

## Cementogenesis is inhibited under a mechanical static compressive force via *Piezo1*

Ying-ying Zhang<sup>a</sup>; Yi-ping Huang<sup>b</sup>; Hua-xiang Zhao<sup>b</sup>; Ting Zhang<sup>c</sup>; Feng Chen<sup>d</sup>; Yan Liu<sup>e</sup>

### ABSTRACT

**Objective:** To investigate whether *Piezo1*, a mechanotransduction gene mediates the cementogenic activity of cementoblasts under a static mechanical compressive force.

**Materials and Methods:** Murine cementoblasts (OCCM-30) were exposed to a 2.0 g/cm<sup>2</sup> static compressive force for 3, 6, 12, and 24 hours. Then the expression profile of *Piezo1* and the cementogenic activity markers osteoprotegerin (*Opg*), osteopontin (*Opn*), osteocalcin (*Oc*), and protein tyrosine phosphatase-like member A (*Ptpla*) were analyzed. *Opg*, *Opn*, *Oc*, and *Ptpla* expression was further measured after using siRNA to knock down *Piezo1*. Real-time PCR, Western blot, and cell proliferation assays were performed according to standard procedures.

**Results:** After mechanical stimulation, cell morphology and proliferation did not change significantly. The expression of *Piezo1*, *Opg*, *Opn*, *Oc*, and *Ptpla* was significantly decreased, with a high positive correlation between *Opg* and *Piezo1* expression. After *Piezo1* knockdown, the expression of *Opg*, *Opn*, *Oc*, and *Ptpla* was further decreased under mechanical stimulation.

**Conclusions:** Cementogenic activity was inhibited in OCCM-30 cells under static mechanical force, a process that was partially mediated by the decrease of *Piezo1*. This study provides a new viewpoint of the pathogenesis mechanism of orthodontically induced root resorption and repair. (*Angle Orthod.* 2017;87:618–624)

**KEY WORDS:** *Piezo1*; Static compressive force; OCCM-30; Cementogenesis

### INTRODUCTION

External root resorption frequently occurs as a result of cementum remodeling during orthodontic treatment. It can affect the long-term viability and stability of teeth.<sup>1</sup> A recent study has demonstrated that external root resorption is centered mainly on the root apex and the root in the compression region.<sup>2</sup> Therefore,

compressive force may play a key role in root resorption during orthodontic processes. Recent studies have illustrated that cementoblasts, force-sensitive cells that react to mechanical compressive forces, play a critical role in the process of root resorption and repair.<sup>3–5</sup> However, information available on the mechanotransduction pathway of cementoblasts exposed to mechanical compressive force is limited. Therefore, understanding how cementoblasts sense and conduct the force would be helpful to determine the root resorption process during orthodontic treatment.

There is growing evidence that compressive forces regulate the cementogenic activity of cementoblasts.<sup>5–8</sup> The osteoprotegerin (*Opg*) and osteopontin (*Opn*) genes associated with cementogenesis are downregulated in cementoblasts under a compressive force. However, the molecular mechanisms by which cementoblasts transduce mechanical force to cementogenic gene expression remain unclear.

Mechanosensitive ion channels located in the plasma membrane sense a mechanical force exerted on the cells, resulting in a change in bilayer tension or the cytoskeleton followed by the activation of downstream signal pathways.<sup>9</sup> Several ion channels have been reported to respond to mechanical stimuli,

<sup>a</sup> MS Student, Department of Orthodontics, Peking University School and Hospital of Stomatology, Beijing, China.

<sup>b</sup> PhD Student, Department of Orthodontics, Peking University School and Hospital of Stomatology, Beijing, China.

<sup>c</sup> Research Assistant, Department of Orthodontics, Peking University School and Hospital of Stomatology, Beijing, China.

<sup>d</sup> Research Associate, Central Laboratory, Peking University School and Hospital of Stomatology, Beijing, China.

<sup>e</sup> Associate Professor, Department of Orthodontics, Peking University School and Hospital of Stomatology, Beijing, China.

Corresponding author: Prof Yan Liu, Department of Orthodontics, Peking University School and Hospital of Stomatology, 22 Zhongguancun South Avenue, Haidian District, Beijing 100081, China (e-mail: yanliuortho@yahoo.com)

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including the transient receptor potential channels and acid-sensing ion 1-3 channels.<sup>10–13</sup> However, they are usually activated by membrane stretching or inflammation, instead of a mechanical compressive force. Recent studies have demonstrated that PIEZO1 (protein), a large evolutionarily conserved transmembrane protein that is richly expressed in the bladder, lungs, colon, and dorsal root ganglia, senses and converts a wide array of mechanical forces into various biological activities.<sup>14–19</sup> A recent study has demonstrated that PIEZO1 transduces static compressive forces and regulates bone remodeling in primary periodontal ligament cells (PDLs).<sup>20</sup> However, it is still not clear whether PIEZO1 mediates the static mechanical compressive force and subsequently regulates cementogenesis in cementoblasts.

Therefore, this study investigated the cementogenic activity in a cementoblast cell line under a static mechanical force and tested whether *Piezo1* gene mediates this process. The findings would aid understanding of the process of external root resorption and help prevent and treat this adverse reaction during orthodontic treatment.

## MATERIALS AND METHODS

### Cell Culture

OCCM-30 cells, a clonal population of immortalized murine cementoblasts,<sup>21</sup> were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Equitech Bio, Texas) supplemented with 100 U/mL penicillin (Gibco) and 100 µg/mL streptomycin (Gibco) at 37°C in a humidified atmosphere consisting of 5% CO<sub>2</sub> in air. To monitor cell morphology, bright-field images were acquired using a Nikon C-HGF1 microscope (Nikon, Tokyo, Japan) at 4×, 10×, and 20× magnification, respectively.

### Mechanical Stimulation: Application of a Static Compressive Force

OCCM-30 cells were seeded into six-well plates. After reaching 70% confluence, a cover glass and a glass bottle containing steel balls were placed on the cells. The compressive force was adjusted to 2.0 g/cm<sup>2</sup> according to previous studies,<sup>22,23</sup> and the force was maintained for 3, 6, 12, and 24 hours.

### Cell Proliferation Assay

A Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was used for the cell proliferation assay according to the manufacturer's instructions. Briefly, after exposure to a static compressive stress for 24 hours, the cells were seeded onto 96-well plates at a

cell density of 1000 cells/well. Cell viability was analyzed at 0, 24, 48, and 72 hours. The CCK-8 working solution was added to the wells and incubated at 37°C for 4 hours. Absorbance was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad, Hercules, Calif).

### Small-interfering RNAs Transfection

Small-interfering RNAs (siRNA) targeting the mouse *Piezo1* transcript and a siRNA negative control (NC) were purchased (Gene Pharma, Suzhou, China). The sequences of the *Piezo1* siRNA were 5'-CCG GCA UCU ACG UCA AAU ATT-3' (sense) and 5'-UAU UUG ACG UAG AUG CCG GUG-3' (antisense). The sequences of the siRNA NC were 5'-UUC UCC GAA CGU GUC ACG UTT-3' (sense) and 5'-ACG UGA CAC GUU CGG AGA ATT-3' (antisense). *Piezo1* siRNA (si-*Piezo1*) and the corresponding NC were transfected into OCCM-30 cells using Avalanche Transfection Reagent (EZ Biosystems, College Park, Md), according to the manufacturer's instructions. After reaching 50% confluence in the six-well plates, the cells were transfected with 100 nM siRNA. After 24 hours, the medium was replaced with fresh DMEM and 10% FBS. The cells were then exposed to the static compressive force and harvested 24 hours later.

### RNA Isolation and Quantitative Reverse-Transcription Polymerase Chain Reaction

The primers were designed using the National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The cells were harvested and total mRNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. Then mRNA was reverse-transcribed into complementary DNA using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, Calif). Quantitative PCR was conducted as described previously.<sup>24</sup> The primer sequences of *Piezo1*, *Opg*, *Opn*, osteocalcin (*Oc*), protein tyrosine phosphatase-like member A (*Ptpla*), and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) are listed in Table 1.

### Western Blot Analysis

Western blot was performed as described previously.<sup>24</sup> Briefly, the cells were lysed in RIPA buffer (Thermo Fisher Scientific, Waltham, Mass). The protein content in the supernatant was determined using the BCA protein assay (Thermo Fisher). The proteins were separated by 6% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The primary antibodies against

**Table 1.** Primers for qRT-PCR

Name	Sense Primer (5'-3')	Antisense Primer (5'-3')
<i>Piezo1</i>	TTCTTCGGGTTGGAGAGGTA	TGTCACCATGTGGTTAAGGATG
<i>Opg</i> <sup>a</sup>	GTGGAATAGATGTCACCCTGTGT	TTGGTCCCAGGCCAAACTGT
<i>Opn</i>	ATGAGATTGGCAGTGATTTG	GTAGGGACGATTGGAGTGA
<i>Oc</i>	AGCAGCTTGGCCAGACCTA	TAGCGCCGGAGTCTGTTCACTAC
<i>Ptpla</i>	AGCCCAGGTATAGGAAGAATGT	CCGCATAACTAACCCAATAGCG
<i>Gapdh</i>	AACGACCCCTTCATTGACCTC	ACTGTGCCGTTGAATTTGCC

<sup>a</sup> *Opg* indicates osteoprotegerin; *Opn*, osteopontin; *Oc*, osteocalcin; *Ptpla*, protein tyrosine phosphatase-like, member A; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase.

PIEZO1 (Abcam, Cambridge, UK) were diluted 1:1000, and those against GAPDH (Santa Cruz Biotechnology, Santa Cruz, Calif) were diluted 1:10,000. The bands were visualized using chemiluminescence (Invitrogen), and the intensities of the bands were quantified using ImageJ software (NIH, Bethesda, Md). The back-

ground was subtracted, and the signal of each target band was normalized to that of the GAPDH band.

### Statistical Evaluation

Statistical analyses were performed using SPSS software (ver. 22.0; IBM Corp, Armonk, NY). Differences between groups were analyzed using Student's *t*-test. Correlations between the expression of *Opg*, *Opn*, *Oc*, *Ptpla*, and *Piezo1* were analyzed using Pearson's correlation coefficient analysis. All data are expressed as mean  $\pm$  standard deviation (SD). A two-tailed value of  $P < .05$  was considered to be statistically significant.

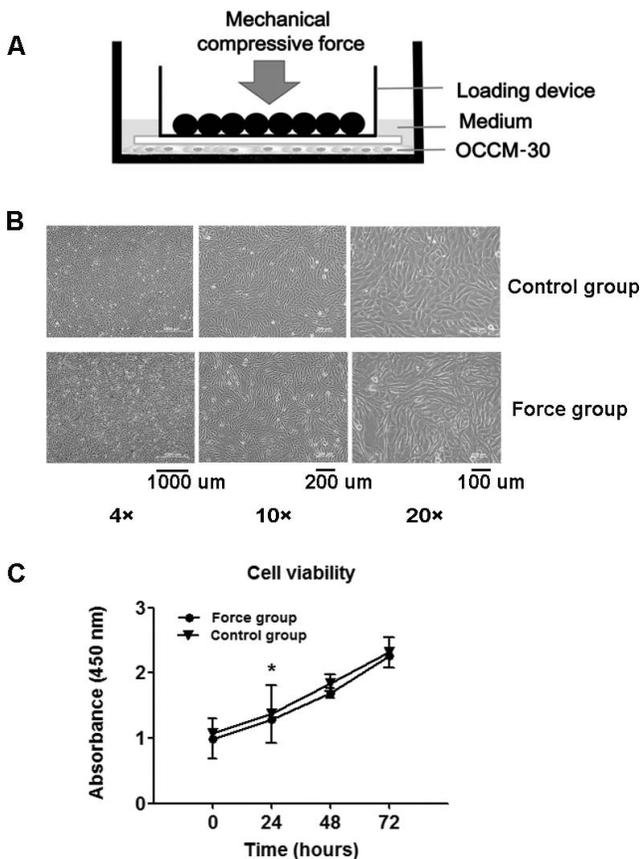
## RESULTS

### Cell Morphology and Proliferation in OCCM-30 Cells Under a Static Mechanical Compressive Force

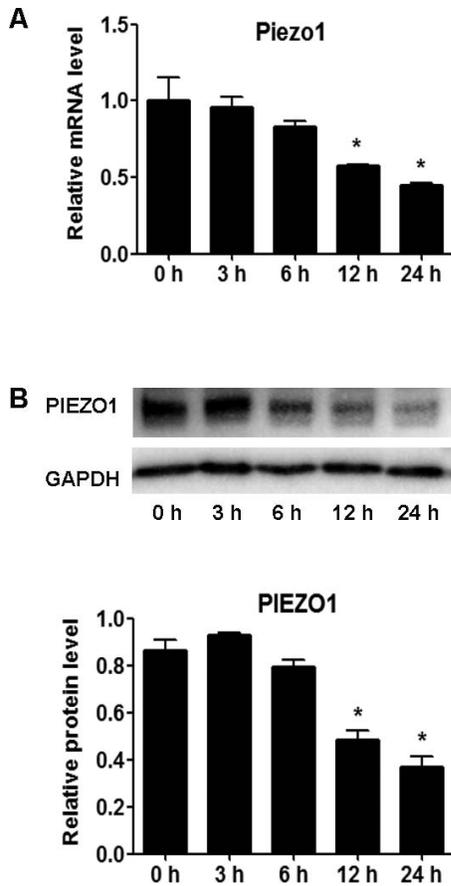
A static mechanical compressive force (Figure 1A) was used to stimulate the OCCM-30 cells. Morphology was observed at 3, 6, 12, and 24 hours, showing no change compared with the control group when the cells were exposed to 2.0 g/cm<sup>2</sup> compressive force (Figure 1B). However, cell morphology started to change and cell apoptosis was observed when the force was greater. Thus, a 2.0-g/cm<sup>2</sup> force was used in subsequent experiments. Cell proliferation was then determined using the CCK-8 assay, after exposure to the static compressive force for 24 hours. The proliferation curve under the static mechanical compressive force was similar to that of the control cells (Figure 1C).

### *Piezo1* Expression Is Decreased Under a Static Mechanical Compressive Force

The expression of *Piezo1* was analyzed by qRT-PCR. The level of *Piezo1* mRNA was decreased significantly in OCCM-30 cells under a static mechanical compressive force compared with that of the control group. It started to decrease at 3 hours and reached the lowest level at 24 hours (Figure 2A). The



**Figure 1.** No significant change in cell morphology or proliferation of OCCM-30 cells under compressive force. (A) Schematic diagram of mechanical stimulation applied on OCCM-30. (B) Cell morphology images of OCCM-30 with and without mechanical stimulation. 4 $\times$  scale bar, 1000  $\mu$ m; 10 $\times$  scale bar, 200  $\mu$ m; 20 $\times$  scale bar, 100  $\mu$ m. (C) CCK-8 assay showing the proliferation curve of OCCM-30 cells with and without mechanical compressive force. Data are shown as mean  $\pm$  SD ( $P < .05$ ).

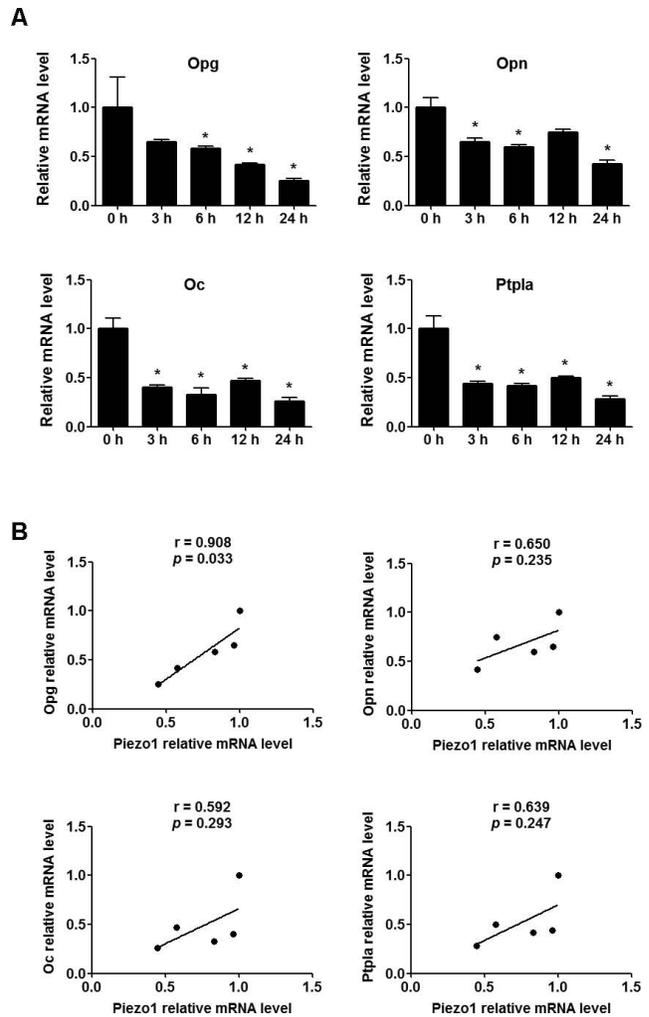


**Figure 2.** *Piezo1* expression was decreased under compressive force. (A) Relative *Piezo1* mRNA expression levels determined by qRT-PCR analysis at 3, 6, 12, and 24 hours under compressive force. (B) Western blot analysis (up) and quantification (down) of protein expression of *PIEZO1* and the internal control *GAPDH* at the indicated time. Data are shown as mean  $\pm$  SD ( $P < .05$ ).

result were confirmed by Western blot showing that *PIEZO1* protein began to decline significantly at 12 hours, and it was at its lowest level at 24 hours (Figure 2B).

**Cementogenesis Is Inhibited in OCCM-30 Under a Static Mechanical Compressive Force**

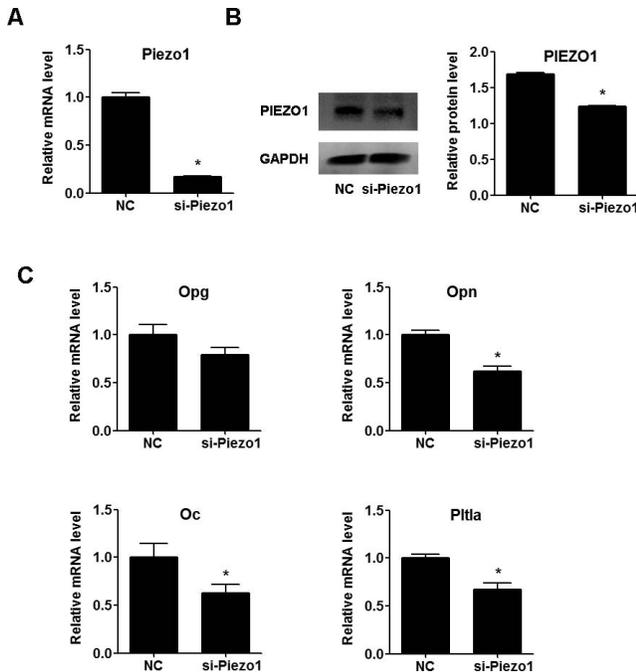
To explore cementogenesis of the OCCM-30 cells after exposure to a force, cementoblastic markers were measured by qRT-PCR. The mRNA expression of *Opg*, *Opn*, *Oc*, and *Ptpla* was decreased time-dependently in the OCCM-30 cells under a static mechanical compressive force, reaching the lowest levels at 24 hours, with a slight oscillation at 12 hours (Figure 3A). Analysis of the correlation between the *Piezo1* and cementogenic markers showed that the expression of *Opg* was highly correlated ( $r = .908$ ) with the *Piezo1* mRNA expression in OCCM-30 under a compressive force, while *Opn*, *Oc*, and *Ptpla* did not show significant correlations (Figure 3B).



**Figure 3.** Cementogenesis was inhibited in OCCM-30 under compressive force. (A) Quantification of mRNA expression of *Opg*, *Ptpla*, *Oc*, and *Opn* measured by qRT-PCR at 3, 6, 12, and 24 hours under compressive force. (B) Correlation between *Opg*, *Opn*, *Oc*, *Ptpla*, and *Piezo1* expression levels at in OCCM-30 under compressive force. Statistical analysis was performed using Pearson's correlation coefficient analysis, with the  $r$  and  $P$  values as indicated. Data are shown as mean  $\pm$  SD ( $P < .05$ ).

**Cementogenesis Is Further Decreased After Knockdown of *Piezo1* in OCCM-30 Cells Under a Mechanical Compressive Force**

To understand the biological function of *Piezo1* in OCCM-30 cells under compressive force, siRNA was constructed specifically for *Piezo1* (si-*Piezo1*) and transfected into the OCCM-30 cells to knock down *Piezo1*. After 24 hours of transfection, the cells were exposed to a mechanical compressive force for another 24 hours before collection. The qRT-PCR and Western blot analysis confirmed an approximately 50% decrease in *Piezo1* expression in the knockdown group (Figure 4A,B). After successful knockdown of *Piezo1*, the *Ptpla*, *Oc*, and *Opn* mRNA expressions

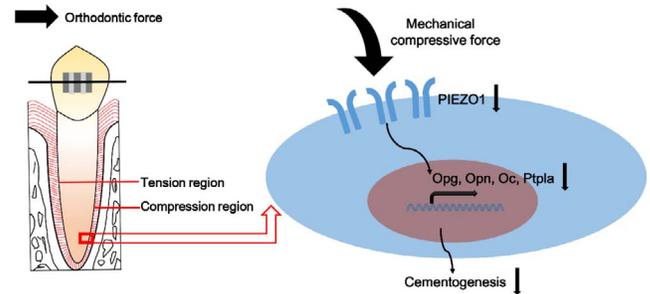


**Figure 4.** Cementogenesis was further decreased after knockdown of *Piezo1* in OCCM-30 cells under compressive force. (A) Relative mRNA expression of *Piezo1* determined by qRT-PCR analysis in the si-*Piezo1* transfection group or its NC group. (B) Western blot analysis (left) and quantification (right) of PIEZO1 protein expression and the internal control GAPDH in the group transfected with si-*Piezo1* or its NC group. (C) Quantification of mRNA expression of *Opg*, *Ptla*, *Oc*, and *Opn* measured by qRT-PCR in the si-*Piezo1* transfection group or its NC group. Results are presented as mean  $\pm$  SD ( $P < .05$ ).

were further significantly downregulated compared with the control group under mechanical force, while the *Opg* expression was only slightly downregulated, not reaching a significant difference (Figure 4C).

## DISCUSSION

In this study, we demonstrated that *Piezo1* was decreased in OCCM-30 cells after exposure to a static compressive force. Although the stress environment between cementum and periodontal ligament during orthodontic processes is similar, a previous study showed that PIEZO1 is increased in PDLs after exposure to a static compressive force.<sup>20</sup> The different responses of PIEZO1 in these two kinds of cells may be due to the distinct characteristics of the target cells. No proliferation difference was found in OCCM-30 cells after exposure to a static compressive force for 24 hours, consistent with a previous study.<sup>8</sup> In the PDLs, however, mechanical stimuli promote cell proliferation by influencing the duration of the S phase and mitotic activity.<sup>25</sup> Moreover, previous studies have shown that the osteoblastic marker expression increases in PDLs under pressure,<sup>26,27</sup> while in the current study,



**Figure 5.** Schematic of *Piezo1* involved in the inhibition of cementogenesis in OCCM-30 cells under compressive force. The teeth primarily affected by orthodontic forces can be divided histologically into two main regions: the compression region and tension region. Under a static compressive force, PIEZO1 was decreased, followed by a decrease in cementoblastic gene expression, resulting in the inhibition of cementogenesis.

these markers were reduced in cementoblast cells. The different characterization of these two cells may result in their different responses to compressive force. In addition, the different response of *Piezo1* indicates that cementoblasts are less sensitive to mechanical compressive forces than PDLs due to the downregulation of this mechanotransduction ion channel. This may partially explain why cement remodeling is less active than alveolar bone remodeling during orthodontic treatment.

Cementoblastic activity was inhibited under compressive force, and the expression of cementogenic markers (*Opn*, *Oc*, and *Ptla*) was decreased under pressure in OCCM-30 cells. This was consistent with previous findings of a remarkable decrease of cementogenic markers in cementoblasts when subjected to compressive forces.<sup>4-6</sup> Cementogenesis was inhibited gradually in a time-dependent manner with a slight oscillation, however, at 12 hours. Some studies regarding the response of *Opn* to the compressive force also displayed a time-dependent oscillation pattern,<sup>28</sup> indicating that many transcription factors may be involved in this process. Also, we found that *Ptla*, *Oc*, and *Opn* were further downregulated significantly under a mechanical force compared with the control group after knockdown of *Piezo1*. These results indicated that the decreased cementogenic activity was partially mediated by *Piezo1* under a mechanical static force.

Another interesting finding was the relationship between *Opg* and *Piezo1*. *Opg* expression was decreased in OCCM-30 under a static compressive force, and it was highly correlated with *Piezo1* mRNA expression. However, after *Piezo1* knockdown, *Opg* mRNA expression was further downregulated slightly but without significant difference under mechanical stimulation. It is possible that *Piezo1* may not directly

regulate *Opg*, and there exist cofactors or intermediates between *Piezo1* and *Opg*. In our previous experiments, we found that transduction of *Opg* plasmids into OCCM-30 cells increases the expression of *Opn*, *Oc*, and *Ptpla*.<sup>4</sup> Also, *Opg* is regulated by many factors, such as BMP2, transforming growth factor  $\beta$ , Wnt signaling pathway, and others.<sup>29</sup> The *Opn*, *Oc*, *Ptpla*, or other molecules may be the intermediates between *Opg* and *Piezo1*. Further study is needed to investigate the possible mechanism.

## CONCLUSIONS

- Cementoblastic gene expression was decreased under a static mechanical compressive force, resulting in the inhibition of cementogenesis, and this process was partially mediated by the decrease of *Piezo1* (Figure 5).
- These findings may provide a new insight into the pathogenesis mechanism of external root resorption and provide molecular targets for the prevention of this adverse reaction.

## ACKNOWLEDGMENT

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