

# Influence of different intensities of vibration on proliferation and differentiation of human periodontal ligament stem cells

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## Abstract

**Introduction:** To understand the effects of low-magnitude, high-frequency (LMHF) mechanical vibration at different intensities on human periodontal ligament stem cell (hPDLSC) proliferation and osteogenic differentiation.

**Material and methods:** The effect of vibration on hPDLSC proliferation, osteogenic differentiation, tenogenic differentiation and cytoskeleton was assessed at the cellular, genetic and protein level.

**Results:** The PDLSC proliferation was decreased after different magnitudes of mechanical vibration; however, there were no obvious senescent cells in the experimental and the static control group. Expression of osteogenesis markers was increased. The expression of alkaline phosphatase (ALP) and osteocalcin (OCN) mRNA was up-regulated at 0.1 g, 0.3 g, 0.6 g and 0.9 g magnitude, with the peak at 0.3 g. The type I collagen (Col-I) level was increased after vibration exposure at 0.1 g, 0.3 g, and 0.6 g, peaking at 0.3 g. The expression levels of both mRNA and protein of Runx2 and osterix (OSX) significantly increased at a magnitude of 0.1 g to 0.9 g, reached a peak at 0.3 g and then decreased slowly. The scleraxis, tenogenic markers, and mRNA expression decreased at 0.05 g, 0.1 g, and 0.3 g, and significantly increased at 0.6 g and 0.9 g. Compared with the static group, the F-actin stress fibers of hPDLSCs became thicker and clearer following vibration.

**Conclusions:** The LMHF mechanical vibration promotes PDLSC osteogenic differentiation and implies the existence of a magnitude-dependent effect of vibration on determining PDLSC commitment to the osteoblast lineage. Changes in the cytoskeleton of hPDLSCs after vibration may be one of the mechanisms of the biological effects.

**Key words:** mechanical vibration, periodontal ligament stem cells, osteogenesis, proliferation, magnitude.

## Introduction

Human periodontal ligament stem cells (hPDLSCs) have been isolated and identified as mesenchymal stem cells (MSCs) since 2004 [1]. It has been shown that hPDLSCs are multipotent cells with features similar to bone marrow and dental pulp MSCs, which are capable of proliferating

and producing different types of tissues such as bone, adipose and tooth associated tissues in specific media [1, 2]. As such, PDLSCs have a dynamic role in maintaining periodontal homeostasis, and are responsible for remodeling and regeneration of periodontal tissues.

It is well known that mechanical stimuli have an important role in bone tissue metabolism and periodontal tissue homeostasis [3–7]. Recently, low-magnitude, high-frequency (LMHF) vibration (e.g., acceleration less than  $< 1 \times g$ , where  $g = 9.81 \text{ m/s}^2$ , at 20–90 Hz) has gained much interest as studies have shown that such mechanical stimulation can positively influence skeletal homeostasis [8–11]. Studies have demonstrated that such kinds of mechanical stimuli exhibit anabolic effects on bone homeostasis in animals [12, 13], postmenopausal osteoporotic women [14], and children with musculoskeletal diseases [15]. However, the underlying mechanism of the anabolic role of LMHF mechanical vibration on bone remains unknown. It has been well accepted that mechanical signals may regulate the direction of stem cell differentiation [16, 17]. With specific regard to mechanical vibration, it has been shown that it has the ability to direct the lineage commitment of bone marrow stromal cells (BMSCs) to the osteoblast lineage [18, 19]. However, little is known about the response of hPDLSCs to any type of mechanical stimulus. While the effect of LMHF mechanical vibration on BMSCs has been established, its potential effect on the remodeling of paradental tissues, including alveolar bone and periodontal ligament (PDL), is currently unknown.

The PDL sensitively mediates the transmission of stress stimuli to the alveolar bone for periodontal tissue remodeling. Derived from PDL, hPDLSCs are surmised to have a similar osteogenic response induced by strain. In this regard, our research group was particularly interested in whether LMHF mechanical vibration exerts an effect on hPDLSC proliferation and differentiation. According to findings from our previous experiments, LMHF mechanical vibration promotes PDLSC osteogenic differentiation and implies the existence of a frequency-dependent effect of vibration on determining PDLSC commitment to the osteoblast lineage. However, the magnitude of the mechanical vibration is vital for the therapy effect. Because mechanical stimuli have also been widely used to enhance the formation and properties of tissue-engineered bone [20], the optimal magnitude and frequency should be made clear. Only when these problems are figured out may it be possible to optimize the clinical application protocol of mechanical vibration to facilitate osteogenesis effectively and safely. Given the fact that the

efficacy of mechanical stimulation is dependent not only on the frequency, but also the magnitude of the applied vibration stimulus [9, 21], we also wanted to establish the optimal parameters of the mechanical stimulus for promoting osteogenic differentiation. To test our hypothesis, we subjected hPDLSCs to LMHF mechanical vibration at a magnitude of 0.05 g to 0.9 g and a frequency of 50 Hz, based on our previous experiments showing that mechanical vibration at frequency of 50 Hz, with a 0.3 g magnitude, was more favorable for hPDLSC osteogenic differentiation. The effect of mechanical vibration on hPDLSC proliferation and osteogenic differentiation potential was assessed at the cellular, genetic and protein levels.

## Material and methods

### Isolation and identification of human periodontal ligament stem cells

Human PDLSCs were isolated and cultured as previously described [22]. Periodontal ligament cells (PDLs) were scraped from the healthy, non-carious premolar tooth roots, extracted from donors aged between 12 and 16 years old for orthodontic reasons with informed consent. The PDLs at passages 2–4 were used for immunomagnetic microbead isolation of PDLSCs via the CD146 microbead kit. The sorted CD146(+) cells were identified as hPDLSCs by immunocytochemical staining using the following antibodies: STRO-1, CD146, CD271 and scleraxis [22]. To investigate the multipotency of hPDLSCs, the isolated cells were tested for the ability to undergo osteogenic and adipogenic differentiation [22]. The passages 3–4 of hPDLSCs were used in the following experiments. Collection and culture of hPDLSCs was approved by the Ethical Committee Board of the West China College of Stomatology, Sichuan University.

### Study design

Cells seeded at  $1 \times 10^5$ /well in a six-well plate were randomly divided into mechanical vibration culture and static culture groups, both of which were cultured in a humidified atmosphere of 5%  $\text{CO}_2$  at 37°C. Cells were incubated overnight in  $\alpha$ MEM with 10% FBS to promote cell attachment. After 24 h, the medium was replaced with  $\alpha$ MEM with 2% FBS for 24 h in order to synchronize the cell cycle. Prior to application of the mechanical stimulus, the culture medium of both groups was replaced with fresh  $\alpha$ MEM with 10% FBS.

### Application of LMHF mechanical vibration strain

Six-well plates culturing hPDLSCs were mounted onto the platform, parallel to the ground, of

a GJX-5 vibration sensor [22] (Beijing Sending Technology, Beijing, China). The hPDLSCs received sinusoidal LMHF mechanical stimuli (magnitude: 0.05 g to 0.9 g, frequency: 50 Hz) perpendicular to the ground for 30 min every 24 h.

### RNA isolation and quantitative real-time RT-PCR

The total RNA of hPDLSCs under different culture conditions was isolated using Trizol reagent (Invitrogen) 2 h after the last stimulus of the fifth day. Following RNA isolation, real-time RT-PCR was carried out using the SYBR-PrimeScript™ RT-PCR Kit (TaKaRa, China). The primer sequences for Runx2, osterix (OSX), Col-I, ALP, OCN, scleraxis and GAPDH were synthesized based on the GenBank database and are presented in Table I.

### Western blots

Total protein for western blot analysis were started to be extracted 12 h after the last stimulation cycle on the fifth day of culture. Forty µg protein extracts were separated on 10% SDS-PAGE gels and subsequently transferred to a PVDF membrane. After blocking, the membranes were probed with primary antibodies raised against osterix and Runx2 (Abcam, Cambridge, MA USA), followed by the addition of a horseradish peroxidase-conjugated secondary antibody (Zhongshan Bio Co., Beijing, China). Immunoreactive proteins were visualized using a chemiluminescence kit (Millipore, Billerica, MA, USA). Band intensities were determined using the ChemiDoc XRS Gel

documentation system and Quantity One software (Bio-Rad, Hercules, CA, USA).

### Cell proliferation assay

After a mechanical vibration stimulus for 3 days, cell proliferation in each group was measured using a cell counting kit (CCK-8; Dojindo Molecular Technologies Inc., Rockville, MD, USA) from 12 h after the last stimulus. The cell viability was shown in direct proportion to the absorbance at 450 nm; therefore, it was calculated as the OD value using a microplate reader (Varioskan Flash; Thermo Fisher Scientific, Waltham, MA, USA).

### Assessment of senescence-associated β-galactosidase staining

The cell senescence of hPDLSCs was tested after mechanical vibration for 5 days, at the ideal intensity and frequency for enhancing PDLSCs' osteodifferentiation based on the results of this and previous works (magnitude: 0.3 g, frequency: 50 Hz). Cell senescence was measured at 12 h after the last stimulus using a senescence-associated β-galactosidase staining kit (Beyotime, China). The senescent cells were observed in an optical microscope and counted from 5 random fields of vision.

### Immunofluorescence analysis

We observed cytoskeletal changes after the mechanical vibration (magnitude: 0.3 g, frequency: 50 Hz), which was more conducive for the osteodifferentiation of PDLSCs, according to the results

Table I. qRT-PCR primers used in this study

Target gene	Primers	Sequence	Fragment size [bp]
Runx2	Forward	5'-CAGATGGGACTGTGGTTACTGT-3'	169
	Reverse	5'-GTGAAGACGGTTATGGTCAAGG-3'	
Osterix	Forward	5'-CTGTGAAACCTCAAGTCTATGGA-3'	69
	Reverse	5'-GCTCTGCAGTCAAGGGAGATG-3'	
GAPDH	Forward	5'-GGAAGGTGAAGTCCGGAGT-3'	229
	Reverse	5'-TGGAAGATGGTGATGGGATT-3'	
ALP	Forward	5'-CAGATGAAGTGGGAGTGCTTGT-3'	115
	Reverse	5'-CTGATGTGGAGTATGAGAGTGACG-3'	
OCN	Forward	5'-CTCACACTCCTCGCCCTATTG-3'	142
	Reverse	5'-GCCTGGTCTTCACTACTCT-3'	
Col-I	Forward	5'-CCACCTGCCTCTGGCTTCT-3'	68
	Reverse	5'-AGCTGTGGAGGAGGTTTCA-3'	
Scleraxis	Forward	5'-GAACACCCAGCCCAAACAGAT-3'	63
	Reverse	5'-TCCTTGCTCACTTTCTCTGGTT-3'	

of our previous studies. Cells were subjected to mechanical vibration continuously for 2 h (magnitude: 0.3 g, frequency: 50 Hz), then fixed with 4% paraformaldehyde, rinsed with PBS 3 times, permeabilized with 0.25% Triton X-100 for 10 min, rinsed with PBS 3 times again, then incubated with 5% BSA at 37°C for 30 min. Next, the cells were incubated with 0.5  $\mu$ M Alexa Fluor 488-conjugated phalloidin (Sigma, USA) at 37°C away from light for 1 h, rinsed twice with PBS, stained with DAPI (Sigma, USA) for 5 min, and finally rinsed twice with PBS. The cells on coverslips were observed using an epifluorescent microscope (Olympus IX70, Japan).

All assays were performed in triplicate, with three independent experiments.

### Statistical analysis

All quantitative data are presented as the mean  $\pm$  standard deviation. Statistical analysis to compare results between groups was carried out by one-way analysis of variance (ANOVA) using a multiple comparison Dunnett post-hoc test, with SPSS software, version 17.0 (SPSS Inc, Chicago, IL, USA). Statistically significant values were defined as  $p < 0.05$ .

## Results

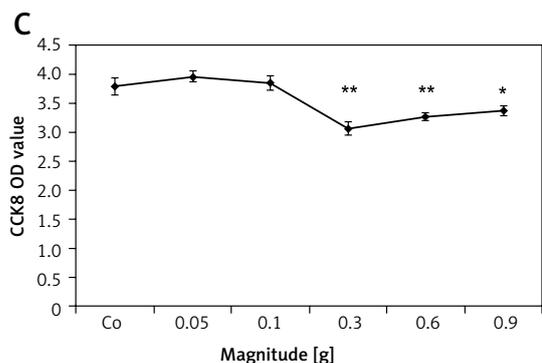
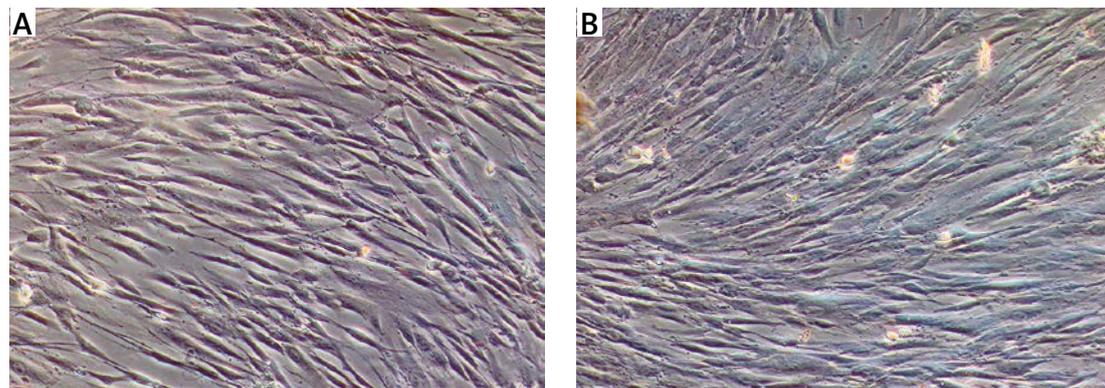
### Effect of mechanical vibration on PDLSC proliferation and cellular senescence

When the acceleration was set equal to or more than 0.3 g, PDLSC proliferation showed a significant

decrease with the largest change in the 0.3 g group, compared to that in the control static group (Figure 1 C). To further confirm the effect of mechanical vibration on hPDLSC vitality, cell senescence was measured. As shown in Figures 1 A and 1 B, no obvious SA- $\beta$ -gal blue positive cell was observed either in the experimental or the control group.

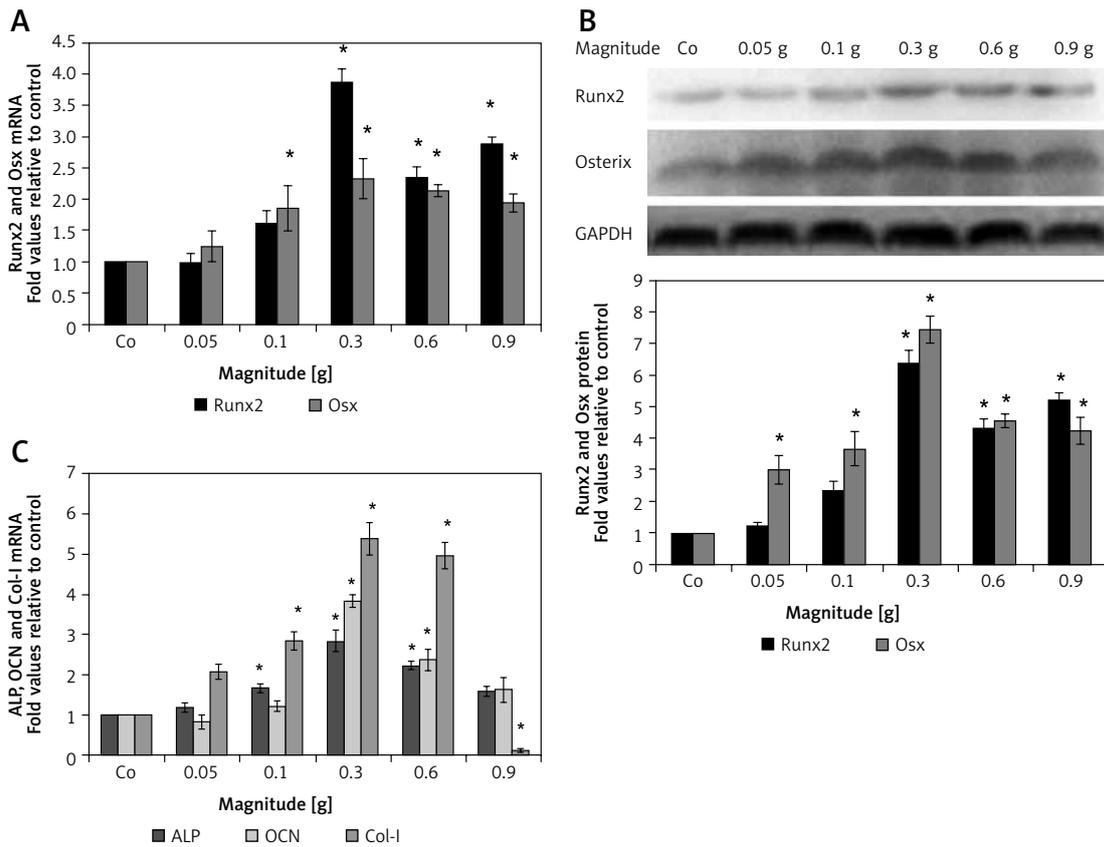
### Osteogenic-specific gene and protein expression levels in PDLSCs stimulated by mechanical vibration at different magnitudes

To investigate the effect of mechanical vibration at different magnitudes on osteogenic differentiation of hPDLSCs, genes associated with osteogenesis, including Runx2, Osx, Col-I, ALP and OCN, were measured by real-time RT-PCR. Compared to the control group, Runx2 mRNA expression slightly increased at 0.1 g, and peaked to approximately 4-fold versus control at 0.3 g (Figure 2 A); the other four mRNAs expression levels showed a common growing trend towards a significant peak at 0.3 g (Osx 2.3-fold, ALP 2.8-fold, OCN 3.8-fold, and Col-I 2.4-fold); then they decreased to a minimum at 0.9 g (Figures 2 A and C). The expression levels of bone specific proteins were further evaluated using western blotting (Figure 2 B). Similar to gene expression data, both Runx2 and Osx protein levels increased in magnitude-dependent manners, with significant peaks at 0.3 g (Runx2 6.4-fold and Osx 7.5-fold), respectively, then decreased to some extent. In summary, the



**Figure 1.** Effect of mechanical vibration on hPDLSC proliferation and senescence. There were no obvious SA- $\beta$ -gal blue positive cells in the static control (A) and the experimental group (B). Scale bars = 200  $\mu$ m for A and B. A trend toward decreased cell proliferation was evident with increased magnitude of stimulation, which was statistically significant at 0.3 g and above, when compared with the stationary control (C)

Bars represent the mean  $\pm$  standard deviation ( $n = 3$ ); \* $p < 0.05$ ; \*\* $p < 0.01$ .



**Figure 2.** The effect of mechanical vibration at different magnitudes on osteogenic gene and protein expression in hPDLSCs. Quantitative PCR results indicate that Runx2 and osterix (Osx) mRNA levels were upregulated by mechanical vibration at 0.1–0.9 g magnitude, as compared to control cells (Co) (A); the mRNA expression levels of ALP, OCN, and Col-I were in a common growing trend towards a significant peak at 0.3 g, then decreased to a minimum at 0.9 g (B); Runx2 and osterix (Osx) protein expression levels increased with increasing magnitude, peaking at a magnitude of 0.3 g compared to control (C)

Each bar represents the mean ± standard deviation (n = 3); \*p < 0.05.

increased protein levels of Runx2 and Osx at 0.1 g to 0.9 g magnitude stimulation were consistent with the changes in their respective mRNA levels.

### Tenogenic-specific gene expression levels in PDLSCs stimulated by mechanical vibration at different magnitudes

The PDLSCs express both osteogenic and tenogenic phenotypes. The mRNA expression level of scleraxis, a tendon-specific transcription factor, decreased at 0.05 g, 0.1 g, and 0.3 g, and significantly increased at 0.6 g and 0.9 g, with a peak increase at 0.9 g (1.97-fold increase versus control; p < 0.05) (Figure 3).

### Changes in the cytoskeleton of PDLSCs after mechanical vibration

In order to examine the effect of mechanical vibration on the cytoskeleton of hPDLSCs as well as to investigate the potential role of the actin cytoskeleton in the osteogenic differentiation in response to mechanical vibration, we observed

cytoskeletal changes after mechanical vibration. Compared to the static group, the F-actin stress fibers of hPDLSCs became thicker and clearer after vibration (Figure 4).

## Discussion

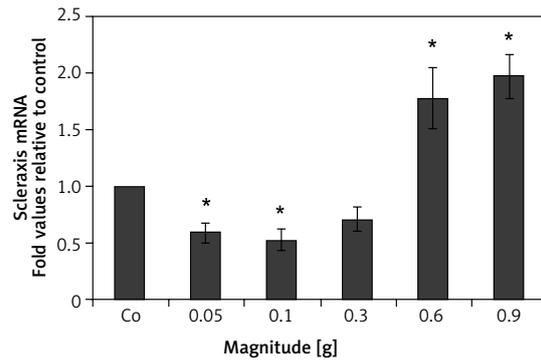
### PDLSC proliferation and cellular senescence

In this work, we successfully isolated hPDLSCs using the CD146 microbead kit. The results of phenotypic detection of hPDLSCs showed that hPDLSCs have similar phenotypes to bone marrow stem cells (BMSCs), with their pluripotency also proven. This suggested that purified mesenchymal stem cells can be obtained from PDL, a readily available source for regenerative dentistry [22]. Subsequently, we examined the potential cellular and molecular regulation of hPDLSCs after mechanical vibration at a magnitude of 0.05 g to 0.9 g and a frequency 50 Hz, in order to test whether the efficacy of mechanical stimulation is dependent on the magnitude of the applied vibration stimulus. In our study, mechanical vibration caused a reduction

of hPDLSC proliferation, which peaked at 0.3 g. Luu *et al.* [18] found that BMSC proliferation was positively influenced by mechanical vibration. Contrastingly, Zhou *et al.* [23] found that BMSC proliferation was decreased after vibration treatment. Senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -Gal) is an established biomarker associated with cellular aging. Senescent cells should be detected as cells showing a cytoplasmic blue precipitate [24]. In the present study, there were no obvious SA- $\beta$ -gal positive cells in the experimental and the static control group. As such, although the decreased PDLSC proliferation observed in our study was confirmed by the cell counting assay, hPDLSCs maintained high vitality after mechanical vibration.

### Osteogenic-specific gene and protein expression levels

Experimental studies have already affirmed the significance of mechanical strain on osteogenic differentiation of BMSCs. Jagodzinski *et al.* [25] found that cyclical stretching significantly up-regulated expression of ALP, OCN, Col-I and Runx2 of human BMSCs. Other studies with 3D-cultured BMSCs [26] also verified cyclic tensile strain-induced osteogenic differentiation. Tang found that hPDLSCs might be sensitive to cyclic tensile strain. The significant increase of Runx2, osterix and Satb2 expression levels may suggest an early response toward osteogenic orientation of hPDLSCs [27]. Studies have revealed that Runx2 and osterix are two essential transcription factors in the osteogenic pathway [28]. Our study aims to investigate the response of hPDLSCs subject to mechanical vibration at different magnitudes. In this study, the expression of both genes was increased by LMHF mechanical vibration in the range of 0.1 g to 0.9 g, which peaked at a magnitude of 0.3 g, albeit to different extents. Based upon the observed enhancement of Runx2 and osterix mRNA expression induced by mechanical



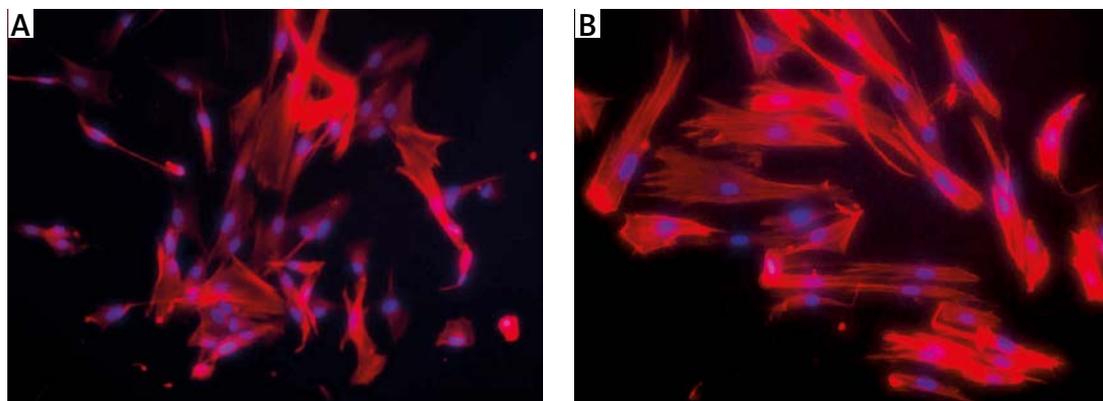
**Figure 3.** The effect of mechanical vibration at different magnitudes on tenogenic gene expression in hPDLSCs. Quantitative PCR results indicate that the mRNA expression level of Scleraxis, a tendon specific transcription factor, decreased at 0.05 g, 0.1 g, and 0.3 g, and significantly increased at 0.6 g and 0.9 g, with a peak increase at 0.9 g

Each bar represents the mean  $\pm$  standard deviation ( $n = 3$ ); \* $p < 0.05$ .

vibration, we conclude that mechanical vibration can affect osteogenesis by increasing the commitment of PDLSCs to the osteogenic lineage. This conclusion is supported by our protein expression data, which were in parallel to mRNA data, implying that the efficacy of vibration was strongly dependent on the magnitude of the applied mechanical stimulation.

The increased matrix synthesis and maturation by mechanical vibration was shown by elevated expression levels of the middle (Col-I, ALP) and late (OCN) osteogenic markers. We interpret our data to indicate that LMHF mechanical vibration drives hPDLSCs to an osteogenic lineage.

The mRNA expression level of scleraxis decreased at 0.05 g, 0.1 g, and 0.3 g, and significantly increased at 0.6 g and 0.9 g. The differences in expression level between tenogenic differentiation markers and osteogenic differentiation markers may have arisen from their unique temporal-specific, frequency-specific and magnitude-specific



**Figure 4.** Change in cytoskeleton of hPDLSCs in response to mechanical vibration. Compared with the static control group (A), the F-actin stress fibers of hPDLSCs became thicker and clearer following vibration stimulation (B)

Scale bars = 100  $\mu$ m for A and B.

regulation. Consistent with our data, Xie *et al.* [11] found that mechanical vibration at 45 Hz, 0.3 g can inhibit trabecular bone resorption, and maintain a high level of matrix quality in the growing skeleton. Lau *et al.* [8] found that when MLO-Y4 osteocytes were subjected to LMHF mechanical vibration, RANKL mRNA expression was decreased most significantly at 60 Hz, 0.3 g. Rubin *et al.* [19] and Luu [18] observed that mechanical vibration at 0.2 g, 90 Hz directed the lineage commitment of BMSCs to the osteoblast lineage. More recently, Zhou *et al.* [23] found that mechanical vibration at 40 Hz, 0.3 g promoted BMSC differentiation of BMSCs seeded on bone-derived scaffolds.

Different regimens of frequencies and intensity of mechanical vibration have been used in cells, animals and human beings, with different results [12, 13, 29]. The ideal frequency and intensity of the stimulus is still not established; although it is known that frequencies that range from 15 Hz to 90 Hz can be strongly anabolic [12], the range of appropriate intensity has not yet been determined. In this regard, our results are in agreement with some previous studies [8, 11, 23], but there are discrepancies with other researchers which we believe reflect the use of different cell types and research models [9, 18, 19]. Some researchers have suggested that the efficacy of mechanical signals that stimulate bone formation are dependent on the applied frequency, rather than the strain magnitude [9, 21]; however, it has been suggested that the degree of cellular response is determined not only by the frequency of the load, but also by the magnitude, rate, and even cycle number of the load [30].

### Changes in the cytoskeleton

In our study, we determined the effect of magnitude at a constant frequency (50 Hz) in order to look for an optimal magnitude to stimulate PDLSCs toward osteogenic differentiation. From our results, mechanical vibration at a magnitude of 0.3 g to 0.6 g was more conducive for the osteodifferentiation of PDLSCs, implying that there is also a magnitude-dependent effect of vibration on determining PDLSCs' commitment to different lineages. What remains unclear is how the mechanical force is recognized by the cells and transduced into a cellular signal that controls transcriptional activity. The physical mechanisms modulating the vibration-induced response have not been identified. Regardless of the physical mechanism involved in transmitting mechanical signals to the cell, the cytoskeleton as the continuous structure between chromosome and cell membrane is likely involved in transmitting mechanical cues within a cell [31]. This hypothesis that the cytoskeleton is intimately involved in transmitting and amplifying

the oscillatory mechanical signal was corroborated by the up-regulation of genes important for cytoskeletal remodeling [32]. It was reported that mechanical force was transferred through the cytoskeleton to the nucleus [33], leading to a biochemical cascade. Previous studies [34] have shown that unidirectional steady flow induced actin stress fiber formation. The purpose of this project was to examine the effect of mechanical vibration on the actin cytoskeleton of hPDLSCs as well as to determine the potential role of the actin cytoskeleton in the osteogenic differentiation in response to vibration. In contrast, the development of thick actin fibers, consistent with the formation of stress fibers, was apparent in cells exposed to a mechanical stimulus. Consistent with Judex [32], the mechanically driven osteogenic commitment of undifferentiated PDLSCs was influenced by the level of cytoskeletal remodeling, which was correlated with the magnitude of applied acceleration. We postulate that the actin cytoskeleton plays a pivotal role in determining the PDLSC mechanical properties and modulation of cellular mechanics at the early stage of stem-cell osteodifferentiation. However, there is a discrepancy in the relationship between osteogenic differentiation of stem cells and skeletal changes. Rodríguez *et al.* [35] reported that the actin cytoskeleton changed from a large number of thin, parallel microfilament bundles extending across the entire cytoplasm in undifferentiated MSCs to a few thick actin filament bundles located at the outermost periphery in differentiated cells. Titushkin discovered that the actin cytoskeleton changed from thick stress fibers in hMSCs into the thinner filamentous network in osteoblasts [36]. Even so, changes in the cytoskeleton of hPDLSCs after vibration may be one of the mechanisms of the biological effects.

In conclusion, our study provides a first glimpse at how hPDLSCs respond to LMHF mechanical vibration at the level of cell proliferation, gene transcription and protein expression. Specifically, we observed a decrease in PDLSC proliferation and an increase in markers of osteogenesis after mechanical vibration stimulation. From our previous results, mechanical vibration at a frequency of 40 Hz to 90 Hz was more conducive for the osteodifferentiation of PDLSCs, implying the existence of a frequency-dependent effect of vibration on determining PDLSC commitment to the osteoblast lineage. This work indicated that mechanical vibration at a magnitude of 0.3 g to 0.6 g with a 50 Hz frequency was more favorable for PDLSC osteogenic differentiation, implying that the osteogenic efficacy of mechanical stimulation is also dependent on the magnitude. In addition, changes in the cytoskeleton of hPDLSCs after vibration may be one of the mechanisms of the biological effects. Therefore, our data provided a range of param-

ters for *in vivo* testing and clinical applications in the future.

More long-term studies *in vitro* and *in vivo* are needed to determine whether vibration at different magnitudes and frequencies than those tested in the current study could become a suitable therapy to enhance periodontal regeneration and accelerate periodontal tissue remodeling.

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The experimental procedure was approved by the Ethical Committee Board of West China College of Stomatology, Sichuan University. The Judgment's reference number is 2009026.

### Conflict of interest

The authors declare no conflict of interest.

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