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GDF15 induced by compressive force contributes to osteoclast differentiation in human periodontal ligament cells

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ABSTRACT

Orthodontic tooth movement (OTM) is initiated by mechanical force and featured as alveolar bone remodeling. Periodontal ligament cells (PDLCs) are one of the major cell components in periodontium and responsible for the signal transduction during OTM. Up to now, the mechanical stress-induced genetic alteration and mechanotransduction mechanisms in PDLCs still remain not fully understood. In this study, we identified a novel compressive force responsive gene, Growth differentiation factor 15 (GDF15), whose expression transcriptionally increased in human periodontal ligament cells (PDLCs) after exposure to the static compressive force *in vitro*. Functional analyses proved that GDF15 could promote osteoclast differentiation of the murine macrophage cell line RAW264.7 cells. Molecular investigation uncovered that GDF15 could promote the expression of several pro-inflammatory cytokines and RANKL/OPG ratio in PDLCs, while knockdown of GDF15 impaired their upregulation induced by compressive force. Additionally, administration of recombinant GDF15 protein stimulated the M1-like polarization of RAW264.7 cells and THP-1 induced macrophages. Mechanistically, siRNA-mediated suppression of GDF15 significantly disrupted the nuclear translocation of NF-κB and ERK phosphorylation in response to compressive force. Finally, Yes-associated protein (YAP) was demonstrated to be the upstream regulator of GDF15 in human PDLCs, implying a force-induced YAP-GDF15 regulation mechanism. Overall, these data suggested important roles of GDF15 in the functional modulation of both PDLCs and osteoclast progenitors in response to compressive force, providing novel insights into the molecular mechanism of mechanotransduction during OTM process.

1. Introduction

Orthodontic tooth movement (OTM) triggered by mechanical force depends on the remodeling of tissues surrounding the roots [1,2]. Accelerating OTM has long been desired for its multiple potential benefits, including shorter treatment duration and reduced side effects [3]. Therefore, it is of great importance to unravel the biological events that occurred during OTM and the underlying mechanisms. At cellular level, Force application first induces apoptosis of the osteocytes on the compression side, and then osteoclastic resorption occurs. On the tension side, new bone forms due to generation of osteoblasts [4–6]. Among these events, bone resorption is considered as a rate-limiting step, with activation of osteoclasts attributed to pro-inflammatory cytokines produced by periodontal ligament cells (PDLCs) such as interleukin 1 (IL-1), interleukin 6 (IL-6), and cyclooxygenase 2 (COX-2), etc. [7]. Importantly, the receptor activator of nuclear factor-κB (RANK)/ligand for the receptor activator of nuclear factor-κB (RANKL)/osteoprotegerin (OPG) axis plays a pivotal role [1]. RANKL secreted by PDLCs could bind on RANK, recruiting the adaptor protein TRAF6 and leading to activation of downstream signaling cascades including protein kinases-mediated NF-κB and AP-1 transcription factor C-FOS, as well as activation of MAPKs such as c-Jun N-terminal kinase (JNK) and p38, which are important mediators of osteoclast differentiation [8].

Originated from myeloid precursors, macrophages can differentiate into osteoclasts under stimulation of M-CSF and RANKL, thereby playing a pivotal role during active osteoclasts formation and bone resorption [9–11]. Macrophages can be polarized into M1 and M2 phenotype. M1 macrophages promote inflammation by producing

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tumor necrosis factor (TNFα), interleukins (IL-1, IL-6), and inducible nitricoxide synthase (iNOS) [12], while M2 macrophages function as anti-inflammatory agents by producing anti-inflammatory products [13]. Recent studies reported increased M1/M2 ratio of macrophage polarization during OTM, indicating an important role of macrophage polarization for the bone remodeling [14]. However, factors that contribute to force induced M1-like polarization are not yet elucidated. Moreover, up to now, the mechanotransduction mechanism in PDLCs are still not clear enough, which promoted us to explore more force-induced genetic changes that lead to the functional alterations of macrophages and PDLCs during mechanotransduction.

Growth differentiation factor 15 (GDF15) is a member of the human transforming growth factor β (TGF-β) superfamily [15]. It is involved in regulation of many signaling pathways including ERK and SMADs like other family members [16–18]. However, the biological role of GDF15 might be unique due to its low amino acid similarity with other members. GDF15 is usually lowly expressed under normal conditions in most tissues, but significantly induced by pathological conditions such as tissue injury or inflammation [19]. GDF15 could upregulate IL-6 expression to promote tumorigenesis of prostate carcinoma cells [20], while its deficiency reduced vascular injury-induced IL-6 upregulation [21], implying the GDF15-mediated positive regulation of IL-6. As to the role of GDF15 in osteoclast differentiation, hypoxia was shown to be able to induce GDF15 expression which promoted NF-κB activation and osteoclastogenesis as a result [22]. Besides, GDF15 was also found to enhance osteoclast differentiation in human peripheral blood monocytes, but decrease the osteogenic differentiation potential of human mesenchymal stem cells (MSC) [23]. Its promotive effect on osteoclast differentiation was also reported in tumor progression [24].

In spite of the critical role of GDF15 in osteoclastogenesis, few studies reported its responsiveness upon mechanical stimuli, especially compressive force stimulation and the consequent biological event. In this study, we aimed to investigate the responsiveness of GDF15 to compressive force in human PDLCs, and its impact on osteoclast differentiation and M1/M2 polarization of macrophages. We further dissect the possible molecular mechanism through which GDF15 promotes osteoclastogenesis, and the upstream regulators orchestrating the up-regulation of GDF15 after force application.

2. Materials and methods

2.1. Cell culture and treatment

The protocol was approved by the Ethical Guidelines of Peking University (PKUSSIRB-201630098) and performed with appropriate informed consents. Human PDLCs were isolated from PDL of normal orthodontic extracted bicuspid, according to previously reported protocols [25,26]. Briefly, the PDL tissues were separated from the mid-third of the root surface and minced into small tissue cubes. Subsequently, the tissue cubes were digested with a solution of 3 mg/mL collagenase (type I) with 4 mg/mL dispase (both from Sigma-Aldrich) in α-minimum essential medium (α-MEM, Hyclone) for 15 min at 37 °C with vigorous shaking. The tissue explants were then plated into culture dishes containing α-MEM supplemented with 10% fetal bovine serum (FBS; Hyclone), 0.292 mg/mL glutamine (Hyclone), 100 units/mL penicillin streptomycin (Hyclone), and 100 mM/L ascorbic acid (Sigma-Aldrich) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. PDLCs were derived from three donors and pooled together. Cells with 4–6 passages were used.

Static compressive force was applied as previously described [27]. When the cells reached 80% confluent in 6-well plates, a layer of glass cover and additional metal weights were placed on top. Cells were subjected to different continuous compressive forces ranging from 0 to 1.5 g/cm^2 for 24 h (h) or at 1.5 g/cm^2 for different durations ranging from 0 to 24 h.

Recombinant human GDF15 protein (rhGDF15) (Peprotech) was added into the medium of RAW264.7 cells or THP-1 induced macrophages, or PDLCs at the concentration of 100 or 200 ng/ml for 48 h.

THP-1 human monocytic cells were used to differentiate into macrophages with RPMI 1640 medium containing 10% fetal bovine serum, 2 mM glutamine, antibiotics, and 50 ng/mL PMA (P1585, Sigma, USA) for 24 h.

To evaluate the influence of YAP on GDF15 expression, the inhibitor of YAP, verteporfin (0, 0.5, 1, 2 μM, MedChemExpress) and the MST1/2 inhibitor XMU-MP-1 (0, 2, 5 μM, Selleck) were added into the medium of PDLCs for 48 h.

2.2. Tartrate-resistant acid phosphatase (TRAP) staining assay

The murine monocytes RAW264.7 cells were induced toward osteoclast differentiation by addition of soluble receptor activator of nuclear factor–κB ligand (sRANKL) (50 ng/mL) and M-CSF (30 ng/mL) in the absence or presence of rGDF15 (100 ng/ml). 4 days later, the cells were fixed and stained for TRAP staining using acid phosphatase kit (387A, Sigma). TRAP-positive multinucleated osteoclasts were counted in 5 visual fields in each well. We calculated the average value of 3 experiments.

2.3. siRNA transfection and quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

For knockdown of genes expression, two double-stranded siRNAs against GDF15 (siGDF15), NF-κB (si NF-κB) and HIF1α (siHIF1α) were chemically synthesized (GenePharma). The sequences of siRNA are as follows: siGDF15: CCAGCUACAAUCCCAUGGU; sip65: GATGAGATCT TCCTACTGT; siHIF1α: CTGATGACCAGCAACTTGA. Transfection of siRNA was performed using the Lipofectamin RNAiMAX (Invitrogen) following the manufacturer's instruction.

For the co-culture experiment, PDLCs were transfected with siGDF15 or the non-sense control siNC, followed by compressive force treatment at 1.5 g/cm^2 for 12 h. The supernatants were collected and used for co-culture with RAW267.4 cells (1:1 mixed with fresh medium). 48 h later RAW264.7 cells were harvested for western blot analysis.

Total RNAs were extracted using Trizol reagent (Invitrogen). Synthesis of first strand cDNA and subsequent quantitative PCR were performed as previously described [28,29]. All RT-qPCR processes were performed three times using GAPDH as the internal control. The primers used in this study are listed in Tables 1 and 2.

2.4. Fractionation, western blot analyses and antibodies

Cultured cells were harvested after washing with ice-cold phosphate-buffered saline and then lysed in extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet p-40, 0.01% protease inhibitor mixture). Cells were fractionated using Nuclease and

Table 1
RT-qPCR primers used in PDLCs and THP-1 induced macrophages.

	Forward primer	Reverse primer
GDF15	caatcccatggtgctcattc	tatgcagtggcagtctttgg
IL-6	aggcactggcagaaaacaac	ttttcaccaggcaagtctcc
IL-8	gtgtgaaggtgcagttttgc	tgtggtccactctcaatcactc
COX-2	aagccttctctaacctctcc	gccctcgcttatgatctgtc
RANKL	atcacagcacatcagagcagaga	aggacagactcactttatgggaac
OPG	gaggcattcttcaggtttgc	gctgtgttgccgttttatcc
IL-1β	tgagcaccttctttcccttc	aggtgcatcgtgcacataag
TNFα	ttcctcagcctcttctccttc	atcactccaaagtgcagcag
ARG	tccaaggtctgtgggaaaag	attgccaaactgtggtctcc
Dectin	acccaagaaaacccatctcc	atgagggcacactacacagttg
IL-4R	caagctcttgccctgttttc	ctggaaaggcatctctttgg
GAPDH	caatgacccctcattgacc	atgacaagcttcccgttctc

Table 2
RT-qPCR primers used in RAW264.7 cells.

	Forward primer	Reverse primer
Nfatc1	tgggagatggaagcaaagac	ttgcggaaaggtggtatctc
TRAP	acttgcgaccattgttagcc	agagggatccatgaagttgc
CK	aaggcagctaaatgcagagg	ttgcatcgatggacacagag
IL-1β	gctgcttccaaacctttgac	ttctccacagccacaatgag
IL-6	ctgatgctggtgacaaccac	tccacgatttcccagagaac
TNF-α	acggcatggatctcaaagac	agatagcaaatcggctgacg
ARG-1	cgcctttctcaaaaggacag	cgcctttctcaaaaggacag
Dectin	aagccaaacatcgtctcacc	cttgaaacgagttggggaag
IL-4R	tccatggtcaacatctccag	atgttgatcgggaagctcag
c-fos	agaaacggagaatccgaagg	tgcaacgcagacttctcatc
gapdh	aacgacccctcattgacctc	actgtgccgttgaatttgcc

Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, MA, USA), according to the manufacturer's protocol.

Western blot analyses were performed as previously described [28]. Antibodies used are as follows: anti-GDF15 (27455-1-AP, Proteintech); anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-47724, Santa Cruz); anti-RANKL (ab45039, Abcam); anti-OPG (ab11994, Abcam); anti-β-actin (#3700, CST); anti-lamin A/C (#4777, CST); anti–NF–κB subunit p65 (10745-1-AP, Proteintech); anti-histone 3 (H3) (17168-1-AP, Proteintech); anti-phospho-ERK (Thr202/Tyr204) (#4370, CST); anti-ERK (#4695 ,CST); anti-CCND1 (sc-450, Santa Cruz).

2.5. Statistical analysis

Statistical analysis was performed with SPSS 13.0. All the data were presented as mean ± SD from 3 independent experiments and assessed by independent 2-tailed Student's *t*-test or 1-way analysis of variance. Statistical significance was considered at $P < 0.05$.

3. Results

3.1. Compressive force induced activation of GDF15 in human PDLCs

In order to dissect the role of GDF15 in mechanotransduction in PDLCs, we first assessed the responsiveness of GDF15 to the compressive force. For that, PDLCs were exposed to compressive force with increasing intensities for 24 h, and then western blot and RT-qPCR analyses were performed to determine the expression changes of GDF15. The results indicated significant increment of GDF15 at both protein and mRNA levels upon force stimuli (Fig. 1A and B). To further consolidate this point, PDLCs were treated with compressive force for different time at certain intensity. The results of RT-qPCR also consistently demonstrated the upregulation of GDF15 (Fig. 1C). Therefore, these data suggested that GDF15 was transcriptionally upregulated by compressive force in human PDLCs.

3.2. GDF15 promotes osteoclast differentiation of RAW264.7 cells

Next, we sought to investigate the functional role of GDF15 induced by force stimuli. Due to the critical role of bone resorption on the compressive side during OTM, we hypothesized that GDF15 secreted by

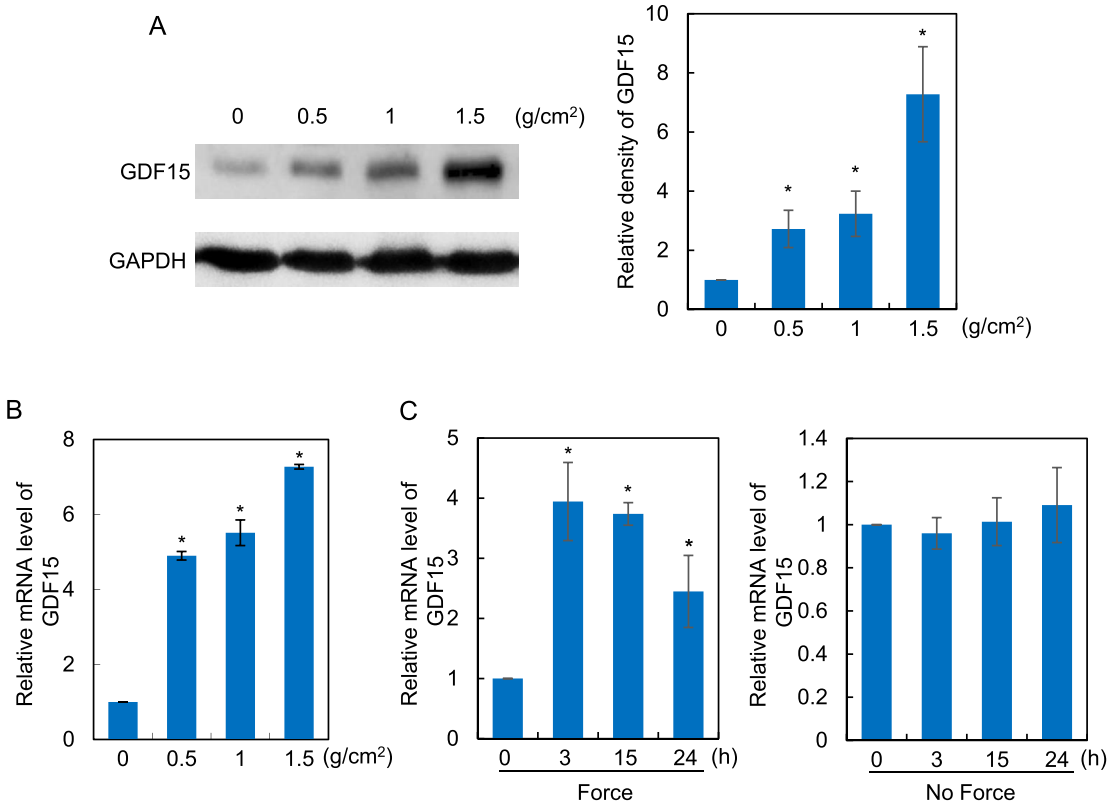

Fig. 1. Compressive force upregulates GDF15 expression in human PDLCs. (A–B) Compressive force induced upregulation of GDF15 in a dose-dependent manner. PDLCs were treated with increasing force intensity for 24 h (h), followed by protein and mRNA extraction. The expression changes of GDF15 at protein level were determined by western blot using centrifuged supernatants (for detection of GDF15) and total proteins (for detection of β-actin) (A). The expression changes of GDF15 at mRNA level were determined by RT-qPCR (B). (C) Forces with different durations induced upregulation of GDF15. PDLCs were treated with varying durations at 1.5 g/cm^2 force (left panel) or without force treatment (right panel) followed by mRNA extraction. Expression change of GDF15 was determined by RT-qPCR. *$p < 0.05$, versus 0 g/cm^2 or 0 h group. ($n = 3$).

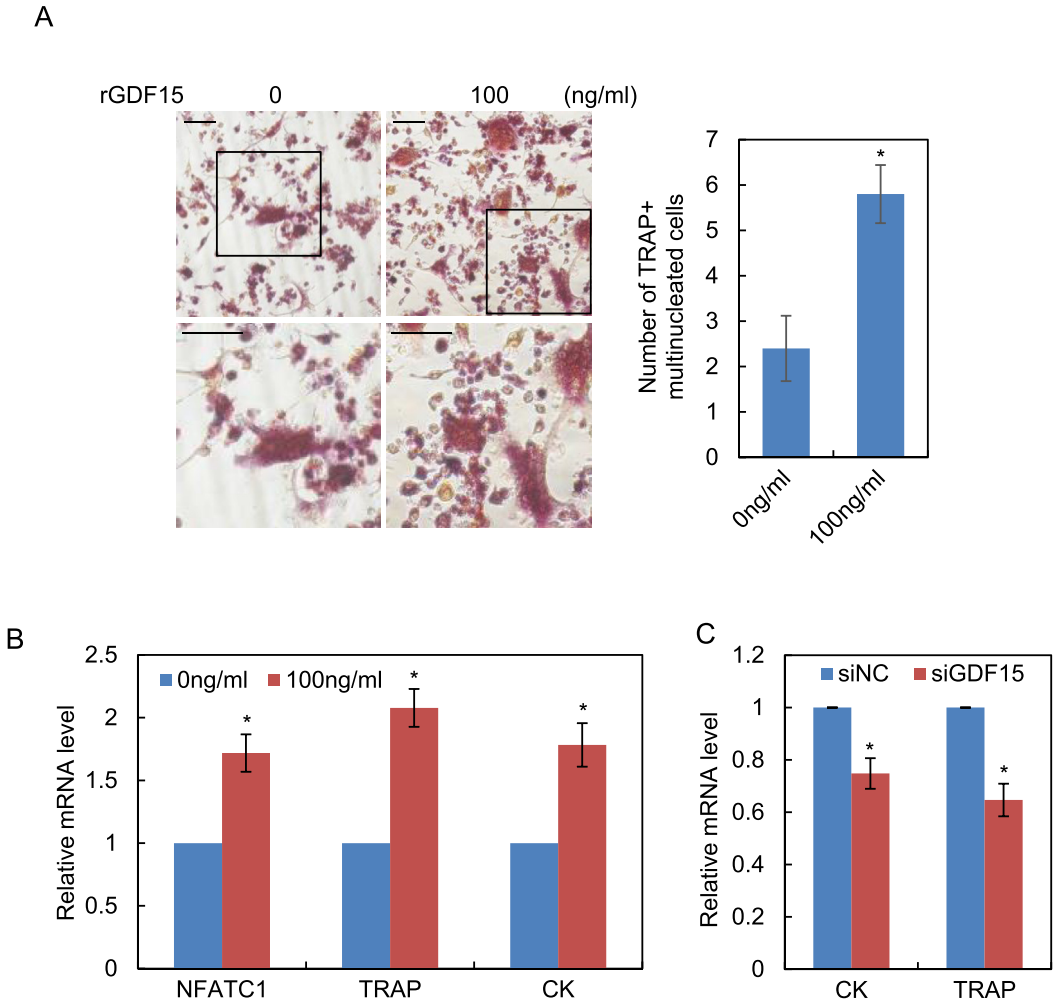

Fig. 2. GDF15 promotes osteoclast differentiation of RAW264.7 cells. (A–B) Administration of rGDF15 enhanced the osteoclastic activity of RAW264.7 cells. RAW264.7 cells were induced to undergo osteoclast differentiation by sRANKL (50 ng/ml) in the absence or presence of rGDF15.4 days later, cells were subjected to TRAP staining (A) and RT-qPCR analyses (B) respectively. The bottom panels show the magnification of the square boxed area in the upper panels. Bar: 100 μm. (C) Knockdown of GDF15 in PDLCs with force loading suppressed osteoclast differentiation marker genes expression. PDLCs were transfected with siRNA against GDF15 (siGDF15) or non-sense control (siNC) and then exposed to compressive force of 1.5 g/cm^2 for 12 h. RAW264.7 cells were co-cultured with supernatants from these PDLCs for 48 h and then subjected to RT-qPCR analyses. *$p < 0.05$ ($n = 3$).

PDLCs might contribute to the osteoclast differentiation of monocytes. To test this, TRAP staining was performed in RANKL-induced RAW264.7 cells in the presence or absence of recombinant GDF15 protein (rGDF15). The results revealed greater number of TRAP positive multinucleated cells in rGDF15 administration cells (Fig. 2A). Besides, RT-qPCR results showed higher expression of osteoclast differentiation marker genes in rGDF15-treated cells than control cells (Fig. 2B). These data underlie the positive regulation of osteoclast differentiation by GDF15.

To explore whether GDF15 played a role in force-induced osteoclast differentiation, we collected supernatants from PDLCs with knockdown of GDF15 or control cells followed by compressive force treatment. RAW264.7 cells were then co-cultured with these supernatants. Results from RT-qPCR showed that the expression of CK and TRAP were all significantly downregulated in GDF15 knocked down cells, indicating an essential role of GDF15 in osteoclast differentiation triggered by compressive force.

3.3. GDF15 is essential for force-induced upregulation of pro-inflammatory cytokines and RANKL/OPG ratio

Having established the promotion effect of GDF15 on osteoclast differentiation, we started out to unravel the underlying molecular mechanisms. PDLCs under compressive force were found to produce a series of pro-inflammatory cytokines, which contribute to the recruitment of osteoclast precursor cells and maturation of osteoclasts. To examine the role of GDF15 in this process, we administered PDLCs with rGDF15. Subsequent RT-qPCR analyses indicated that the expression of IL-6, IL-8 and COX2 was enhanced in GDF15 added PDLCs (Fig. 3A). On the contrary, when the expression of GDF15 was knocked down by siRNA, the force-induced upregulation of these inflammation factors was significantly impaired (Fig. 3B).

Among the inflammation factors, RANKL expression could elicit downstream signaling including NF-κB and MAPK, while OPG was a negative regulator of osteoclastogenesis due to its competent binding with RANKL for RANK [30]. Therefore RANKL/OPG was found to be upregulated and play key roles during OTM. We administered PDLCs with rGDF15 and found that the RANKL/OPG ratio was upregulated upon GDF15 administration (Fig. 4A). In contrast, when GDF15 expression was knocked down by siRNA transfection, the upregulation of RANKL/OPG induced by compressive force was significantly attenuated (Fig. 4B). Collectively, these data pointed to the indispensable role of GDF15 in full activation of inflammation factors and RANKL/OPG induced by compressive force.

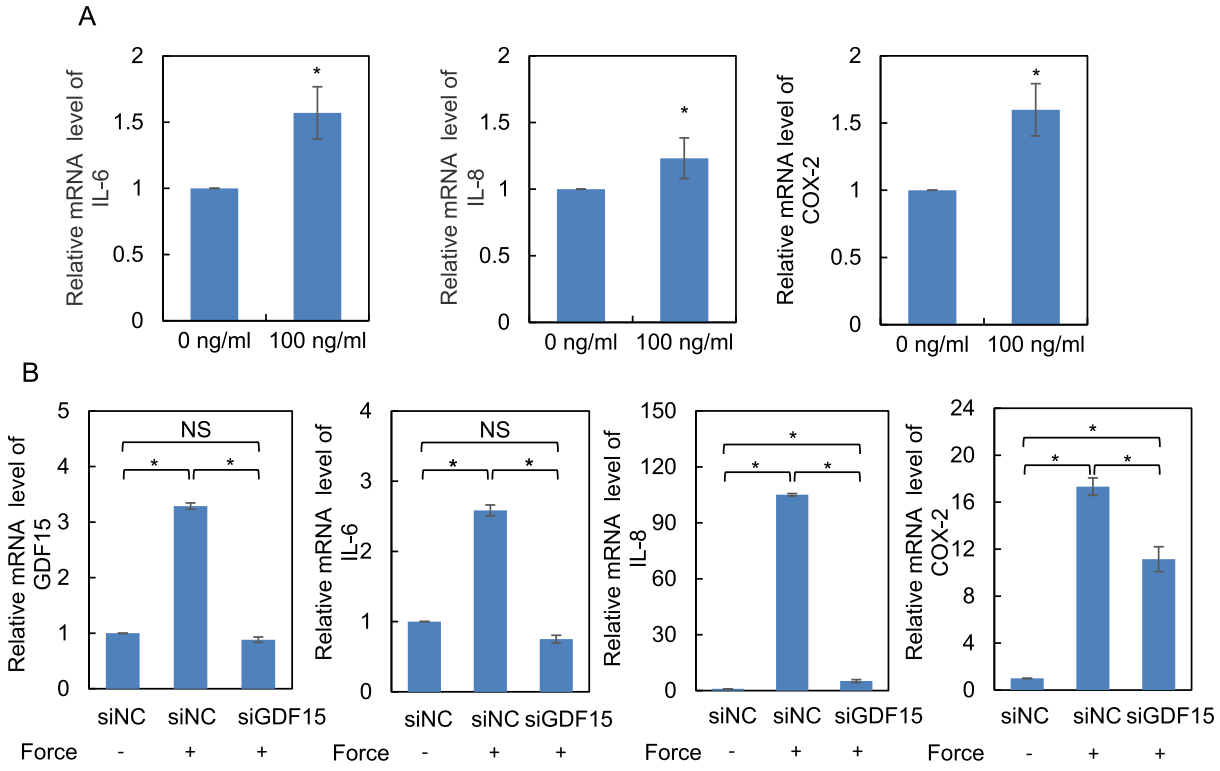

Fig. 3. GDF15 is important for the upregulated expression of pro-inflammatory cytokines induced by compressive force. (A) PDLCs were treated with rGDF15 for 48 h, followed by mRNA extraction and RT-qPCR analyses. (B) PDLCs were transfected with siRNA against GDF15 (siGDF15) or non-sense control (siNC) and then exposed to compressive force of 1.5 g/cm^2 for 12 h. RT-qPCR analyses were performed for expression changes of indicated genes. *p < 0.05 (n = 3).

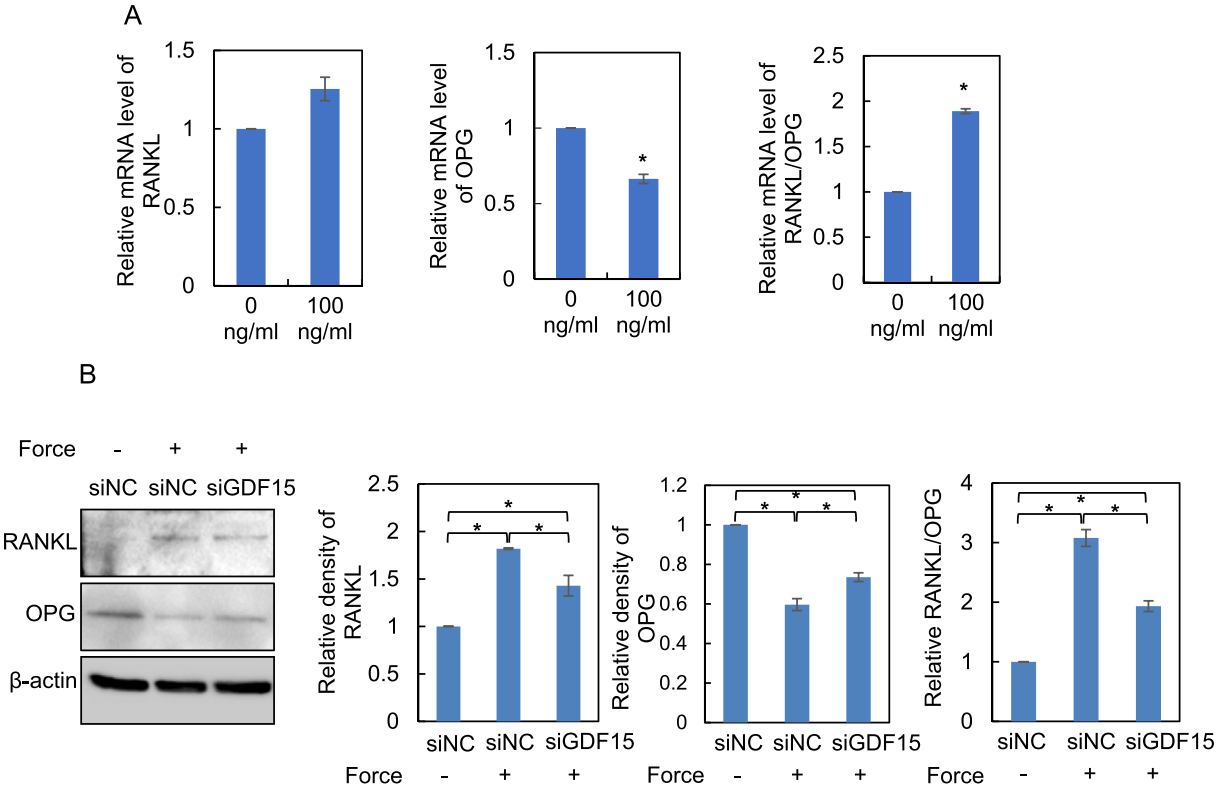

Fig. 4. GDF15 is essential for the force-induced elevated RANKL/OPG. (A) PDLCs treated with rGDF15 for 48 h were subjected to RT-qPCR analyses for expression changes of RANKL and OPG. (B) PDLCs were transfected with siRNA against GDF15 (siGDF15) or non-sense control (siNC) and then exposed to compressive force of 1.5 g/cm^2 for 12 h. Western blot analyses were performed for expression changes of RANKL and OPG. *p < 0.05 (n = 3).

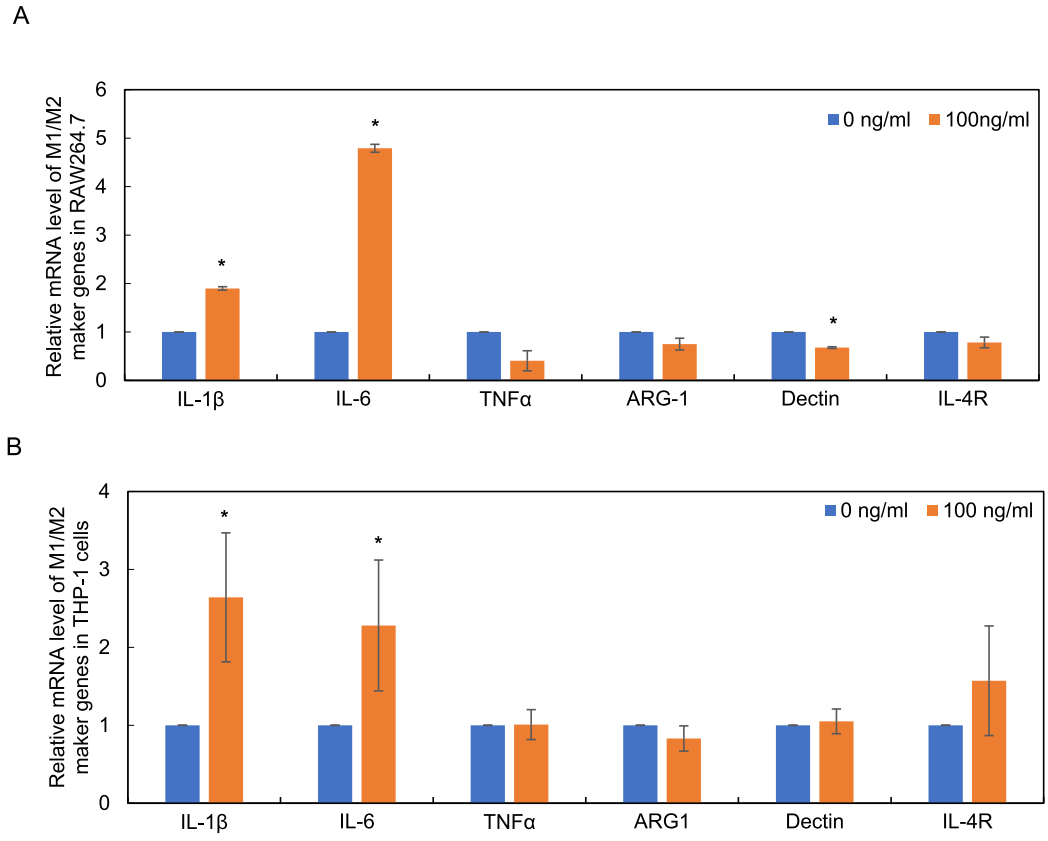

Fig. 5. GDF15 promotes M1-like macrophage polarization. (A) RAW264.7 cells were cultured with or without rGDF15 for 48 h. Then mRNA was extracted and RT-qPCR analyses were performed for expression changes of M1/M2-related genes. (B) THP-1-induced macrophages were cultured with or without rGDF15 for 48 h. Then mRNA was extracted and RT-qPCR analyses were performed for expression changes of M1/M2-related genes. *$p < 0.05$ ($n = 3$).

3.4. GDF15 promotes the M1-like macrophage polarization

Recent studies reported that compressive force could induce macrophage polarization toward M1, which play important role in osteoclast differentiation of OTM [14]. We asked if GDF15 participate in regulation of the macrophage polarization. To clarify this point, we administered RAW264.7 cells with rGDF15. RT-qPCR results showed that the M1 marker genes IL-1β and IL-6 expressions were enhanced by GDF15 addition, while the expression of M2 marker gene Dectin was reduced, indicating that GDF15 promoted the M1-like macrophage polarization in RAW264.7 (Fig. 5A). Similar results were obtained from experiments in THP-1 induced macrophages, with IL-1β and IL-6 up-regulated and M2 marker genes unchanged upon GDF15 treatment (Fig. 5B). These results suggested that GDF15 could promote the M1-like macrophage polarization in both RAW264.7 and THP-1 induced macrophages.

3.5. GDF15 contributes to the activation of NF-κB and ERK pathway under compressive force

Next, we began to explore the underlying mechanism through which GDF15 promoted the expression of pro-inflammatory cytokines. We first focused on NF-κB, which played central roles during cellular response to mechanical stimuli and osteoclast differentiation. Previous studies have demonstrated the activation of NF-κB upon force application both *in vitro* and *in vivo* [31]. We speculated that GDF15 might contribute to the activation of NF-κB in PDLCs, thereby promoting the transcription of downstream pro-inflammatory genes. To verify this point, GDF15 was knocked down in PDLCs before application of compressive force. Western blot was performed to evaluate the NF-κB distribution changes in the nuclear and cytoplasm. The results in Fig. 6A showed that compressive force induced the nuclear entry of NF-κB. However, when GDF15 expression was suppressed, the force-induced nuclear entry of NF-κB was weakened, as manifested by increased NF-κB in the cytoplasm and reduced NF-κB in the nuclear. NF-κB activation in macrophages is important for osteoclast differentiation, so we tested the impact of GDF15 on NF-κB in RAW264.7 cells. As expected, the nucleus NF-κB was more abundant in GDF15-treated cells, while the cytoplasmic NF-κB was diminished on the contrary (Fig. 6B). These results suggested that GDF15 contributes to force-induced NF-κB activation.

Besides NF-κB, MAPK/ERK is another important pathway in osteoclast differentiation. Given that GDF15 was reported to be able to activate ERK pathway like other members of TGF-β family, we hypothesized that GDF15 also contributed to the force-induced ERK activation. To prove this point, RAW264.7 cells were co-cultured with supernatants from PDLCs with GDF15 manipulation and force application. Western blot analysis revealed that force-induced phosphorylation of ERK was decreased upon GDF15 depletion (Fig. 6C). Moreover, administration of GDF15 also enhanced the expression of C-FOS and CCND1, the two classical downstream target genes of ERK (Fig. 6C and D). These data together verified the role of GDF15 in regulation of NF-κB and ERK pathway in response to compressive force.

3.6. GDF15 is regulated by YAP in PDLCs

Having clarified the signaling pathway regulated by GDF15, we sought to delineate the upstream signaling molecules responsible for

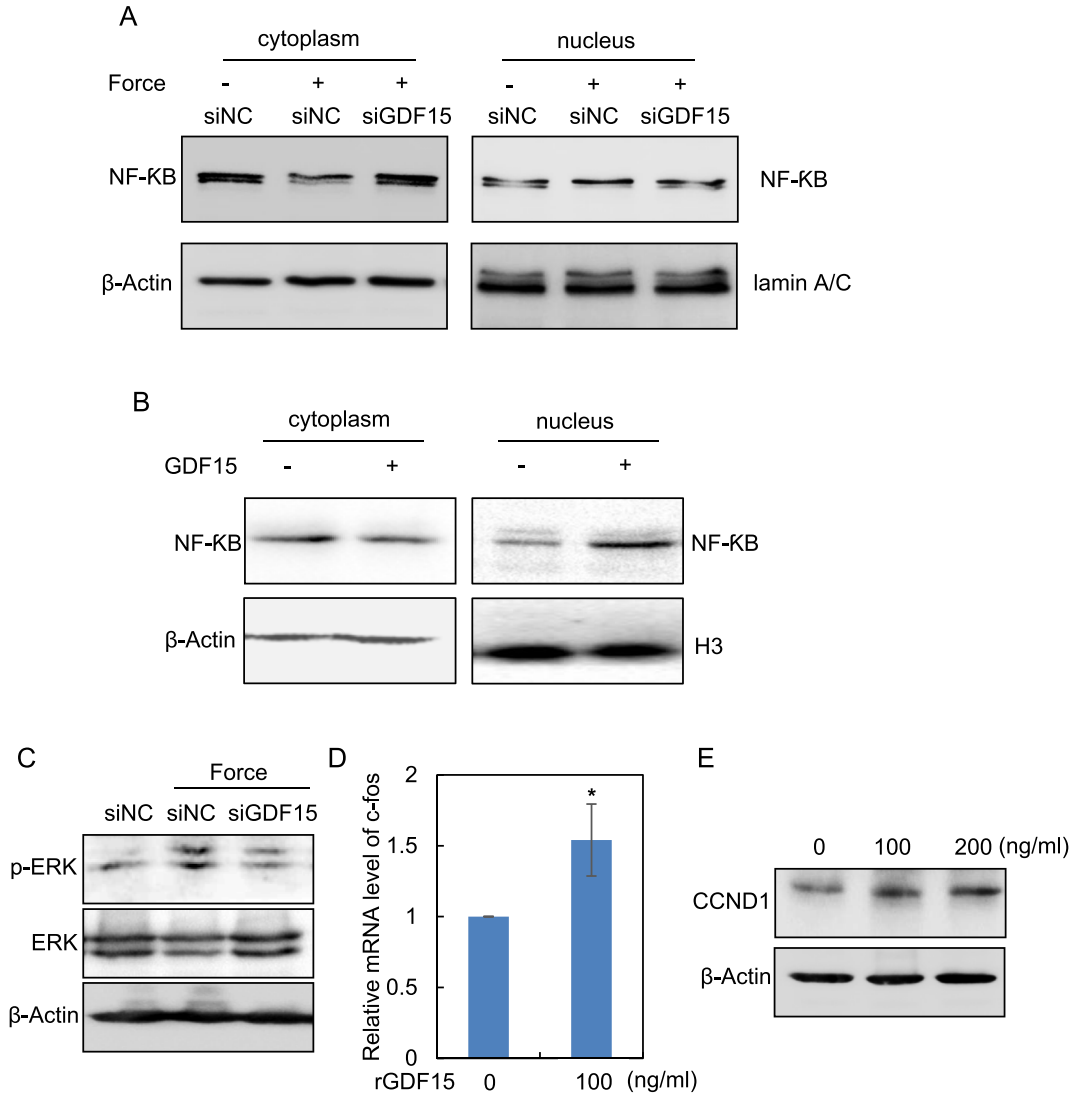

Fig. 6. GDF15 is involved in NF-κB and ERK activation in response to compressive force. (A) PDLCs were transfected with siRNA against GDF15 (siGDF15) or non-sense control (siNC) and then exposed to compressive force of 1.5 g/cm^2 for 12 h. The cells were harvested and subjected to nuclear and cytoplasmic lysates fractionation and western blot analyses. Lamin A/C and β-actin served as loading controls of nuclear and cytoplasmic proteins, respectively. (B) RAW264.7 cells were induced by sRANKL with or without rGDF15 for 48 h. Then the cells were harvested and subjected to nuclear and cytoplasmic lysates fractionation and western blot analyses. Histone 3 (H3) and β-actin served as loading controls of nuclear and cytoplasmic proteins, respectively. (C) PDLCs were transfected with siRNA against GDF15 (siGDF15) or non-sense control (siNC) and then exposed to compressive force of 1.5 g/cm^2 for 12 h. RAW264.7 cells were co-cultured with supernatants from these PDLCs for 48 h and then subjected to western blot analyses. β-actin served as a loading control. (D) RAW264.7 cells administered with rGDF15 or not were subjected to RT-qPCR analyses for C-FOS expression. (E) RAW264.7 cells administered with increasing dosage of rGDF15 were subjected to western blot analyses for expression changes of CCND1. β-actin served as a loading control. *p < 0.05 (n = 3).

force-induced upregulation of GDF15. GDF15 was previously reported to be regulated by NF-κB and HIF-1α , so we knocked down the expression of the two respectively. However, RT-qPCR results showed no obvious alteration of GDF15 (Fig. 7A and B), indicating GDF15 was not regulated by NF-κB or HIF-1α in PDLCs.

YAP/TAZ, as the vital node in Hippo signaling, plays a pivotal role in mechanotransduction process. Since YAP was found to repress GDF15 expression in breast cancer cells [32], we speculated that GDF15 could also be negatively regulated by YAP in PDLCs. Our earlier study demonstrated that compressive force could lead to decreased expression of YAP [33], which could result in depression and upregulation of GDF15. We treated PDLCs with verteporfin, the inhibitor of YAP [34], and found that the protein level of GDF15 prominently increased after YAP inhibition (Fig. 7C), which validated the negative regulation of GDF15 by YAP. Additionally, treatment with XMU-MP-1 [35], the inhibitor of MST1/2 suppressed the expression of GDF15 (Fig. 7D). Since MST1/2 is a kinase responsible for the phosphorylation of endogenous MOB1, LATS1/2 and YAP, its inhibition would lead to activation of YAP [36]. Therefore, this indirectly demonstrated that YAP activation could suppress GDF15 expression.

4. Discussion

So far, most studies about GDF15 were regarding its role in tumor progression. It has been shown to be upregulated in several aggressive tumor types and correlated with poor prognosis and patient survival [37]. However, its role in the occurrence or progression of other diseases is still poorly understood.

There are existing studies on the role of GDF15 during osteoclast differentiation. Through microarray analysis GDF15 was found to be

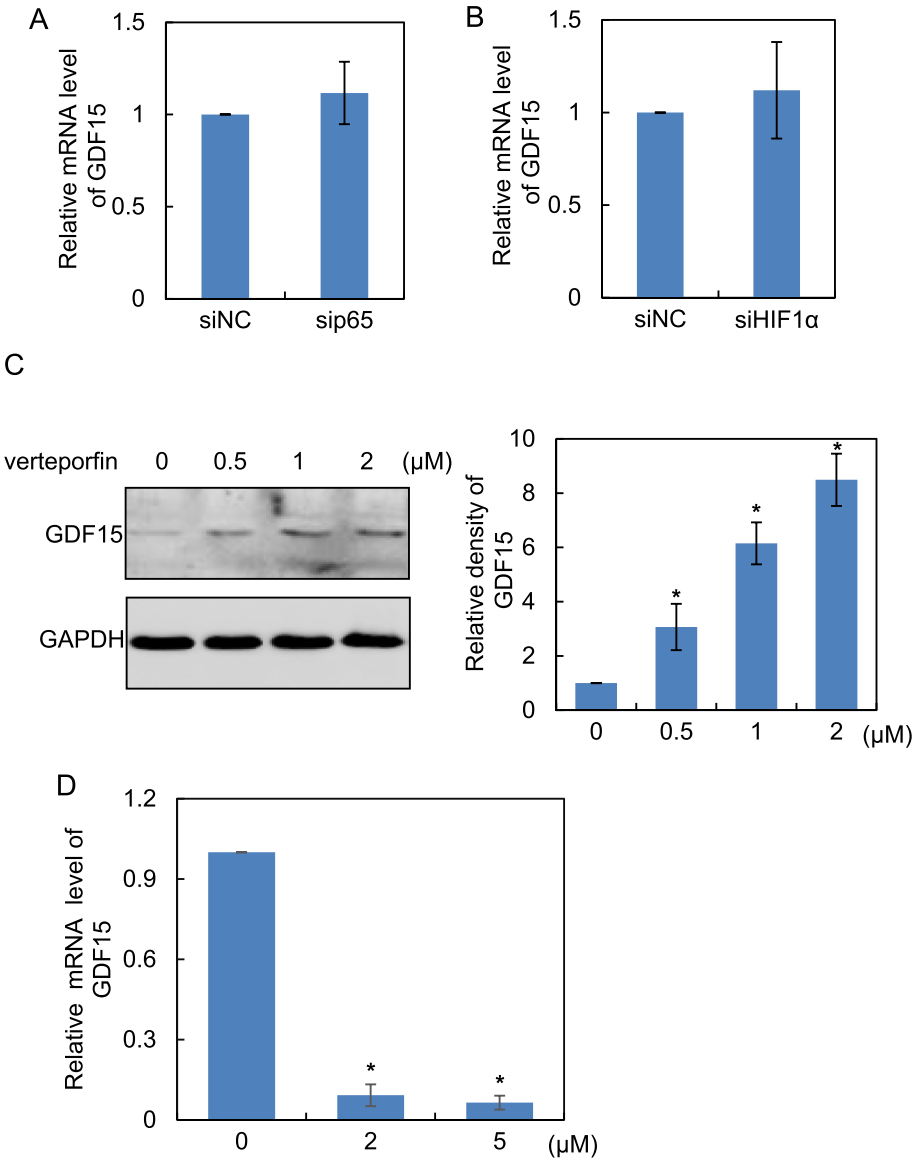

Fig. 7. Hippo signaling regulates GDF15 expression in human PDLCs. (A) PDLCs were transfected with siRNA against p65 (the subunit of NF-κB) (sip65) or non-sense control (siNC). Then mRNA was extracted for RT-qPCR analyses. (B) PDLCs were transfected with siRNA against HIF-1α (siHIF1α) or non-sense control (siNC). Then mRNA was extracted for RT-qPCR analyses. (C) PDLCs were treated with increasing dosage of verteporfin for 48 h and harvested for western blot analyses. GAPDH served as a loading control. (D) PDLCs were treated with increasing dosage of XMU-MP-1 for 48 h and harvested for RT-qPCR analyses. *p < 0.05 (n = 3).

induced in osteocytic cells under hypoxia, which further promotes osteoclast differentiation though activation of NF-κB [22]. Besides, GDF15 was shown to promote osteoclast formation in prostate cancer metastasizing to bone [24]. The experiments in human peripheral blood monocytes also confirmed the promotion effect of GDF15 on osteoclast differentiation and its high serum levels are associated with multiple myeloma bone disease [23]. Additionally, knockdown of GDF15 in RPMI-8226 human multiple myeloma cells was found to inhibit osteoclastic differentiation through inhibiting the RANKL-Erk1/2 signaling pathway [38]. These studies suggested the positive regulation of osteoclastogenesis mediated by GDF15. However, whether GDF15 is involved in osteoclast differentiation triggered by mechanical cues was not yet reported. One study in pancreatic cancer cells showed that solid stress stimulates fibroblasts activation and strongly upregulates GDF15 expression. Our study consistently discovered the elevated expression of GDF15 in human PDLCs in response to compressive force, and its essential role in force-induced osteoclastogenesis.

Regarding the molecular mechanism through which GDF15 promotes osteoclast differentiation, previous finding demonstrated that NF-κB could be activated by GDF15 in osteocytic cells under hypoxia. NF-κB was also activated in rats applied with orthodontic stimuli, suggesting its important role in OTM. Our study found that GDF15 was indispensable for the activation of NF-κB in PDLCs. Therefore, on one hand, GDF15 activates NF-κB in PDLCs, thereby upregulates the downstream target genes' expression such as IL-6 and IL-8, which are important inflammation factors facilitating osteoclastogenesis. On the other hand, NF-κB in the monocytic macrophages was also activated by GDF15, which further contributes to M1-like polarization as well as osteoclast differentiation of monocytic macrophages. Mechanistically, GDF15 was found to activate TGF-β-activated kinase 1 (TAK1) and consequently led to prolonged activation of NF-κB in Enteropathogenic Escherichia coli-infected epithelial cells and ovarian cancer cells [39,40]. It remained to be investigated in our future work whether TAK1 was essential for GDF15-induced NF-κB activation under mechanical force, or there might be other unknown mediators for GDF15-induced NF-κB activation.

Hippo signaling is crucial in transduction of mechanical signaling. After the perception of mechanical strains, actin cytoskeleton and Rho GTPases would act on the core kinase components of Hippo pathway, including the Set20-like kinase 1/2 (MST1/2) and the large tumor suppressor 1/2 (LATS1/2) [36], which further lead to the sequestration and degradation of Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) in cytoplasm, and such inactivation will eventually lead to the cessation of transcription of their target gene. In breast cancer cells, YAP was found to repress GDF15 expression by recruiting EZH2 to the promoter region of GDF15 [32]. Our study proved that GDF15 was upregulated by YAP inhibition, whereas inhibition of MST1/2, the negative regulator of YAP activity resulted in suppression of GDF15.

Some limitations also exist in our study. First, the force application model used in our study is 2D culture which could not exactly mimic the *in vivo* situation. Further studies are needed to explore the role of GDF15 using specific materials and equipment to apply mechanical forces in 3D culture of PDLCs. Secondly, orthodontic forces are known to elicit hypoxia attributed to occlusion of the periodontal ligament vessels on the pressure side and decreasing of the blood perfusion. Not only does hypoxia affects cell proliferation or apoptosis, but it also contributes to bone remodeling process [41]. In our study, the force application model we used could not exclude the involvement of hypoxia. However, the qPCR analysis in Fig. 7B indicated no significant impact on GDF15 expression upon knockdown of HIF1α, one of the master transcription factors upregulated in response to hypoxia. Therefore, GDF15 was probably not induced by hypoxia in our model. In the future we will examine the distinct role of hypoxia and mechanical force using the oxygen sensing plates.

In summary, our results highlighted GDF15 as an important mediator for compressive-force induced osteoclast differentiation, thus providing new insights into molecular mechanisms of osteoclastogenesis mediated by PDLCs during OTM.

Contribution of the authors

Shuo Li performed the experiments, analyzed the data and wrote the paper. Qian Li contributed to the funding collection, designed and performed the experiments, analyzed the data and wrote the paper. Ye Zhu contributed to data collection and analyses. Wei Hu contributed to the funding collection and supervised the project.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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References

[1] V. Krishnan, Z. Davidovitch, Cellular, molecular, and tissue-level reactions to orthodontic force, Am. J. Orthod. Dentofacial Orthop. 129 (2006) 469 e461-432.
[2] B. Lee, Force and tooth movement, Aust. Orthod. J. 23 (2007) 155.
[3] Y. Li, L.A. Jacox, S.H. Little, C.C. Ko, Orthodontic tooth movement: the biology and clinical implications, Kaohsiung J. Med. Sci. 34 (2018) 207–214.
[4] S. Moin, Z. Kalajzic, A. Utreja, J. Nihara, S. Wadhwa, F. Uribe, R. Nanda, Osteocyte death during orthodontic tooth movement in mice, Angle Orthod. 84 (2014) 1086–1092.
[5] T. Matsumoto, T. Iimura, K. Ogura, K. Moriyama, A. Yamaguchi, The role of osteocytes in bone resorption during orthodontic tooth movement, J. Dent. Res. 92 (2013) 340–345.
[6] S.S. Baloul, Osteoclastogenesis and osteogenesis during tooth movement, Front Oral Biol. 18 (2016) 75–79.
[7] D.S. Amarasekara, H. Yun, S. Kim, N. Lee, H. Kim, J. Rho, Regulation of osteoclast differentiation by cytokine networks, Immune Netw. 18 (2018) e8.
[8] J.H. Park, N.K. Lee, S.Y. Lee, Current understanding of RANK signaling in osteoclast differentiation and maturation, Mol. Cells 40 (2017) 706–713.
[9] S.L. Teitelbaum, Bone resorption by osteoclasts, Science 289 (2000) 1504–1508.
[10] D.H. Yang, M.Y. Yang, The role of macrophage in the pathogenesis of osteoporosis, Int. J. Mol. Sci. 20 (2019).
[11] M. Pereira, E. Petretto, S. Gordon, J.H.D. Bassett, G.R. Williams, J. Behmoaras, Common signalling pathways in macrophage and osteoclast multinucleation, J. Cell Sci. 131 (2018).
[12] D.C. Dale, L. Boxer, W.C. Liles, The phagocytes: neutrophils and monocytes, Blood 112 (2008) 935–945.
[13] S. Gordon, Alternative activation of macrophages, Nat. Rev. Immunol. 3 (2003) 23–35.
[14] D. He, X. Kou, R. Yang, D. Liu, X. Wang, Q. Luo, Y. Song, F. Liu, Y. Yan, Y. Gan, Y. Zhou, M1-like macrophage polarization promotes orthodontic tooth movement, J. Dent. Res. 94 (2015) 1286–1294.
[15] M.R. Bootcov, A.R. Bauskin, S.M. Valenzuela, A.G. Moore, M. Bansal, X.Y. He, H.P. Zhang, M. Donnellan, S. Mahler, K. Pryor, B.J. Walsh, R.C. Nicholson, W.D. Fairlie, S.B. Por, J.M. Robbins, S.N. Breit, MIC-1, a novel macrophage inhibitory cytokine, is a divergent member of the TGF-beta superfamily, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 11514–11519.
[16] S. Li, Y.M. Ma, P.S. Zheng, P. Zhang, GDF15 promotes the proliferation of cervical cancer cells by phosphorylating AKT1 and Erk1/2 through the receptor ErbB2, J. Exp. Clin. Cancer Res. 37 (2018) 80.
[17] N. Urakawa, S. Utsunomiya, M. Nishio, M. Shigeoka, N. Takase, N. Arai, Y. Kakeji, Y. Koma, H. Yokozaki, GDF15 derived from both tumor-associated macrophages and esophageal squamous cell carcinomas contributes to tumor progression via Akt and Erk pathways, Lab. Investig. 95 (2015) 491–503.
[18] J. Xu, T.R. Kimball, J.N. Lorenz, D.A. Brown, A.R. Bauskin, R. Klevitsky, T.E. Hewett, S.N. Breit, J.D. Molkentin, GDF15/MIC-1 functions as a protective and antihypertrophic factor released from the myocardium in association with SMAD protein activation, Circ. Res. 98 (2006) 342–350.
[19] K. Unsicker, B. Spittau, K. Krieglstein, The multiple facets of the TGF-beta family cytokine growth/differentiation factor-15/macrophage inhibitory cytokine-1, Cytokine Growth Factor Rev. 24 (2013) 373–384.
[20] K.H. Tsui, Y.L. Chang, T.H. Feng, L.C. Chung, T.Y. Lee, P.L. Chang, H.H. Juang, Growth differentiation factor-15 upregulates interleukin-6 to promote tumorigenesis of prostate carcinoma PC-3 cells, J. Mol. Endocrinol. 49 (2012) 153–163.
[21] G.A. Bonaterra, S. Zugel, J. Thogersen, S.A. Walter, U. Haberkorn, J. Strelau, R. Kinscherf, Growth differentiation factor-15 deficiency inhibits atherosclerosis progression by regulating interleukin-6-dependent inflammatory response to vascular injury, J. Am. Heart Assoc. 1 (2012) e002550.
[22] E. Hinoi, H. Ochi, T. Takarada, E. Nakatani, T. Iezaki, H. Nakajima, H. Fujita, Y. Takahata, S. Hidano, T. Kobayashi, S. Takeda, Y. Yoneda, Positive regulation of osteoclastic differentiation by growth differentiation factor 15 upregulated in osteocytic cells under hypoxia, J. Bone Miner. Res. 27 (2012) 938–949.
[23] M. Westhrin, S.H. Moen, T. Holien, A.K. Mylin, L. Heickendorff, O.E. Olsen, A. Sundan, I. Turesson, P. Gimsing, A. Waage, T. Standal, Growth differentiation factor 15 (GDF15) promotes osteoclast differentiation and inhibits osteoblast differentiation and high serum GDF15 levels are associated with multiple myeloma bone disease, Haematologica 100 (2015) e511–514.
[24] S. Wakchoure, T.M. Swain, T.A. Hentunen, A.R. Bauskin, D.A. Brown, S.N. Breit, K.S. Vuopala, K.W. Harris, K.S. Selander, Expression of macrophage inhibitory cytokine-1 in prostate cancer bone metastases induces osteoclast activation and weight loss, The Prostate 69 (2009) 652–661.
[25] T. Iwata, M. Yamato, Z. Zhang, S. Mukobata, K. Washio, T. Ando, J. Feijen, T. Okano, I. Ishikawa, Validation of human periodontal ligament-derived cells as a reliable source for cytotherapeutic use, J. Clin. Periodontol. 37 (2010) 1088–1099.
[26] W. Zheng, S. Wang, D. Ma, L. Tang, Y. Duan, Y. Jin, Loss of proliferation and differentiation capacity of aged human periodontal ligament stem cells and rejuvenation by exposure to the young extrinsic environment, Tissue Eng. A 15 (2009) 2363–2371.
[27] H. Kanzaki, M. Chiba, Y. Shimizu, H. Mitani, Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor kappaB ligand up-regulation via prostaglandin E2 synthesis, J. Bone Miner. Res. 17 (2002) 210–220.
[28] Y. Zhang, D. Zhang, Q. Li, J. Liang, L. Sun, X. Yi, Z. Chen, R. Yan, G. Xie, W. Li, S. Liu, B. Xu, L. Li, J. Yang, L. He, Y. Shang, Nucleation of DNA repair factors by FOXA1 links DNA demethylation to transcriptional pioneering, Nat. Genet. 48 (2016) 1003–1013.
[29] Y. Zhang, J. Liang, Y. Li, C. Xuan, F. Wang, D. Wang, L. Shi, D. Zhang, Y. Shang, CCCTC-binding factor acts upstream of FOXA1 and demarcates the genomic response to estrogen, J. Biol. Chem. 285 (2010) 28604–28613.
[30] W.J. Boyle, W.S. Simonet, D.L. Lacey, Osteoclast differentiation and activation, Nature 423 (2003) 337–342.
[31] J. Zuo, L.A. Archer, A. Cooper, K.L. Johnson, L.S. Holliday, C. Dolce, Nuclear factor kappaB p65 phosphorylation in orthodontic tooth movement, J. Dent. Res. 86 (2007) 556–559.
[32] T. Wang, B. Mao, C. Cheng, Z. Zou, J. Gao, Y. Yang, T. Lei, X. Qi, Z. Yuan, W. Xu, Z. Lu, YAP promotes breast cancer metastasis by repressing growth differentiation factor-15, Biochim. Biophys. Acta (BBA) - Mol. Basis Dis. 1864 (2018) 1744–1753.
[33] Q. Li, G. Han, D. Liu, Y. Zhou, Force-induced decline of TEA domain family member 1 contributes to osteoclastogenesis via regulation of Osteoprotegerin, Arch. Oral Biol. 100 (2019) 23–32.
[34] Y. Liu-Chittenden, B. Huang, J.S. Shim, Q. Chen, S.J. Lee, R.A. Anders, J.O. Liu, D. Pan, Genetic and pharmacological disruption of the TEAD-YAP complex suppresses the oncogenic activity of YAP, Genes Dev. 26 (2012) 1300–1305.
[35] F. Fan, Z. He, L.L. Kong, Q. Chen, Q. Yuan, S. Zhang, J. Ye, H. Liu, X. Sun, J. Geng,

L. Yuan, L. Hong, C. Xiao, W. Zhang, X. Sun, Y. Li, P. Wang, L. Huang, X. Wu, Z. Ji, Q. Wu, N.S. Xia, N.S. Gray, L. Chen, C.H. Yun, X. Deng, D. Zhou, Pharmacological targeting of kinases MST1 and MST2 augments tissue repair and regeneration, Sci. Transl. Med. 8 (2016) 352ra108.

[36] Z. Meng, T. Moroishi, K.L. Guan, Mechanisms of Hippo pathway regulation, Genes Dev. 30 (2016) 1–17.

[37] P.J. Emmerson, K.L. Duffin, S. Chintharlapalli, X. Wu, GDF15 and growth control, Front. Physiol. 9 (2018) 1712.

[38] M. Yuan, J. Chen, Z. Zeng, Knockdown of macrophage inhibitory cytokine-1 in RPMI-8226 human multiple myeloma cells inhibits osteoclastic differentiation through inhibiting the RANKL-Erk1/2 signaling pathway, Mol. Med. Rep. 14 (2016) 5199–5204.

[39] H.J. Choi, J. Kim, K.H. Do, S.H. Park, Y. Moon, Prolonged NF-kappaB activation by a macrophage inhibitory cytokine 1-linked signal in enteropathogenic Escherichia coli-infected epithelial cells, Infect. Immun. 81 (2013) 1860–1869.

[40] K.H. Kim, S.H. Park, K.H. Do, J. Kim, K.U. Choi, Y. Moon, NSAID-activated gene 1 mediates pro-inflammatory signaling activation and paclitaxel chemoresistance in type I human epithelial ovarian cancer stem-like cells, Oncotarget 7 (2016) 72148–72166.

[41] A. Niklas, P. Proff, M. Gosau, P. Romer, The role of hypoxia in orthodontic tooth movement, Int. J. Dentistry 2013 (2013) 841840.