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Force-induced decline of TEA domain family member 1 contributes to osteoclastogenesis via regulation of Osteoprotegerin

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ABSTRACT

Objective: This study aims to investigate the responsiveness of transcription factor TEA domain family member 1 (TEAD1) to mechanical force and its impact on osteoclastogenesis as well as expression of Osteoprotegerin (OPG), an inhibitor for osteoclastogenesis playing crucial roles in mechanical stress-induced bone remodeling and orthodontic tooth movement (OTM).

Methods: We first analyzed the correlation between several transcription factors and OPG expression in human periodontal ligament cells (PDLCs). Then dynamic expression changes of TEAD1 with force application were analyzed due to its high correlation with OPG. Loss-of-function experiments were performed to demonstrate the role of TEAD1 in regulation of RANKL/OPG, as well as osteoclastogenesis by tartrate-resistant acid phosphatase (TRAP) staining. Combination of bioinformatics analyzes and chromatin immunoprecipitation assay was utilized to investigate occupancy of TEAD1 on the enhancer elements of OPG and the dynamic change in response to force stimuli. Involvement of Hippo signaling in regulation of OPG was further demonstrated by pharmacologic inhibitors of several components.

Results: Expression of TEAD1 highly correlates with that of OPG and decreases in response to mechanical force in human PDLCs. Knockdown of TEAD1 downregulates expression of OPG and promotes osteoclast differentiation. Mechanical force induced decreased binding of TEAD1 on an enhancer element ~22 kilobases upstream of OPG promoter. OPG was also affected by pharmaceutical disruption of Hippo signaling pathway.

Conclusions: TEAD1 is a novel mechano-responsive gene and plays an important role in force-induced osteoclastogenesis, which is dependent, as least partially, on transcriptional regulation of OPG.

1. Introduction

Periodontal ligament is the soft connective tissue lying between cementum and the alveolar bone. It plays crucial roles in providing vascular supply and nutrients, as well as maintaining bone homeostasis (Beertsen, McCulloch, & Sodek, 1997). Periodontal ligament cells are mainly composed of heterogeneous fibroblasts that include osteogenic progenitor cells. Besides, there are cementum cells, macrophages and lymphocytes in the periodontal ligament (Jiang et al., 2016). During mechanical stimuli-induced orthodontic tooth movement (OTM), compressive force applied on the periodontal ligament could induce osteoclastogenesis through secretion of a series of pro-inflammatory cytokines including interleukin 1 (IL-1), interleukin 6 (IL-6), and cyclooxygenase 2 (COX-2), etc. 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 and is considered to be a rate-limiting determinant for OTM (Yamaguchi, 2009). OPG inhibits osteoclastogenesis as a decoy receptor to prevent the interaction between RANK and its ligand RANKL in bone homeostasis and osteoporosis (Boyle, Simonet, & Lacey, 2003; Simonet et al., 1997). Expression of OPG was reported to be downregulated both *in vivo* and *in vitro* after force application. (Li et al., 2011, 2013; Li, Zhang, Wang, Li, & Zhang, 2015; Nishijima et al., 2006; Toygar, Kircelli, Bulut, Sezgin, & Tasdelen, 2008) Moreover, local OPG gene transfer to periodontal tissue could inhibit OTM (Kanzaki et al., 2004), underlying the importance of OPG in osteoclastogenesis during OTM. However, up to now, its upstream regulators responding to mechanical cues remain elusive.

Mechanical forces are sensed primarily at integrin–extracellular matrix and cell–cell adhesion sites at cell surface. Then the information

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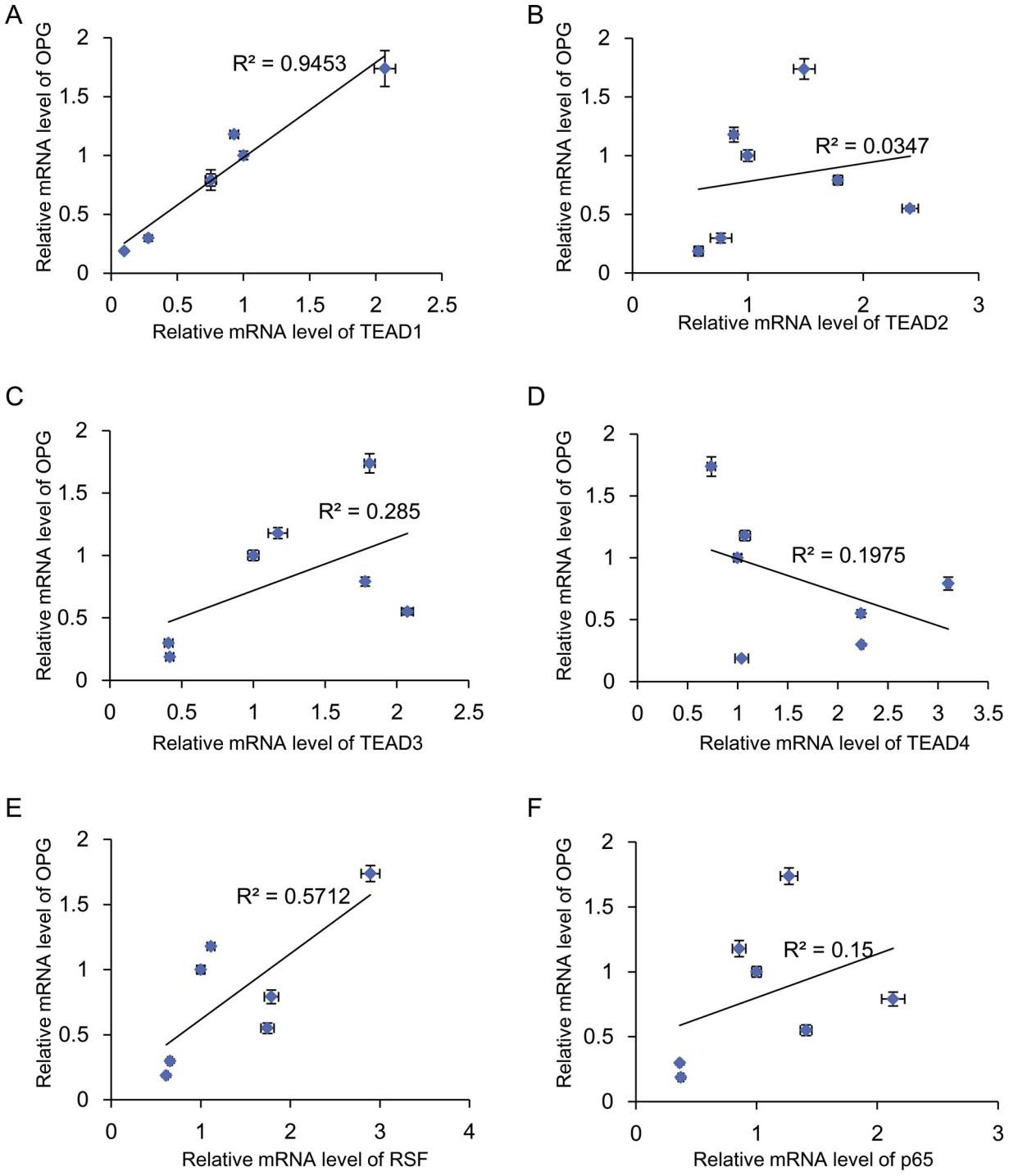


Fig. 1. Expression of TEAD1 correlates with OPG in human PDLCs. The total mRNA of freshly isolated PDLCs from 7 donors was collected and subjected to quantitative reverse transcription PCR (RT-qPCR). The gene expression correlations between each indicated transcription factor and OPG were displayed as a scatter plot, respectively. (n = 3).

is transmitted through mechanosensory systems that include stretch-activated ion channels, integrins and adherens junctions, adaptor proteins such as vinculin and talins, focal adhesion kinase, and the SRC-family kinases that connect the extracellular mechanical world to the F-actin cytoskeleton. (Uhler & Shivashankar, 2017) Notably, these mechanosensory proteins and cytoskeleton remodeling could induce changes in some signaling pathways, such as Hippo signaling cascade through interaction with its components and further cause downstream transcriptional changes and cellular behavioral alterations (Panciera, Azzolin, Cordenonsi, & Piccolo, 2017).

The Hippo cascade with established functions in organ size control and tissue homeostasis is emerging as a mechanotransduction pathway (Meng, Moroishi, & Guan, 2016; Yu, Zhao, & Guan, 2015). 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) (Meng et al., 2016). The translation of physical cues into biochemical reactants through Hippo signaling thus would 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 (Meng et al., 2016). When these two transcriptional coactivators are shuttled into nucleus, they could interact with the TEA domain (TEAD) family transcription factors to regulate a broad spectrum of downstream genes with diverse roles in self-renew of stem cell, cell proliferation and fate determination (Zanconato, Cordenonsi, & Piccolo, 2016).

In mammals, four highly conserved TEAD transcription factors have been identified as TEAD1, TEAD2, TEAD3 and TEAD4 (Xiao, Davidson, Matthes, Garnier, & Chambon, 1991). Though TEADs can be detected in almost any eukaryotes,(Kaneko & DePamphilis, 1998) their expression patterns are distinct and each of them has a unique function in controlling both physiologic processes and oncogenic malignancies (Jacquemin et al., 1998; Kaneko, Cullinan, Latham, & DePamphilis, 1997; Zhou et al., 2016). Moreover, though TEADs' function as transcription factors require interaction with coactivatiors such as YAP/TAZ and p160, aberrant transcriptional levels of TEADs are found in various types of cancer which correlate with poor clinical outcome (Pobbati & Hong, 2013), indicating the important role of TEADs in cell functional maintenance. In addition, YAP/TAZ has been reported to be involved in regulation of osteoblast and osteoclast differentiation, though the conclusions are still controversial (Hong et al., 2005; Kegelman et al., 2018; Zaidi et al., 2004), raising the possibility that Hippo signaling might also be involved in mechanical stress-induced bone remodeling process. Therefore, we believe it necessary to investigate whether TEADs family and YAP/TAZ could transcriptionally respond to mechanical stress and mediate downstream target genes regulation regarding osteoclast differentiation.

In this study, we identified TEAD1 as a novel mechano-responsive gene in human PDLCs. We showed that TEAD1 decreased upon force stimuli, correlating with the expression of OPG. Loss-of-function experiments indicated TEAD1-mediated regulation of RANKL/OPG and osteoclastogenesis of co-cultured RAW264.7 cells. We further dissected the molecular basis for TEAD1′s function with physical cues induced dynamic binding on a distal enhancer element of OPG in human PDLCs.

2. Materials and methods

2.1. Cell lines, cell culture and treatments

Human PDLCs were isolated from PDL of normal orthodontic extracted bicuspid, according to previously reported protocols with slight modification (Iwata et al., 2010; Zheng et al., 2009). Tissues were obtained under approved guidelines set by Peking University Ethical Committee with informed donor consent. Briefly, the PDL tissues of 7 donors 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. Cells were used in this study with 4 to 6 passages. In Figs. 2–5, PDLCs isolated from 3 of the 7 individuals were pooled together and used.

Static compressive force was applied as previously described. (Mitsui et al., 2005) A layer of glass cover and additional metal weights were placed on top of an 80% confluent cell layer in 6-well plates. 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.

To evaluate the influence of Hippo signaling on OPG expression, the following inhibitors were used in this study: the JNK inhibitor SP600125 (Selleck), the MST1/2 inhibitor XMU-MP-1 (Selleck, final concentration 2 μM), and the YAP/TAZ activity inhibitor verteporfin (MedChemExpress).

2.2. Flow cytometry analyses

Cells were washed with PBS, detached with 0.25% trypsin, and fixed with 75% ethanol overnight. After treatment with 1 mg/ml RNase A (Sigma) at 37 °C for 30 min, cells were resuspended in 0.5 ml of PBS and stained with propidium iodide in the dark for 30 min. Fluorescence was measured with a flow cytometry system (BD Biosciences). The cell cycles were analyzed using the Modfit software.

2.3. Co-culture of PDLCs and RAW 264.7 or human peripheral blood mononuclear cells (PBMCs) and TRAP staining

PDLCs were seeded into 6-well plates and transfected with siRNAs against TEAD1 or the non-sense control siRNA. Then the cells were subjected to compressive force of 1.5 g/cm^2 for 24 h, and RAW264.7 cells were added to the well. After 7 days, the cells were fixed and stained for TRAP staining using acid phosphatase kit (387 A, Sigma). TRAP-positive multinucleated osteoclasts were counted in 5 visual fields in each well (n = 3). We calculated the average value of 3 experiments. Human PBMCs were primarily derived from periphery blood. Then the PBMCs were co-cultured with PDLCs and subjected to TRAP staining 21 days later.

2.4. Fractionation, western blotting 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, pH 8.0. 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet p-40, 0.01% protease inhibitor mixture). Cells were fractionated using Nuclease and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, MA, USA), according to the manufacturer's protocol.

Western blotting analyses were performed as previously described (Zhang et al., 2016). Antibodies used are as follows: anti-TEAD1 (13283-1-AP, Proteintech); anti-OPG (ab11994, Abcam); anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-47724, Santa Cruz); anti-RANKL (ab45039, Abcam); anti-Phospho-YAP (Ser127) (#13008, Cell Signaling Technology, (CST)); anti-Phospho-YAP (Ser397) (#13619, CST), anti-YAP/TAZ (#8418, CST); anti-β-actin (#3700, CST) ; anti-lamin A/C (#4777, CST).

2.5. siRNA transfection, plasmid transfection and quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Two double-stranded siRNAs against TEAD1 and the scrambled control siRNA (siNC) were chemically synthesized (GenePharma). The sequences of siRNA are as follows: siTEAD1-1: CGATUUGUAUACCGA AUAA; siTEAD1-2: GAAAGGUGGCUUAAAGGAA. Transfection of siRNA was performed using the Lipofectamin RNAiMAX (Invitrogen) following the manufacturer's instruction.

The TEAD1 overexpression plasmid pRK5-Myc-TEAD1 and the empty control vector pRK5 were purchased from Addgene. Transfection of plasmid was performed using Lipofectamine LTX (Invitrogen) following the manufacturer's instruction.

Total RNAs were extracted from PDLCs using Trizol reagent (Invitrogen). Synthesis of first strand cDNA and subsequent quantitative PCR were performed as previously described. All qRT-PCR processes were performed three times using GAPDH as the internal control. The primers used in this study are as listed below: GAPDH forward (F): caatgacccttcattgacc, GAPDH reverse (R): atgacaagcttcccgttctc; RANKL F: ATCACAGCACATCAGAGCAGAGA, RANKL R: AGGACAGACTCACT TTATGGGAAC; OPG F: gaggcattcttcaggtttgc, OPG R: gctgtgttgccgttttatcc; TEAD1 F: cttgccagaaggaaatctcg, TEAD1 R: ccccagcttgttatgaatgg; TEAD2 F: ttttggtctggaggatctgg, TEAD2 R:

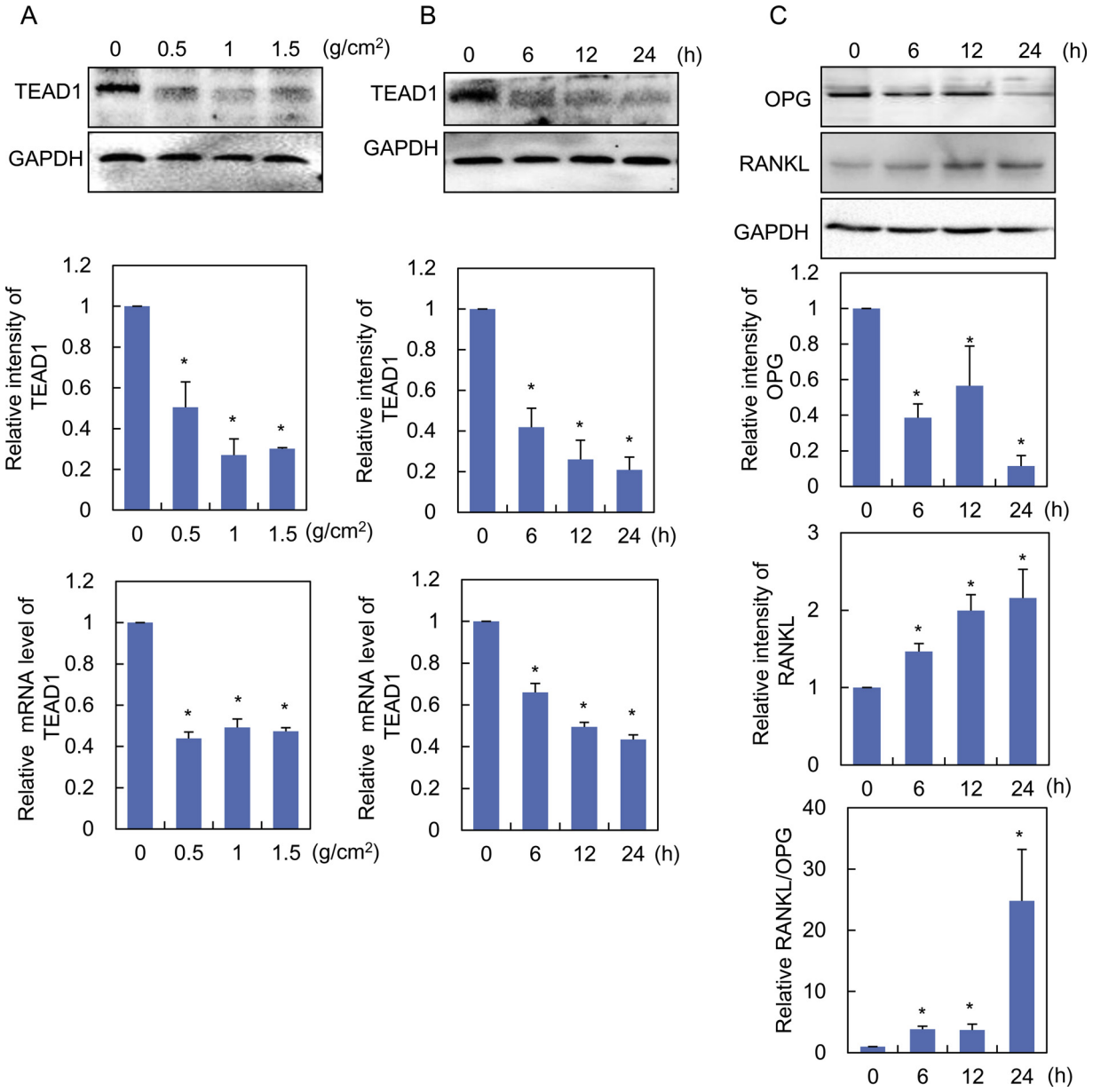


Fig. 2. Compressive force decreases expressions of TEAD1 in human PDLCs. (A) Forces with different intensity induced downregulation of TEAD1. PDLCs were treated with increasing force intensity for 24 h, followed by total proteins and mRNA extraction. Expression changes of TEAD1 were determined by western blot (top and middle) and qRT-PCR (bottom), respectively. (B) Force induced downregulation of TEAD1 in a time-dependent manner. PDLCs were treated with varying durations at 1.5 g/cm^2 force, followed by total proteins and mRNA extraction. Expression changes of TEAD1 were determined by western blot (top and middle) and qRT-PCR (bottom), respectively. (C) Protein levels of RANKL and OPG changed with force application. Western blotting analysis of RANKL and OPG in PDLCs exposed to compressive force of 1.5 g/cm^2 for prolonged time durations (F). GAPDH serves as a loading control. Data represent mean ± SD from three independent experiments. *P < 0.05. (n = 3).

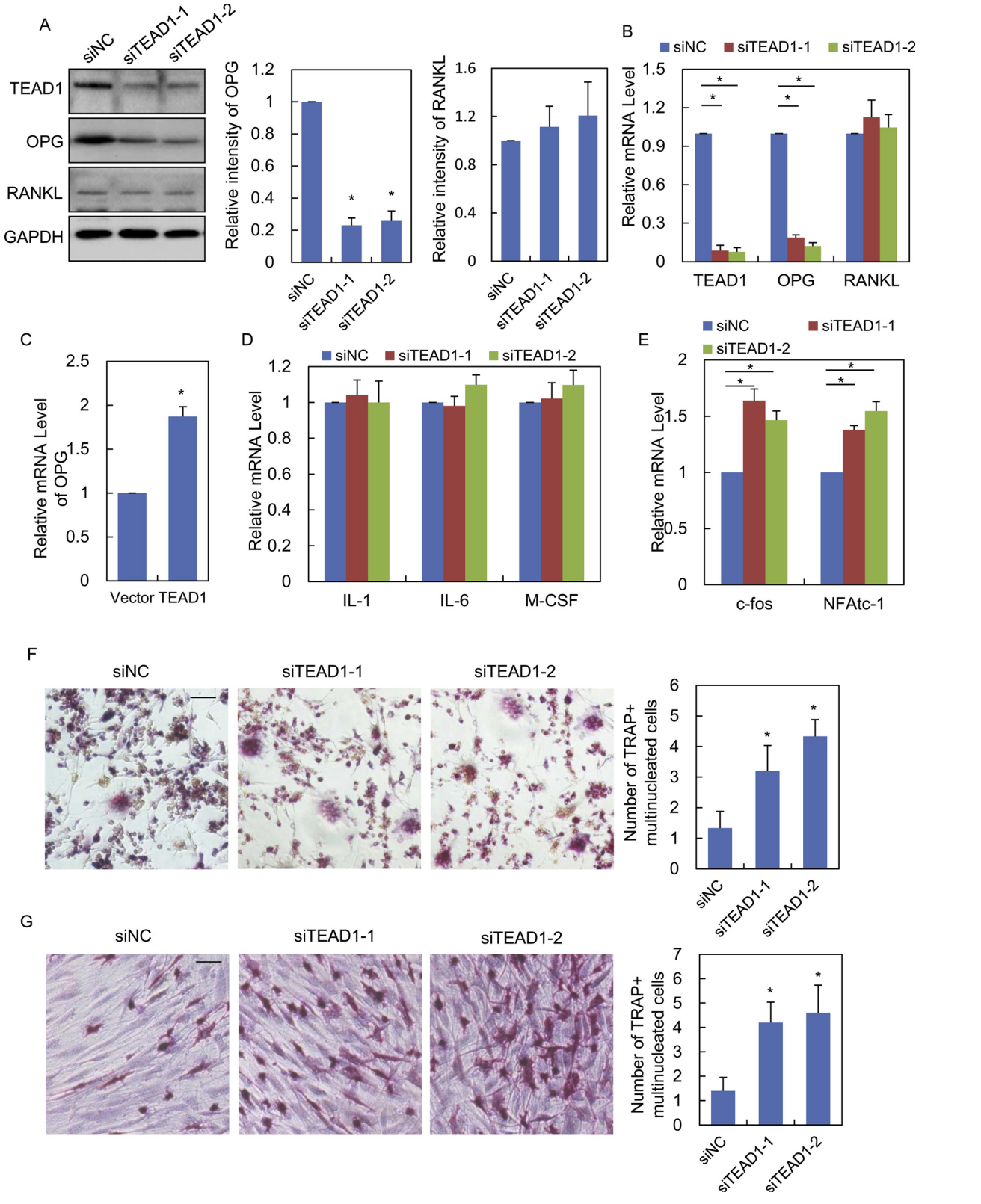
atgggggagtcagtgacaag ; TEAD3 F: agatgtacggccgaaatgag, TEAD3 R: ttttctcgtccgagtcttcc; TEAD4 F: tcatccacaagctcaagcac TEAD4 R: tcatccacaagctcaagcac; SRF F: Gccactggctttgaagagac, SRF R: tgctaggtgctgtttggatg; NF-ƙB subunit p65 F: Tgggaatccagtgtgtgaag, p65 R: aaggggttgttgttggtctg. IL-6 F: aggcactggcagaaaacaac, IL-6 R: ttttcaccaggcaagtctcc; IL-1 F: tgcctgagatacccaaaacc, IL-1 R: gtttggatgggcaactgatg; colony stimulating factor 1(M-CSF) F: ttgtcaaggacagcaccatc, M-CSF R: ttctgggacccaattagtgc; m (mouse) c-fos F: agaaacggagaatccgaagg, mc-fos R: tgcaacgcagacttctcatc; m nuclear factor of activated T cells 1 (Nfatc1) F: tgggagatggaagcaaagac, mNfatc1 R: ttgcggaaaggtggtatctc; mgapdh F: aacgacccttcattgacctc, mgapdh R: actgtgccgttgaatttgcc.

2.6. Bioinformatics analysis

The genome-wide DNase I sensitivity data for periodontal ligament cells was retrieved from the ENCODE project (Consortium, 2012). We used homer (Heinz et al., 2010) software to scan the whole genome for TEAD motifs.

2.7. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay was performed using the SimpleChIP Enzymatic Chromatin IP (Immunoprecipitation) Kit (#9002, CST) according to manufacturer's instruction. The antibody used was TEAD1 (#12292, CST). The precipitated DNA was quantified by qPCR using primers, which were designed according to the 5 regions



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potentially bound by TEAD1 as uncovered by bioinformatics analysis: primer1F: TGCCTAATGCTGTTGACTGG; primer1R: TTCCATCTGGTGG TGGAAAG; primer2F: TTCCACTTTGTGGTGAGGTG; primer2R: AAAA GAGATGGTGCCCAACC; primer3 F: GTGACTGCAAGGGCATTTTAC; primer3R: GCGTCTTTAGTTGTGGACTGG; primer4F: TGTGCTTGTGTC TCCTCCAC; primer4R: TCTGGGACACACTCCAACTG; primer5 F: TGA GCTCATGTTCTCCAAGG; primer5R: TGGGAGTGTTGGCTTTTAGG.

2.8. Statistical analyses

All the data were presented as mean ± standard deviation (SD) of three independent experiments. Statistical analysis was performed

Fig. 3. TEAD1 knockdown results in decreased expression of OPG, as well as promoted osteoclast differentiation. (A–B) Knockdown of TEAD1 by siRNA led to downregulation of OPG. PDLCs were transfected with two independent siRNAs against TEAD1 (siTEAD1-1, siTEAD1-2), or the non-sense control siRNA (siNC). Then cells were harvested for western blotting (A) and qRT-PCR analyses (B) of indicated genes. GAPDH serves as a loading control. (C) Overexpression of TEAD1 upregulated OPG expression. PDLCs were transfected with the plasmid overexpressing TEAD1 (TEAD1), or the empty control vector (Vector). Then mRNA was extracted and subjected to qRT-PCR analysis of OPG expression. (D) Knockdown of TEAD1 had no effect on the expression of IL-1, IL-6 and M-CSF. PDLCs were transfected with siRNA as in (A) and subjected to qRT-PCR analysis. (E) Knockdown of TEAD1 in PDLCs promoted activation of co-cultured RAW264.7 cells. RAW264.7 cells were co-cultured with supernatants from PDLCs transfected with siRNAs against TEAD1 (siTEAD1-1, siTEAD1-2) or non-sense control (siNC), followed by force stimulation for 24 h. 3 days later, RAW 264.7 cells were harvested and subjected to qRT-PCR analyses for expression of c-fos and NFATc-1. (F–G) TEAD1 knockdown promoted force-induced osteoclast differentiation in co-culture systems. PDLCs were transfected with siRNA as in (A), followed by 24 h's force application at 1.5 g/cm^2 and subsequent co-culture with RAW264.7 (F) or human PBMCs (G) for 7 or 21 days. Then cells were subjected to TRAP staining. Scale bar: 100 μm. Data represent mean ± SD from three independent experiments. (n = 3) *P < 0.05.

using Student *t*-test or one-way analysis of variance (ANOVA). P values are specified in the relevant figure legend. Statistical significance was considered at P < 0.05.

3. Results

3.1. Correlated down-regulation of OPG and TEAD1 expressions in response to compressive force

As stated above, OPG has been well recognized as a critical inhibitor for osteoclastogenesis with extensive implications in mechanical cues induced orthodontic tooth movement (Yamaguchi, 2009), yet little molecular insight on its upstream linkage toward known mechanosignaling pathways has been documented. To address this, we collected periodontal ligament cells (PDLCs) from 7 donors (Q. Li, Ma, Zhu, Zhang, & Zhou, 2017), and extracted their mRNA to test the expression correlation between OPG and a panel of transcription factors with established mechanotransduction functions such as serum response factor (SRF), p65 in nuclear factor-κB (NF-κB) pathway (Mendez & Janmey, 2012), TEAD1, TEAD2, TEAD3 and TEAD4 in Hippo pathway. The mRNA levels were measured by quantitative reverse transcription PCR (qRT-PCR), and the statistical analysis revealed a significant expression correlation between OPG and TEAD1 compared to other candidates (Fig. 1A-F).

To further probe the expression changes of OPG and TEAD1 in response to physical strains, we then exposed the PDLCs to compressive mechanical stress ranging from 0 to 1.5 g/cm^2. Western blotting results indicated that when the external force was applied stronger, the protein level of TEAD1 decreased to a lower extent (Fig. 2A, top and middle panels). qRT-PCR results revealed a consistent decline of their mRNA levels (Fig. 2A, bottom panel). A time-course analysis for their protein and mRNA expression changes further consolidated this point, since

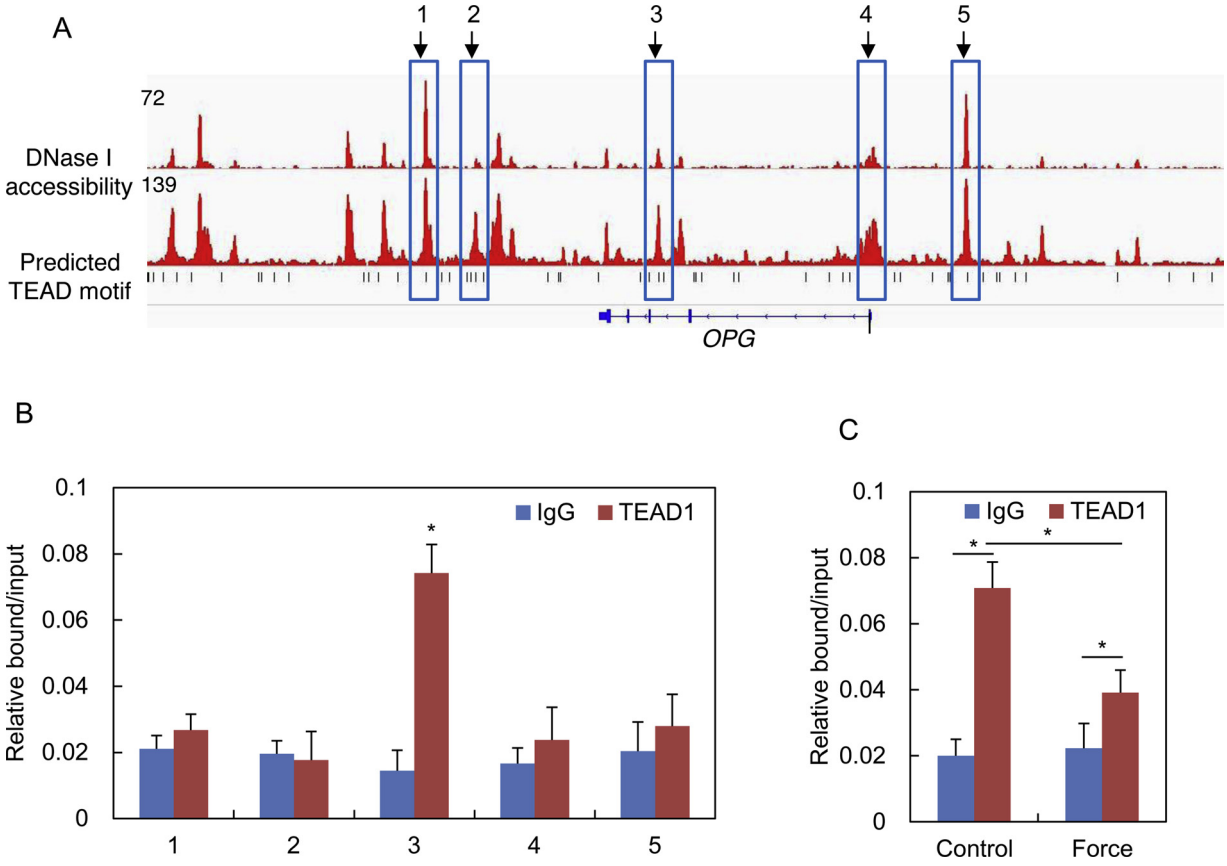

Fig. 4. Identification of a distal enhancer occupied by TEAD1 at the OPG locus. (A) Schematic representation of DNase I sensitivity data surrounding the *OPG* locus from two replicates of periodontal ligament cells. The data was retrieved from the ENCODE project (Consortium, 2012). TEAD binding motif was predicted by use of homer software (Heinz et al., 2010). (B) Chromatin immunoprecipitation assay coupled with quantitative PCR was performed in PDLCs. Enrichment of TEAD1 on the five candidates from (A) was determined. (C) TEAD1's occupation on the distal enhancer decreases with force application. PDLCs were exposed to compressive force of 1.5 g/cm^2 for 24 h. Enrichment of TEAD1 on the indicated element in the unstrained (Control) or strained cells (Force) was determined using chromatin immunoprecipitation assay. Data represent mean ± SD from three independent experiments. (n = 3) *P < 0.05.

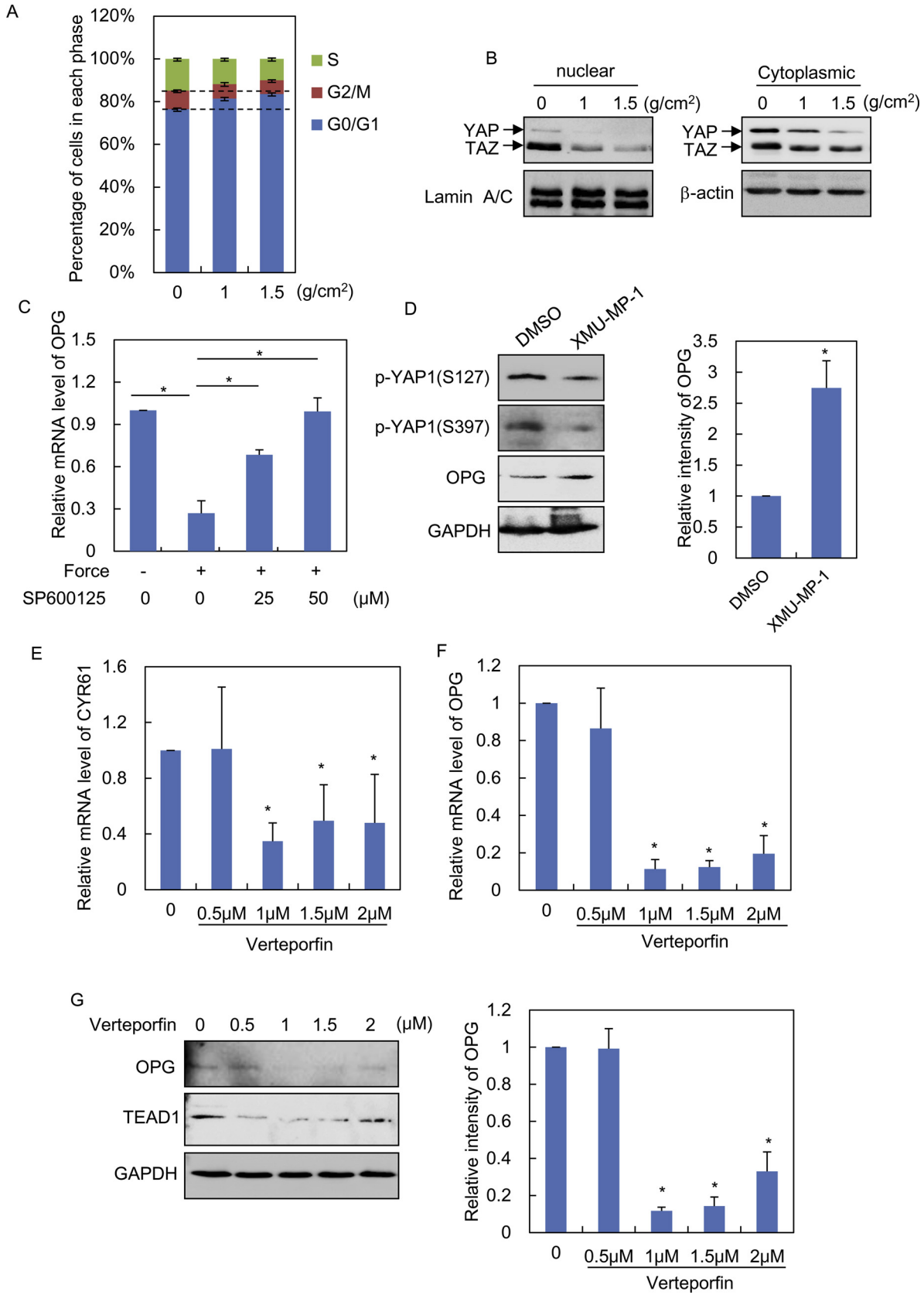

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Fig. 5. Hippo signaling pathway regulates OPG expression. (A) Flow cytometry analysis of PDLCs subjected to increased force intensity for 24 h. The percentages of cells in each phase were shown as average from 3 independent experiments. (B) Compressive force decreased expression of YAP/TAZ both in the nucleus and in the cytoplasm. PDLCs were exposed to increasing force intensity for 24 h, and then the cells were harvested and subjected to nuclear and cytoplasmic lysates fractionation and western blotting analyses. Lamin A/C and β-actin served as loading controls of nuclear and cytoplasmic proteins, respectively. (C) SP600125 attenuated force-induced reduction of OPG. PDLCs were treated with JNK-specific inhibitor SP600125 at indicated concentrations for 24 h. The cells were then exposed to compressive force of 1.5 g/cm^2 for 6 h. The mRNA level of OPG was measured by qRT-PCR. (D) OPG expression was promoted by administration of XMU-MP-1. PDLCs were treated with MST1/2-specific inhibitor XMU-MP-1 for 48 h. The protein level of OPG and phosphorylated YAP1 (p-YAP1(S127), p-YAP1(S397)) were determined by western blot. (E–G) OPG was repressed by administration of Verteporfin. PDLCs were administered with increasing dosage of Verteporfin for 48 h. Then total cell lysates and mRNA were extracted and subjected to qRT-PCR analysis (E, F) and western blotting (G), respectively. GAPDH served as a loading control. Data represent mean ± SD from three independent experiments. (n = 3) *P < 0.05.

their inhibition by mechanical strains was more prominent when the force were applied for a longer time (Fig. 2B). We also observed the elevated RANKL, and declined OPG expression induced by compressive force (Fig. 2C), implying a high correlation between TEAD1 and OPG in response to compressive force.

3.2. TEAD1 regulates OPG expression and osteoclast differentiation

Having established the correlation between TEAD1 and OPG in both unstressed and force-stressed conditions, we speculated that TEAD1 might modulate expression of OPG via functioning as a bone fide transcription factor for OPG. If so, we could expect that OPG expression would decrease in the unstrained PDLCs when TEAD1 is depleted. Indeed, after transfection of PDLCs with two independent siRNAs against TEAD1 or control siRNA, we observed that the expression of OPG in TEAD1 depleted cells was significantly lower than that in the control cells at both protein level and mRNA level (Fig. 3A, B). However, the expression of RANKL was not affected (Fig. 3A, B). Conversely, overexpression of TEAD1 led to increased OPG expression in the unstrained cells (Fig. 3C). These data suggested that knockdown of TEAD1 suppressed OPG expression, whereas overexpression of it promoted OPG expression. Besides RANKL/OPG, there are other pro-inflammatory cytokines which play important roles in osteoclast differentiation, such as colony stimulating factor 1(M-CSF), IL-1, IL-6, etc. qPCR results indicated that knockdown of TEAD1 had no significant impact on expression of these genes, underlying the specific regulation of OPG by TEAD1 (Fig. 3D).

Given the critical role of OPG in bone resorption during OTM, we next explored whether TEAD1 could play a role in regulating force-induced osteoclastogenesis. To this end, PDLCs were first transfected with siRNA against TEAD1, and then exposed to compressive loading, followed by co-culture with the murine macrophage cell line RAW264.7. As shown in Fig.3E, knockdown of TEAD1 in force-stimulated PDLCs upregulated the expression of osteoclast marker genes c-fos and NFATc1 in co-cultured RAW264.7 cells. More importantly, the numbers of TRAP positive osteoclasts in the two groups with TEAD1 knockdown (siTEAD1-1,-2) were significantly higher than that of control group (siNC) (Fig. 3F). To consolidate this point, we took advantage of human PBMCs and co-cultured them with PDLCs. The results of TRAP staining kept in accordance with that of the RAW264.7 cells (Fig. 3G). These results indicated that force-induced decline of TEAD1 contributed to osteoclastogenesis in co-culture systems.

3.3. Occupation of TEAD1 on a distal enhancer at the OPG locus decreases with force application

To thoroughly delineate the molecular basis for TEAD1-mediated transcriptional regulation of OPG, we next sought to determine the binding site of TEAD1 on the OPG locus. Since transcription factor binding sites are enriched in open chromatin context, which can be probed by their high sensitivity to DNase I digestion, we thus took advantage of the genome-wide DNase I sensitivity data from the ENCODE (Consortium, 2012) project for its epigenomic annotations of periodontal ligament cells to narrow down the number of possible regulatory elements in the OPG locus. With further restriction using TEAD motif information (Heinz et al., 2010), we finally obtained five potential TEAD bound enhancers in a ~200 kilobases window surrounding the OPG locus (Fig. 4A).

We then performed chromatin immunoprecipitation assay to examine the enrichment pattern of TEAD1 on these candidates. The results in unstrained PDLCs revealed that the site ~22 kilobases from OPG transcription start site was a genuine TEAD1 bound element compared to a negative control region and other candidates (Fig. 4B). Furthermore, in line with the decreased OPG expression in response to physical cues (Fig. 2), application of compressive force resulted in the deceased binding of TEAD1 at this site (Fig. 4C). From these results, it can be proposed that compressive force induced declined binding of TEAD1 on the enhancer element of OPG, leading to transcriptional downregulation of OPG.

3.4. Hippo signaling pathway is involved in regulation of OPG

TEAD1 is one of the major nuclear effectors in Hippo signaling cascade (Meng et al., 2016). Given the critical function of Hippo pathway in mechanosignaling, we hypothesized that Hippo signaling might be affected by compressive force and participate in regulation of downstream biological events such as proliferation and osteoclastogenesis. Flow cytometry analyses suggested that compressive force suppressed the PDLCs' proliferation, as manifested by elevated percentage of G0/G1 PDLCs and decreased S phase cells along with increased force intensity (Fig. 5A). This raised the possibility that compressive force led to changes in YAP/TAZ activity thereby retarded the cells' growth. Indeed, fractionation experiment demonstrated the force-induced downregulation of YAP/TAZ both in the nucleus and the cytoplasm. (Fig. 5B) We then asked whether inactivation of key Hippo components or inhibition of its upstream c-Jun N-terminal kinase (JNK) could impair the responsiveness of OPG to physical cues. For this purpose, PDLCs were treated with JNK specific inhibitor SP600125 prior to exposure to compressive force loading. By comparison of the qRT-PCR results in strained versus unstrained cells, we observed that, contrary to the significantly decreased expression of OPG in Hippo-proficient cells, such downregulation was severely blunted in PDLCs treated with increasing dosage of SP600125 (Fig. 5C). Next, we examined the alteration of OPG in PDLCs treated with XMU-MP-1, which has been shown to be able to block MST1/2 kinase activity, and reduce the phosphorylation of endogenous MOB kinase activator 1 A (MOB1), LATS1/2 and YAP (Fan et al., 2016). Western blot analysis demonstrated the prominent reduction of YAP phosphorylation by XMU-MP-1, while protein level of OPG was inversely elevated (Fig. 5D), underlying the negative regulation of Hippo signaling on OPG expression.

As the nuclear effector of Hippo signaling, TEAD family transcription factors function critically relying on recruitment of YAP/TAZ during mechanotransduction. So it can be postulated that inhibition of YAP/TAZ activity would impair the transcription of downstream target genes of TEADs including OPG. To testify this point, we treated PDLCs with increasing dosage of verteporfin, a specific inhibitor of YAP/TAZ activity capable of disrupting the association between YAP/TAZ and TEADs (Liu-Chittenden et al., 2012). As the well-recognized target gene of YAP/TAZ, cellular communication network factor 1 (CCN1, CYR61) expression showed profound decrease upon verteporfin administration,

indicating that YAP/TAZ activity was effectively inhibited (Fig. 5E). As expected, the expression of OPG was also downregulated along with verteporfin administration, even to a greater extent than CYR61 (Fig. 5F, G). The administration of verteporfin also inhibited TEAD1 expression, similar with reports in the cancer cell lines (Wei et al., 2017). These results demonstrated that transcription of OPG was inhibited by blockage of YAP/TAZ activity.

4. Discussion

OTM is featured as an alveolar bone remodeling process. On the tension side, new bones are formed by the osteoblasts; while bone resorption occurs by the osteoclasts on the compression side (Baloul, 2016). The osteoclast differentiation is initiated by RANKL secreted by osteoblasts. Binding of RANKL on RANK recruits the adaptor protein TNF receptor associated factor 6 (TRAF6), 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 mitogen activated protein kinases (MAPKs) such as c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase. These factors have been shown to play critical roles during osteoclast differentiation (Park, Lee, & Lee, 2017). OPG usually acts as an inhibitor of osteoclastogenesis due to its competition with RANKL for the binding of RANK. Moreover, it was also reported to promote the separation of osteoclasts adhering to the bone matrix from the bone surface (O'Brien, Williams, & Marshall, 2001). OPG expression was demonstrated to be decreased in PDLCs subjected to compressive force as well as gingival crevicular fluid during OTM (Kim, Park, Park, Lee, & Kang, 2013; Li et al., 2015; Nishijima et al., 2006), while the underlying mechanisms are not reported yet to the best of our knowledge. Our study provided evidence that the Hippo signaling component, TEAD1 decreases in response to compressive force stimuli, which contributes to reduction of OPG and thereby promoting osteoclastogenesis of co-cultured osteoclast precursors.

Besides its role in mediating osteoclastogenesis during OTM, aberrant RANKL/OPG could lead to a variety of diseases involving bone metabolism, such as osteoporosis, rheumatoid arthritis, multiple myeloma and periodontitis (Barbato et al., 2015; Phetfong et al., 2016; Walsh & Choi, 2014). Meta-analyses revealed that OPG polymorphisms are associated with bone mineral density and osteoporosis (Sun et al., 2014, Wang et al., 2013); circulating OPG levels are elevated in rheumatoid arthritis (Wang et al., 2017); OPG concentration is also associated with the presence and severity of peripheral arterial disease in type 2 diabetes mellitus (Demkova, Kozarova, Malachovska, Javorsky, & Tkac, 2018). These findings indicate important roles of OPG in maintaining bone homeostasis. As regard to molecular mechanisms regulating OPG expression, estrogen was reported to stimulate OPG expression at transcriptional level through the estrogen receptor (ER), and at post-transcriptional level via miRNA (Hofbauer et al., 1999; Jia, Zhou, Zeng, & Feng, 2017). Other transcription factors of OPG include homeobox family members (Hox) (Wan, Shi, Feng, & Cao, 2001), smad (Thirunavukkarasu et al., 2001), transcription factor 4 (TCF-4) (De Toni et al., 2008) and GATA binding protein 3 (GATA3) as previously reported (Kao & Stankovic, 2015). Our study demonstrated that TEAD1 positively regulates OPG expression, through binding on a potential enhancer element ˜22 kilobases away from OPG promoter, and this occupancy declines with force application. Therefore, we identified TEAD1 as a novel regulator of OPG during mechanotransduction. Future work should be carried out as to uncover the precise binding site of TEAD1 on the enhancer element, as well as whether the binding site possesses enhancer activity examined by reporter assay. Given the critical role of OPG in the occurrence of bone metabolism-related diseases, it is plausible to speculate that TEAD1-mediated OPG regulation might also be involved in pathogenesis of these diseases.

Hippo cascade has been identified as a mechanotransduction signaling pathway responsive to different kinds of physical cues, including cell matrix stiffness, cell geometry, cell density, cell adhesion and the external mechanical stimuli (Meng et al., 2016). Mechanical signals, in most scenarios, modulate phosphorylation events of the core kinase cascade with YAP/TAZ phosphorylation finally affected, which further influences the nuclear localization of YAP/TAZ and the recruitment of TEADs transcription factors (Meng et al., 2016; Schroeder & Halder, 2012). It is previously reported that static equiaxial strain could induce dynamic nuclear YAP localization in human PDLCs (Huelter-Hassler, Tomakidi, Steinberg, & Jung, 2017). However, transcriptional changes of Hippo components in response to compressive force and their impact on osteoclastogenesis are still not clear. Our study for the first time identified TEAD1 as a compressive force responsive gene in human PDLCs, which decreases upon force application and correlates with the expression of OPG. OPG expression was also affected by pharmaceutical interference of Hippo components, suggesting that Hippo signaling might be implicated in osteoclastogenesis during OTM. Future work should be performed to unravel the upstream molecular mechanisms causing the transcriptional decline of TEAD1, which are likely to be orchestrated by epigenetic changes upon force application.

In summary, our results highlighted an intriguing mechanism by which OPG is regulated by force-induced decline of TEAD1, thus providing new insights into molecular mechanisms of osteoclastogenesis mediated by PDLCs during OTM.

Conflict of interest

The Authors declare that they have no conflict of interest related to this study.

Contribution of authors

Qian Li designed the study, acquired and analyzed the data, drafted the article and gave final approval of the version to be submitted.

Gaofeng Han contributed to acquisition of data, revised the paper critically, and gave final approval of the version to be submitted.

Dawei Liu contributed to analysis and interpretation of data, revised the paper critically for important intellectual content, and gave final approval of the version to be submitted.

Yanheng Zhou contributed to conception of the study, revised the paper critically for important intellectual content, and gave final approval of the version to be submitted.

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