

Functional role of mechanosensitive ion channel Piezo1 in human periodontal ligament cells

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ABSTRACT

Objective: To evaluate the function of Piezo1, an evolutionarily conserved mechanically activated channel, in periodontal ligament (PDL) tissue homeostasis under compressive loading.

Materials and Methods: Primary human PDL cells (hPDLs) were isolated, cultured, and then subjected to 2.0 g/cm² static compressive loading for 0.5, 3, 6, and 12 hours, respectively. The expressions of Piezo1 and osteoclastogenesis marker gene were assessed by semiquantitative reverse transcription–polymerase chain reaction. In addition, Piezo1 inhibitor, GsMTx4, was used to block the function of Piezo1, and tumor necrosis factor- α was also used as a positive control. After 12 hours of compressive loading the PDLs were co-cultured with murine monocytic cell line RAW264.7. Immunofluorescence, western blot, enzyme-linked immunosorbent assay, and tartrate-resistant acid phosphatase staining were also used to test the potency of PDLs to induce osteoclastogenesis and the activation of nuclear factor (NF)- κ B.

Results: Piezo1, cyclooxygenase-2, receptor activator of NF- κ B ligand, and prostaglandin E2 were significantly upregulated under static compressive stimuli. GsMTx4 repressed osteoclastogenesis in the mechanical stress–pretreated PDLs-RAW264.7 co-culture system. Furthermore, NF- κ B signaling pathway was involved in the mechanical stress–induced osteoclastogenesis.

Conclusions: Piezo1 exerts a transduction role in mechanical stress–induced osteoclastogenesis in hPDLs. (*Angle Orthod.* 2015;85:87–94.)

KEY WORDS: Piezo channel; Periodontal ligament; Mechanical loading; Osteoclastogenesis; NF- κ B

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INTRODUCTION

Periodontal ligament (PDL) tissue, which lies between tooth cementum and alveolar bone, is of fundamental importance in bone homeostasis. Proper orthodontic forces and occlusal forces lead to a balanced bone remodeling. Previous studies showed that PDL cells (PDLs) can perceive the mechanical signals and transfer them to the progenitor cells, which can differentiate into osteoclasts or osteoblasts.^{1–4} Recently, we found that the expression of potential osteoclastogenesis inducers was upregulated in a compressed PDLs model, including receptor activator of nuclear factor κ B Ligand (RANKL), cyclooxygenase-2 (Cox2), while the expression of osteoprotegerin (OPG) was downregulated.^{5,6} Furthermore, prostaglandin E2 (PGE2) may increase RANKL mRNA expression in compressed PDLs.³ However, the mechanism by which PDLs sense and transduce mechanical signals is still unclear.

Recently, Coste⁷ and Coste et al.⁸ found that Piezo channel is an evolutionarily conserved ion channel family involved in mechanotransduction. It contains 24–32 transmembrane regions. Many other researchers^{9–12}

also reported the Piezo channel to be a key player in cellular response to mechanical stimuli in different organs. PDLCs are mechanosensitive cells, so it is hypothesized that Piezo might also play a role in the response of PDLCs to mechanical stimuli.

Nuclear factor (NF)- κ B is important in regulating cellular responses to mechanical stress.^{13–15} The signals induced by proinflammatory cytokines such as tumor necrosis factor (TNF)- α and other stimuli, are transmitted to the nucleus through nuclear translocation of NF- κ B.¹⁶ Then nuclear NF- κ B binds to consensus sequences of proinflammatory genes to initiate mRNA transcription.¹⁷ Mechanical strain augments inflammation and osteoclastogenesis; we speculated that mechanical stimulus-induced osteoclastogenesis might be associated with NF- κ B activation, which might in turn be due to altered Piezo1 expression. Therefore, this study aimed to examine the function of Piezo1 in human PDLCs (hPDLCs) under mechanical stress and to explore its putative downstream signaling pathway.

MATERIALS AND METHODS

Cell Culture

hPDLCs were isolated from the root surface of healthy extracted premolars for orthodontic reasons. This study was approved by the Research Ethics Committee of West China Hospital, Sichuan University, PR China. Written informed consent was obtained from all of the patients. The periodontal tissue was digested with 2 mg/mL collagenase type I (Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS) for 30 minutes at 37°C and then centrifuged at 400 g for 8 minutes at room temperature. Cells were then resuspended in α -minimum essential medium (α -MEM) (Hyclone, Shanghai, China) supplemented with 20% fetal bovine serum (FBS) (Gibco, Logan, UT, USA), 100 U/mL penicillin, and 100 μ g/mL streptomycin (Hyclone, Shanghai, China) and incubated at 37°C in a 5% CO₂ atmosphere. After reaching confluence, culture medium was changed with α -MEM containing 10% FBS. The cells obtained between the third and fifth passages were used for all experiments.

Murine monocytic cell line RAW264.7 (Sciencell, Carlsbad, CA, USA) was maintained in α -MEM containing 10% FBS with addition of 100 U/mL penicillin and 100 μ g/mL streptomycin at 37°C in a 5% CO₂ atmosphere.

Mechanical Stimulation: Application of Static Compressive Force

Prior to mechanical strain the suspension of hPDLCs (1×10^5 cells in 2 mL media/well) was drip-

ped into a 6-well plate, and 72 hours were given to fully stabilize the PDLCs. The PDLCs were compressed continuously using the same compression method described previously. This method comprises a modification of the method described by Hiroyuki and Li.^{3,5,18} Briefly, a cover glass and a glass bottle are placed on the monolayer hPDLCs. The compressive force is adjusted by adding lead granules to the bottle to allow PDLCs to be subjected to 2.0 g/cm² of compressive force, and the force is maintained for 0.5, 3, 6, or 12 hours, respectively.^{3,18} These time points were selected according to the method described in our previously published article.¹⁹ Two and a half microliters of GsMTx4 (Abcam, San Francisco, CA, USA), a specific inhibitor of cationic mechanosensitive ion channels, was added, and 0.8 ng/mL of TNF- α (R&D, Shanghai, China) was added to the indicated groups.²⁰

Immunofluorescence

After mechanical stimulation for 0.5 hours, the hPDLCs were rinsed with PBS twice and fixed with 4% paraformaldehyde (Hyclone, Beijing, China) and then treated with 0.2% Triton X-100 (MP Biomedicals, Santa Ana, CA, USA) for 10 minutes at room temperature. Slices were treated with 5% normal goat serum in PBS for 1 hour at room temperature to block nonspecific immunoreaction. After thorough rinsing, the slices were incubated with primary antibodies of Piezo1 (Novus Biologicals, Littleton, CO, USA) and subsequently with anti-rabbit secondary antibody conjugated with Alexa 488 (diluted 1:2000; Invitrogen, Carlsbad, CA, USA) to accomplish immunofluorescence. Then the slices were counterstained with Hoechst 33342 to visualize nuclei (Thermo Scientific, Shanghai, China).

Reverse Transcription–Polymerase Chain Reaction

After hours of compressive stress, total RNA was extracted from three replicas using the reagent TRIzol. Semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed and analyzed to assess the expression of Piezo1, RANKL, OPG, and Cox2. The oligonucleotide sequences of the primers were shown in Table 1. RT-PCR was carried out using the RNA PCR kit on a FTC-2000 Real Time PCR system (Funglyn Biotech Inc, Shanghai, China). The $2^{-\Delta\Delta Ct}$ method was used for analysis of gene expression, as described in other studies.^{21,22} Briefly, equation ΔCt was Ct of each target gene transcript – Ct of Gapdh, and $\Delta\Delta Ct$ was calculated using the following formula: $\Delta\Delta Ct = \Delta Ct$ [target gene in experiment group] – ΔCt [target gene in control group], then the relative fold difference was calculated using the formula $2^{-\Delta\Delta Ct}$.

Table 1. Primer Sequences for Reverse Transcription–Polymerase Chain Reaction (RT-PCR) Analyses

Target	Forward	Reverse	Annealing Temperature, °C
Piezo1	GGCAACTTCCTCACCAAGA	GGGTATTTCTTCTGTCTCT	54
Cox2	AAGCCTTCTTAACCTCTCC	GCCCTCGCTTATGATCTGTC	54
RANKL	ACCGACATCCCATCTGGTTCCC	GCCATCCTGATTAACCTATTAGT	54
OPG	TTGAAATGGCAGTTGATTCCTTT	TATCCTCTTTCTCAGGGTGCTTG	54
Gapdh	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTGGGCCAT	54

Western Blot Analysis

For extraction of whole cellular proteins, PDLCs were rinsed with cold PBS twice and then lysed with cell RIPA buffer (added to 1 mM PMSF, phenylmethylsulfonyl fluoride), on ice for 30 minutes. For fractions of cytosolic and nuclear proteins, an extraction reagent kit (NE-PER Nuclear and Cytoplasmic Extraction Reagents, Thermo, Shanghai, China) was used according to the manufacturer's recommendations. Proteins were boiled in Laemmli sample buffer (Invitrogen, Carlsbad, CA, USA) and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membrane. The membranes were incubated with primary antibodies of Piezo1, NF- κ B, β -actin, and histone 3 (Santa Cruz, Dallas, TX, USA) overnight; this step was followed by incubation for 1 hour with secondary antibody (diluted 1:2000; Bioss, Boston, MA, USA). The membrane was detected with the aid of enhanced chemiluminescent detection reagents using a fluorescence imaging system (Clinx Science Instruments, Beijing, China).

PGE2 Measurement by Enzyme-Linked Immunosorbent Assay

The amount of secreted PGE2 in the cell culture supernatant was determined using a PGE₂ enzyme-linked immunosorbent assay kit (Mybiosource, Saint Louis, MO, USA), per the manufacturer's protocol.

Co-Culture of RAW264.7 and PDLCs

In order to test the ability of stressed-induced PDLCs to induce osteoclastogenesis, we co-cultured the RAW264.7 cells with PDLCs after compressive stimulation or TNF- α treatment.²³ After 12 hours of compressive stimulus, the glass bottle was removed carefully. We subsequently co-cultured 1×10^5 RAW264.7 cells with PDLCs for 4 days in 10% FBS α -MEM.

Tartrate-Resistant Acid Phosphatase Staining

After co-culturing of PDLCs and RAW264.7 for 4 days, cells were rinsed twice with PBS and fixed with 4% paraformaldehyde, then were stained for

tartrate-resistant acid phosphatase (TRAP) activity by using an acid phosphatase leukocyte kit (Sigma, Saint Louis, MO, USA) per the manufacturer's protocol. Multinucleated cells having three or more nuclei were counted as osteoclasts, and three researchers counted independently under low-power microscopy (4 \times). After training in calibration, they randomly picked three view fields in each slice and calculated the average number of the osteoclasts under one view field.

Statistical Evaluation

Quantitative data were analyzed for statistical difference using a Mann-Whitney test at a significance level of $P < .05$ ($n = 3$ for each group). The values are expressed as the means \pm the standard error of the mean (SEM).

RESULTS

Mechanical Loading Induces Both Osteoclastogenesis Marker Gene and Piezo1 Upregulation

As expected, exposure to mechanical stimuli significantly increased Cox2 expression over 12 hours in a time-dependent manner (Figure 1A). Furthermore, RANKL mRNA in tested hPDLCs at 12 hours was significantly higher than that measured at the earlier time point (Figure 1B). In contrast, OPG mRNA in loaded or controlled hPDLCs showed no significant difference, at least in our system (Figure 1C), resulting in an increased ratio of RANKL to OPG.

Consistent with increased osteoclastogenesis-related genes, the Piezo1 mRNA profile in loaded hPDLCs increased at 0.5 hours and lasted 12 hours. Immunostaining and Western blot further confirmed the enhancement of Piezo1 production in tested hPDLCs (Figures 2A–F and 3A).

GsMTx4 Suppresses NF- κ B Activation

To investigate the intracellular mechanisms by which Piezo1 may facilitate mechanical stress-induced osteoclastogenesis responses, we evaluated the nuclear translocation of NF- κ B, the major transcription factor involved in the signal transduction of proinflammatory cytokines. Both cytoplasmic and nuclear NF- κ B

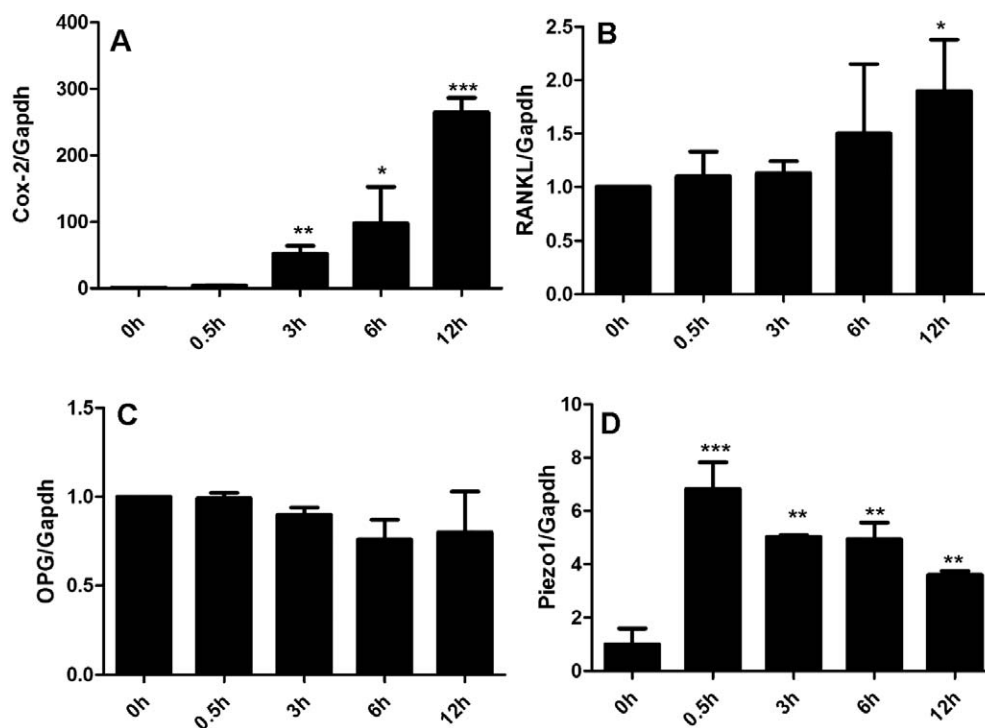


Figure 1. Mechanical stress is associated with increased osteoclastogenesis and Piezo1 activation in hPDLCs. (A) Cox-2 mRNA expression significantly increased after 3 hours of loading. (B) RANKL mRNA significantly increased after 12 hours of loading. (C) OPG mRNA expression did not change after mechanical stress. (D) Piezo1 mRNA expression significantly increased after 0.5 hours of loading and over the tested 12 hours. Values show as mean ± SEM. n = 3. * $P = .05$; ** $P = .01$; *** $P = .001$; **** $P = .0001$.

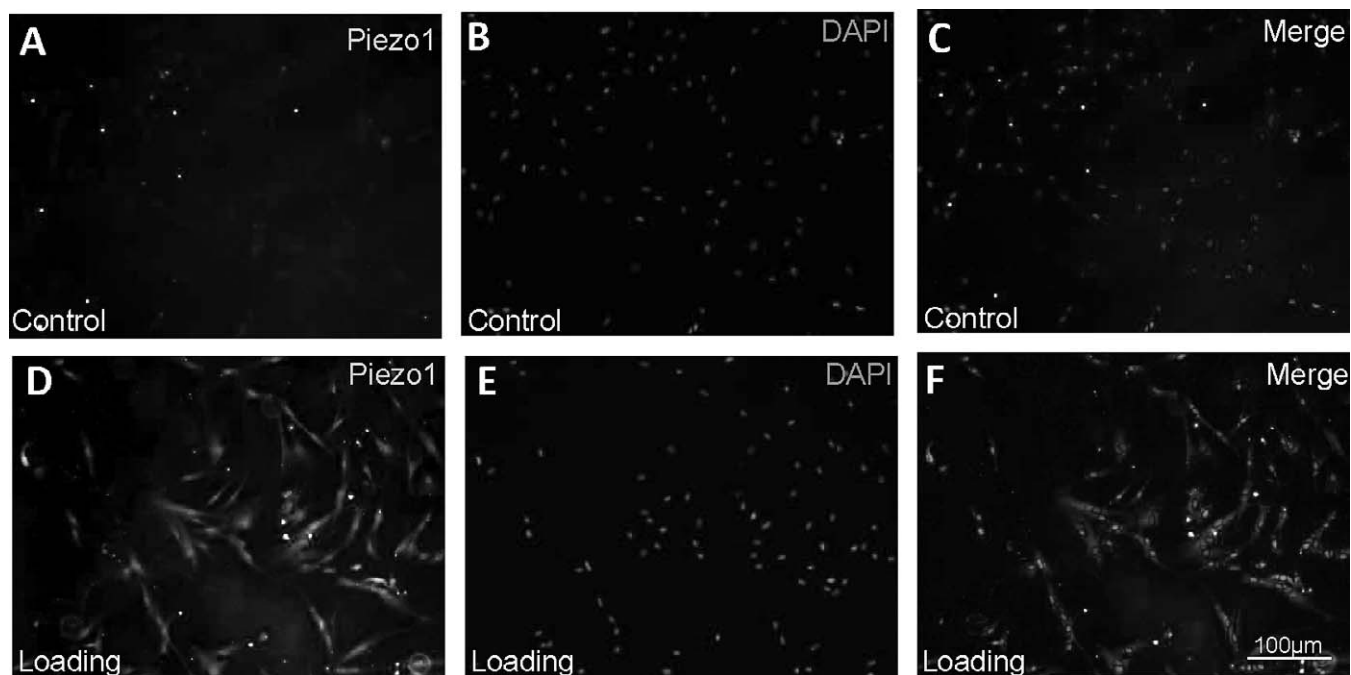


Figure 2. Piezo1 expression in isolated hPDLCs. Piezo1 expression is much stronger in hPDLCs loaded for 3 hours (D–F) than in normal hPDLCs (A–C). Scale bar: 100 μm.

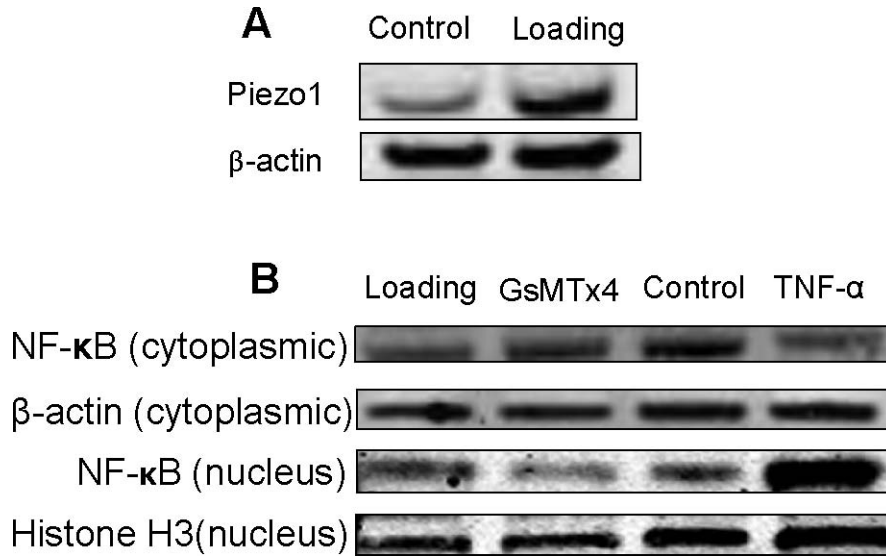


Figure 3. Western blot analysis of Piezo1 and NF-κB in hPDLCS. (A) Western blot analysis showing the effect of mechanical stress on Piezo1. (B) Western blot analysis showing the effect of GsMTx4 and TNF-α on nuclear NF-κB; Western immunoblots probed with antibodies against NF-κB from nuclear and cytosolic proteins. Histone 3 and β-actin were used as internal controls.

were investigated using Western immunoblots. Nuclear NF-κB was significantly increased 3 hours after mechanical loading. Addition of TNF-α served as a positive control. GsMTx4 suppressed the increase of

nuclear NF-κB induced by mechanical loading. In contrast, cytoplasmic NF-κB decreased after mechanical loading, which was recovered by GsMTx4 (Figure 3B).

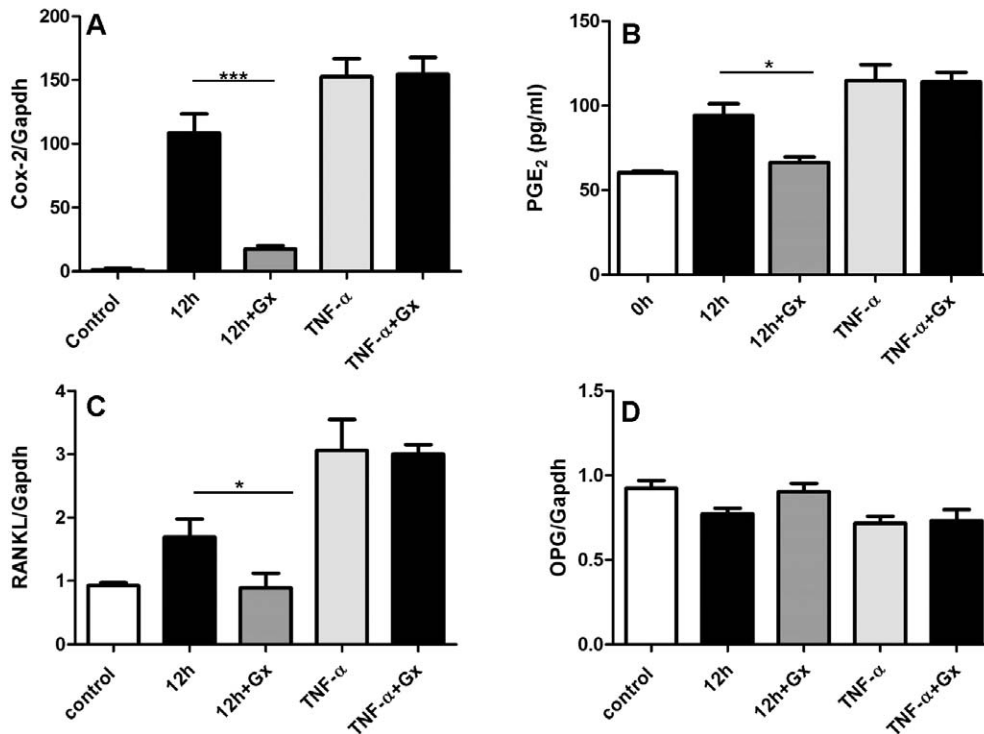


Figure 4. Piezo1 inhibitor, GsMTx4 (Gx), treatment is associated with reduced mechanical stress-induced osteoclastogenesis but not with TNF-α-induced osteoclastogenesis. Changes of mRNA profile of Cox2 (A), RANKL (C), OPG (D), and protein production of PGE2 (B) in hPDLCS after mechanical stress (12 hours) or TNF-α induction with or without addition of Gx. Values shown as mean ± SEM. n = 3, * P = .05; ** P = .01; *** P = .001; **** P = .0001.

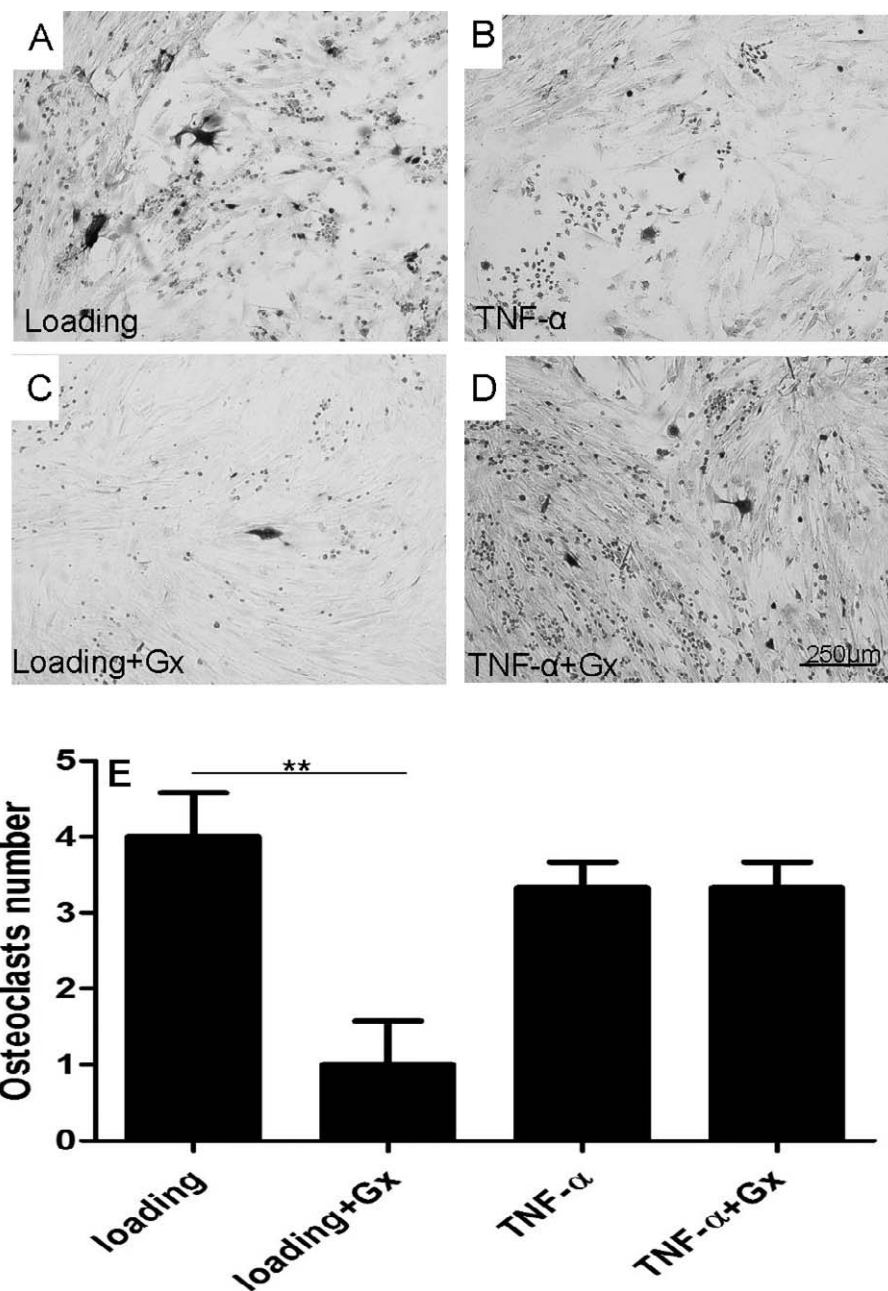


Figure 5. TRAP staining analyzes the effects of Gx on mechanical stress–induced and TNF- α –induced osteoclastogenesis in a PDL-RAW264.7 co-culture system. (A,C) Gx reduced numbers of osteoclast-like (TRAP⁺) cells in mechanical stress–pretreated PDL-RAW264.7 co-culture system. (B,D) Gx did not alter numbers of osteoclast-like cells in a TNF- α –pretreated PDL-RAW264.7 co-culture system. (E) Quantification of TRAP staining results. Values show as mean \pm SEM. Scale bar = 250 μ m. * $P = .05$; ** $P = .01$; *** $P = .001$; **** $P = .0001$.

GsMTx4 Inhibits Osteoclastogenesis

The effects of GsMTx4 on osteoclastogenesis were then explored by measuring Cox2, RANKL, and OPG mRNA amounts as well as synthesized PGE2 amount. GsMTx4 significantly reduced the mechanical stress induced, but it did not influence TNF- α –induced up-regulation of Cox2 and RANKL mRNA (Figure 4A,C). Consistently, GsMTx4 also inhibited mechanical stress–induced PGE2 synthesis but not TNF- α –induced PGE2 synthesis (Figure 4B). However, OPG

mRNA shows no significant difference among all groups (Figure 4D). Furthermore, we also tested the formation of osteoclast-like cells after 4 days of co-culture. As shown in Figure 5A, mechanically loaded PDLs induced the differentiation of RAW264.7 into osteoclast-like cells (shown as multinucleated TRAP-positive cells in image), and GsMTx4 inhibited the effect of mechanical stimulus. However, GsMTx4 could not affect the function of TNF- α in inducing osteoclastogenesis. The quantification of osteoclasts numbers

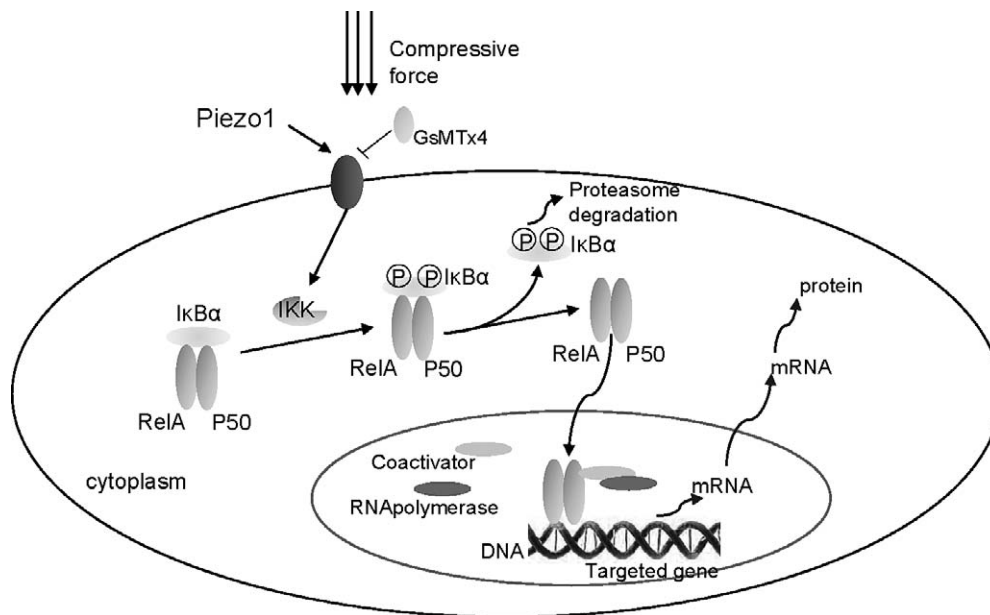


Figure 6. Mechanical stress activates NF- κ B signaling via Piezo1-dependent pathway.

shows GsMTx4 inhibits the effect of mechanical stress by 65% (Figure 5B). Thus, those observations indicate that GsMTx4 exerts a restraining effect on mechanical stress-induced osteoclastogenesis.

DISCUSSION

Orthodontic force is believed to be sensed and transduced by PDLs. Progenitor cells in the periodontium then differentiate into osteoclasts and osteoblasts, thereby causing bone remodeling and tooth movement. In the present study, we first investigated whether osteoclastogenesis-associated genes are activated following mechanical force administration in hPDLs. We tested time-course mRNA profiles of Cox2, RANKL, and OPG. As many other studies and our previous microarray data show, osteoclastogenesis inducer gene expression increased and osteoclastogenesis inhibitors dropped down under static mechanical compression.^{5,6} In the present study, Cox2 and RANKL mRNA also increased significantly after hours of mechanical stress. However, in the present study OPG mRNA was not changed in loaded hPDLs.

Next, we tested the role of Piezo channels in PDL tissues. To the best of our knowledge, few reports have involved study of the role of Piezo channels, although there are several studies that mention other ion channels in PDL tissues, K⁺ channels, like TREK-1.^{24,25} Thus, in the present study, we tested time-course Piezo1 gene expression in primary hPDLs under compressive loading. We found that Piezo1 mRNA increased after 0.5 hours of mechanical loading

and lasted for 12 hours. Immunofluorescence and Western blot analysis reconfirm the increases of Piezo1 expression in hPDLs after stimulation.

NF- κ B transcription factors are involved in highly conserved signaling pathways that respond to proinflammatory cytokines and mechanical signals through their nuclear translocation and subsequent activation of a plethora of genes.^{13–15} NF- κ B consists of the transactivation subunit RelA/p65 and the DNA-binding subunits.¹⁶ We demonstrate that mechanical stress induces nuclear translocation of NF- κ B, which affects the expression of target genes such as Cox2 and RANKL (Figure 6), while the underlying mechanism remains unknown. We also used Piezo1 channel inhibitor (GsMTx4) to block the function of Piezo1. After utilizing GsMTx4, mechanical stress-induced NF- κ B nuclear translocation and osteoclastogenesis-associated genes such as Cox2, RANKL were restrained. In addition to the inhibition of Cox2 and RANKL gene expression, we also found that static mechanical compression promotes the secretion of PGE₂, which is consistent with the findings of previous studies^{26,27} that claim that PGE₂ has a role in osteoclastogenesis.^{26,27} Furthermore, GsMTx4 can also restrain the upregulation of PGE₂.

In the present study, administration of GsMTx4 also diminished the potency of mechanically loaded but not TNF- α -treated hPDLs differentiating RAW264.7 into osteoclast-like cells.

One limitation to this study worth noting is that GsMTx4 is not specific for Piezo1, although this inhibitor indeed can only block mechanosensitive ion channels, but not all ion channels. Thus, further study with RNA interference is needed to reconfirm it.

In conclusion, we found that Piezo1 exerted a transduction role in mechanical stimuli-induced osteoclastogenesis with the involvement of NF- κ B activation. A better understanding of the biological behavior of PDLs responding to mechanical stress might facilitate the development of pharmacological therapy for accelerating orthodontic tooth movement or preventing postorthodontic treatment relapse.

CONCLUSIONS

- The present data demonstrate for the first time that PDL exposure to mechanical loading is associated with the activation of mechanically sensitive ion channel Piezo1. The pharmacological blockade of Piezo1 by GsMTx4 might inhibit the potency of PDLs inducing osteoclastogenesis.
- Our results indicate that osteoclastogenesis induced by mechanical stress is transduced by Piezo1 ion channel and mediated by NF- κ B signaling pathway.

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