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ORIGINAL ARTICLE

Impact of PIEZO1-channel on inflammation and osteoclastogenesis mediated via periodontal ligament fibroblasts during mechanical loading

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Abstract

The identification of mechanosensitive ion channels and their importance in innate immunity provides new starting points to elucidate the molecular mechanisms of orthodontic tooth movement. The mechanosensitive electron channel PIEZO1 (Piezo Type Mechanosensitive Ion Channel Component 1) may play a crucial role in orthodontic tooth movement. To investigate the role of the PIEZO1 channel, periodontal ligament fibroblasts (PDLF) were subsequently treated with a PIEZO1 inhibitor (GsMTx) with simultaneous pressure application or with an activator (JEDI2) without mechanical strain. The expression of genes and proteins involved in orthodontic tooth movement was examined by RT-qPCR, Western blot and ELISA. In addition, the effect on PDLF-mediated osteoclastogenesis was investigated in a coculture model using human monocytes. Inhibition of PIEZO1 under pressure application caused a reduction in *RANKL* (receptor activator of NF-kB ligand) expression, resulting in decreased osteoclastogenesis. On the other hand, activation of PIEZO1 without mechanical strain downregulated OPG (osteoprotegerin), resulting in increased osteoclastogenesis. PIEZO1 appears to play a role in the induction of inflammatory genes. It was also shown to influence osteoclastogenesis.

KEYWORDS

mechanosensitive electron channels, orthodontic tooth movement, periodontal ligament fibroblasts, PIEZO1

INTRODUCTION

In addition to aesthetic and functional aspects, orthodontic treatment fulfils a preventive role. Tooth misalignments can create niches that are difficult to clean with the risk of increased plaque accumulation, which favours the development of caries [1], gingivitis [2], and periodontal disease [3]. Orthodontic tooth movement achieved using appliances can resolve these dental malocclusions and thus reduce the risk of dental caries, among others.

Remodelling processes take place in the periodontal ligament in response to the application of an orthodontic force

to a tooth crown, which creates pressure and tension zones [4]. While mainly alveolar bone is resorbed in pressure zones, apposition of alveolar bone occurs in tension zones. Through these remodelling processes, the desired position of the tooth is achieved.

The largest population of periodontal ligament cells are fibroblasts (PDLF) [5, 6]. PDLF perform an important function in the maintenance of tissue homeostasis. In addition, they have regulatory functions in immune defence, as they can produce cytokines under inflammatory conditions [4]. PDLF are mechanosensitive cells, as they respond to compressive strain applied, for example, by an orthodontic appliance [4, 5, 7]. PDLF secrete, among others, proinflammatory cytokines and can thereby promote bone remodelling. Proinflammatory cytokines include tumor necrosis factor (TNF), interleukin-1-beta (IL1B) and interleukin 6 (IL6). Furthermore, PDLF produce prostaglandin-endoperoxide-synthase-2 (PTGS2). PTGS2 induces the production of prostaglandins from arachidonic acids [8]. The release of cytokines and prostaglandins causes a sterile inflammation in compression areas [9]. This sterile inflammation initiates the production of receptor activator of NF-kB ligand (RANKL) by osteoblasts, osteocytes, and PDLF, among others [10]. RANKL can bind to the membrane-bound RANK receptor on the preosteoclast surface. As a result, hematopoietic osteoclast precursor cells can differentiate into active, bone-resorbing osteoclasts [10]. To prevent excessive bone resorption, the RANKL decoy receptor osteoprotegerin (OPG) is expressed. OPG is produced by osteoblasts, macrophages and PDLF and can also bind to RANK, preventing further differentiation into active osteoclasts and associated bone resorption [10].

The mechanosensitive PIEZO1 (Piezo Type Mechanosensitive Ion Channel Component 1) channel is localized on the cell membrane of mechanosensitive PDLF [11]. Mechanosensitive ion channels possess a critical role in mediating mechanotransduction. By stretching the cell membrane, mechanosensitive ion channels open and ions can flow into the cell interior [12]. PIEZO1 is expressed primarily in non-sensory tissues. Increased prevalence of PIEZO1 channels is found in the lung, bladder, and skin [13]. Structural PIEZO channels can be divided into two regions: one is a peripheral module and the other is a pore-forming module [14]. The two modules are connected by an intracellular bar [15]. The peripheral module is composed of three homologous propeller-like helix structures. These three propeller-like helix structures are each composed of 38 transmembrane regions [16]. It is currently thought that the peripheral module performs the role as a mechanotransducer. When pressure is applied, stretching of the rotor blades occurs and the channel opens. The pore module crosses the cell membrane. While in the resting state the pore module is open in the PIEZO1 channel, it is closed in the PIEZO2 channel [16].

The PIEZO1 channel has been shown to be involved in bone remodelling, as bone is very mechanosenstive [17]. Cell-specific deletion of PIEZO1 in osteocytes interferes with osteogenesis [18], while deletion in mesenchymal stem cells affects the activity of osteoclasts [19]. The identification of mechanosensitive PIEZO channels and their role in innate immunity and bone remodelling may provide new starting points to clarify the molecular mechanisms of orthodontic tooth movement. In this study, the role of the PIEZO1 channel was investigated by inhibiting and activating it. We hypothesize that PIEZO1 is critically involved in the regulation of PDLF induced osteoclastogenesis by impairing the sterile Inflammation.

MATERIAL AND METHODS

Animal experiment

To test the expression of *Piezo1* mRNA in the periodontal tissue after orthodontic treatment, an animal experiment was conducted in compliance with the ARRIVE guidelines. The first molar on the left side of the upper jaw in eight male C57Bl/6N wild-type mice (age: 8-10 weeks; Charles River Laboratories; sample size was calculated with G-Power version 3.1.9.2 based on Mann-Whitney test) was moved by inserting an elastic band (diameter: 0.3 mm; Inwaria) which was left in situ for 7 days (approval no. AZ55.2.2-2532-2-567-13) [20]. The right side was not treated and served as the control side. The mice were fed tap water and a standard diet (V1535; ssniff). This was softened after the onset of orthodontic tooth movement to facilitate feed intake. Seven days after insertion of the elastic band the mice were euthanized, the upper jaw was immediately transferred to liquid nitrogen and stored at -80˚C until RNA was isolated from the periodontal ligament and gene expression of *Piezo1* was analysed by RT-qPCR.

Cell culture experiments

General cell culture conditions

The respective experiments were performed with a pool of human PDLF. These were derived from the root surface of extracted caries-free wisdom teeth, which had been extracted for medical reasons from a total of six patients. All experiments were approved by the ethics committee of the University of Regensburg (approval no. 12-170-0150). Likewise, informed consent to use the teeth for these research purposes was obtained from the patients. To prevent undesired contamination, cell culture work was always performed under sterile conditions. PDLF were cultured in DMEM high

glucose (Sigma-Aldrich) supplemented with 10% foetal calf serum (FCS; PAN Biotech), 1% L-glutamine (GE Healthcare), 1% antibiotic/antimycotic solution (Sigma-Aldrich) and 100 μM ascorbic acid (Sigma-Aldrich) until they were used for the experiments at passages 4 to 6. After isolation, PDLF were cultured in RPMI medium 1640 with GlutaMax (Gibco), 10% FCS (PAN Biotech), 1% antibiotic/antimycotic solution (Sigma-Aldrich), 100 μM ascorbic acid (Sigma-Aldrich) and transferred to either 6-well (Omnilab) or 24-well cell culture plates (Greiner) for experiments.

Experiments for PIEZO1 mRNA expression after different compression periods

To determine the time kinetics of *PIEZO1* gene expression, 70,000 PDLF per well on a 6-well plate were seeded in complete medium. After 24 h of pre-incubation, a sterile glass plate (2 g/cm^2) was placed on the cells for 0.5, 1, 2, 4, 6, 24, or 48 h, respectively [7, 21]. RNA was isolated and gene expression of *PIEZO1* was analysed by RT-qPCR. This experiment was repeated three times in triplicates ($n = 9$).

Influence of the PIEZO inhibitor GsMTx4

To examine the effect of the PIEZO inhibitor GsMTx4 under pressure conditions, PDLF were either seeded in a 24-well plate (25,000 PDLF per well) or on a 6-well plate (100,000 PDLF per well). After 24 h of preincubation, GsMTx4 (4 μM; Tocris) was added. GsMTx4 is derived from tarantula venom and alters the membrane properties of the cell and thus impairs the function of mechanosensitive channels [22].

Pressure was applied through sterile zirconia plates (2 g/cm^2) for further 48 h [23]. This experiment was repeated two times in biological triplicates ($n = 6$).

Influence of the PIEZO activator JEDI2

PIEZO1 was activated under control conditions without additional compressive strain by JEDI2. JEDI2 causes PIEZO1 activation via the extracellular side of its peripheral leaf-like structure, leading to channel opening and Ca^{2+} influx [15]. As JEDI2 has to be dissolved in dimethyl sulfoxide (DMSO), the same amount of DMSO was used for the controls. PDLF were either seeded in a 24-well plate (25,000 PDLF per well) or a 6-well plate (100,000 PDLF per well). After 24 h of preincubation, JEDI2 (1 mM; Tocris) was added. One hour later, pressure was applied through sterile zirconia plates (2 g/cm^2) for further 48 h. This experiment was repeated two times in biological triplicates ($n = 6$).

Coculture experiments

To investigate the influence of PIEZO1 on osteoclastogenesis, a coculture experiment using the monocytic THP1 cells (TiB-202, ATCC) was performed. Initially, 25,000 PDLF per well were seeded into a 24-well plate, as described above for each experiment. Simultaneously, for the differentiation of THP1 cells into macrophages, the cells were seeded at a density of 5×10^6 cells in 10 ml RPMI medium 1640 with GlutaMax (Gibco) and 20% FCS (PAN Biotech) per dish. To allow the cells to settle to the bottom of the dish, phorbol-12-myristate-13-acetate (PMA, Millipore) was pipetted onto the THP1 cells after 1 h. The THP1 cells were cultured in the incubator for 72 h, whereas either the activator or inhibitor were added to the PDLF after 24 h. Pressure application was performed another hour later using zirconia plates. Thereafter, the PDLFs were kept in the incubator for another 48 h. Differentiated THP1 cells were scraped from the dish, counted using the Neubauer counting chamber, and 100,000 cells were pipetted to each of the differently treated PDLF. This was followed by re-incubation for 72 h in an incubator. To detect differentiated osteoclasts TRAP staining was performed. This experiment was repeated three times in biological triplicates or duplicates ($n = 9/6$).

RNA isolation

In the mouse experiment, RNA was extracted from the first upper molar and adjacent tissue of both the treated and control side of the jaw after pulverization in a bone mill (Retsch) using the RNeasy Mini Kit (74,104, Qiagen) according to the manufacturer's instructions.

RNA from cell culture experiments was isolated with the TRIZOL method. After a total incubation time of 72 h, RNA from PDLF was isolated using 250 μl RNA Solv Reagent (VWR). After the addition of 100 μl of chloroform, cells were centrifuged at 13,000 rpm for 15 min. The aqueous phase was transferred to 500 μl isopropanol (VWR) and stored at -80˚C overnight. By washing the RNA twice with 80% ethanol and subsequent centrifugation at 13,000 rpm for 10 min, an RNA pellet was obtained which was then dried for 30 min. Finally, it was resuspended in 10 μl of nuclease-free water (Carl Roth).

The extracted RNA from tissue and cell culture was quantified by photometric measurement using the nanophotometer (Implen).

cDNA synthesis

An equal amount of RNA was diluted with nuclease-free water (Carl Roth) and subsequently mixed with 4.5 μl of a

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TABLE 1 Overview of target and reference genes used for mouse samples

Gene	Gene name	5′-forward-primer-3′	5′-reverse-primer-3′
Eef1a1	Eukaryotic translation elongation factor 1 alpha 1	AAAACATGATTACAGGCACATCCC	GCCCGTTCTTGGAGATACCAG
Piezo1	Piezo type mechanosensitive ion channel component 1	TTCTGGGACAAAACGGTAGCC	AGCCTGGTGGTGTTAAAGATGTC

TABLE 2 Overview of target and reference genes used for human samples experiments

Gene	Gene name	5′-forward-primer-3′	5′-reverse-primer-3′
IL1B	Interleukin-1-beta	ATGACCTGAGCACCTTCTTTCCCT	GCATCGTGCACATAAGCCTCGTTA
IL6	Interleukin 6	TGGCAGAAAACAACCTGAACC	CCTCAAACTCCAAAAGACCAGTG
OPG	Osteoprotegerin	TGTCTTTGGTCTCCTGCTAACTC	CCTGAAGAATGCCTCCTCACAC
PPIB	Peptidyl-prolyl-cis-trans-isomerase-B	TTCCATCGTGTAATCAAGGACTTC	GCTCACCGTAGATGCTCTTTC
PIEZO1	Piezo type mechanosensitive ion channel component 1	ACCTGCGTCATCATCGTGTG	AGTTGGTGCTGTTGGGGAAG
PTGS2	Prostaglandin-endoperoxide-synthase-2	GAGCAGGCAGATGAAATACCAGTC	TGTCACCARAGAGTGCTTCCAAC
TNF	Tumor necrosis factor alpha	GAGGCCAAGCCCTGGTATG	CGGGCCGATTGATCTCAGC
RANKL	Receptor activator of NF-kB ligand	ATACCCTGATGAAAGGAGGA	GGGGCTCAATCTATATCTCG
RPL22	Ribosomal protein L-22	TGATTGCACCCACCCTGTAG	GGTTCCCAGCTTTTCCGTTC

prepared master mix for cDNA synthesis. The master mix was composed of the following components: 1× M-MLV reverse transcriptase buffer (Promega), 0.1 nmol oligodT (Thermo Fisher Scientific), 0.1 nmol random hexamer (Thermo Fisher Scientific), 40 U RNase inhibitor (Thermo Fisher Scientific), and 200 U reverse transcriptase (Promega). The samples were then heated at 37˚C for 1 h. To terminate the reaction, reverse transcriptase was inactivated by heating at 95˚C for 2 min.

RT-qPCR

RT-qPCR was performed in the Realplex2 (Eppendorf). In each well of a 96-well plate (Biozym), 1.5 μl of diluted cDNA and 8.5 μl of a primer mix were pipetted. The primer mix consisted of the following components: 0.25 μl each of forward and reverse primers (Tables 1 and 2), 5 μl Luna Universal qPCR Master Mix (New England Biolabs) and 3 μl of nuclease-free water (Carl Roth). The 96-well plate was then covered with foil and briefly centrifuged. The following program was used for RT-qPCR: 95˚C for 2 min, 45 cycles of 95˚C for 10 s each, 60˚C for 20 s, 72˚C for 8 s. Finally, primer specificity was checked by melting curve analysis. For the determination of gene expression, the formula $2^{-\Delta Cq}$, with $\Delta Cq = Cq$ (target gene)—Cq (geomean of two reference genes) was used. *Eef1a1* was used as reference gene for mouse samples (Table 1). For the pressure experiments, a combination of *RPL22* and *PPIB* was used as a reference (Table 2), since previously performed experiments showed that these genes are constantly expressed under the prevailing conditions [24].

Immunoblotting

Cellytic reagent (C2978; Sigma-Aldrich) was mixed with a proteinase inhibitor Thermo Fisher) and 100 μl of this mixture were pipetted on each well of a 6-well plate. Protein quantification was performed using Roti-Quant (Carl Roth) according to the manufacturer's instructions and then measured at an absorbance length of 595 nm. Equal amounts of protein were added to a loading buffer and heated at 70˚C for 7 min. Protein samples were separated on an 8% polyacrylamide gel and transferred from to a polyvinylidene fluoride membrane (Carl Roth) using a tank blot procedure. The membrane was immersed for 1 h in 5% milk powder (Carl Roth) in TBS-T followed by overnight incubation of the membrane with the primary antibody PTGS2 (1:2500; Thermo Fisher Scientific) or ACTIN (1:1000; Sigma-Aldrich). After washing, the membrane was incubated with the secondary antibody (1:5000; Rockland Immunochemicals). Luminata Crescendo Western HRP substrate (Sigma-Aldrich) was used to detect

antibody binding using the VWR genoplex (VWR) through the Geno Capture program.

Enzyme-linked immunosorbent assays (ELISA)

To detect OPG in the cell culture supernatant of the PDLF, a commercially available ELISA was performed according to the manufacturer's instructions (Thermo Fisher Scientific).

Tartrate resistant acid phosphatase (TRAP) staining

To investigate the differentiation of THP1 cells into osteoclasts, TRAP staining was performed. For this purpose, the cells were washed twice with PBS. This was followed by incubation with 10% glutaraldehyde (Carl Roth) at 37˚C for 15 min to fix the cells. After washing again twice with PBS, staining of the cells was performed for 10 min at 37˚C using TRAP staining solution. The TRAP staining solution was previously prepared using the following components: 3 mg Fast Red Violet LB stain per ml TRAP buffer (50 ml 0.1 M acetate buffer, 10 ml 0.3 M sodium tartrate, 1 ml 10 mg/ml naphthol AS-MX phosphate, 100 μl Triton X-100 [all components from Sigma-Aldrich]). After removal of the TRAP staining solution, the cells were washed again with PBS, followed by counting of TRAP positively stained osteoclasts under a light microscope (Olympus-IX50; Olympus).

Statistics

To identify any possible outliers, a ROUT analysis (maximum desired false discovery rate Q = 5%) was performed, which revealed no outliers. The data set regarding the mice experiment was analysed using a paired *t*-Test. Data sets from the cell culture experiments were tested with a Welch-corrected ANOVA followed by Dunnett's T3 multiple comparison test. In the graphs, each point represents one data point. The horizontal line represents the mean value, the vertical lines represent the standard errors.

RESULTS

Gene expression of *PIEZO1* in the periodontal ligament after mechanical strain

First, we tested the expression of *Piezo1* mRNA in the periodontal ligament of the orthodontically treated jaw side or the untreated control side using a paired design in a mouse model. Insertion of an orthodontic appliance led to an elevated *Piezo1* gene expression in the periodontal ligament (Figure 1A). Next, we tested the effect of different periods of compressive strain on the gene expression of *PIEZO1* in human PDLF. Already after 1 h of compression, there was a significantly higher expression of *PIEZO1* than in the untreated control, which remained significantly elevated over the entire 48-h period studied (Figure 1B). This indicates a possible role of mechanosensitive PIEZO1 channels during orthodontic treatment and compressive force treatment of PDLF.

Impact of the PIEZO1 inhibitor GsMTx4 on the expression of inflammatory genes with compressive strain

To test this, we investigated the effect of the PIEZO1 inhibitor GsMTx4 in combination with pressure application on the expression of the inflammatory genes TNF, IL6 and PTGS2 by PDLF. Compressive strain enhanced gene expression of all investigated inflammatory genes (Figure 2). Treatment with GsMTx4 under simultaneous pressure application diminish this effect compared to the compressed cells without GsMTx4 treatment. For *PTGS2*, there was still a tendency for higher gene expression after GsMTx4 treatment and pressure application (Figure 2C). This effect on *PTGS2* mRNA expression could also be observed on the protein level.

Impact of the PIEZO1 inhibitor GsMTx4 on bone remodeling with compressive strain

Bone resorption is essential for orthodontic tooth movement and is mediated by osteoclasts. Osteoclastogenesis is controlled by expression of RANKL and its decoy receptor OPG. *OPG* gene expression was significantly lower with compressive strain without and with PIEZO1 inhibition (Figure 3A). This was also observed on the protein level when analysing OPG secretion (Figure 3B). Elevated *RANKL* gene expression due to compressive strain was significantly diminished by GsMTx4 treatment (Figure 3C). This was also visible in the coculture model, where more $TRAP^{+}$ cells were visible after pressure application (Figure 3D). This effect was lower using GsMTx4, indicating a regulatory role of PIEZO1 channel on PDLF-induced osteoclastogenesis.

Impact of the PIEZO1 activator JEDI2 on the expression of inflammatory genes without compressive strain

Inhibition of PIEZO1 under combined compressive strain already gave some hints on a possible role of PIEZO1 on

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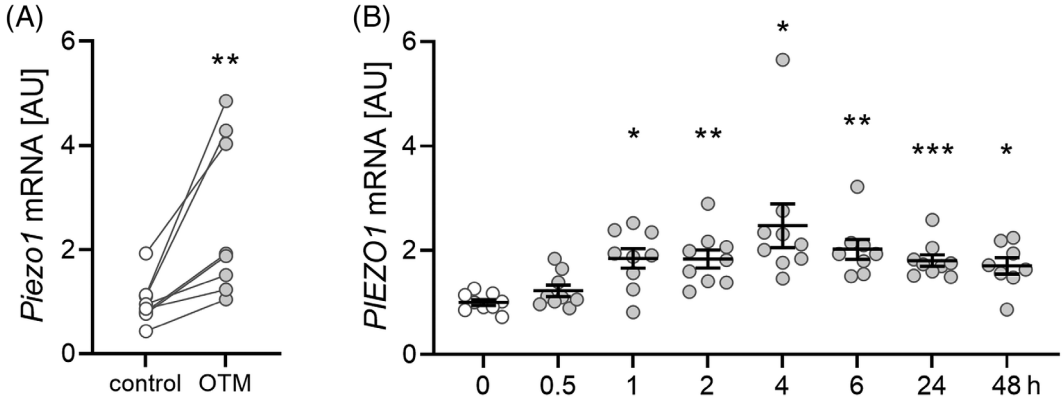

FIGURE 1 *Piezo1* mRNA in the periodontal ligament without and with OTM ($n = 8$; A) and in PDLF subjected to compressive strain for different periods ($n \geq 8$; B) Statistics: (A) paired *t*-Test; (B) Welch-corrected ANOVA followed by Dunnett's T3 post-hoc tests; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. ANOVA, analysis of variance; OTM, orthodontic treatment; Piezo1, Piezo Type Mechanosensitive Ion Channel Component 1.

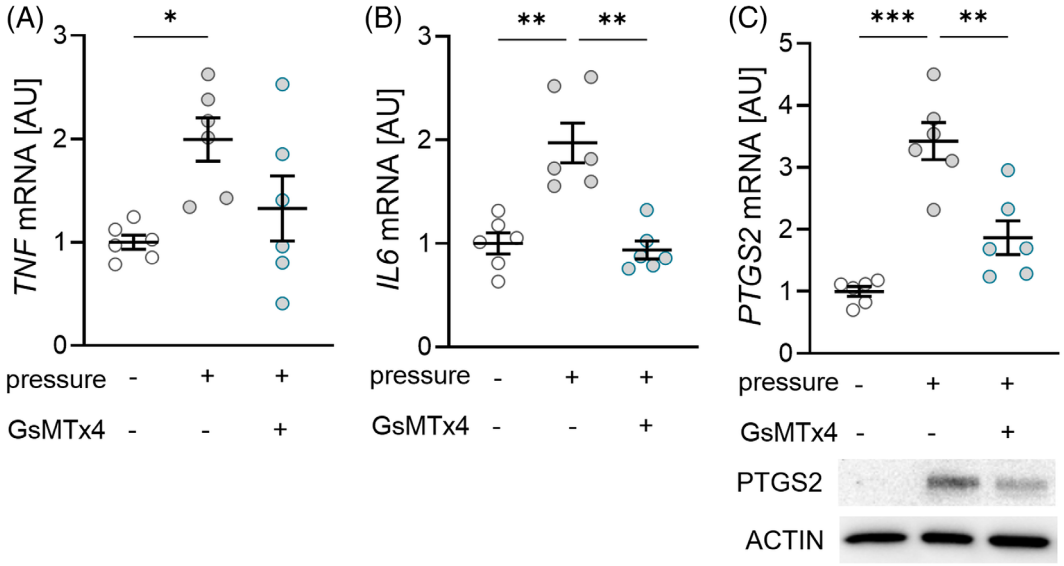

FIGURE 2 Gene expression of *TNF* (A) and *IL6* (B) as well as gene and protein expression of PTGS2 (C) in PDLF exposed to compressive strain and GsMTx4; $n = 6$. Statistics: Welch-corrected ANOVA followed by Dunnett's T3 post-hoc tests; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. ANOVA, analysis of variance; IL6, interleukin 6; PDLF, periodontal ligament fibroblasts; PTGS2, prostaglandin-endoperoxide-synthase-2; TNF, tumor necrosis factor.

PDLFs. Next, we used a PIEZO1 activator without additional compressive strain and investigated the effects on inflammatory genes (Figure 4). As expected, *TNF* gene expression was higher after pressure application. Treatment with JEDI2 under control conditions failed to enhance *TNF* gene expression (Figure 4A). Elevated *IL6* gene expression was observed with compressive strain and JEDI2 treatment (Figure 4B). Compressive strain also enhanced *PTGS2* gene and protein expression (Figure 4C). Addition of PIEZO1 activator JEDI2 had a massive effect on *PTGS2* gene expression without compressive force, which was significantly higher than in the control and the compressed group (Figure 4C).

Impact of the PIEZO1 activator JEDI2 on bone remodeling without compressive strain

Last, we tested the effect of JEDI2 on osteoclastogenesis. We observed a lower *OPG* gene expression after treatment with JEDI2 which were similar to that of compressive strain (Figure 5A). When analysing the OPG secretion in the cell culture supernatant, we also detected a lower OPG secretion due to JEDI2 treatment. But JEDI2 did not lower the OPG secretion to the same extent as compressive strain (Figure 5B). We detected no inducing effects of JEDI2 on *RANKL* gene expression (Figure 5C). However, the number of $TRAP^{+}$ cells

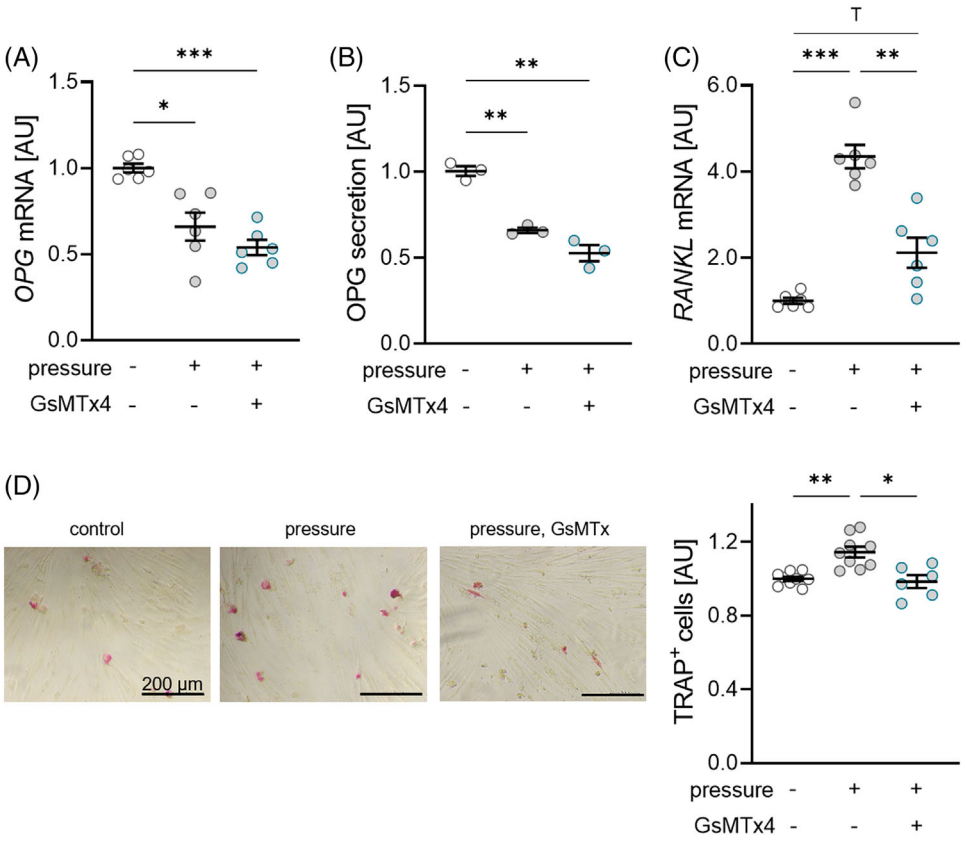

FIGURE 3 (A) Gene expression ($n = 6$) and (B) protein secretion ($n = 3$) of OPG and (C) *RANKL* mRNA expression ($n = 6$) in PDLF exposed to compressive strain and (D) GsMTx4 as well as PDLF-induced osteoclastogenesis assessed by determination of $TRAP^+$ cells in a coculture model ($n \geq 6$). Statistics: Welch-corrected ANOVA followed by Dunnett's T3 post-hoc tests; $^{T}p < 0.1$, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. ANOVA, analysis of variance; OPG, osteoprotegerin; PDLF, periodontal ligament fibroblasts; RANKL, receptor activator of NF-kB ligand.

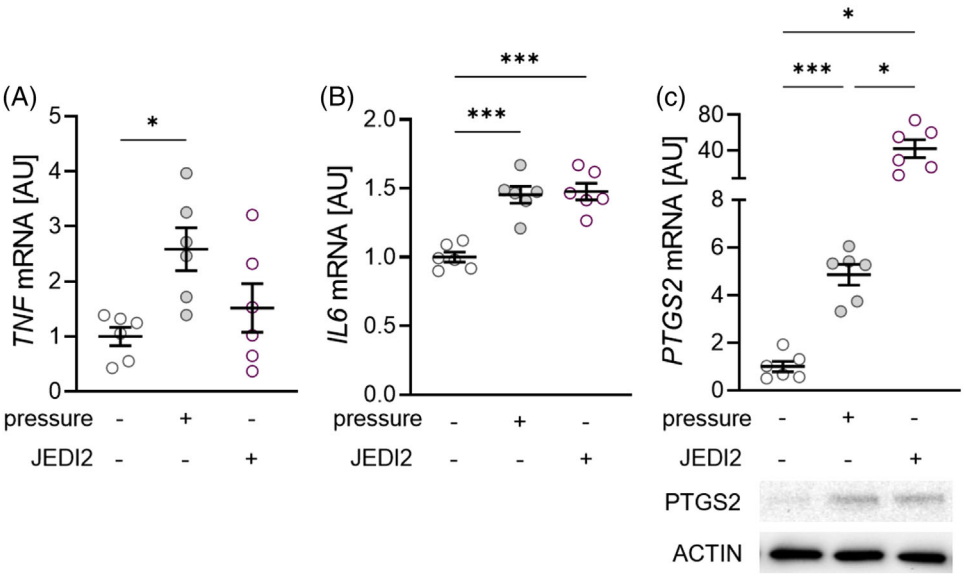

FIGURE 4 (A) Gene expression of *TNF* and (B) *IL6* as well as (C) gene and protein expression of PTGS2 in PDLF; $n = 6$. Statistics: Welch-corrected ANOVA followed by Dunnett's T3 post-hoc tests; $^{*}p < 0.05$; $^{***}p < 0.001$. ANOVA, analysis of variance; IL6, interleukin 6; PDLF, periodontal ligament fibroblasts; PTGS2, prostaglandin-endoperoxide-synthase-2; TNF, tumour necrosis factor.

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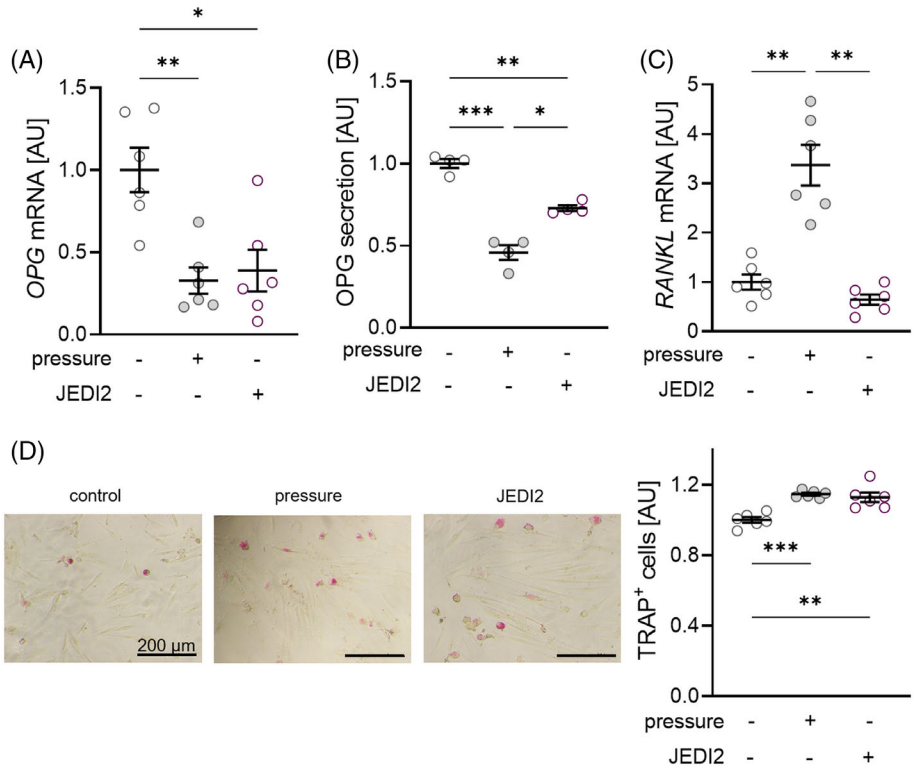

FIGURE 5 (A) Gene expression ($n = 6$) and (B) protein secretion ($n = 3$) of OPG as well as (C) *RANKL* mRNA expression ($n = 6$) and PDLF-induced osteoclastogenesis assessed by determination of $TRAP^+$ cells in a (D) coculture model ($n = 6$). Statistics: Welch-corrected ANOVA followed by Dunnett's T3 post-hoc tests; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. ANOVA, analysis of variance; OPG, osteoprotegerin; PDLF, periodontal ligament fibroblasts; RANKL, receptor activator of NF-kB ligand.

in the coculture model were higher than seen for the untreated control with activation of PIEZO1 using JEDI2 at a similar level as that by compressive strain (Figure 5D). These data indicate a regulatory role of PIEZO1 on osteoclastogenesis.

DISCUSSION

The present findings demonstrate that *PIEZO1* mRNA levels are higher in PDLF following compressive force treatment and in mice after orthodontic tooth movement, than in controls unexposed to force. Furthermore, it was shown that PIEZO1 impacted on the induction of inflammatory genes and PDLF-mediated osteoclastogenesis, which both can influence orthodontic treatment.

This study was mainly conducted in PDLF subjected to compressive force treatment and chemical inhibition or activation of PIEZO1. This is the major limitation of this study, as orthodontic tooth movement is a multicellular event based on the interaction of PDLF, immune cells, osteoclasts and osteoblasts. Nevertheless, PDLF comprise the main cell population in the periodontal ligament, which are exposed to the mechanical forces during orthodontic treatment. They react to the mechanical stress with increased expression of inflammatory mediators like TNF, IL6, and PTGS2 and receptor activator of NFk-B, which is critically involved in osteoclastogenesis [4, 21]. To investigate the function of PIEZO1 in compressed PDLF, PIEZO1 was further inhibited by GsMTx4 [12, 25, 26] and activated by JEDI2 [15]. GsMTx4 is a non-selective inhibitor of the PIEZO1 channel, reducing the cation influx into the cell by 70% while JEDI2 promotes Ca^{2+} influx thereby mimicking the activated PIEZO1 channel [15].

The observed higher *PIEZO1* mRNA levels in PDLF after compressive force treatment are in line with the findings reported in other publications [11, 27]. This upregulation of gene expression can be an indicator of a possible role of PIEZO1 in the transmission of mechanical stimuli in the cell.

Pressure application promotes enhanced expression of inflammatory genes and led to higher osteoclastogenesis [4, 21]. Simultaneous pressure application and inhibition of PIEZO1 by GsMTx4 downregulated the inflammatory mediators *IL6* and *PTGS2* as well as *RANKL* levels. In addition, PDLF-mediated osteoclastogenesis was significantly reduced. These data are in line with those of Jin et al. [11] indicating that PIEZO1 does not exert its bone resorption-promoting mode of action via TNF, as no fluctuations in TNF expression were seen upon inhibition of PIEZO1.

Activation of PIEZO1 without mechanical strain led to lower OPG expression and secretion, resulting in increased osteoclastogenesis. The inflammatory genes *IL6* and *PTGS2*

were also significantly upregulated without additional pressure application and activation of PIEZO1 by JEDI2. To date, the effects of PIEZO1 activation on the expression profile of PDLF have not been described in the literature. The present findings show that activation of PIEZO1 has an impact on osteoclastogenesis and the induction of inflammatory genes. The underlying signal transduction process of PIEZO1 has not yet been elucidated.

Wang et al. [28] investigated the role of PIEZO1 on the osteogenic differentiation of periodontal stem cells focussing on the Notch signalling pathway. Upon activation of the Notch pathway, transcription of some genes involved in bone metabolism and angiogenesis, among others, can be regulated [29]. Wang et al. [28] concluded that under tensile stress, PIEZO1 expression is upregulated, which can activate the Notch signalling pathway.

In relation to the findings presented here, the relationship between PIEZO1 expression and Notch signalling in compressed PDLF would be of interest. Furthermore, animal experiments may help to clarify the role of PIEZO1 during orthodontic tooth movement. This study demonstrated a role of PIEZO1 in the induction of inflammatory genes and on PDLF-mediated osteoclastogenesis. Inhibition of PIEZO1 under pressure application caused a reduction in RANKL expression, resulting in decreased osteoclastogenesis. On the other hand, activation of PIEZO1 without pressure application downregulated OPG, resulting in increased osteoclastogenesis.

AUTHOR CONTRIBUTIONS

Conceptualization: Agnes Schröder, Christian Kirschneck, Peter Proff; **Methodology**: Agnes Schröder, Christian Kirschneck; **Validation**: Agnes Schröder, Eva Paddenberg, Christian Kirschneck; **Formal analysis**: Agnes Schröder, Christian Kirschneck; **Investigation**: Katharina Neher, Bernhard Krenmayer, Eva Paddenberg, Agnes Schröder; **Resources**: Gerrit Spanier, Peter Proff; **Data curation**: Agnes Schröder, Christian Kirschneck, Eva Paddenberg; **Writing—original draft preparation**: Agnes Schröder, Christian Kirschneck, Eva Paddenberg; **Writing—review and editing**: Peter Proff, Gerrit Sapnier; **Visualization**: Agnes Schröder, Eva Paddenberg, Christian Kirschneck; **Supervision**: Christian Kirschneck, Peter Proff.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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