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Research article

Intermittent compressive force regulates human periodontal ligament cell behavior via yes-associated protein

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HIGHLIGHTS

- YAP plays role as a mechanosensitive transcriptional activator of human PDL cells in response to ICF.
- ICF activates YAP and its target genes to promote cell proliferation and osteogenic differentiation of human PDL cells.
- Loss of YAP enhances adipogenic differentiation of human periodontal ligament cells.

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ABSTRACT

Intermittent compressive force influences human periodontal ligament (PDL) cell behavior that facilitates periodontal tissue regeneration. In response to mechanical stimuli, Yes-associated protein (YAP) has been recognized as a mechanosensitive transcriptional activator that regulates cell proliferation and cell fate decisions. This study aimed to investigate whether compressive forces influence cell proliferation and cell fate decisions of human PDL cells via YAP signaling. YAP expression was silenced by shRNA. The effect of YAP on cell proliferation, adipogenesis and osteogenesis of PDL cells under ICF loading were determined. Adipogenic differentiation bias upon ICF loading was confirmed by fourier-transform infrared spectroscopy (FTIR). The results revealed that ICF-induced YAP promotes osteogenesis, but it inhibits adipogenesis in PDL cells. Depletion of YAP results in PDL cells that are irresponsive to ICF and, therefore, the failure of the PDL cells to undergo osteogenic differentiation. This was shown by a significant reduction in calcium deposited in the CF-derived osteoblasts of the YAP-knockdown (YAP-KD) PDL cells. As to control treatment, reduction of YAP promoted adipogenesis, whereas ICF-induced YAP inhibited this mechanism. However, the adipocyte differentiation in YAP-KD cells was not affected upon ICF treatment as the YAP-KD cells still exhibited a better adipogenic differentiation that was unrelated to the ICF. This study demonstrated that, in response to ICF treatment, YAP could be a crucial mechanosensitive transcriptional activator for the regulation of PDL cell behavior through a mechanobiological process. Our results may provide the possibility of facilitating PDL tissue regeneration by manipulation of the Hippo-YAP signaling pathway.

1. Introduction

Mechanoregulation influence stem cell fate differentiation in response to physical and mechanical cues, including extracellular matrix (ECM) stiffness, cell surface topography, and extrinsic forces [1]. After sensing external cues from the surrounding ECM through cell surface receptor, the membrane associated protein, and ion channels (a process called mechanosensing), these stimuli are translated into signals through the intracellular pathway via a process called mechanotransduction [2]. Several evidences demonstrated that ECM stiffness, as well as, mechanical force influence on cell fate decision of mesenchymal stem cells (MSCs). For example, the high matrix stiffness enhances intergrins clustering and focal adhesion to modulate F-actin polymerization, resulting in activation of RUNX2-induced osteogenesis [3]. Besides the ECM

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stiffness, mechanical force plays an important role in cell behavior through cell-cell and cell-ECM interactions, resulting in activation of cytoskeletal-associated protein such as focal adhesion and Rho GTPase signaling [4]. The regulation of cell behavior through these mechanobiological processes depends on the mode of the force and cell type [5].

The periodontal ligament (PDL) is a specialized connective tissue within the tooth socket that connects the tooth to the alveolar bone. The PDL is responsible for transferring mechanical forces experienced by the tooth during occlusal and orthodontic tooth movements to the surrounding bone [6]. Numerous load-applied models have been established using PDL tissue-derived cells to investigate the influence of mechanical forces on the homeostasis of the periodontium, including the PDL and alveolar bone. Recent studies have demonstrated that the application of different forces leads to different mechanobiological responses in human PDL cells. The force types include continuous compressive force, mechanical vibration, static mechanical stretch, fluid shear stress, cyclic tension, and hydraulic pressure [7, 8, 9, 10, 11]. The intermittent compressive force facilitates periodontal tissue homeostasis by regulating PDL cell activity. In response to intermittent compressive force, PDL cells can function as mechanosensor by stimulating receptor activator of nuclear factor kappa B ligand (RANKL) via interleukin-1β (IL-1β), thereby activating bone remodeling [12]. Previous studies have shown that ICF promotes the expression of sclerostin *(SOST)* and periostin *(POSTN)* in human PDL cells through TGF-β1 signaling pathway, which may facilitate periodontal tissue remodeling [13].

Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) are downstream mediators of the Hippo signaling pathway. Both have recently been found to play major roles in cell proliferation, cell apoptosis, cell differentiation, thereby regulated tissue growth during organogenesis and tissue regeneration [14, 15]. Active YAP localized in the nucleus binds to the TEAD transcription factor to induce downstream target gene expression, including genes regulating cell proliferation and anti-apoptosis regulatory genes [16]. In canonical Hippo-YAP signaling, YAP is negatively regulated by the Hippo core kinases, LATS1 and LATS2. Once there is cell-cell contact signaling, Hippo-core kinases become activated and are able to phosphorylate YAP, resulting in cytoplasmic retention and degradation. Therefore, cell proliferation was inhibited [17]. Recently, a non-canonical YAP pathway has been demonstrated. It has been shown that YAP is responsive to physical and mechanical cues, such as ECM rigidity, mechanical stress, and adhesive area [18, 19, 20]. Upon receiving mechanical stimuli through the actin-cytoskeleton network, YAP and TAZ respond to signals by influencing changes in the physical and structural features of the cells [21, 22]. Previous study demonstrated the expression of YAP in dental epithelial and oral mesenchymal tissues. The overexpression of YAP in the dental epithelium affects tooth morphogenesis and the patterning of enamel knots [23]. In contrast, the small tooth germ with reduced epithelial cell proliferation was detected in YAP knockout mice [24], suggesting the essential role of YAP in tooth formation and surrounded mesenchymal tissues including periodontium.

The role of YAP in the maintenance of bone homeostasis by promoting osteogenesis and suppressing adipogenesis has been previously demonstrated. An *in vivo* study showed that YAP is required to promote cell proliferation and differentiation of osteoblasts and to suppress the adipogenic potential of bone marrow-derived mesenchymal stem cells (BM-MSCs), thereby maintaining bone mass [25]. Research has revealed that overexpression of YAP using small molecules promotes osteogenic differentiation, while suppression of YAP induces adipogenic differentiation of human MSCs [26]. The potential of YAP to promote osteogenic differentiation has recently been reported in human PDL cells upon cyclic strength loading [27]. However, the role of YAP under compressive force in the fate decision of human PDL cells has not yet been examined.

In this study, we investigated whether PDL cells acts as a mechanosensor in response to ICF via the Hippo-YAP pathway. The effect of YAP knockdown on cell proliferation and cell fate decisions was determined in human PDL cells under intermittent compressive force. We expect that our findings will lead to the elucidation of the role of YAP in PDL cell behavior in response to mechanical stimuli, which may facilitate the development of strategies for future clinical applications in periodontal tissue regeneration.

2. Materials and methods

2.1. Isolation and cell culture of PDL cells

Healthy human periodontal tissues were obtained from at least 3 donors at the Department of Surgery, Faculty of Dentistry, Chulalongkorn University. Informed consent for sample collection was obtained from all patients. The cell isolation protocol was approved by the ethics committee for human research (HRBC-DCU 2018-093). PDL cells were isolated from freshly extracted third molars removed for orthodontic reasons as previously described [28]. Briefly, periodontal tissues attached around the middle third of the root surface were gently scraped and cut into small pieces prior plating on 35-mm tissue culture dishes to allow cell migration out of the explant tissues. Isolated cells were cultured in growth medium containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml amphotericin B. Cells were incubated at 100% humidity in an atmosphere of 5% CO_2 at 37 °C. The culture medium was changed every two days. All cell culture reagents were purchased from Gibco (Carlsbad, CA).

2.2. Compressive force treatment

For compressive force stimulation, PDL cells were seeded in 6-well culture plates at a density of 3.5×10^5 cells/well. Cells were serum-starved at 37 °C, 100% humidity, and 5% CO_2 for 8 h prior to mechanical loading. Compressive force loading was performed as described previously [29, 30]. Briefly, a plastic cylinder with metal coins was placed on the surface of the culture medium to generate compressive stress on the cell monolayer culture at the bottom of the plate. For the intermittent compressive force loading, a loading force of 1.5 g/cm^2 was applied to the cells at a frequency of 0.23 Hz. As to continuous compressive force loading, a continuous load was applied to the cells with a force of 1.5 g/cm^2 (Figure 1A).

For small molecule treatment, dobutamine hydrochloride (DH) (St. Louis, MO, USA) was used as the YAP inhibitor. Working concentrations of 5, 10, and 20 μM of DH were prepared from 20 mmol/l stock solution. The final concentration of 10 μM of DH was added to the medium. PDL cells were pretreated with DH for 30 min prior to intermittent compressive force loading.

2.3. Osteogenic differentiation of human PDL cells

For osteogenic differentiation induction, human PDL cells were seeded at a density of 5×10^4 cells per 1 of 6-well plates (Corning, Corning, NY, USA). The cells were cultured in osteogenic medium, which was DMEM high glucose (Gibco) supplemented with 50 mg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), 100 nM dexamethasone (Sigma-Aldrich), and 10 mM β-glycerophosphate (Sigma-Aldrich). The cells were incubated at 100% humidity and 5% CO_2 at 37 °C. The culture medium was changed every two days. The cells were cultured in osteogenic medium for 14 days prior to harvesting for osteoblast characterization, including alkaline phosphatase activity and osteogenic-specific gene expression analysis by quantitative real-time polymerase chain reaction (qRT-PCR). Calcium deposition ability was evaluated on day 14 after osteogenic induction. The cells were fixed with 4% formaldehyde for 15 min and stained with 40 mM Alizarin Red S solution (Sigma-Aldrich) for 20 min at room temperature. Quantification was performed by elution in 10% acetic acid with subsequent incubation at room temperature for 30 min. After centrifugation, the supernatant was collected and prepared in triplicates in a 96-well plate. The absorbance was

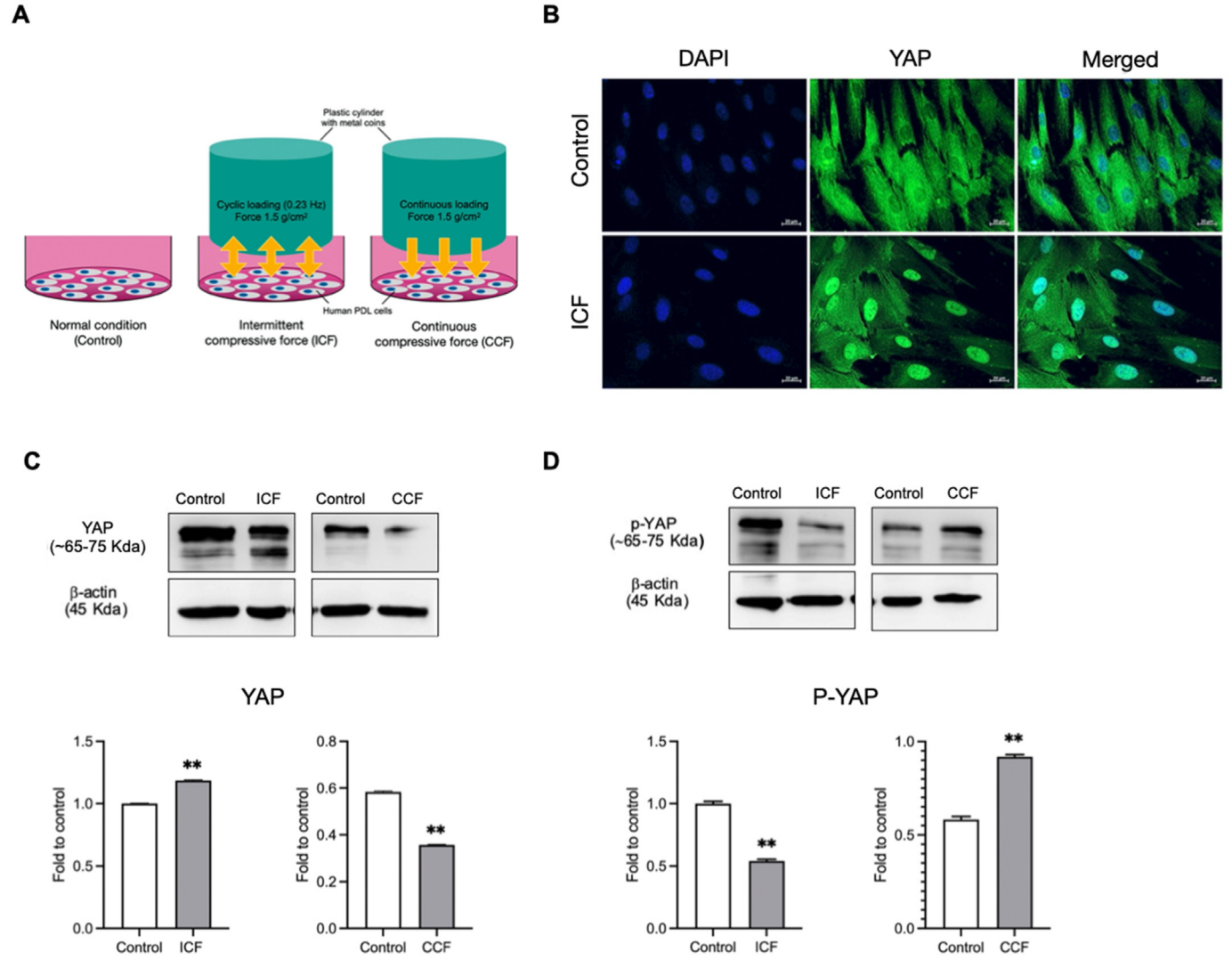

Figure 1. Expression of YAP in human PDL cells under compressive force loading. Schematic diagram of compressive force treatment in human PDL cells. (A) Experimental groups including controls (normal condition without compressive force loading), PDL cells with intermittent compressive force at 0.23 Hz, 1.5 g/cm^2 (ICF), and PDL cells with continuous loading force at 1.5 g/cm^2 (CCF). (B) Immunostaining of YAP-specific antibody (green) demonstrated the nuclear localization of YAP in PDL cells under ICF stimulation. DAPI (blue) was used to stain DNA. Scale bar, 20 μm. (C) Western blotting assay showed the activities of YAP in PDL cells under ICF and CCF. See Supplementary figure 5A–B for uncropped gel images. Quantification of YAP protein levels confirmed a significant increase in YAP expression with ICF, but a decreased YAP expression with CCF. (D) Quantification of P-YAP protein levels confirmed a significant decrease with ICF, but an increase with CCF. Band intensity was normalized to β-actin. The bars represent mean ± SEM. N = 3 independent experiments; *$P < 0.05$; **$P < 0.01$ considered significant.

measured at 405 nm using a spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). For the alkaline phosphatase staining at day 14 of the osteogenic differentiation period, the culture medium was removed from culture dish. The cells were rinsed with phosphate buffered saline and then stained with an alkaline phosphatase staining kit as per the manufacturer's protocol (SK-5100; Vector Laboratories, Burlingame, CA, USA). The stained, differentiated cells were observed under an inverted fluorescence microscope.

2.4. Adipogenic differentiation of human PDL cells

For adipogenic differentiation induction, human PDL cells were seeded at a density of 5 × 10^4 cells per 1 in 6-well plates (Corning) in complete culture medium. After 24 h, the cells were cultured for 21 days in an adipogenic differentiation medium. This comprised DMEM high-glucose (Gibco) supplemented with 1 μM dexamethasone (Sigma-Aldrich), 0.5 μM isobutylmethylxanthine (Sigma-Aldrich), 5 μg/ml insulin, and 50 μM indomethacin (Sigma-Aldrich). The medium was changed every 2–3 days. Adipogenic differentiation of PDL cells was determined by qRT-PCR for adipogenic-specific gene expression. Cells were assessed for intracellular lipid accumulation by using 0.5% (w/v) Oil Red O (Sigma Aldrich) in isopropanol for 30 min at RT. For the quantification of Oil Red O staining, the dye was eluted with 100% isopropanol by incubating cells with isopropanol for 10 min at RT. Solution aliquots of 200 μl/well were transferred into a 96- well plate. The absorbance was measured at 510 nm using a spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA).

2.5. Lentiviral shRNA transduction

For viral packaging, HEK293T cells were transfected with pLV (shYAP1) EGFP using Lipofectamine 3000 (Life Technologies, Carlsbad, CA, USA). The transfected HEK293T cells were incubated in 100-mm culture dishes at 37 °C and 5% CO_2 for 72 h. The medium containing pLentivirus was harvested, filtered through a 0.45 μm filter, and used for pLentivirus transduction. For viral transduction, human PDL cells were

incubated with 200 μl of viral supernatant and 8 μg/ml polybrene in culture medium for 24 h, and the medium was refreshed. Positive PDL cells expressing EGFP were evaluated under an inverted fluorescence microscope. The reduction in YAP expression was confirmed by qRT-PCR and Western blot analysis.

2.6. Scratch wound healing assay

After intermittent compressive force loading for 24 h, the proliferative ability of PDL cells was evaluated using a scratch wound assay. Cells were scratched with a P1000 pipette tip to create an artificial wound before being washed with culture media to remove cell debris. The cells were cultured in a fresh medium and maintained for 48 h. Wound widths were observed under an inverted microscope and measured using ImageJ software at 0, 6, 24, and 48 h of incubation. The percentage wound closure was calculated using the following equation 1.

$$\text{Wound closure } (\%) = \frac{A_0 - A_t}{A_0} \times 100$$

Equation 1: *"A_0" is the area of the wound measured immediately after scratching, and "At" is the area of the wound measured after the scratch has been performed for t hours.*

2.7. Flow cytometry analysis

Annexin V staining were performed and acquired via flow cytometry according to the manufacturers' instructions. The PDL Cells were stained with PE-conjugated annexin-V and 7-aminoactinomycin D (7-ADD) (BD Biosciences; San Jose, CA, USA) in the binding buffer in the presence of Ca2+ for 15 min. The cells were then harvested by mechanical scraping and analyzed using a FACS Canto cytometer (BD Biosciences).

2.8. Immunofluorescence staining

After fixing for 30 min in 4% cold paraformaldehyde at room temperature, the cells were incubating with 0.2% Triton in phosphate-buffered saline (PBS) for 10 min, and incubated for 2 h with primary antibody; anti-human KI-67 rabbit IgG antibody (1:100) (Abcam, Cambridge, UK) and anti-human YAP rabbit IgG antibody (1:250) at 37 °C. The cells were washed 3 times with PBS and incubated with anti-rabbit IgG Rhodamine conjugated (1:1000) and anti-rabbit IgG Alexa Fluor-488 conjugated (1:1000) (Cell Signaling Technology, Danvers, MA) for 1 h at 37 °C, and was covered with an antifade mounting medium containing DAPI. Isotype control staining was performed in parallel with the negative control. For quantification, a digital camera was used to capture 5 non-overlapping randomly field (×100) of each sample and showed as the percentage of KI67-positive cells per total cell counts. Each experiment was performed 3 times. The staining pattern was examined with an Axio Observer Z1 and ZEN pro (Zeiss International, Oberkochen, Germany).

2.9. RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent and RNA (1 μg) was converted into cDNA using a reverse transcriptase enzyme kit (Promega, Madison, WI, USA). qRT-PCR was performed using FastStart SYBR Green Master Essential DNA (Roche Applied Science, Indianapolis, IN, USA) with a LightCycler 96 (Roche Diagnostic, Mannheim, Germany), with GAPDH used as the reference gene for all experimental quantifications. The primer sequences are listed in Table 1.

2.10. Western blot analysis

Protein extraction was performed using cell lysis buffer (10x RIPA; Cell Signaling Technology, Danvers, MA, USA) containing protease inhibitors (Roche Life Science) supplemented with protease inhibitors (Roche Life Science). Total proteins were electrophoresed on 7% SDS-PAGE gels and the separated proteins were transferred to PVDF membranes (Merck Millipore, Billerica, MA, USA). Membranes were incubated with primary antibodies at 4 °C overnight. The primary antibodies used were as follows: anti-YAP, anti-phosphorylated YAP (Cell Signaling Technology), diluted 1:1000, and anti-β-actin peroxidase (ACTB; Sigma-Aldrich), diluted 1:25 000. The membrane was washed three times with Tris-buffered saline/Tween before incubation with peroxidase-conjugated, species-appropriate antibodies at a 1:5000 dilution at room temperature for 2 h. The signals were visualized by chemiluminescence (Merck Millipore).

2.11. Synchrotron fourier-transform infrared spectroscopy analysis (FTIR)

Differentiated PDL cells were harvested at day 21 of the adipogenic differentiation culture. Prior to a synchrotron fourier-transform infrared spectroscopy (FTIR) measurement, the cells were dissociated into single cells by enzymatic trypsinization and washed 3 times with phosphate buffer saline. The pellets of 4×10^5 cells were dropped onto infrared-transparent, 2-mm thick, barium fluoride windows. They were subsequently air-dried and washed with distilled water to avoid culture medium contamination.

Synchrotron FTIR spectra were acquired using a Hyperion 2000 FTIR microscope (Bruker Optics, Billerica, MA, USA). It had a liquid-nitrogen-cooled mercury-cadmium-telluride detector coupled to a Bruker Vertex 70 V FTIR spectrometer (Bruker Optics). The spectrometer was connected to an infrared microspectroscopy beamline (BL4.1 IR Spectroscopy and Imaging) at the Synchrotron Light Research Institute (SLRI, Nakhon Ratchasima, Thailand). Data were collected using an aperture size of 10 × 10 μm. The spectra were acquired in reflectance mode over

Table 1. Primer sequences used for quantitative real-time polymerase chain reaction.

Gene	Forward primer sequences	Reverse primer sequences
GAPDH	5′- TCATGGGTGTGAACCATGAGAA-3′	5′-GGCATGGACTGTGGTCATGAG-3′
LIN28A	5′- GAGCATGCAGAAGCGCAGATCAA-3′	5′- TATGGCTGATGCTCTGGCAGAAG-3′
LIN28B	5′- GCCCCTTGGATATTCCAGTC-3′	5′- TGACTCAAGGCCTTTGGAAG-3′
OCT4	5′- GCAACCTGGAGAATTTGTTCCT-3′	5′- AGACCCAGCAGCCTCAAAATC-3′
REX1	5′- TGGGAAAGCGTTCGTTGAGA-3′	5′- CACCCTTCAAAAGTGCACCG-3′
SOX2	5′- ACCAGCTCGCAGACCTACAT-3′	5′- ATGTGTGAGAGGGGCAGTGT-3′
KI67	5′- CGTTTGTTTCCCCAGTGTCT-3′	5′- CTCCCTGCCCCTTTCTATTC-3
YAP1	5′- CAGGTTGGCAGATGGCAAAG-3′	5′- TGTTGTCTGATCGATGTGATTGA-3′
OSX	5′- CTCGTCTGACTGCCTGCCTAG-3′	5′- GCGTGGATGCCTGCCTTGTA3-3′
RUNX2	5′- CGGGCTACCTGCCATCAC-3′	5′- GGCCAGAGGCAGAAGTCAGA-3′
COL1A1	5′- GTGCTAAAGGTGCCAATGGT-3′	5′- ACCAGGTTCACCGCTGTTAC-3′
PPARG	5′- CCTATTGACCCAGAAAGCGATT-3′	5′- CATTACGGAGAGATCCACGGA-3′
ADIPOQ	5′- TATCCCCAACATGCCCATTCG-3′	5′- TGGTAGGCAAAGTAGTACAGCC-3′

the range of 4000 to 800 cm^{-1} and at a spectral resolution of 6 cm^{-1}, with 64 scans co-added. The spectral acquisition and instrument control were performed using OPUS 7.5 software (Bruker Optics). Spectral changes in the functional groups were evaluated at the integral area of each peak, especially the region of amide I protein (1700–1600 cm^{-1}), amide II protein (1600–1500 cm^{-1}), CH stretching from lipid (3000–2800 cm^{-1}), C=O ester lipid (1750–1700 cm^{-1}), and $PO43^{-}$ or nucleic acid (1200–900 cm^{-1}). Spectra from each sample group were analyzed using principal component analysis (PCA). Preprocessing of the spectral data was performed using baseline correction. Data were normalized using extended multiplicative signal correction of spectral regions from 3000 to 2800 cm^{-1}, and 1800–900 cm^{-1}, using Unscrambler 10.1 software (CAMO Software, Oslo, Norway).

2.12. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). SEM was calculated based on the number of individual donors employed in each experiment. Cells from at least three different donors, without pooling, were subjected to each experiment. Statistical analyses of significance were performed using Student's t-test for comparing treatment and control groups. One-way ANOVA was used for multiple-group comparisons, and a post hoc test for individual comparison. Statistical significance was set at $P < 0.05$. Analyses were performed using the GraphPad Prism software (GraphPad Prism, San Diego, CA, USA).

3. Results

3.1. Effects of compressive forces on YAP signaling

To determine the effects of compressive forces on YAP in human PDL cells, we first determined YAP expression after intermittent compressive force (ICF) and continuous compressive force (CCF) loading. Human PDL cells were collected for qRT-PCR and Western blot analysis to determine YAP expression and activity, respectively. The results showed the nuclear translocation of YAP in PDL cells treated with ICF (Figure 1B). The active form of YAP increased when treated with ICF, whereas it decreased when CCF was applied (Figure 1C). In addition, the inactive form of phosphorylated YAP (p-YAP) decreased with ICF, whereas it increased with CCF (Figure 1D). These results indicate that ICF induced the expression of YAP and its activity in human PDL cells as determined by phosphorylation state of YAP.

3.2. Intermittent compressive force activates YAP transcriptional activity

To investigate whether PDL cells respond to ICF via the YAP signaling cascade, YAP-knockdown (YAP-KD) PDL cells were generated using short hairpin RNA (shRNA) targeting YAP (shYAP). A wild-type PDL cell line carrying an empty plasmid backbone was used as control. Suppression of YAP protein expression was confirmed by Western blot (Figure 2A). Thereafter, YAP-KD cells were loaded under intermittent compressive force (ICF) for 24 h. The loss of YAP did not affect the cell viability, nor did induce cell apoptosis in non-ICF and ICF stimulation (Supplementary Figure 1). The mRNA expression of *YAP1* and its downstream target genes, including *CYCLIN-D1, C-MYC*, and *CTGF*, was determined after ICF loading (Figure 2B,C). The results showed that the gene expression of *YAP1, CYCLIN-D1*, and *C-MYC* was significantly upregulated in PDL cells treated with ICF (Control ICF) compared to the control. The mRNA expression levels of *YAP*, *CYCLIN-D1, C-MYC*, and *CTGF* were downregulated in both the YAP-KD and YAP-KD ICF cells compared with the control and control ICF groups, respectively. Interestingly, in a comparison between the with- and without-ICF conditions, the same gene expression levels were higher in YAP-KD ICF cells than in YAP-KD cells alone. These results clearly indicated that ICF could activate the

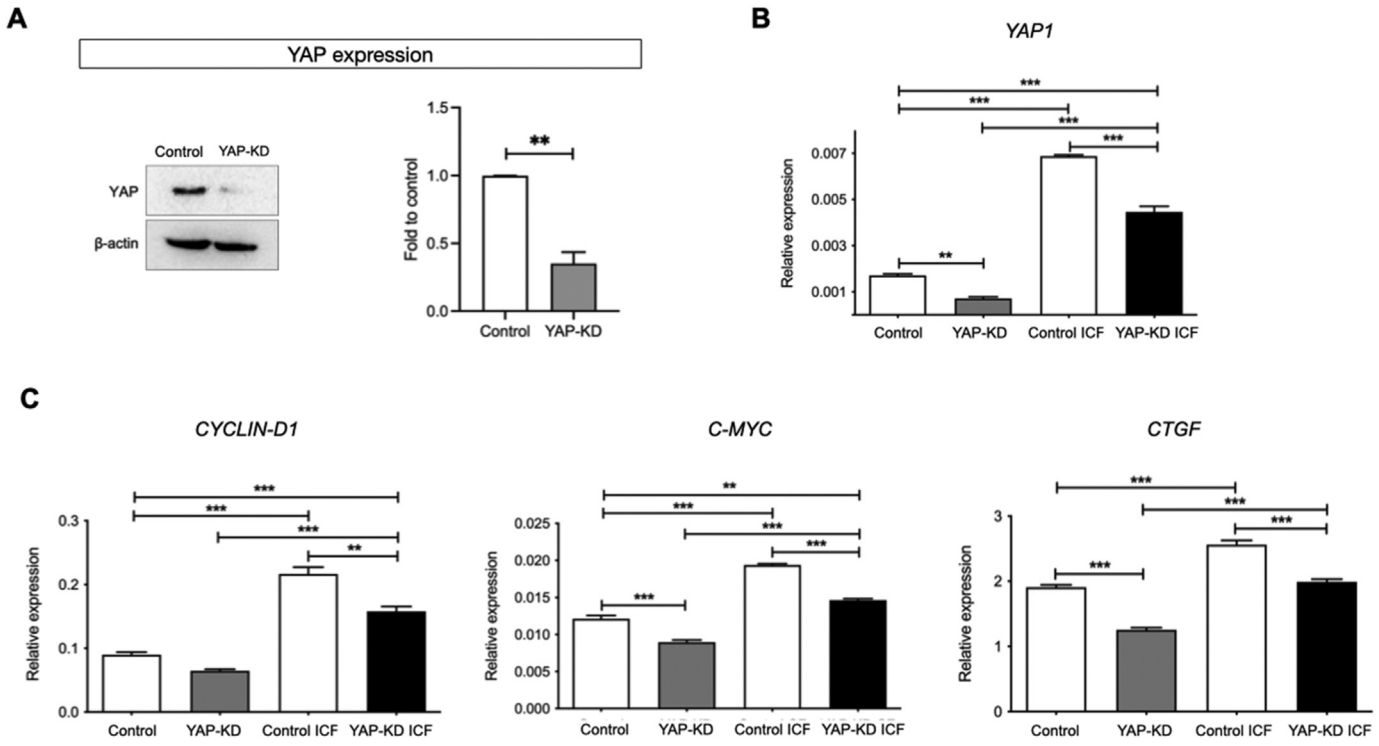

Figure 2. mRNA expression of YAP and its downstream target genes in YAP knockdown PDL cells under ICF loading. (A) The protein expression level of YAP in human PDL cells decreased after YAP knockdown by shRNA. Quantification of YAP protein levels confirmed a significant depletion in YAP expression in YAP knockdown (YAP-KD) PDL cells. See Supplementary figure 5C for uncropped gel images. (B–C) The relative gene expression level of *YAP1*, *CYCLIN-D1*, *C-MYC*, and *CTGF* were examined in YAP-KD and YAP-KD ICF cells. Data are expressed as transcript abundance ($2^{-\Delta Cq}$ relative to GAPDH). All qRT-PCR results are mean ± SEM comparative Ct values (n = 3); $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ considered significant.

transcriptional activity of YAP, as seen by the upregulation of YAP downstream target genes upon ICF treatment. In addition, these results suggest the role of YAP as a mechanosensing protein responsible for translating ICF signaling into cellular changes.

3.3. Intermittent compressive force increases cell proliferation via YAP signaling

To determine whether compressive force influences cell proliferation in human PDL cells, cells were loaded with ICF and CCF and subsequently collected for mRNA expression analysis using qRT-PCR. The results showed that the mRNA expression levels of cell proliferation-associated markers, including *LIN28a*, *LIN28b*, *OCT4*, *REX1*, *SOX2*, and *KI67,* were significantly upregulated with ICF, but there were no differences between cells in the CCF group and the control (Figure 3A). These results implied that compressive force influences the expression of cell proliferation-associated genes in a load-type manner. Moreover, mRNA expression of *LIN28A*, *LIN28b*, *OCT4*, *REX1*, *SOX2*, and *KI67* was significantly decreased in YAP-KD cells compared with the control under ICF loading (Figure 3B). These results were consistent with those observed when the pharmaceutical drug dobutamine hydrochloride (DH), a known YAP inhibitor, was used (Supplementary Figure 2). The immunofluorescence analysis showed that ICF significantly increased the percentage of KI67-positive cells compared to the control. While the loss of YAP decreased the percentage of KI67-positive cells compared to the control with and without ICF (Figure 3C,D).

Next, we investigated whether ICF influences the proliferation of human PDL cells via YAP expression using *in vitro* wound healing assay. The results showed that the proportional reduction in wound area increased gradually over time, with wounds nearly sealed 48 h after incubation in the control group (Figure 4A). In comparison, YAP-KD ICF cells presented a lower percentage of wound reduction than the control ICF cells (Figure 4B). A decreased percentage of wound reduction was also observed in the DH-treated PDL cells (Supplementary Figure 3).

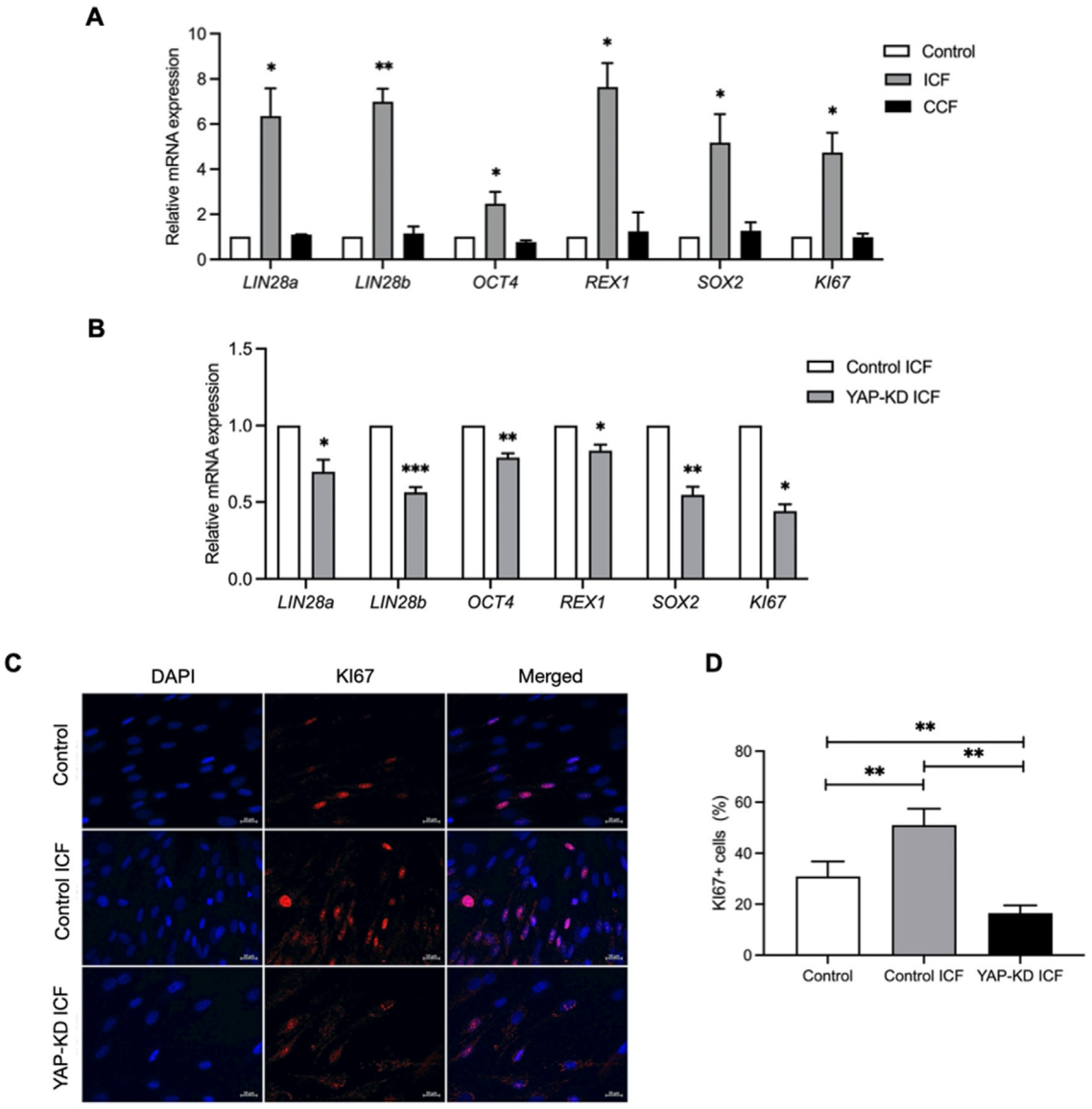

Figure 3. mRNA expression levels of cell proliferation-associated marker in YAP-KD cells under ICF loading. (A) mRNA expressions for *LIN28a*, *LIN28b*, *OCT4*, *REX1*, *SOX2* and *KI67* in control PDL cells (Control), PDL cells stimulated with ICF (ICF) and PDL cells stimulated with CCF (CCF) was analyzed by qRT-PCR. (B) A qRT-PCR analysis demonstrated the downregulation of *LIN28a*, *LIN28b*, *OCT4*, *REX1*, *SOX2* and *KI67* expression levels in ICF-treated YAP-KD cells (YAP-KD ICF) compared to ICF-treated PDL cells (Control ICF). (C) Immunostaining for the proliferation marker KI67 (red) and nuclei marker DAPI (blue) in YAP-KD ICF. Scale bar, 20 μm. (D) The percentage of ki-67-positive cells in YAP-KD ICF was lower than both the control and control ICF. Data are mean and SEM from N = 3 independent experiments; $*P < 0.05$; $**P < 0.01$, $***P < 0.001$ considered significant.

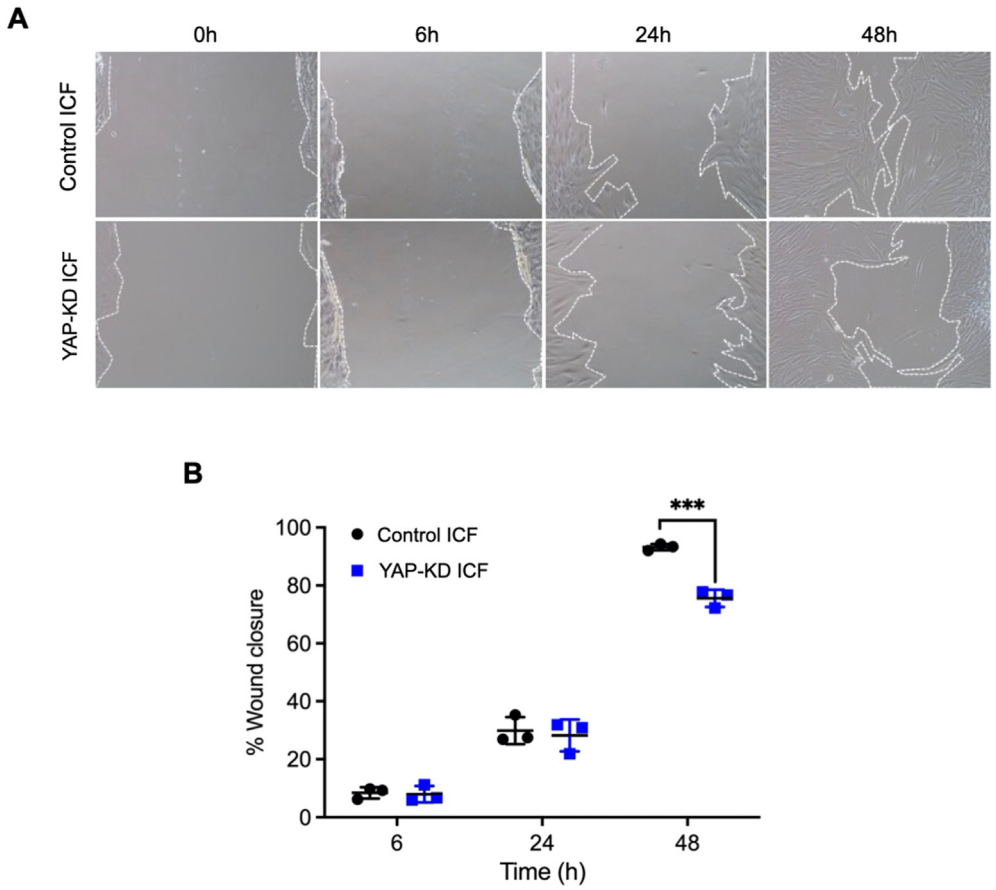

Figure 4. Silencing of YAP decreased cell proliferation of human PDL cells under ICF loading. (A) After ICF loading, a scratch wound assay was performed, and the wound area was observed under inverted microscope by ImageJ software at incubation periods of 0, 6, 24, and 48 h. (B) The percentage of wound closure was analyzed and presented as mean ± SEM. Data shown are representative of at 3 independent experiments; ***$P < 0.001$ considered significant.

Taken together, these results suggest that ICF influences human PDL cell proliferation via YAP signaling, as demonstrated by the decreased transcriptional activity of YAP target genes and delayed wound closure in the absence of YAP expression.

3.4. Increased YAP activity upon ICF loading affects cell fate decision and differentiation

We further explored whether ICF influenced the osteogenic differentiation ability of human PDL cells via YAP signaling. YAP-KD cells were treated with ICF for 24 h and subjected to osteogenic differentiation for 14 days. Cells were analyzed for mRNA expression of osteogenic-specific markers, including *RUNX2*, *OCN*, and *COL1A1*, by qRT-PCR. The protein expression of the osteogenic-specific gene *COL1A1* was determined by Western blot analysis as well as by alizarin red S staining of a calcified bone matrix. The results showed that the gene expression levels of *OCN* and *COL1A1* in the YAP-KD treated with intermittent compressive force were significantly downregulated compared with those in the control treatment (Figure 5A**).** The protein level of COL1 was markedly increased in the ICF-induced PDL cells, whereas it was decreased in the YAP-KD cells both with and without ICF treatment (Figure 5B). Osteogenic differentiation was confirmed by alkaline phosphatase (ALP) enzymatic activity staining, which was lower in the YAP-KD ICF cells than in the controls (Supplementary Figure 4). Quantitative measurement of bone matrix mineralization using alizarin red S staining showed markedly increased secretion of mineralized bone matrix in the control ICF compared with the control. However, suppression of YAP significantly decreased mineral deposition both with and without ICF loading, compared to each control condition (Figure 5C).

We further determined whether ICF influenced the adipogenic differentiation ability of human PDL cells via YAP expression. To test this, YAP-KD cells were induced to undergo adipogenic differentiation so that we could examine their adipogenic differentiation potential by mRNA expression analysis using qRT-PCR and measurement of lipid droplets stained with Oil Red O. After 21 days, the mRNA expression of adipogenic-specific markers, *PPARG* and *ADIPOQ*, was significantly upregulated in the YAP-KD and YAP-KD ICF cells, compared to each control condition. However, we found no significant difference between the control ICF and control groups (Figure 6A).

Oil Red O staining was performed to observe intracellular lipid droplets, which were visualized using bright-field microscopy. Interestingly, increased accumulation of cells with lipid droplets was clearly observed in YAP-KD cells. It has been shown that increasing YAP activity could promote differentiation bias towards the osteogenic lineage while inhibiting adipogenic lineage commitment [26]. In this study, the result showed that indirect upregulation of YAP activity by applying ICF to normal PDL cells (Control ICF) significantly reduced lipid droplet formation when compared with the untreated control (Figure 6B-C). Interestingly, quantitative analysis of Oil Red O showed no significant difference in lipid accumulation in YAP-KD and YAP-KD ICF, and higher amounts of lipid droplets of YAP-KD ICF were still observed when compared to control ICF. This result suggests that the lack of YAP in PDL cells is irresponsive to mechanical forces.

To further confirm that YAP is an important mechanosensing signaling molecule, the reduction of YAP could lead to other distinct changes in the biological response in the cells, regardless of the presence of ICF. The overall changes in the global biomolecules were studied using synchrotron FTIR analysis. Under similar culture conditions as ICF, cells

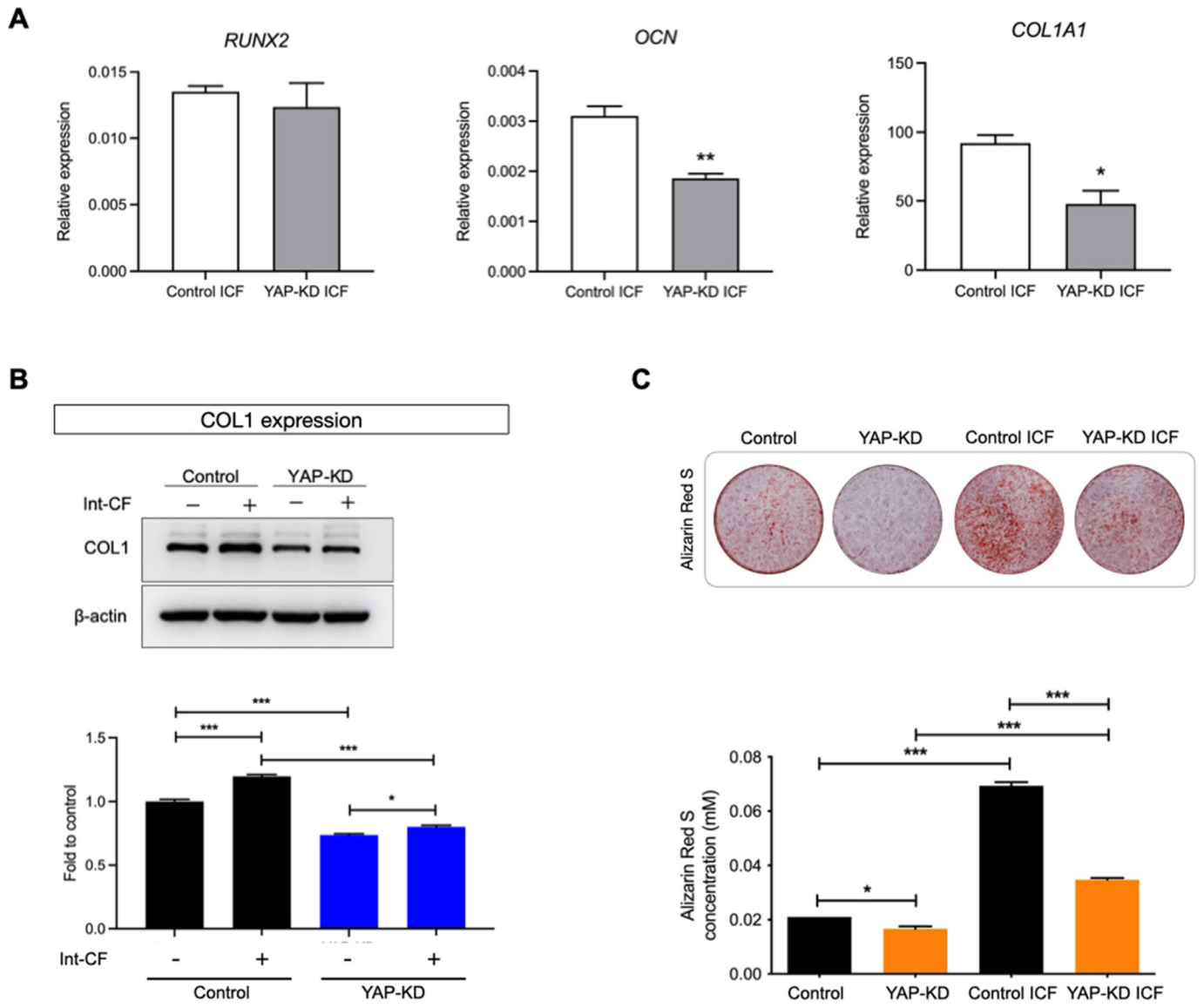

Figure 5. Suppression of YAP inhibits osteogenic differentiation of human PDL cells under ICF loading. (A) The relative mRNA expression of osteogenic specific markers including *RUNX2*, *OCN* and *COL1A1* were examined in YAP-KD and YAP-KD ICF cells. (tern blot analysis showed the protein expression levels of COL1 in YAP-KD and YAP-KD (B) Western blot analysis showed the protein expression levels of COL1 in YAP-KD and YAP-KD ICF cells. See Supplementary figure 5D for uncropped gel images. (C) Matrix mineralization was examined using Alizarin Red S staining. The quantitative Alizarin Red S concentration was analyzed by absorbance measurement. Error bars indicate SEM. Data shown are representative of at least 3 independent experiments; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ considered significant.

lacking YAP showed clear changes in global cell biomolecule structure when compared to control ICF. It revealed a relatively higher lipid level in the YAP-KD ICF cells than in the control ICF, which was presented as integral areas for the prominent lipid (Figure 7A-C). The PCA score plot of all recorded FTIR spectra of the control ICF and YAP-KD ICF cells demonstrated a clear separation of the two-cell type (Figure 7D-E). This result supports the idea that YAP is an important signaling molecule that regulates adipogenic differentiation in human PDL cells. Rescue of YAP activity by applying ICF to YAP-depleted cells did not completely mimic the control phenotype.

4. Discussion

YAP has been recognized as a downstream activator of the Hippo signaling pathway, which plays an essential role in cell proliferation and apoptosis. YAP has also been shown to be a key regulator in mechanotransduction process that controls cellular activities in response to mechanical stimuli [31]. *In vitro* compressive force models have been shown to influence cell proliferation and osteogenic differentiation in several cell types, including osteoblasts [32], stem cells from human exfoliated deciduous teeth [33], and PDL cells [34]. Nevertheless, the role of YAP in the response to compressive force has not yet been elucidated.

The distinct influence of compressive force types has been stated in regulating biological activities of human PDL cells. Previous study demonstrated that intermittent force potentially enhanced IL-1β-mediated RANKL expression compared with continuous force, suggesting the role in bone remodeling [12]. Subsequently, *HES1* mRNA expression was increased after ICF stimulation, but not in CCF, indicated the different effect among intermittent and continuous force regarded to Notch signaling pathway in human PDL cells [30]. The mRNA expression profile analysis demonstrated the influence of ICF on gene expressions compared to CCF stimulation in human PDL cells. The OSX mRNA expression was increased in PDL cells after ICF treatment, while it was

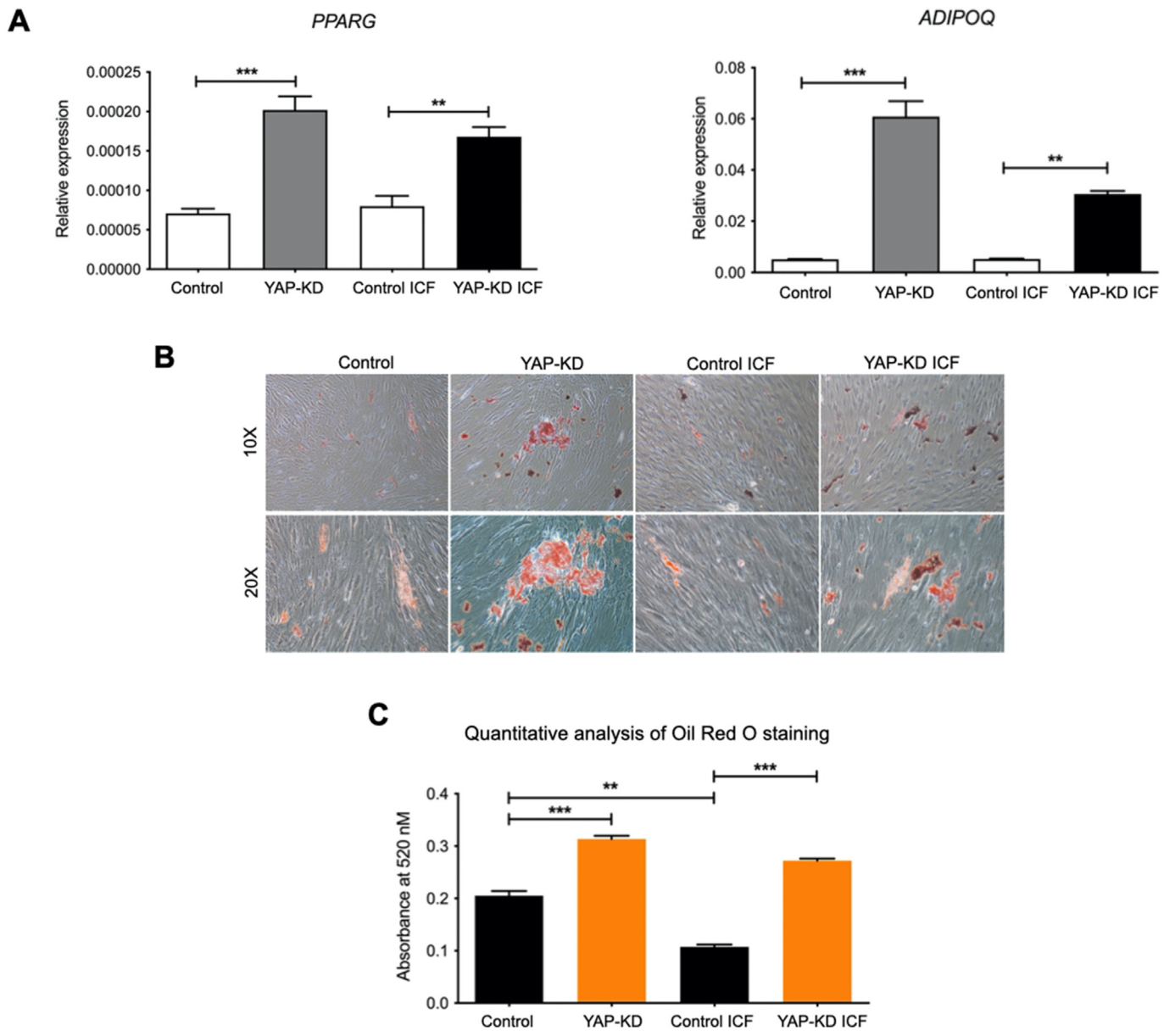

Figure 6. Suppression of YAP enhanced adipogenic differentiation of human PDL cells under ICF loading. (A) Gene expressions for *PPARG* and *ADIPOQ* in control, YAP-KD, control ICF and YAP-KD ICF cells were analyzed by qRT-PCR. (B) Accumulation of lipid droplets was detected using Oil red O staining. (C) Absorbance measurement was performed for quantitative analysis. Data are mean and SEM from N = at least 3 independent experiments; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ considered significant.

attenuated in CCF treated PDL cells. In addition, ICF-treated PDL cells showed a greater ability of matrix mineralization than CCF, indicating the effect of different compressive force types on osteogenic differentiation capacity of PDL cells [35]. In current study, we initially demonstrated that the expression level of YAP in PDL cells can be significantly stimulated by ICF, but not by CCF. This suggests that the role of YAP also depends on the loading manner of compressive force.

Intermittent compressive force activates YAP activity and its downstream target genes, which commonly contribute to cell cycle and proliferation. Suppression of YAP using inducible shRNA knockdown hinders the influence of intermittent compressive force-induced pluripotent factors, cell proliferation, and osteogenic differentiation of human PDL cells. However, YAP suppression has the potential to drive PDL into the adipogenic lineage. Overall, our findings indicate the role of YAP as a mechanosensitive regulator required for cell proliferation and fate determination in human PDL cells (Figure 8).

It is well known that periodontal tissue responds to mechanical stimuli conducted either physiologically or non-physiologically, for example, orthodontic tooth movements. Upon mechanical loading during tooth movement, specialized cells located within the PDL tissue transfer mechanical signals from the tooth to the surrounding alveolar bone. These cells are also activated to maintain periodontal tissue homeostasis [36, 37]. According to previous studies, the application of interval loading as an intermittent compressive stress is a mechanical model that can provide suitable physiological tooth movement during mastication by activating PDL cells [38, 39]. On the other hand, continuous compressive force loading is represented as orthodontic tooth movement on the bone-resorption side [40, 41]. The suture compression force promotes osteoclastogenesis at the compressive side of the PDL cells, resulting in bone resorption and decreased bone density [42]. Taken together, these findings suggest that different types of compressive force exert distinct PDL cell responses in alveolar bone remodeling.

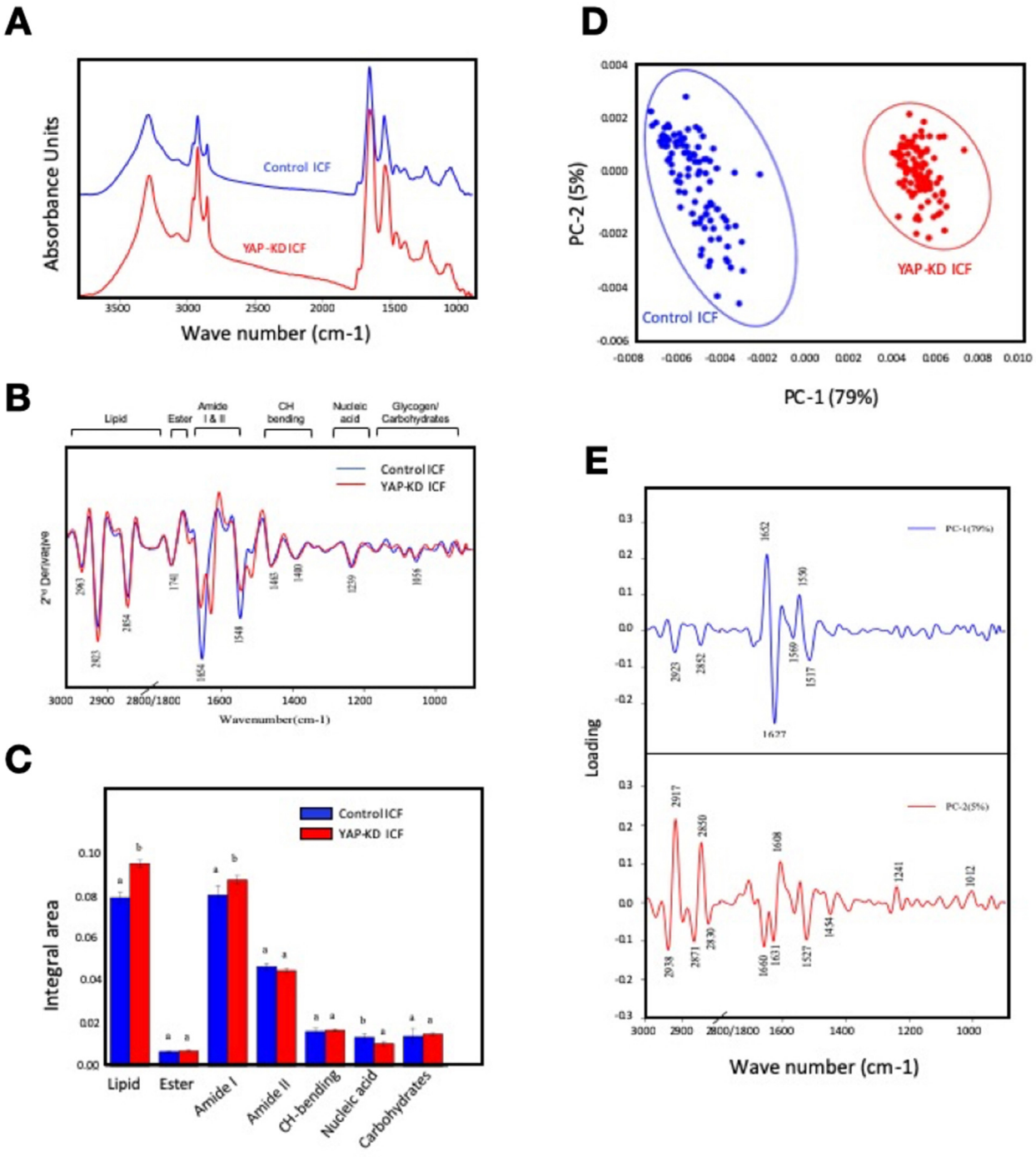

Figure 7. Synchrotron FTIR signatures indicated adipogenic phenotypes of differentiated YAP-KD cells under ICF loading. (A) A PCA analysis was used to separate the spectra differences of the control ICF and YAP-KD ICF cells into the ranges of 3000–2800 cm^{-1} and 1800–900 cm^{-1}. (B) Second derivative spectral from control ICF and (C) PCA was performed on the second derivative spectra from the control ICF and YAP-KD ICF cells. (D) 2D PCA score plot of all recorded FTIR spectra of control CF and YAP-KD ICF cells. (E) The CH stretching from lipids at 2917, 2850 and 1241 cm^{-1} from nucleic acid were heavily loaded for the positive PC2 loading plot (F) Score loading of PC1 (upper) and PC2 (lower) to identify the variable corresponding to wavelength number. Blue dots represent control; red dots represent YAP-KD ICF cells. Eclipses depicted in the plot define the confidence level with which 95% of the data are allocated.

YAP and its coactivator TAZ (YAP/TAZ) have been reported to promote the expression of genes that regulate pluripotency in mouse embryonic stem cells, such as *OCT4*, *NANOG*, and *SOX2*. Activated YAP enhances the expression of pluripotent transcriptional regulators by binding interactions with TEAD2 (TEA/ATTS domain) transcription factors, leading to the self-renewal capacity of mouse ESCs. However, silencing YAP expression leads to loss of pluripotency, resulting in cells undergoing differentiation [43, 44]. Apart from conventional mesenchymal stem cell markers, human PDL cells have been shown to express several pluripotent factors such as *OCT4*, *NANOG*, *SOX2*, and *REX1* [45,46]. Previous study showed that STAT3 activated Oct4/Sox2 expression in dental pulp and PDL cells under co-cultured system both *in vitro* and *in vivo*. Theses indicated that pluripotency of dental-derived cells can be modulated through cell-cell communication [47]. Our findings demonstrated that YAP knockdown interfered with the expression level of *OCT4*, *SOX2*, and *REX1* in ICF-induced PDL cells. The result was also confirmed by using small molecule treatment

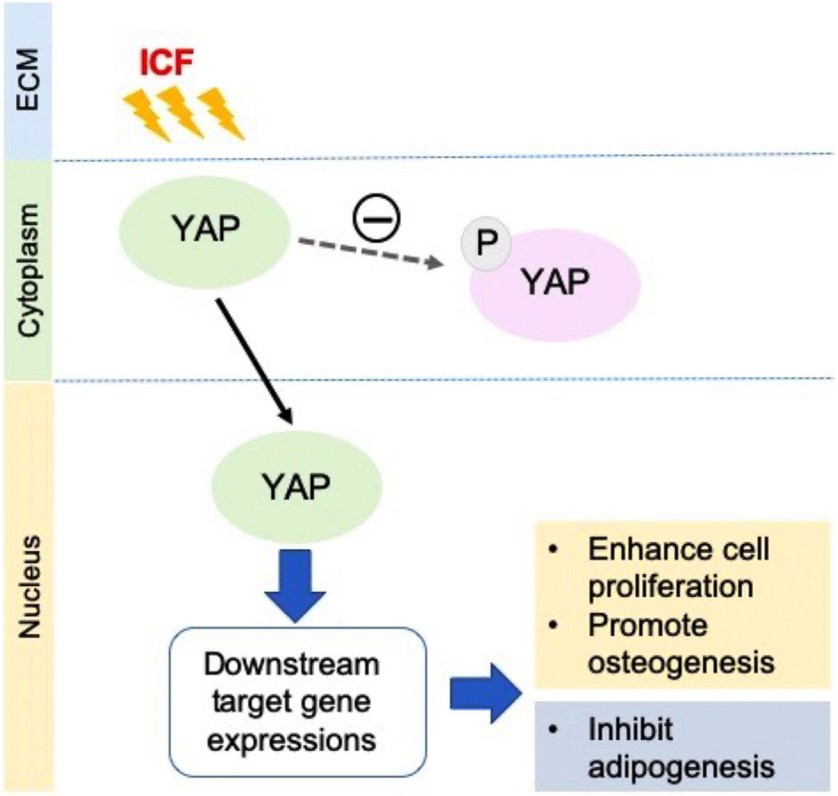

Figure 8. Schematic diagram of the human PDL cell behavior in response to ICF via YAP signaling. ICF inhibit YAP phosphorylation in cytoplasm, then YAP translocate into nucleus and activate its downstream target gene expressions. These signaling cascade is require for cell proliferation and cell fate decisions by promoting osteogenesis and inhibiting adipogenesis in human PDL cells.

(Supplementary figure 2). Dobutamine hydrochloride (DH), beta-1 adrenergic agonist has been potentially inhibit nuclear translocation of YAP, thereby recognized as YAP signaling inhibitor [48]. Previous study demonstrated that dobutamine could attenuate osteogenic differentiation, while promote adipogenic differentiation of human MSCs via YAP signaling [49]. Collectively, we thus suggested that YAP may associated to the pluripotency of PDL cells in response to intermittent compressive force.

In response to mechanical cues (e.g. ECM stiffness, cell-cell or cell-ECM interactions), cells receive extrinsic stimuli from the surrounding extracellular matrix (ECM) via cell surface receptor, integrins. Integrin clustering activates adhesion complexes and interacts with cytoskeletal components, such as focal adhesion kinase (FAK). The actin cytoskeleton can modulate YAP/TAZ through canonical and non-canonical Hippo pathways. For example, in canonical pathway, the deformation of the cytoskeleton-generated transforming protein Ras homolog family member A (RhoA) and MAPK, thereby regulating the Hippo core kinases (MST1/2 and LATS1/2), which in turn result in modulate phosphorylation and localization of YAP/TAZ [50, 51]. In response to ECM stiffness, FAK-mediated cytoskeleton showed to modulate YAP/TAZ in non-canonical pathway via plasma membrane protein and several signal transducers including Caveolin-1 (CAV-1), GTPases, MAP4K and JNK. The mechanical force has also showed to modulate the same mechanosensing machineries as ECM stiffness, such as RAP2 and MAP4Ks, to regulate YAP/TAZ cascade [52]. Beside the role as mechanosensing protein in response to mechanical cues, YAP also plays an important role in cell communication, which generates intrinsic force and is transmitted to neighboring cells through cell-cell junctions, such as cadherin receptors [53].

Since cell-based bone tissue engineering technology is promising for clinical bone tissue regeneration by providing the three dimensional (3D) microenvironment as *in vivo*, the signaling pathway that mediated in 3D environment has been widely studied compared to the conventional 2D system [54, 55]. Unlike 2D system, cells in 3D culture may not always increase cell spreading when increased matrix stiffness. The cellular response to matrix stiffness in 3D system has showed to be dependent on the types and matrix properties such as physical crosslink of hydrogel [56], material viscoelasticity [57] and degradability of a matrix [58]. In this context, the YAP-mediated cellular response to hydrogel stiffness and its degradability has revealed to be different among 2D and 3D systems. Recent study demonstrated that high stiffness in 2D MSC culture induced YAP/TAZ nuclear localization, correlated with increase cell spreading than low and medium stiffness group. Whereas high stiffness 3D hydrogel reduced YAP/TAZ nuclear localization and has lower cell spreading than low and medium stiffness. In addition, degradable hydrogel showed to enhance YAP/TAZ nuclear translocation and increased cell spreading compared to cells in non-degradable hydrogels, suggesting the influence of hydrogel degradability on cellular response in 3D microenvironment [59].

The influence of intermittent compressive force on PDL cell behavior through the mechanotransduction pathway has recently been revealed using bioinformatic profiling. A study showed that intermittent compressive force upregulated genes involved in ECM-receptor interaction, focal adhesion, and TGF-β signaling pathways, which facilitate the osteogenic differentiation potential of PDL cells [35]. Similarly, our study also demonstrated that intermittent compressive force could enhance the proliferation and osteogenic differentiation of human PDL cells via the YAP signaling cascade. Moreover, intermittent compressive force rescued the osteogenic differentiation potential of YAP-KD PDL cells; this was confirmed by Western blot analysis and calcium deposition, as shown in Figures 5B and 5C. Overall, these findings suggest that intermittent compressive force influences PDL cell behavior through the mechanotransduction pathway.

Previous studies have demonstrated the role of YAP/TAZ in cell lineage commitment in osteogenic and adipogenic phenotypes through the focal adhesion activated-cytoskeleton rearrangement resulting from ECM cues

(e.g., ECM stiffness, substrate mechanics, and cell spreading) [60, 61, 62]. Despite the cell surface topography or substrate rigidity, YAP-regulated cell commitment to the osteogenic lineage is also dictated by mechanical stimuli. Shear stress enhances the nuclear accumulation of YAP in a RHOA-dependent manner, enhancing the osteogenic differentiation of MSCs. Conversely, the fate of MSCs results in a commitment to the adipogenic lineage through a loss of YAP activity caused by low mechanical loading [63]. Recently, the role of YAP in osteogenesis was demonstrated in human PDL cells stimulated with cyclic stretch forces [27]. Likewise, our study found that intermittent compressive force regulated the adipo-osteogenic cell decision of PDL cells in a YAP-dependent manner, as suppression of YAP led to osteogenesis inhibition and enhanced adipogenesis. Although further studies are needed to examine the mechanisms by which ICF activates the YAP pathway, our data indicated that YAP could be a mechanosensitive transcriptional activator for the regulation of PDL cell behavior in response to intermittent compressive force. These results suggest that manipulation of the Hippo-YAP signaling pathway may facilitate periodontal tissue regeneration.

5. Conclusions

Considering the underlying mechanisms of mechanical forces regulating PDL cell behavior may provide key insights into periodontal tissue homeostasis. Our findings indicated that YAP in PDL cells could act as a mechanosensing protein that regulates PDL cell differentiation. Intermittent compressive force induces YAP expression in PDL cells and causes differentiation bias towards the osteogenic lineage. In contrast, loss of YAP alters PDL cell commitment to the adipogenic lineage. This can explain the importance of mechanical loading in the PDL tissue to prevent alveolar bone loss. Taken together, we propose that the Hippo-YAP signaling pathway may be involved in physiological tooth movements to maintain periodontium homeostasis. Moreover, we speculate that the direct manipulation of YAP may control cell fate differentiation into osteogenic and adipogenic lineages of human PDL cells, thereby facilitating the application of YAP in periodontal tissue regeneration.

Ethics approval and consent to participate

The human PDL cell isolation protocol was approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University (HRBC-DCU 2018-093).

Declarations

Author contribution statement

Nuttha Klincumhom; Chanchao Lorthongpanich: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Kanjana Thumanu; Praphasri Septham; Wutthikiat Phomyu: Performed the experiments; Analyzed and interpreted the data.

Surapol Issaragrisil: Contributed reagents, materials, analysis tools or data.

Prasit Pavasant: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration interests statement

The authors declare no conflict of interest.

Additional information

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