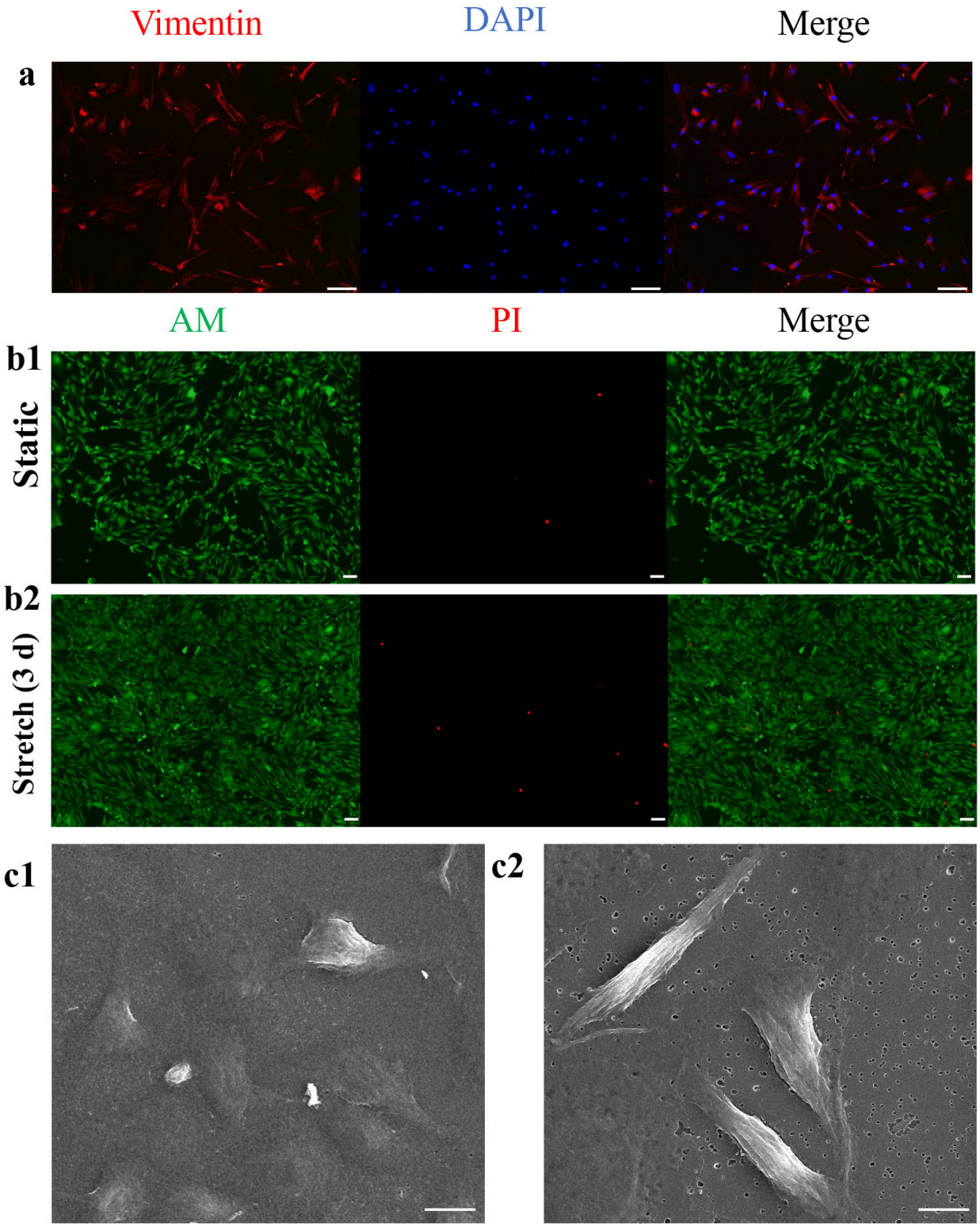

Fig. 1. Cell type identification and application of cyclic stretch to cells. (**a**) Vimentin immunolabelling showed that more than 90 % of the cells extracted from the tissues of the PLL were fibroblasts (scale bar: 50 μm). (**b**) AM/PI staining showed that stretching culture did not increase cell mortality (scale bar: 50 μm). (**c**) Cells before and after stretching were scanned by SEM (scale bar = 20 μm).

Stretching stimulation induced actin cytoskeleton reorganisation and YAP nuclear translocation

To investigate how the cytoskeleton changed to respond to mechanical stretching, OPLL-derived primary cells were subjected to cyclic mechanical stretching for 9 h/d at a 5 % amplitude. Subsequently, the F-actin was stained by phalloidin. Cells displayed a markedly different cell morphology following dynamic culture. Stretching loading caused cellular elongation, while the static cells showed a flattened, pancake-like shape, indicating that stretching induced actin cytoskeleton reorganisation in these cells (Fig. 3**a**). The remodelling of the cytoskeleton led to a change in the location of the protein YAP. 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. 3**c**). When the stretching force lasted for 9 h, the distribution of YAP in the nucleus and cytoplasm was approximately equal. When the cells were subjected to continuous 3 d (9 h/d) dynamic stretch culture, YAP was predominantly distributed in the nucleus. To measure the shape change of cells, a concept of cell aspect ratio was introduced. 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 measurements demonstrated that the cells that were cultured dynamically exhibited a higher aspect ratio compared with the static group (Fig. 3**b**). This observation was further confirmed by RT-qPCR and Western blot analysis. Cell elongation increased the expression of *YAP* as well as of the dephosphorylated YAP protein. At the meantime, the expression of *RhoA*, a cytoskeleton regulation gene, was up-regulated with the increase of cell stretch (Fig. 3**d**,**e**). This indicated that cytoskeletal changes activated the expression of RhoA, which in turn increased the downstream YAP expression.

Taken together, the results indicated that OPLL-cells could respond to mechanical stretch by adjusting cytoskeleton organisation. Driven by

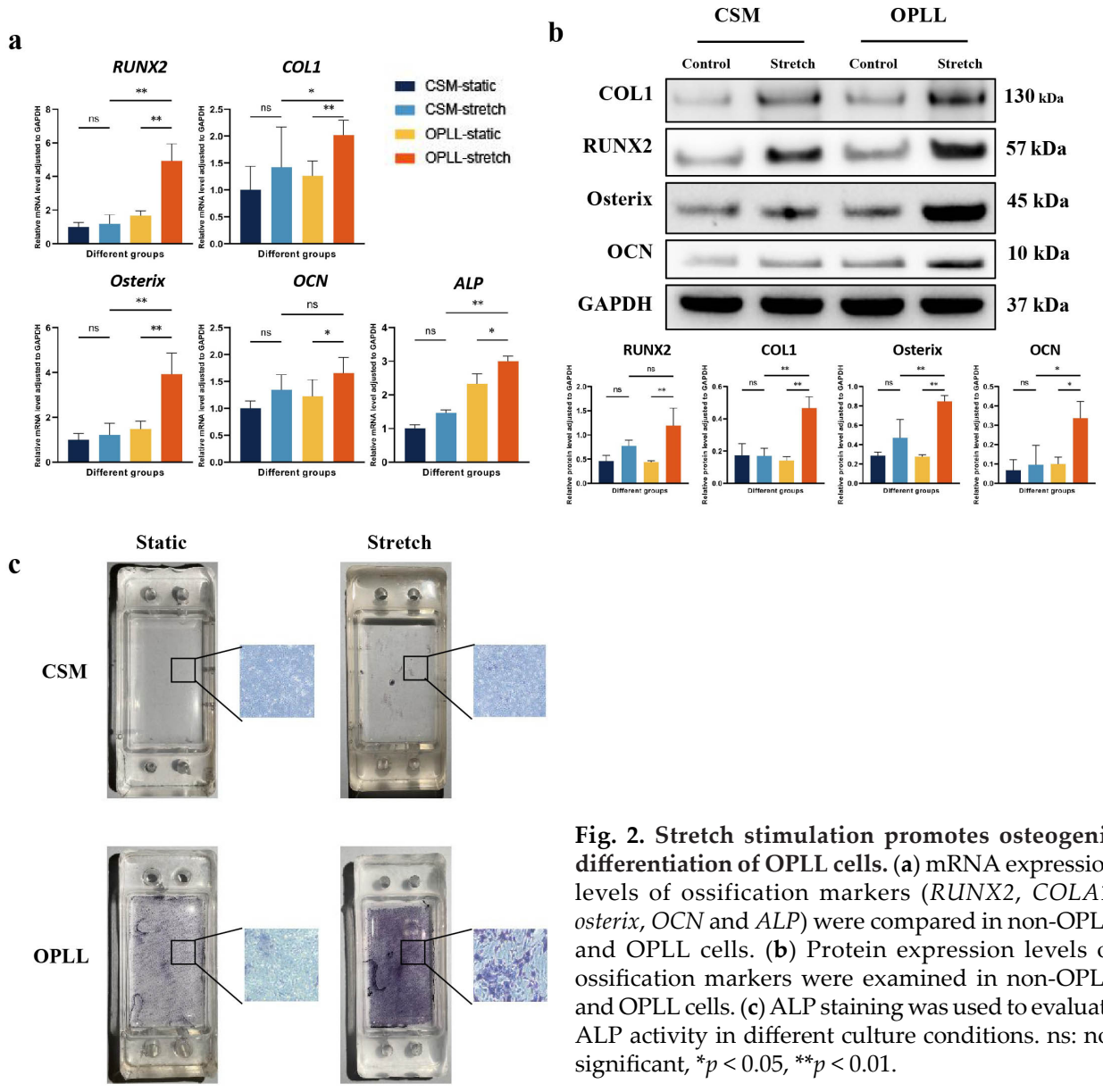

Fig. 2. Stretch stimulation promotes osteogenic differentiation of OPLL cells. (**a**) mRNA expression levels of ossification markers (*RUNX2*, *COLA1*, *osterix*, *OCN* and *ALP*) were compared in non-OPLL and OPLL cells. (**b**) Protein expression levels of ossification markers were examined in non-OPLL and OPLL cells. (**c**) ALP staining was used to evaluate ALP activity in different culture conditions. ns: not significant, *$p < 0.05$, **$p < 0.01$.

such cytoskeletal morphological changes, YAP is translocated from the cytoplasm to the nucleus to participate in downstream transcriptional regulation.

Activation status of Hippo and Wnt pathways after stretch stimulation

YAP, as a key gene of crosstalk between the Hippo and Wnt/β-catenin pathway, has been shown to dephosphorylate itself and enter the nucleus to regulate the expression of osteogenic genes following mechanical stimulation. The present study detected the expression of key genes involved in these two pathways. The results showed that the expression of *LATS*, *MST* and *TEAD* in OPLL cells did not change significantly following mechanical stimulation (Fig.4**a**,**b**), while the expression of β-catenin increased significantly (Fig.4**c**,**d**). Western blot analysis reached a similar result to RT-qPCR, with no significant changes in the expression of LATS and TEAD, indicating no significant changes 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 pathways.

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. 5**a**). Immunofluorescence results showed good transduction efficiency for both lentiviruses (Fig. 5**b**). According to the results of RT-qPCR and Western blot, the best transduction sequence was selected for subsequent experimental study (Fig. 5**c**,**d**). After successful transduction, cells were subjected to stretch stimulation with described parameters to evaluate the effect of tensile mechanical stimulus upon 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 showed decreased responses to mechanical stimulation. Compared with one gene alone, knockdown of two genes at the

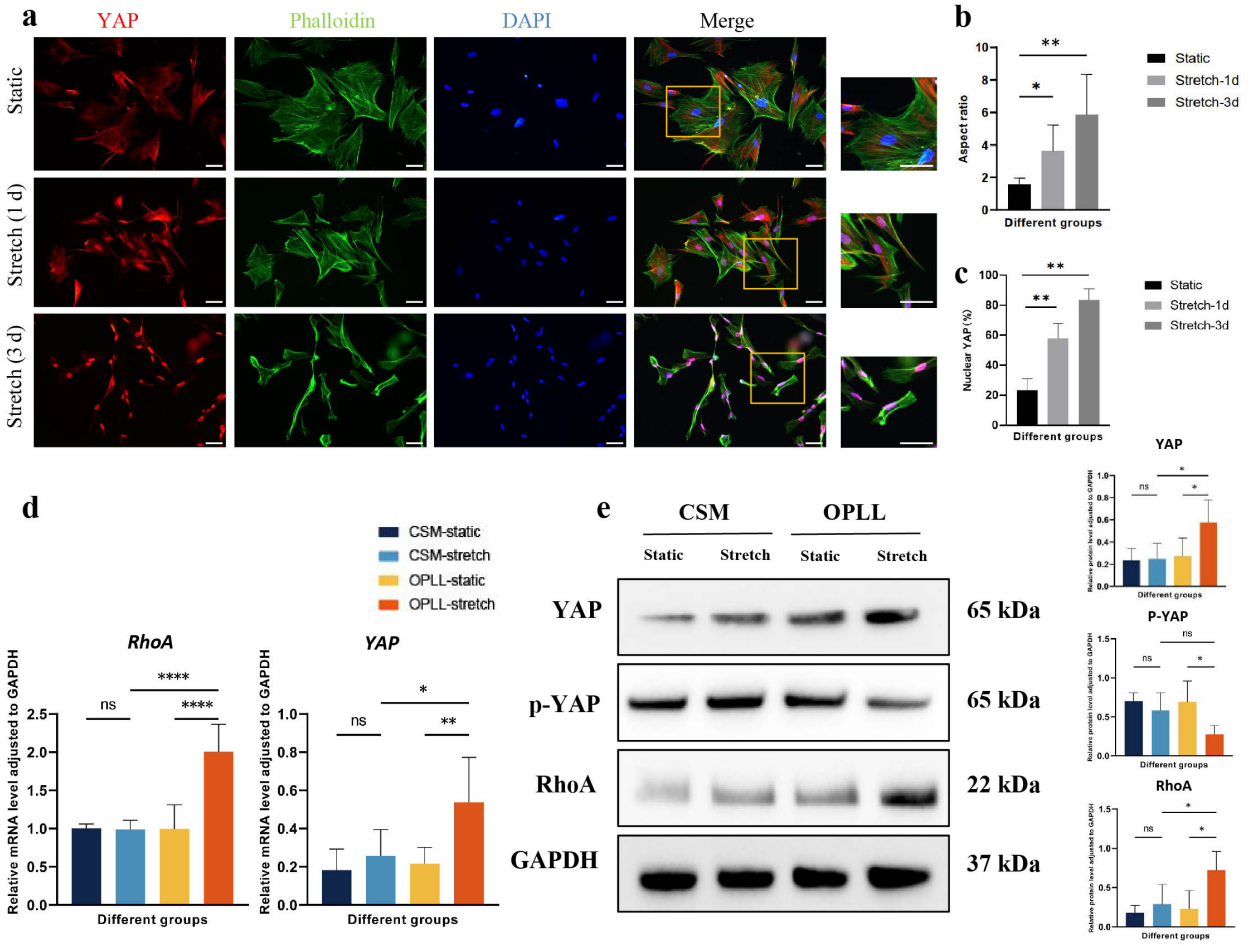

Fig. 3. Stretching stimulation induced actin cytoskeleton reorganisation and YAP nuclear translocation. (**a**) The expression of YAP protein in OPLL-derived primary cells of the PLL gradually shifted from cytoplasm to nucleus after receiving stretching stimulation (scale bar: 50 μm). (**b**) Ratio of long axis to short axis after stretching. (**c**) Proportion of YAP nuclear expression during cell stretching. (**d**,**e**) Gene and protein expression levels of YAP and RhoA. ns: not significant, *$p < 0.05$, **$p < 0.01$, ****p < 0.0001.

same time induced lower expression of osteogenic markers (Fig. 5**e**,**f**). These findings suggested that the downregulation of YAP or β-catenin expression will weaken the osteogenic differentiation of OPLL cells driven by stretch mechanical stimulation.

Discussion

OPLL, a disease characterised 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 vertebrae, 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. Moreover, 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 are subjected to mechanical stretching stimulation, the expression of osteogenic genes is upregulated, while there is no significant change in non-OPLL cells (Bhatt *et al.*, 2007; Yang *et al.*, 2011b). According to the authors, tensile stress may increase the expression of connexin43 and abnormally activate multiple signalling pathways in OPLL-derived primary cells (Chen *et al.*, 2017; Chen *et al.*, 2016; Sugita *et al.*, 2020). Furthermore, endoplasmic reticulum stress response as well as disorders of ATP, prostaglandin I2 and endothelin metabolism are 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 responsible for the increase in 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

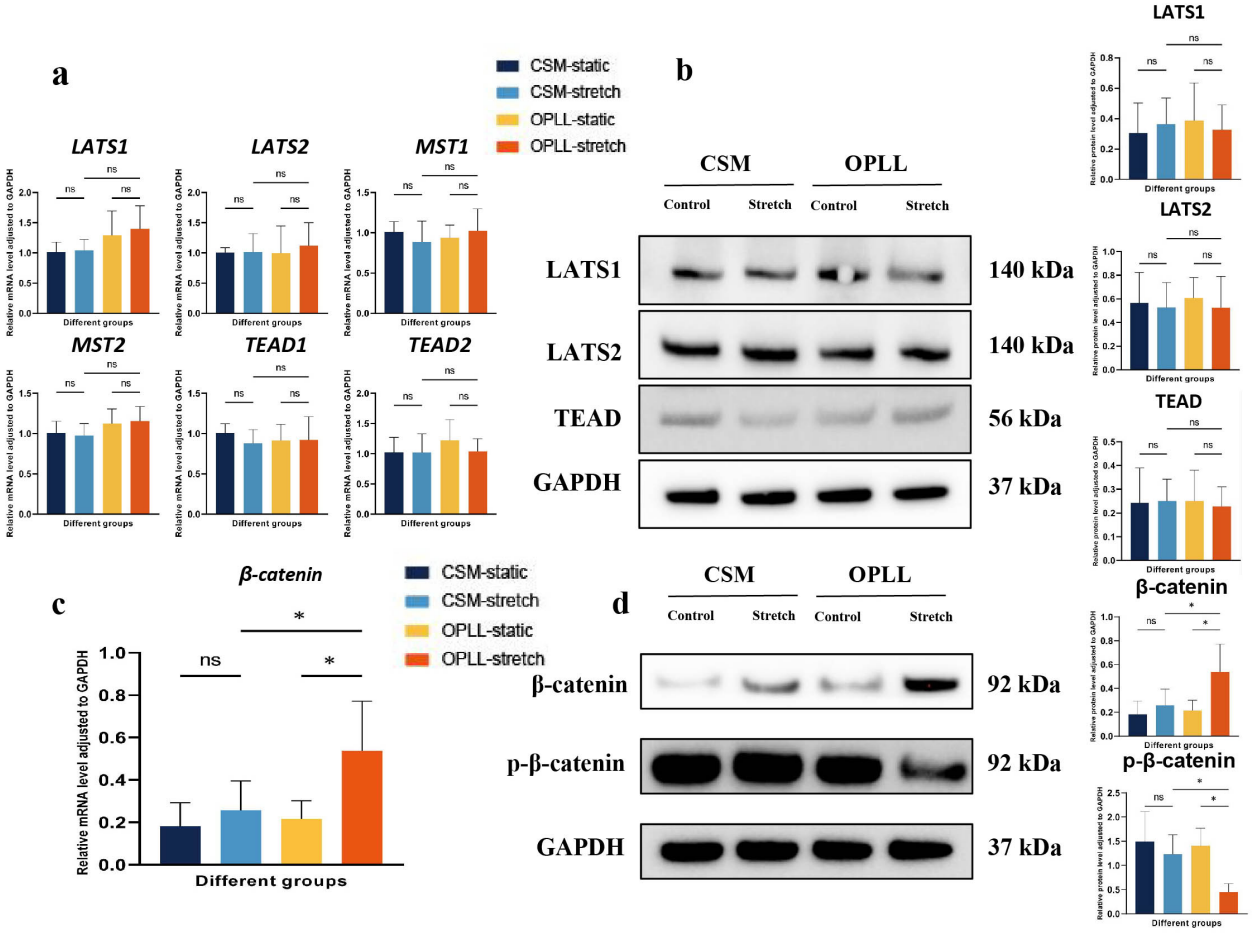

Fig. 4. Knockdown of YAP or β-catenin reduced osteogenic gene changes in OPLL cells induced by tensile mechanical stimulation. (**a**-**d**) Dephosphorylated YAP activated Wnt/β-catenin pathway to synergistically regulate cell osteogenic differentiation, which was independent of Hippo pathway. ns: not significant, *$p < 0.05$.

signals are converted into biological signals that can be recognised by cells. Based on these questions, the present study was conducted.

The present study not only revealed the possible mechanism of osteogenic differentiation of OPLL cells after tensile mechanical stimulation but also elucidated how cells sense mechanical 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 organisation of the F-actin cytoskeleton (Totaro *et al.*, 2018). Results suggested that when cells received tensile mechanical stimulation, the cytoskeleton remodelled and YAP was dephosphorylated, thus allowing its transfer from the cytoplasm to the nucleus. After entering the nucleus, YAP bound RUNX2 or activated Wnt/β-catenin pathway to promote cell osteogenic differentiation, with these biological effects being independent of the Hippo pathway.

YAP, as one of the essential mechanosensitive transcriptional activators (Aragona *et al.*, 2020; Zhong *et al.*, 2013a; Zhong *et al.*, 2013b), plays an important role in the osteogenic differentiation of ligament

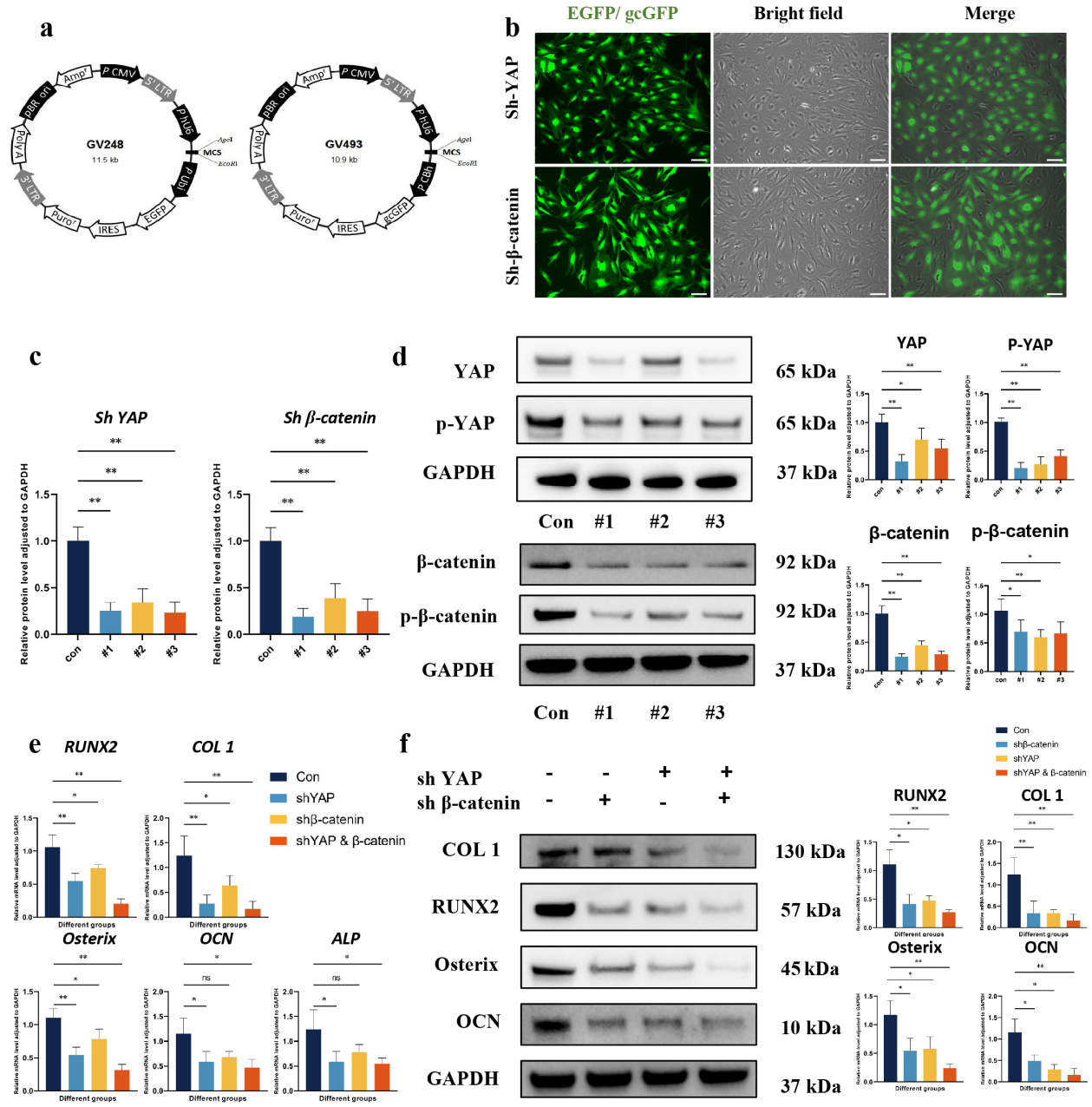

Fig. 5. Activation status of Hippo and Wnt pathways after stretch stimulation. (**a**) Plasmid construction diagram. (**b**) The transduction efficiency was determined by immunofluorescence after lentivirus transduction (scale bar: 50 μm). (**c**,**d**) Knockdown efficiency of transduced cells was detected at gene and protein levels. (**e**,**f**) Changes in gene and protein levels of osteogenic markers after transduced cells were subjected to mechanical stretch stimulation. ns: not significant, *$p < 0.05$, **$p < 0.01$.

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 own entry into the nucleus and gradually getting degraded 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 that the stretching amplitude of 5-10 % promotes cell differentiation towards the 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 into downstream transcriptional regulation (Chen *et al.*, 2018b; Cui *et al.*, 2015; Dong *et al.*, 2021). Cui *et al.* (2015) confirmed that YAP begin to transfer from the cytoplasm to the nucleus when 5 % tensile stress is applied for 3 h in human umbilical vein endothelial cells. When the dynamic culture time is extended to 6 h, YAP is almost completely expressed in the nucleus (Cui *et al.*, 2015). These results are consistent with the present study, where 9 h stretching stimulation promoted cytoskeleton remodelling and increased 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 adhesions. Changes in cell surface proteins induce the remodelling of F-actin and the alteration of the cytoskeletal structure, which triggers the activation of the Rho pathway. The activated RhoA will contact the non-phospho YAP, prompting it to enter into the nucleus, 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 signal transduction and YAP loses 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 changed from flat to long spindle, the location of YAP protein expression also changed. In addition, changes in the cytoskeleton alter the mechanical connection between the cell membrane and the nuclear envelope. Elosegui-Artola *et al.* (2017) showed that a force applied to the nucleus directly drives YAP nuclear translocation by decreasing the mechanical restriction of nuclear pores to molecular transport. When cells are exposed to a high matrix stiffness environment, a mechanical connection between the nucleus and the cytoskeleton is established 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 affect the nuclear expression of YAP. Super-resolution imaging

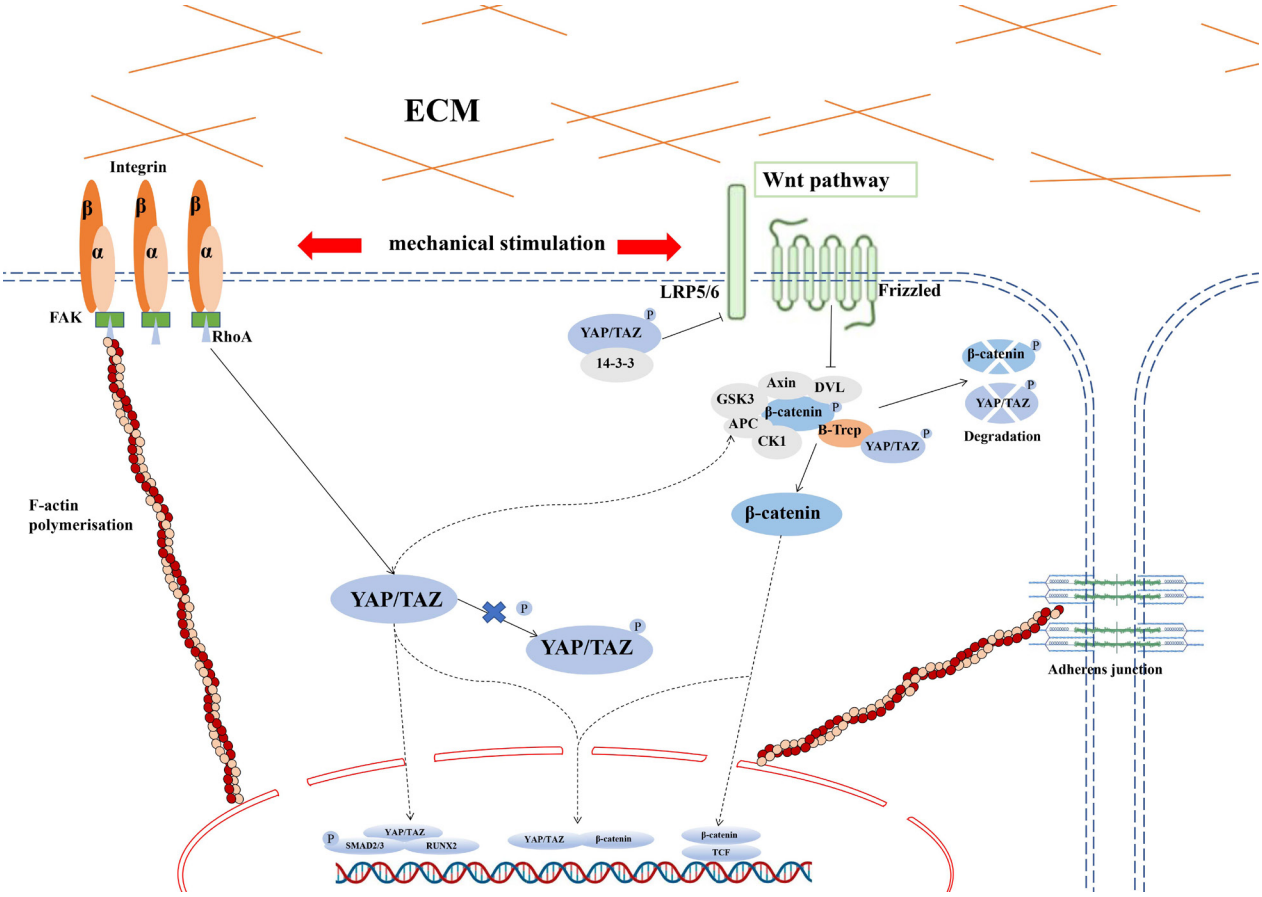

Fig. 6. Mechanism of osteogenic differentiation induced by stretching stimulation in OPLL-derived primary cells.

showed that a different cell density affects the cellular location of YAP expression and a high cell density inhibits YAP entry into the nucleus (Gao *et al.*, 2020). Moreover, the open state of PIEZO1, a mechanically sensitive protein, could also affect the activation of YAP. Pathak *et al.* (2014) showed that knocking out *PIEZO1* inhibits the stretched-stimulation-induced nuclear transfer of YAP in neural stem cells.

Similar to previous research, the present study found that the dephosphorylation of YAP induced by stretching stimulation was involved in downstream regulation, and this progress was dependent on the Wnt/β-catenin rather than the Hippo pathway (Elosegui-Artola *et al.*, 2017; Li *et al.*, 2019). YAP activity is essential for maintaining β-catenin nuclear expression and YAP-Wnt/β-catenin axis is one of the important links in maintaining bone homeostasis and inhibiting cell adipogenic differentiation (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 degrade progressively in the cytoplasm. The regulation of β-catenin through a cytoplasmic destruction complex forms the crux of the Wnt signalling cascade (Schaefer *et al.*, 2018). When active YAP dissociates 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 to the present study. First, all PLL tissues were too fragmented to distinguish the specific anatomical structures, so it was difficult to conduct HE or immunofluorescence staining to verify the experimental results at the tissue level. Second, limited by the function of the custom-made uniaxial dynamic apparatus, only sinusoidal stretching mode could be carried on. Therefore, the cells did not have any rest during the stretching process, which may not be consistent with normal cervical physiological activity. Third, the diagnosis of disease was based on imaging examination and clinical symptoms, which might not have been accurate for some people who carried the susceptibility gene but did not develop the disease and were classified as the control group. Finally, due to the difficulty in constructing an animal model of local heterotopic ossification of the PLL caused by mechanical stimulation, the experiment was only conducted *in vitro*.

Conclusion

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

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The authors have no conflict of interest to declare.

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