

## Original Article

# Cyclic tension promotes osteogenic differentiation in human periodontal ligament stem cells

Tao Shen<sup>1,2\*</sup>, Lin Qiu<sup>3\*</sup>, Huijun Chang<sup>1,4\*</sup>, Yanchun Yang<sup>1</sup>, Congxiang Jian<sup>1</sup>, Jian Xiong<sup>1</sup>, Jixiang Zhou<sup>1</sup>, Shiwu Dong<sup>5</sup>

<sup>1</sup>Department of Stomatology, Southwest Hospital, Third Military Medical University, Chongqing, China; <sup>2</sup>Department of Stomatology, Out-Patient Department of 61251 PLA Troops, Qinhuangdao, China; <sup>3</sup>Department of Stomatology, General Hospital of Beijing Military Area Command, Beijing, China; <sup>4</sup>Department of Stomatology, Out-Patient Department of Sichuan Military Area Command, Chengdu, China; <sup>5</sup>Department of Biomedical Materials Science, School of Biomedical Engineering, Third Military Medical University, Chongqing, China. \*Equal contributors.

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**Abstract:** Orthodontic forces result in alveolar bone resorption and formation predominantly on the pressure and tension sides of the tooth roots, respectively. Human periodontal ligament stem cells (PDLSCs) have demonstrated the capacity to differentiate into osteoblasts, and they play important roles in maintaining homeostasis and regenerating periodontal tissues. However, little is known about how PDLSCs contribute to osteoblastogenesis during orthodontic tooth movement on the tension side. In this study, we applied a 12% cyclic tension force to PDLSCs cultured in osteoinductive medium. The osteogenic markers Runx2, ALP, and OCN were detected at the mRNA and protein levels at different time points using real-time PCR and western blot analyses. We discovered that the mRNA and protein levels of Runx2, ALP and OCN were significantly up-regulated after 6, 12 and 24 hours of mechanical loading on PDLSCs compared to levels in unstimulated PDLSCs ( $P < 0.05$ ). This study demonstrates, for the first time, the effects of mechanical tensile strain on the osteogenic differentiation of PDLSCs, as examined with a Flexcell FX-4000T Tension Plus System. Our findings suggested that cyclic tension could promote the osteogenic differentiation of PDLSCs. Furthermore, the effects of orthodontic force on alveolar bone remodeling might be achieved by PDLSCs.

**Keywords:** Periodontal ligament, stem cells, stress, cell differentiation, osteoblasts, biomechanics

## Introduction

Orthodontic tooth movement is induced by mechanical stimuli and is facilitated by remodeling of the periodontal ligament (PDL) and alveolar bone. The forces applied to the teeth are transmitted through the PDL to the alveolar bone, leading to bone deposition on the tension side and bone resorption on the pressure side. Previous studies have indicated that the progenitor cells in the PDL could differentiate into osteoblasts [1-4] and that mechanical tension forces on PDL cells could induce osteoblastogenesis-supporting activity [5-8]. Clinically, ankylosed teeth and dental implants that lack PDL cannot be moved by orthodontic treatment [9]. Therefore, PDL cells likely play critical roles in periodontal and osseous remodeling during orthodontic tooth movement, and some

precursor cells in the PDL could maintain homeostasis and the regeneration of periodontal tissue.

Human periodontal ligament stem cells (PDLSCs), which were first isolated from PDL tissues in 2004 [1], are a unique cell population with mesenchymal stem cell (MSC) properties. These PDLSCs possess the capacity for self-renewal and the potential for multilineage differentiation. Recent studies have reported that PDLSCs express the MSC-associated surface markers STRO-1, CD29, CD44, CD90, CD105, CD146, and CD166 in addition to an array of cementoblastic/osteoblastic markers [1, 2, 10]. PDLSCs also exhibited osteogenic characteristics under defined culture conditions *in vitro*, and they demonstrated the capacity to generate cementum/PDL-like structures *in vivo* [1-4],

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suggesting the involvement of PDLSCs in alveolar bone remodeling. Additionally, mechanical strain could stimulate MSCs to differentiate into osteoblasts [11-13], which are vital for bone formation in distraction osteogenesis. Thus, it is likely that PDLSCs are capable of developing into osteoblasts under mechanical tension force.

However, the majority of studies on PDLSCs have mainly focused on their proliferation and differentiation in response to various biological factors and chemical molecules [3, 4, 14, 15]. Studies of the effects of mechanical strain on the osteogenic differentiation of PDLSCs are rare.

Here, we postulated that PDLSCs exposed to a mechanical tensile force such as that which occurs during orthodontic tooth movement would differentiate into osteoblasts, leading to subsequent bone deposition. To test this hypothesis, we applied cyclic tension force to PDLSCs under osteogenic induction conditions, and we monitored the expression of Runx2, ALP and OCN by real-time PCR and western blot analyses after 6, 12, and 24 hours of loading. In addition, the levels of CD146, a differentiation marker in the development of osteoblasts, were determined.

### Materials and methods

#### *Ethics statement*

Informed written consents were obtained from all patients who participated in this study. Tissue specimens were obtained for investigation purposes only. The protocols for the use of human tissues in this study were approved by the Ethics Committee of Southwest Hospital, Third Military Medical University, China.

#### *PDLSC isolation and culture*

Disease-free first premolars ( $n = 14$ ), extracted for orthodontic purposes, were collected from 8 patients aged 12-24 years old at the Department of Stomatology of Southwest Hospital. All of the participants provided written and informed consent prior to tooth extraction, according to the guidelines of Third Military Medical University.

PDL tissues were gently separated from the middle third of the root with a scalpel and were

then digested in a solution of 1 mg/mL collagenase type I (Gibco-Invitrogen, Carlsbad, CA, USA) for 40 minutes. Then, the pellet PDL cells and tissues were obtained after centrifugation and plated in six-well plates (Corning Inc., Corning, NY, USA) and were incubated at 37°C in 5% CO<sub>2</sub> with alpha-modified Eagle's Medium ( $\alpha$ -MEM; Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% (V/V) fetal bovine serum (FBS; Gibco-Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), 100 mM ascorbic acid (Sigma, Saint Louis, MO, USA), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin (Gibco-Invitrogen, Carlsbad, CA, USA). When the PDL cells reached 60% confluence, they were trypsinized, and a single-cell suspension was prepared for isolation of the PDLSCs.

After the single-cell suspension was harvested, the cells were seeded into a 96-well culture plate, ensuring that each well had no more than one cell. The single-cell wells were selected and marked under the microscope after 8 to 12 hours, and the cells were cultured in  $\alpha$ -MEM containing 10% (V/V) FBS supplemented with antibiotics at 37°C in a 5% CO<sub>2</sub> incubator. When they reached 40% confluence, the cells in the marked holes were expanded and passaged. The colony-derived PDLSCs at 4 to 6 passages were used for the study. The medium was changed every 3 days throughout the experiments.

#### *Characterization*

To assess their colony-forming efficiency, 200, 500 and 800 cells were plated onto separate 100-mm culture dishes with  $\alpha$ -MEM growth medium. After 14 days, the cultures were fixed with pure methanol and were stained with Giemsa stain. Cell aggregates were observed under a microscope, and aggregates of more than 50 cells were counted as colonies.

Flow cytometric analysis was used to determine the cell phenotype and the cell cycle of the PDLSCs. Cultured cells were detached as single cell suspensions and were resuspended in blocking buffer for 30 min. A total of  $3 \times 10^6$  cells were incubated with antibodies (20  $\mu$ L of CD146, 20  $\mu$ L of STRO-1, BioLegend, San Diego, CA, USA) and isotype control antibodies (15  $\mu$ L, BioLegend, San Diego, CA, USA) for 1 hour on ice. After washing, the cells were ana-

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**Table 1.** Primer sequences for qPCR

Gene	Primer sequence
Runx2	Forward: 5-GCAGTCCCAAGCATTTCAT-3
	Reverse: 5-CACTCTGGCTTTGGGAAGAG-3
ALP	Forward: 5-CCACGTCTTCACATTGGTG-3
	Reverse: 5-AGACTGCGCCTGGTAGTTGT-3
OCN	Forward: 5-GTGCAGAGTCCAGCAAAGGT-3
	Reverse: 5-AGACTGCGCCTGGTAGTTGT-3
CD146	Forward: 5-CTGCTGAGTGAACCACAGGA-3
	Reverse: 5-TCAGGTCATGCAACTGAAGC-3
$\beta$ -actin	Forward: 5-CATTAAGGAGAAGCTGTGCT-3
	Reverse: 5-GTTGAAGGTAGTTTCGTGGA-3

lyzed using a flow cytometer (BD Bioscience, Bedford, MA, USA). For cell cycle evaluation,  $3 \times 10^6$  cells were prepared and treated with pre-cooled 75% ethanol overnight at 4°C. Then, the cells were stained with propidium iodide for 30 min on ice, and flow cytometric analysis was performed to investigate the fractions of cells that were in the G0/G1, G2, and S phases of the cell cycle.

To test the differentiation potential of the PDLSCs, osteogenic and adipogenic differentiation tests were performed. The cells were plated in growth medium at  $2 \times 10^4$  cells/cm<sup>2</sup> on 24-well plates. Upon reaching 100% confluence, the growth medium was changed to either osteogenic or adipogenic differentiation medium (Cyagen Biosciences, Santa Clara, CA, USA), and the manufacturer's protocols were followed. The control groups were treated with standard PDLSC culture medium as described above. After 3 weeks, the cells were fixed with 4% formalin and were stained with Alizarin red and Oil red O dyes (Sigma, Saint Louis, MO, USA), respectively.

### *Application of cyclic strain*

PDLSCs were cultured in a 6-well, flexible-bottomed culture plate coated with type I collagen (Sigma, Saint Louis, MO, USA). When 100% confluence, PDLSCs were serum-starved overnight, and osteoinductive medium was added. Then, cyclic tension force (12% deformation) was applied to the PDLSCs at a frequency of 0.1 Hz (5-s stretch and 5-s relaxation) for 6, 12 and 24 hours using a Flexcell FX-4000T Tension Plus System (Flexcell International Corporation, Hillsborough, NC, USA). The control cultures were maintained under identical culture condi-

tions but without mechanical stimulation. Then, the cells were collected, and their mRNA and protein levels were investigated.

### *Real-time quantitative polymerase chain-reaction assay (qPCR)*

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) after performing mechanical loading on the PDLSCs. cDNA synthesis was performed with a RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The primers used are listed in **Table 1**. The qPCR reactions were performed using SYBR Green PCR Master Mix and an ABI 7500 Real-time PCR Detection System (Applied Biosystems, Grand Island, NY, USA). Critical threshold (CT) values obtained from qPCR were analyzed using the  $2^{-\Delta\Delta CT}$  method for relative quantification of each target gene [16]. The mRNA expression changes were calculated after normalization to  $\beta$ -actin as an endogenous control.

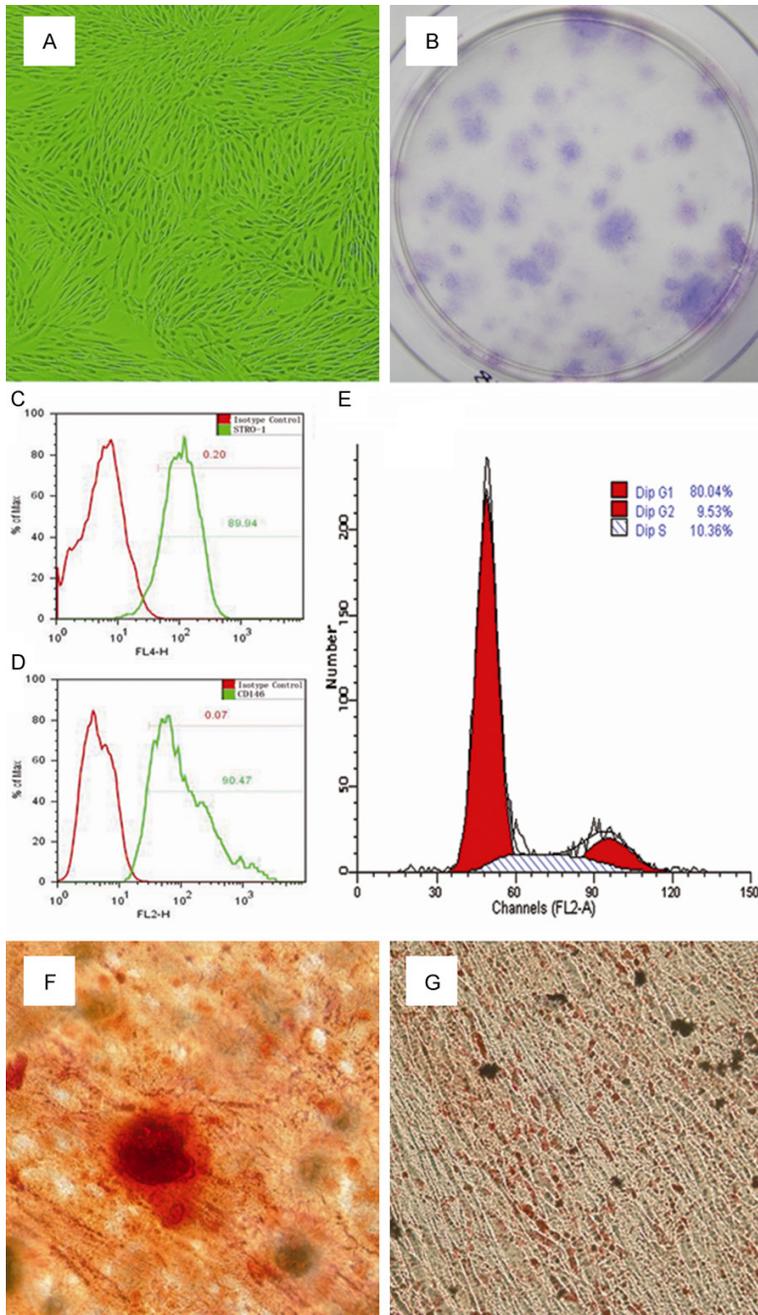
### *Western blot*

Total protein was extracted with a Total Protein Extraction Kit (ProMab Biotechnologies, Richmond, CA, USA) and was measured using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein extracts were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were blotted onto nitrocellulose membranes (Pierce, Rockford, IL, USA). The blots were incubated with primary antibodies overnight at 4°C, followed by incubation with a secondary antibody at room temperature for 1 hour. The primary antibodies specific to Runx2, ALP, OCN and CD146 were purchased from Santa Cruz Biotechnology (USA). The blots were developed with an enhanced chemiluminescence system (Pierce, Rockford, IL, USA), and the integrated optical density (IOD) values of each blot were analyzed using Gel Pro software, version 4.0 (Media Cybernetics, Rockville, MD, USA). GAPDH was run as a reference protein.

### *Statistical analysis*

All of the data are presented as the mean  $\pm$  SD, and all of the experiments were repeated at least three times ( $n = 3$ ). Two-way ANOVA was

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**Figure 1.** Isolation and characterization of PDLSCs. (A) PDLSCs obtained by the cloning method retained a typical fibroblastic spindle shape and had the ability to form adherent clonogenic cell clusters. (B) Representative images of colonies formed by PDLSCs at low seeding density and stained with Giemsa stain after 14 days of culture. (C-E) Cells were sorted by flow cytometry and were analyzed using Cell Quest software (BD, USA). (C) The percentage of the STRO-1-positive cell population was identified (89.94%). (D) The proportion of CD146-positive cells was identified (90.47%). (E) The fractions of cells in the G0/G1, G2, and S phases of the cell cycle were identified (G1 = 80.04%, G2 = 9.53%, S = 10.36%). (F, G) Cultured PDLSCs formed Alizarin red-positive calcium deposits (F) and Oil red O-positive lipid clusters (G) after 3 weeks of culture in osteogenic and adipogenic induction medium, respectively.

performed for statistical comparisons using SPSS software, version 13.0 (IBM Corporation, Armonk, NY, USA). *P*-values < 0.05 were considered statistically significant.

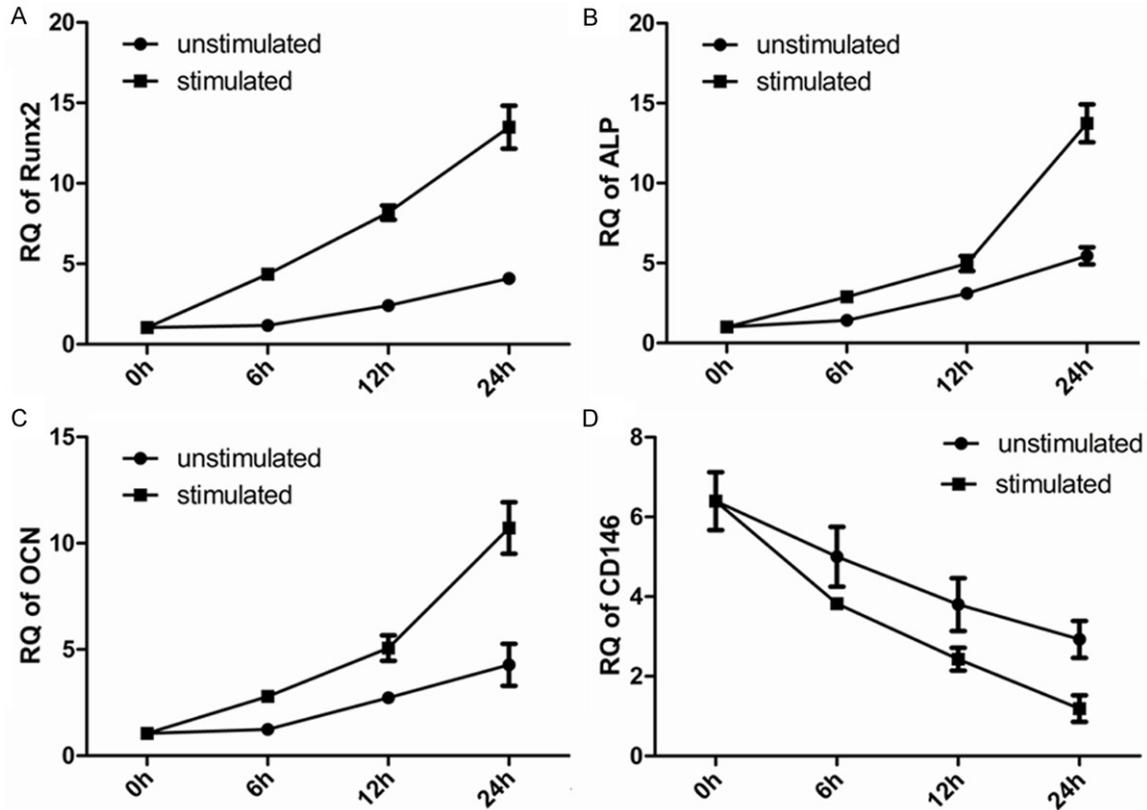
### Results

The obtained cells retained a typical fibroblastic spindle shape and had the ability to form adherent clonogenic cell clusters (**Figure 1A, 1B**). Flow cytometry was used to characterize the cell phenotype and cell cycle. The results showed that the isolated cells were positive for the PDLSC markers STRO-1 and CD146 (**Figure 1C, 1D**), and most of the cells were in the G0/G1 phase of the cell cycle (**Figure 1E**). After the PDLSCs were cultured with osteoinductive medium for 3 weeks, calcium deposits were observed with Alizarin red staining (**Figure 1F**); under adipogenic induction, the PDLSCs developed into Oil red O-positive lipid-laden droplets (**Figure 1G**).

**Figure 2A-C** shows the time course of mRNA expression changes in the PDLSCs in response to cyclic tension force. The expression of Runx2, ALP, and OCN mRNA, detected by qPCR, was up-regulated in both mechanically stimulated and unstimulated cultures in a time-dependent manner; however, the mechanically stimulated cultures showed higher mRNA expression of these genes (*P* < 0.05). The protein levels determined by western blot were consistent with the changes in mRNA expression (*P* < 0.05) (**Figure 3A-D**).

As shown in **Figure 2D**, in the cells without mechanical stim-

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**Figure 2.** Results of real-time quantitative polymerase chain-reaction assay (qPCR). qPCR was performed using total RNA obtained from mechanically stimulated and unstimulated cultures. The relative quantities (RQs) of the target genes were calculated by the  $2^{-\Delta\Delta CT}$  method, and the results showed statistically significant ( $P < 0.05$ ) up- (A, Runx2; B, ALP; C, OCN) or down-regulation (D, CD146) of mRNA expression in response to exposure to cyclic mechanical tension of 12%.  $\beta$ -actin was used as the endogenous control in three independent experiments. Values are expressed as the mean  $\pm$  SD.

ulation, CD146 expression decreased over time, whereas the expression of CD146 in cultures under cyclic strain decreased more significantly than that in unstimulated cells ( $P < 0.05$ ). After 12 hours of loading, the mRNA expression of CD146 showed a statistically significant decrease ( $P < 0.05$ ) in both groups, and similar results were observed for the protein levels (Figure 3A, 3E).

### Discussion

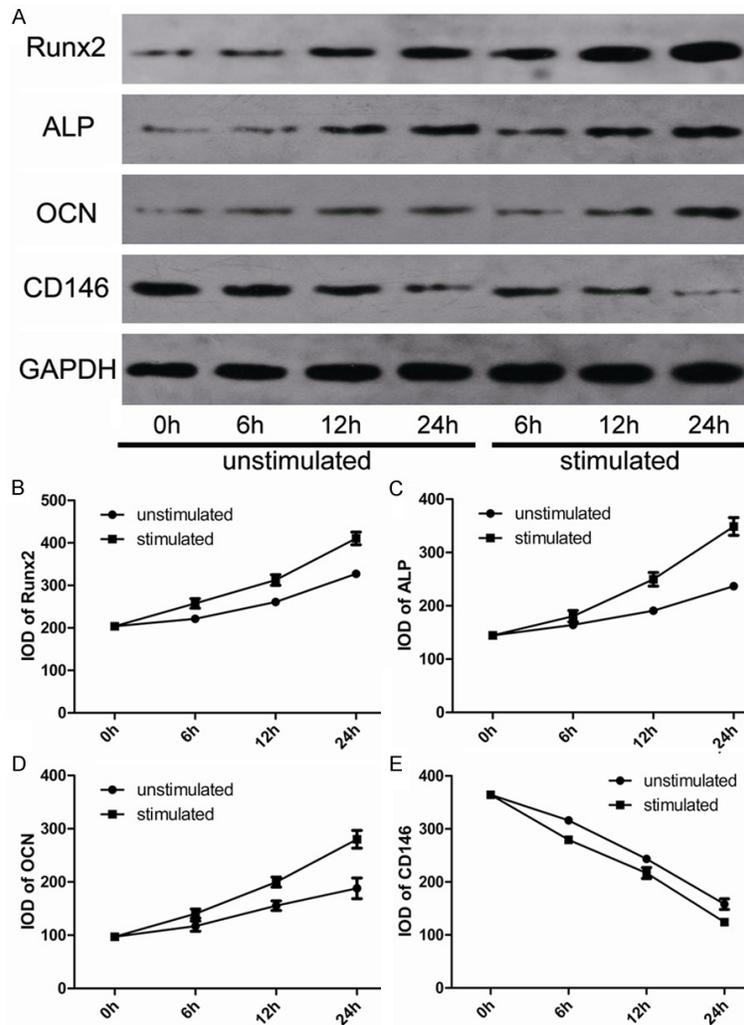
Orthodontic tooth movement is achieved by alveolar bone remodeling. PDLSCs have great potential for osteogenic differentiation, and they play important roles in periodontal tissue repair and in tissue regeneration [1, 2, 4, 17]. However, the role of PDLSCs in osteoblastogenesis during orthodontic tooth movement is unclear. In this experiment, cyclic tension force was applied to PDLSCs cultured in osteoinduc-

tive medium, and the results showed that cyclic tension force could promote the up-regulation of the osteogenic markers Runx2, ALP, and OCN in PDLSCs.

In this study, we developed a new protocol by modifying the limiting dilution technique to isolate a pure population of PDLSCs. The single-cell-derived colony cultures were characterized by the expression of the PDLSC markers STRO-1 and CD146 as well as by their capacity for multipotent differentiation [1]. The cells we obtained possessed the same properties as PDLSCs, which suggested that the cells that we used in this experiment were reliable and available.

Mechanical force is known to be a stimulus for alveolar bone remodeling. The effects of mechanical stimulation on cells are dependent on the device, magnitude, duration, and fre-

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**Figure 3.** Results of the western blot analysis. PDLSCs were cultured in osteoinductive medium for 6, 12 and 24 hrs in the presence or absence of cyclic tension of 12%. Then, western blot assays were performed, and the integrated optical density (IOD) values were analyzed. (A) The results from one representative independent experiment out of three are shown. (B-E) IOD value analyses showed a statistically significant ( $P < 0.05$ ) increase (A, Runx2; B, ALP; C, OCN) or decrease (D, CD146) in the expression of proteins of interest in a time-dependent manner. The expression levels were normalized to that of GAPDH. The data are expressed as the mean  $\pm$  SD of three independent experiments.

quency [9, 18-20]. In our study, we used a Flexcell FX-4000T Tension Plus System, a computer-regulated bioreactor that uses vacuum pressure to apply defined, controlled, and cyclic deformation to growing cells in vitro, which has been the most frequently utilized cell culture mechanostimulus system [21]. The strain magnitude of 12% was based on finite element analysis data [22]; larger stretching could induce the osteogenic lineage differentiation of tendon stem cells [20]. The tensile loading fre-

quency (0.1 Hz) and duration (6, 12 and 24 hours) in this experiment were determined from the literature on PDL cell stress tests [6, 7, 23].

Runx2 is a key transcription factor that functions in the modulation of osteogenic differentiation of MSCs and in bone formation [24, 25]. In addition, Runx2 is a target of mechanical signals in osteoblastic cells, and it also controls the expression of major osteoblast-specific genes, such as ALP, OCN, type I collagen, bone sialoprotein, osteopontin, and collagenase-3 [24, 27]. Several studies [24, 25] have established that Runx2-deficient mice completely lack bone formation due to the arrest of osteoblast maturation, and overexpression of Runx2 induced nonosteogenic cells to express osteoblast-related genes in vitro. Additionally, mechanical stimuli could lead to increased expression of Runx2, which is a target of mechanical signals in osteoblastic cells [5, 25]. Accordingly, in our study, the onset of Runx2 expression represented the early stage of PDLSC osteogenic lineage commitment. Our results indicated that increased Runx2 expression, in a time-dependent manner under mechanical tension, marked the beginning of the osteogenic differentiation of PDLSCs.

ALP is an indicator of new bone formation, and it has been commonly studied as an early marker of the osteogenic differentiation of MSCs in tension force experiments. Some studies have found that mechanical tension increased the ALP expression of PDL cells [7, 8]; however, other studies have reported that mechanical stretching resulted in decreases in ALP activity [6, 28]. A possible explanation for the discrepancy in these findings might be differences in mechanical deformation, loading style, dura-

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tion, and cell culture conditions. Considering that orthodontic force leads to bone deposition on the tension side and that ALP is an indicator of new bone formation, the expression of ALP would be expected to increase when strain is applied [8]. In this model, we demonstrated that the ALP expression of PDLSCs was enhanced by cyclic mechanical stretching, suggesting the beginning of induction of osteogenic differentiation.

OCN is an osteoblast-specific marker, and it is present during the late stages of osteoblast differentiation [29]. In this study, we observed that the OCN expression by PDLSCs under mechanical tension was significantly increased, suggesting the termination of osteogenic differentiation and the onset of mineralization. The changes in OCN expression also suggested that cyclic strain could promote the differentiation of PDLSCs toward osteogenic lineage commitment. Furthermore, our observations showed that the up-regulation of OCN expression tended to correlate positively with the expression of Runx2; these findings are consistent with the conclusions of earlier studies that reported that OCN is the target gene of Runx2 and is regulated by Runx2 [24-26].

Another antigen that we evaluated was CD146, which is considered to be a marker for MSCs, and its expression may be linked to multipotency. MSCs with greater differentiation potential expressed higher levels of CD146 on the cell surface [30]. In this study, we found that CD146 was expressed in PDLSCs before the induction of osteogenic differentiation. When PDLSCs were cultured in osteoinductive medium, the CD146 expression of both mechanically stimulated and unstimulated cultures began to decrease in a time-dependent manner. Therefore, it is likely that CD146 was expressed during PDLSC proliferation, and its expression decreased when the PDLSCs differentiated toward osteoblast-like cells. CD146 could be considered a transient marker for the early differentiation of PDLSCs toward an osteogenic lineage, and the CD146 expression level decreased with the loss of the multi-lineage potential of PDLSCs under mechanical stimulation.

In this study, we observed that under cyclic tension force, the expression of Runx2, ALP and OCN by PDLSCs was significantly increased and the expression of CD146 was decreased at

both the mRNA and protein levels in a time-dependent manner. Our results strongly suggested that cyclic strain could promote the differentiation of PDLSCs towards osteogenic lineage commitment. The results also suggested that the expression of Runx2, ALP and OCN by the PDLSCs indicated the early-stage differentiation of PDLSCs toward osteogenic lineage commitment.

Our investigation demonstrated that mechanical stimulation promoted the osteogenic differentiation of PDLSCs. It was previously reported that both cyclic tension and osteoinductive medium could enhance the osteogenic commitment of human MSCs, but cyclic tension was a stronger differentiation factor than osteoinductive medium [11]. In this study, the expression of osteogenic markers by PDLSCs cultured with both osteoinductive medium and cyclic strain was higher than that of cells cultured only with osteoinductive medium. This finding suggested that PDLSCs are mechanically sensitive cells and that cyclic tension force was an important regulator that induced the osteoblastogenesis of the PDLSCs.

In summary, we observed that cyclic tension force exerted an influence on the up-regulation of the osteogenic markers Runx2, ALP, and OCN in PDLSCs, suggesting that mechanical stimulation could enhance the osteogenic differentiation of these periodontal stem cells. The up-regulated expression of Runx2, ALP, and OCN by the PDLSCs also strongly suggested direct differentiation toward osteogenic lineage commitment. Therefore, PDLSCs might be considered a source of osteoblasts and could play a pivotal role in bone remodeling during orthodontic tooth movement. Due to their osteogenic potential, PDLSCs could potentially be used for the reconstruction of periodontal tissues during orthodontic therapy. However, the mechanotransduction mechanism [18] by which mechanical loading is transduced into a cascade of cellular and molecular events is unknown. Thus, a better understanding of the cellular signaling pathway during the mechanical osteoinductive response of PDLSCs is essential for future improvements in orthodontic therapy.

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### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Jixiang Zhou, Department of Stomatology, Southwest Hospital, Third Military Medical University, Chongqing, China. Fax: +86-23-68754387; E-mail: ortho\_zhou@163.com; Dr. Shiwu Dong, Department of Biomedical Materials Science, School of Biomedical Engineering, Third Military Medical University, Chongqing, China. Fax: +86-23-68771270; E-mail: dongshiwu@163.com

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