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## ABSTRACT

The sympathetic nervous system (SNS) regulates bone resorption through  $\beta$ -2 adrenergic receptor (Adrb2). In orthodontic tooth movement (OTM), mechanical force induces and regulates alveolar bone remodeling. Compressive force-associated osteoclast differentiation and alveolar bone resorption are the rate-limiting steps of tooth movement. However, whether mechanical force can activate Adrb2 and thus contribute to OTM remains unknown. In this study, orthodontic nickel-titanium springs were applied to the upper first molars of rats and Adrb1/2<sup>-/-</sup> mice to confirm the role of SNS and Adrb2 in OTM. The results showed that blockage of SNS activity in the jawbones of rats by means of superior cervical ganglion ectomy reduced OTM distance from 860 to 540  $\mu$ m after 14 d of force application. In addition, the injection of nonselective Adrb2 agonist isoproterenol activated the downstream signaling of SNS to accelerate OTM from 300 to 540  $\mu$ m after 7 d of force application. Adrb1/2<sup>-/-</sup> mice showed significantly reduced OTM distance (19.5  $\mu$ m) compared with the wild-type mice (107.6  $\mu$ m) after 7 d of force application. Histopathologic analysis showed that the number of Adrb2-positive cells increased in the compressive region of periodontal ligament after orthodontic force was applied on rats. Mechanistically, mechanical compressive force upregulated Adrb2 expression in primary-cultured human periodontal ligament cells (PDLCs) through the elevation of intracellular Ca<sup>2+</sup> concentration. Activation of Adrb2 in PDLCs increased the RANKL/OPG ratio and promoted the peripheral blood mononuclear cell differentiation to osteoclasts in the cocultured system. Upregulation of Adrb2 in PDLCs promoted osteoclastogenesis, which accelerated OTM through Adrb2-enhanced bone resorption. In summary, this study suggests that mechanical force-induced Adrb2 activation in PDLCs contributes to SNS-regulated OTM.

**KEY WORDS:** orthodontics, biomechanical phenomena, beta-2 adrenergic receptors, sympathetic nervous system, bone remodeling, osteoclast.

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# Force-induced Adrb2 in Periodontal Ligament Cells Promotes Tooth Movement

## INTRODUCTION

$\beta$ -2 adrenergic receptor (Adrb2), 1 of the 3 postsynaptic  $\beta$  adrenergic receptors, is important in sympathetic nervous system (SNS)-regulated bone remodeling that involves osteoblast-mediated bone formation and resorption (Takeda *et al.*, 2002; Elefteriou *et al.*, 2005).  $\beta$  adrenergic receptor agonist isoproterenol (ISO) treatment triggers an osteoclastogenic response to accelerate bone loss (Takeda *et al.*, 2002). Adrb2<sup>-/-</sup> mice show a higher bone mass phenotype than the wild type (WT) by decreasing the expression of osteoclast differentiation factor RANKL in osteoblast progenitor cells (Elefteriou *et al.*, 2005). Bones are mechanosensitive organs remodeled with functional mechanical loading (Biewener, 1985; Huiskes *et al.*, 2000). In orthodontic tooth movement (OTM), alveolar bone remodeling is induced and regulated by constant exertion of mechanical force. Tooth movement is started and facilitated by the compressive force-associated osteoclast formation and bone resorption (Immermann and Compere, 1954). Force-stimulated local aseptic periodontal inflammation has an important function in the adaptive response of bone to OTM (Cooper and Sims, 1989; Garlet *et al.*, 2007; Iwasaki *et al.*, 2009). Recent studies report that selective Adrb2 antagonist butoxamine reduces SNS-accelerated OTM by preventing alveolar bone loss (Kondo *et al.*, 2013; Sato *et al.*, 2014). However, whether mechanical force can directly affect the activity of Adrb2 and thus regulate bone remodeling in OTM remains unclear. Moreover, the contributions of SNS and Adrb2 signaling in bone mechanoadaptive response remain controversial (de Souza *et al.*, 2005; Marenzana *et al.*, 2007).

The periodontal ligament (PDL) is a layer of soft tissue between the tooth and the alveolar bone that provides the necessary microenvironment for cells involved in the alveolar bone remodeling of OTM. Mechanical force acts on PDL cells (PDLCs) and induces the secretion of inflammatory factors, such as PGE2 (Kang *et al.*, 2010), and osteoclastogenesis by upregulating RANKL (Kanzaki *et al.*, 2002). As PDL is derived from neural crest cells (Dupin and Sommer, 2012), PDLCs may express neural receptor Adrb2. Therefore, force-induced Adrb2 in PDLCs may be involved in the bone remodeling of OTM.

In this study, we hypothesize that force-induced activation of Adrb2 in PDLCs may contribute to osteoclast-mediated OTM. To address this hypothesis, we explored whether orthodontic force could induce the activation of Adrb2 in PDLCs and consequently promote OTM through enhanced osteoclastogenesis in the OMT model of rats and Adrb1/2<sup>-/-</sup> mice. We further examined the underlying mechanism of force-induced activation of Adrb2 in primary-cultured human PDLCs.

## MATERIALS & METHODS

### Animals and Treatments

Sprague-Dawley rats (male, 8 wk old), *Adrb1/2<sup>-/-</sup>* mice (003810, male, 8-10 wk old; Jackson Laboratory), and WT mice (129, male, 8-10 wk old) were used in this study. All the animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Peking University (LA2013-92).

To set up the OTM animal model, orthodontic nickel-titanium coiled springs (0.2 mm in thickness, 1 mm in diameter, 5 mm in length; Smart Technology, Beijing, China) were ligated between the right maxillary first molar and the incisors of the rats (Appendix Fig. 1A; Dunn *et al.*, 2007). The springs were activated to deliver an orthodontic force of 60 g.

We designed independent experiments and chose different time periods to observe OTM distance because of the different purposes of each experiment and our preliminary data. To test the role of SNS on OTM, the right superior cervical ganglions of the rats were removed or sham-operated in 2 groups: force group and force + superior cervical ganglionectomy (SCGx) group ( $n = 8$ ). The effectiveness of SCGx was confirmed by ipsilateral eyelid ptosis (Matthews and Quilliam, 1964) (Appendix Fig. 2). Three days after the operation, orthodontic force was applied to both groups for 14 d. To confirm the role of *Adrb2* in OTM, 1 d after the application of orthodontic force, 2 groups of rats (force and force + ISO,  $n = 10$ ) were intraperitoneally injected with vehicle or nonselective *Adrb2* agonist ISO (20 mg/kg bodyweight; 15627, Sigma), respectively, for 6 consecutive days.

Orthodontic force was applied to the 2 groups of mice (*Adrb1/2<sup>-/-</sup>* group and WT group,  $n = 14$ ) to reveal the osteoclastogenic function of *Adrb2* in OTM. The spring (1 mm in length) was bonded between the molar and the incisors of the mice by using flowable restorative resin (3M ESPE, USA) to deliver a force of 30 g for 7 d (Appendix Fig. 1B).

### Tartrate-resistant Acid Phosphatase Staining

The medium number of transverse serial sections (4.5  $\mu\text{m}$ ) from the corresponding group was stained for tartrate-resistant acid phosphatase (TRAP) using a leukocyte acid phosphatase kit (387A, Sigma) according to the manufacturer's protocol. TRAP-positive multinucleated (> 3 nuclei) cells that attached to the alveolar bone surface (Parfitt *et al.*, 1987) mesial to the middle palatal roots were counted ( $n = 5$ ).

### Cell Culture and Treatments

All protocols used to obtain human tissue samples were approved by the Ethical Guidelines of Peking University. The protocols were performed with appropriate informed consent (PKUSSIRB-201311103). Human PDLCs were isolated from PDL of normal orthodontic extracted bicuspid as previously reported (Seo *et al.*, 2004). PDLCs isolated from 3 different individuals were pooled together and used in this study with 3 to 4 passages. Human orofacial bone marrow-derived cells (OMCs) were isolated using a previously described method (Yamaza *et al.*, 2011).

PDLCs were treated with various doses of ISO (0-1  $\mu\text{M}$ ) alone or combined with *Adrb2* selective antagonist ICI 118,551 (0.01-0.1  $\mu\text{M}$ ; I127, Sigma) for 6 hr to investigate the promotion of osteoclast differentiation. To knock down *Adrb2* expression, PDLCs were transfected with *Adrb2* small interfering (si) RNA and scramble siRNA for 48 hr before ISO stimulation.

### Mechanical Stimulation on Primary-cultured Cells

Static compressive force was applied to PDLCs and OMCs *in vitro* using the uniform method (Mitsui *et al.*, 2005) in a culture medium containing 6 mM of  $\text{Ca}^{2+}$ . In brief, 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 2  $\text{g}/\text{cm}^2$  for 6 hr or at 1.5  $\text{g}/\text{cm}^2$  for different durations ranging from 0 to 12 hr.

To reveal the role of intracellular  $\text{Ca}^{2+}$  in force-induced *Adrb2* upregulation, PDLCs were pretreated with intracellular calcium chelator BAPTA-AM (5  $\mu\text{M}$ ; A1076, Sigma) for 30 min in 6 mM of  $\text{Ca}^{2+}$  medium and subjected to compressive force (1.5  $\text{g}/\text{cm}^2$ , 6 hr).

### Peripheral Blood Mononuclear Cell and PDLC Co-culture

PDLCs were seeded into 12-well plates at  $5 \times 10^3$  cells/well.  $\text{CD11b}^+$  peripheral blood mononuclear cells were added to PDLCs at  $5 \times 10^5$  cells/well after 1 d. The cells were co-cultured in a medium containing M-CSF (30 ng/mL) and sRANKL (50 ng/mL; Eleftheriou *et al.*, 2005). The cells were then treated with 0.1  $\mu\text{M}$  of ISO or vehicle. After 3 wk, the cells were fixed and stained for TRAP. TRAP-positive multinucleated (> 3 nuclei) cells were counted in 5 visual fields at  $20\times$  magnification in each well ( $n = 3$ ).

### Statistical Analyses

Data were presented as mean  $\pm$  standard deviation. Statistical analyses were performed using Student's *t*-test or 1-way analysis of variance (SPSS 19.0);  $p < .05$  was considered statistically significant.

## RESULTS

### SNS-regulated OTM Partially Depends on *Adrb2*

To confirm the function of SNS in OTM, the superior cervical ganglia of the rats were removed to block SNS activity in the jawbones. The tooth movement distance on the force-applied side increased to 860  $\mu\text{m}$  after 14 d of OTM in the force group. Tooth movement distance of the SCGx group (540  $\mu\text{m}$ ) decreased compared with that of the force group ( $p < .05$ ; Fig. 1A). Hematoxylin and eosin staining showed the integrity of the periodontal tissue around the 2 molars after tooth movement (Fig. 1), which suggests that orthodontic force-induced tooth movement partially depends on SNS.

To reveal whether *Adrb2* contributed to SNS-regulated OTM, *Adrb2* was activated by ISO in the rat OTM animal model. Force-induced increase in tooth movement distance in the force

group (300  $\mu\text{m}$ ) was further promoted by the administration of ISO (540  $\mu\text{m}$ ,  $p < .001$ , Appendix Fig. 1D). Orthodontic force was applied to *Adrb1/2<sup>-/-</sup>* and WT mice. The tooth movement distance on the force-applied side increased to 107.6  $\mu\text{m}$  after 7 d of OTM in WT mice (Fig. 1B). However, tooth movement distance significantly decreased in the knockout mice (19.5  $\mu\text{m}$ ) compared with that in WT ( $p < .001$ ). *Adrb2* promotes osteoclastogenic response, and *Adrb1* contributes to osteoblastogenic response (Pierroz *et al.*, 2012). We suggest *Adrb2* to play an important role in OTM as compressive force-associated bone resorption in the direction of the tooth movement limits the rate of OTM (Masella and Meister, 2006).

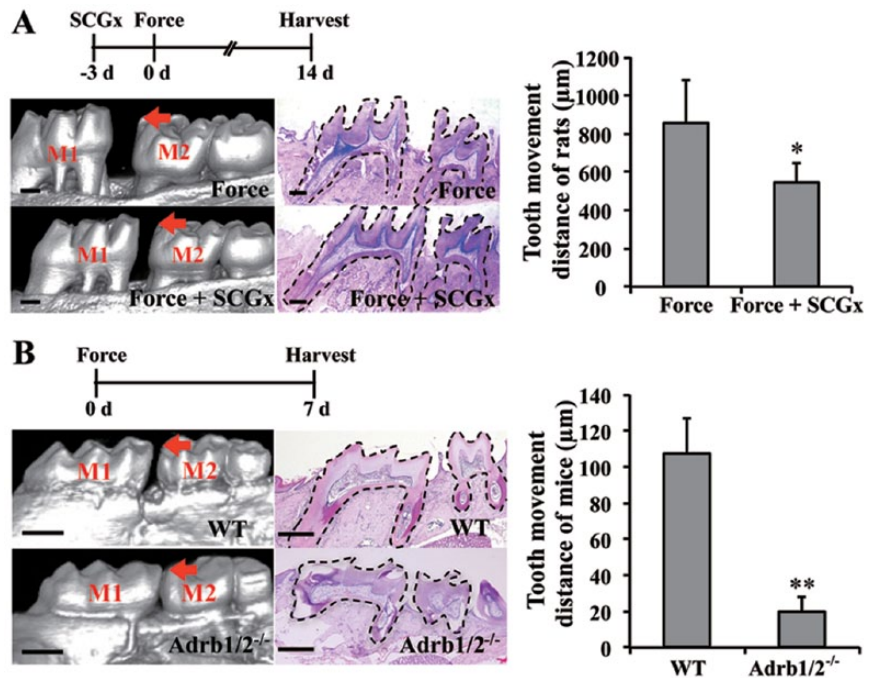
### Mechanical Force Upregulates the ADRB2 Expression in PDLs

Histopathologic examination revealed *Adrb2*-positive fibroblast-like PDLs in the compressive region of PDL in the force group after the application of orthodontic force for 7 d in rats. Few positive cells were observed in the control group (Figure 2A). This finding suggests that mechanical stimulation could upregulate *Adrb2* expression in PDLs.

*Adrb2* expression has been detected in osteoblasts. However, we found that orthodontic force had more significant effects on PDLs than OMCs in terms of upregulating *Adrb2* expression (Appendix Figure 3A). To confirm this phenomenon, PDLs and OMCs were subjected to static compression *in vitro*. The upregulation of *Adrb2* was higher in PDLs than in OMCs after the application of force (Figure 2B). Moreover, *Adrb2* expression in PDLs showed an increasing trend with the increase in force-treated intensity or time (Figure 2C, D), whereas no significant increase was found in OMCs (Appendix Figure 3B, C). The results were consistent with *in vivo* observation, confirming that *Adrb2* is expressed in PDLs and that mechanical force upregulates the expression of *Adrb2* in PDLs.

### Force-induced ADRB2 Upregulation Partially Depends on the Increase in Intracellular Ca<sup>2+</sup> Concentration

We further explored the underlying mechanism of force-induced *Adrb2* upregulation in PDLs. Figure 3A, B shows the dynamic changes in the intracellular Ca<sup>2+</sup> concentration in the same field of view. Before force or exogenous Ca<sup>2+</sup> was applied, intracellular Ca<sup>2+</sup> concentration could barely be detected. After separately applying force or treating the cells with Ca<sup>2+</sup>, we found no evident change in intracellular Ca<sup>2+</sup> concentration. In comparison, the green intracellular Ca<sup>2+</sup> signal was significantly



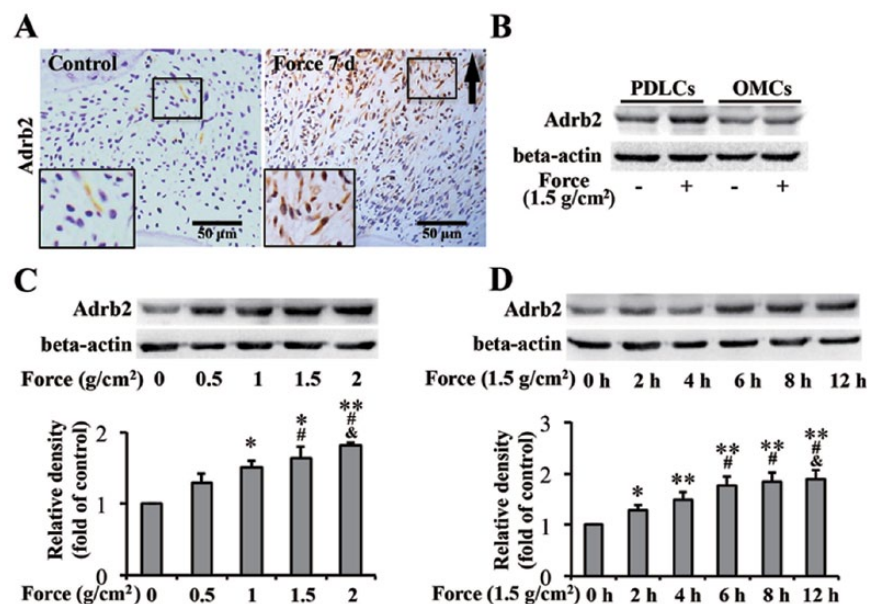
**Figure 1.** Sympathetic nervous system-regulated orthodontic tooth movement (OTM) partially depends on  $\beta$ -2 adrenergic receptor (*Adrb2*). (A, B) Analysis of the OTM distance by micro-computed tomography scanning and hematoxylin and eosin staining. (A) Superior cervical ganglionectomy (SCGx) decreased OTM distance after 14 d of force application in rats ( $n = 8$ ). (B) OTM distance decreased in the *Adrb1/2<sup>-/-</sup>* group compared to the WT group after 7 d of force application in mice ( $n = 14$ ). WT, wild type. Hematoxylin and eosin staining shows the integrity of the periodontal tissue around the 2 molars after tooth movement. The bar indicates 500  $\mu\text{m}$ . M1, the first molar. M2, the second molar. Arrows indicate OTM distance. \* $p < .05$ . \*\* $p < .001$ .

enhanced after a combined treatment of force and exogenous Ca<sup>2+</sup> was applied. BAPTA-AM, used to suppress intracellular Ca<sup>2+</sup> concentration, decreased the force-induced upregulation of *Adrb2* protein (Figure 3C). Therefore, the force-induced increase in intracellular Ca<sup>2+</sup> concentration has an important function in the force-induced *Adrb2* upregulation.

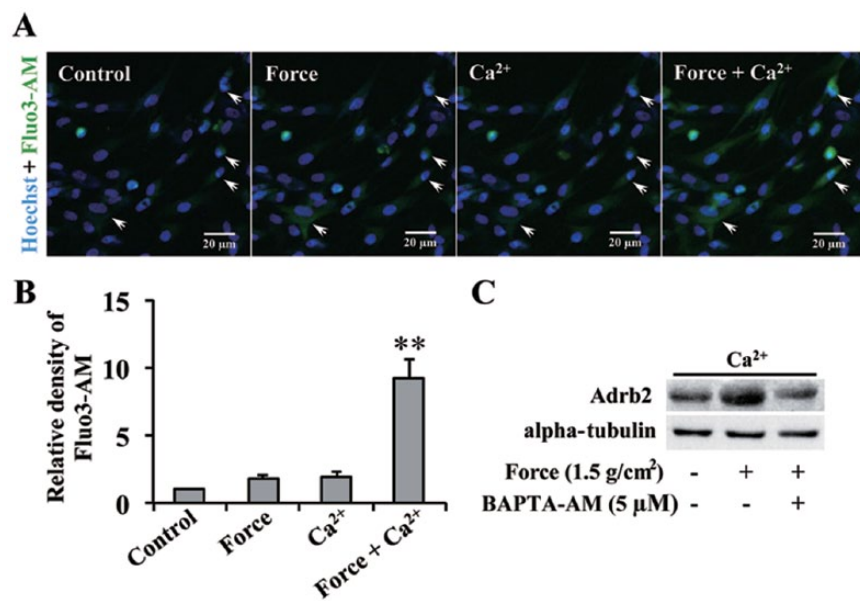
### Adrb2 Activation in PDLs Promotes RANKL/OPG Ratio and Osteoclast Differentiation

Although mechanical force upregulated the expression of *Adrb2*, the function of force-induced *Adrb2* in PDLs remains unclear. RANKL mRNA was upregulated by ISO treatment. ICI 118,551 partially reversed the ISO-induced upregulation of RANKL. The OPG mRNA showed a different trend from that of RANKL. The mRNA ratio of RANKL/OPG increased when the cells were treated with ISO and decreased when treated with ICI 118,551 together (Figure 4A).

To confirm the enhancing effect of *Adrb2* on the mRNA ratio of RANKL/OPG, we used *Adrb2* siRNA to knock down *Adrb2* expression in PDLs (Appendix Figure 4). After endogenous *Adrb2* was suppressed, the upregulation of the mRNA ratio of RANKL/OPG by ISO was almost eliminated (Figure 4B). Western blot showed that ISO treatment increased the expression of RANKL but was reversed when ICI 118,551 was added.



**Figure 2.** Mechanical force upregulates  $\beta$ -2 adrenergic receptor (Adrb2) expression in periodontal ligament cells (PDLs). **(A)** Immunohistochemistry of Adrb2. Orthodontic force increased the number of Adrb2-positive PDLs in rats. Arrows indicate the direction of tooth movement. Large boxed areas show higher-magnification views of the small boxes. **(B-D)** Western blot of Adrb2. **(B)** Higher Adrb2 expression was induced in PDLs than in orofacial bone marrow-derived cells *in vitro* after the application of force for 6 hr. **(C)** Adrb2 expression in PDLs was upregulated with increasing force-treated intensity for 6 hr. \* $p < .05$  vs. 0 g/cm<sup>2</sup> group. # $p < .05$  vs. 0.5 g/cm<sup>2</sup> group. & $p < .05$  vs. 1 g/cm<sup>2</sup> group. \*\* $p < .01$  vs. 0 g/cm<sup>2</sup> group. **(D)** Adrb2 expression in PDLs was upregulated as force-treated time was prolonged. \* $p < .05$  vs. 0-hr group. # $p < .05$  vs. 2-hr group. & $p < .05$  vs. 4-hr group. \*\* $p < .01$  vs. 0-hr group. Beta-actin served as internal control for equal loading. The data are representative of 3 independent experiments.



**Figure 3.** Force-induced  $\beta$ -2 adrenergic receptor (Adrb2) upregulation in periodontal ligament cells (PDLs) depends on intracellular Ca<sup>2+</sup> concentration. **(A)** Immunofluorescence of intracellular Ca<sup>2+</sup>. Fluo3-AM indicates intracellular Ca<sup>2+</sup> (green). Nuclei were counterstained with Hoechst (blue). Arrows indicate the dynamic changes in the fluorescence intensity of cells. **(B)** Relative density of intracellular Ca<sup>2+</sup> signal. Force-stimulated PDLs in HBSS with 10 mM of Ca<sup>2+</sup> presented significantly higher intracellular Ca<sup>2+</sup> concentration compared with the control (Ca<sup>2+</sup>-free HBSS), Force (Ca<sup>2+</sup>-free HBSS), and Ca<sup>2+</sup> groups. \*\* $p < .01$ . **(C)** Western blot of Adrb2. Force-induced Adrb2 upregulation was reversed by BAPTA-AM. Alpha-tubulin served as internal control for equal loading. The data are representative of 3 independent experiments.

The increase in RANKL expression became subtle after Adrb2 siRNA transfection was performed, although the same ISO dose was also administered (Figure 4C).

Furthermore, we showed that force-induced RANKL upregulation in PDLs was partially decreased by Adrb2 antagonist ICI 118,551 pretreatment (Figure 4D). Therefore, force-induced upregulation of RANKL partially depended on Adrb2 in PDLs.

We used a PDLs/CD11b<sup>+</sup> peripheral blood mononuclear cells coculture system to further validate the enhancing capacity of Adrb2 in PDLs in osteoclast differentiation. The ISO-stimulated group yielded more TRAP-positive multinucleated cells. This finding indicates that PDLs could promote osteoclast differentiation *in vitro* through Adrb2 signaling ( $p < .001$ ; Figure 4E).

### Orthodontic Force-induced Osteoclast Differentiation Partially Depends on Adrb2

TRAP staining was conducted to confirm the function of Adrb2 in osteoclast differentiation during OTM. TRAP-positive multinucleated cells were barely detected in the specific position of the control group in rats. However, the number of TRAP-positive multinucleated cells increased after orthodontic force was applied ( $p < .05$ ). ISO treatment further increased the orthodontic force-induced osteoclast differentiation ( $p < .05$ ; Figure 5A, B). The number of orthodontic force-induced TRAP-positive multinucleated cells decreased in Adrb1/2<sup>-/-</sup> mice compared with that in WT mice ( $p < .05$ ; Figure 5C, D). Our data suggested that osteoclast differentiation in OTM partially depended on force-induced Adrb2.

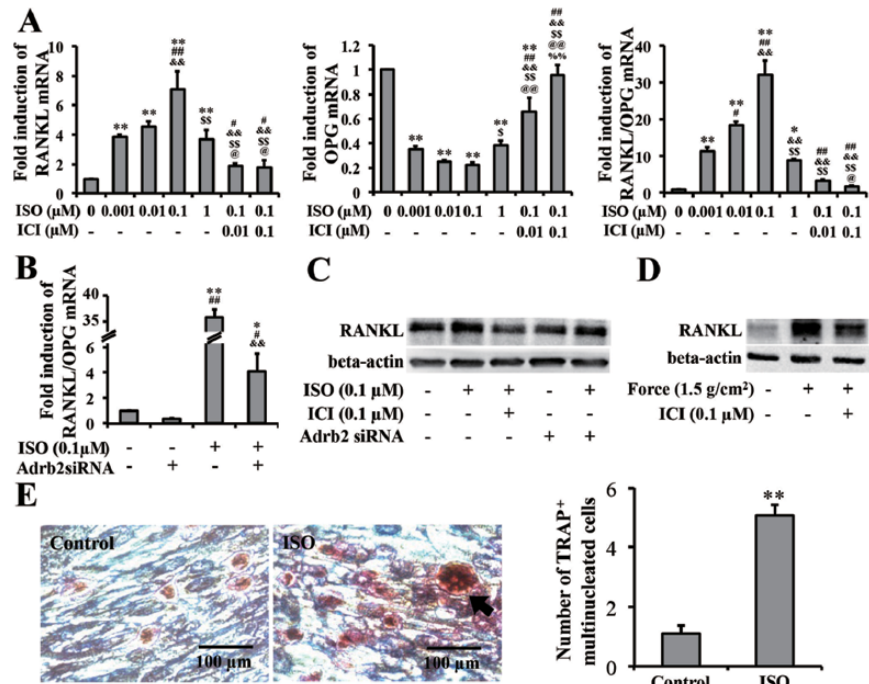
## DISCUSSION

We demonstrated that mechanical force-induced Adrb2 expressed in PDLs contributed to SNS-regulated OTM by modulating osteoclast differentiation. First, OTM was reduced by SNS blockade and accelerated by ISO injection in rats. Adrb1/2<sup>-/-</sup> mice also showed reduced OTM distance. Second, force induced the activation of Adrb2 in PDLs through the increase in intracellular Ca<sup>2+</sup> concentration. Third, the activation of Adrb2 in PDLs promoted osteoclast differentiation

by increasing the RANKL/OPG ratio. Our results provide new insights to understand force-induced remodeling of alveolar bone in OTM.

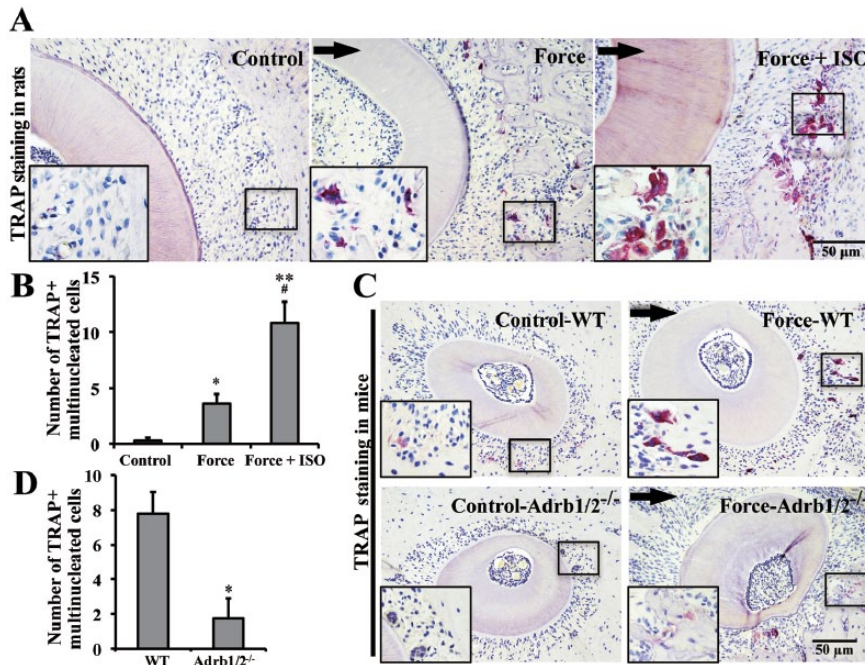
Force-induced Adrb2 contributed to osteoclast differentiation and SNS-regulated OTM. In this study, the effect of sympathectomy on OTM was demonstrated for the first time by directly removing the superior cervical ganglion. Consequently, OTM distance was decreased by SCGx. Our result is consistent with that of a previous study in which sympathetic signaling is found to regulate experimental tooth movement (Kondo *et al.*, 2013). ISO promoted osteoclast differentiation and OTM in rats. Adrb1/2<sup>-/-</sup> mice showed a decrease in osteoclast differentiation and OTM distance. Although OTM distance may also be affected by other SNS-regulated activities, such as masticatory movement and alveolar bone density (Passatore and Roatta, 2007; Sato *et al.*, 2014), our data provide a novel mechanism by which SNS-regulated OTM is mediated by force-induced Adrb2 activation. In previous studies, animal models were used to investigate the function of SNS in bone mechanoadaptive responses, such as tail suspension and sciatic neurectomy. However, hardly any SNS involvement was found (de Souza *et al.*, 2005; Marenzana *et al.*, 2007; Marenzana and Chenu, 2008). Orthodontic force, as an exerted mechanical stimulus, could induce the corresponding change in alveolar bone remodeling visually indicated by the distance of tooth movement. Thus, the OTM animal model used in this study was relatively effective in examining the bone mechanoadaptive responses. The regulatory function of SNS and Adrb2 in bone remodeling under mechanical microenvironment was supported by our results. Therefore, the mechanism by which these responses affect the OTM process should be determined.

Adrb2 is known to be expressed in osteoblasts and various osteogenic cell lines, such as UMR 106, SaoS-2, and MG63, serving as the mediator of SNS regulated bone remodeling (Aitken *et al.*, 2009; Eleftheriou *et al.*, 2005; Wong, 1979). We found that Adrb2 was highly expressed in PDLs after 7 d of orthodontic force application *in vivo*. In addition, Adrb2 expression was further confirmed in isolated and expanded PDLs derived from the neural crest where the SNS originated. PDL-derived stem cells express neural crest cell-specific markers and can be induced to form neural crest-like cells (Coura *et al.*, 2008; Dupin and Coelho-Aguiar, 2013; Fortino *et al.*, 2014; Xiaojie *et al.*, 2014), which implies a close connection



**Figure 4.**  $\beta$ -2 adrenergic receptor (Adrb2) in periodontal ligament cells (PDLs) promotes osteoclast differentiation. (A, B) Real-time polymerase chain reaction data of RANKL/OPG mRNA ratio in PDLs. (A) ISO upregulated RANKL/OPG mRNA ratio, which was reversed by ICI. ICI, ICI 118,551; ISO, isoproterenol. \* $p < .05$  vs. the control group. # $p < .05$  vs. 0.001- $\mu$ M ISO group. &#p < .05 vs. 0.01- $\mu$ M ISO group. \$ $p < .05$  vs. 0.1- $\mu$ M ISO group. @ $p < .05$  vs. 1- $\mu$ M ISO group. % $p < .05$  vs. 0.1- $\mu$ M ISO + 0.01- $\mu$ M ICI group. \*\* $p < .01$  vs. the control group. ## $p < .01$  vs. 0.001- $\mu$ M ISO group. && $p < .01$  vs. 0.01- $\mu$ M ISO group. \$\$ $p < .01$  vs. 0.1- $\mu$ M ISO group. @@ $p < .01$  vs. 1- $\mu$ M ISO group. %% $p < .01$  vs. 0.1- $\mu$ M ISO + 0.01- $\mu$ M ICI group. (B) Pretreatment of Adrb2 siRNA downregulated ISO-induced RANKL/OPG mRNA ratio. \* $p < .05$  vs. the control group. # $p < .05$  vs. 0.1- $\mu$ M ISO group. &# $p < .05$  vs. Adrb2 siRNA group. \*\* $p < .01$  vs. the control group. ## $p < .01$  vs. 0.1- $\mu$ M ISO group. && $p < .01$  vs. Adrb2 siRNA group. (C, D) Western blot of RANKL in PDLs. (C) RANKL protein expression depends on Adrb2 in PDLs. RANKL expression was evaluated by Western blot. (D) Force-promoted RANKL expression was partially decreased by Adrb2 antagonist ICI pretreatment. Beta-actin served as the internal control for equal loading. (E) TRAP staining of osteoclasts in PBMCs cocultured with PDLs. Arrows represent TRAP-positive multinucleated osteoclasts. Treatment with ISO increased osteoclasts. OSC, osteoclasts.  $n = 3$ . \*\* $p < .01$ . The data are representative of 3 independent experiments.

between PDLs and SNS in Adrb2 expression. Static compressive force *in vitro* upregulated the expression of Adrb2 in PDLs as force was increased and force-treated time was prolonged. A previous study reported that glucocorticoids increase Adrb2 expression in osteoblasts (Ma *et al.*, 2011). In this study, we showed that mechanical stimulation could also promote Adrb2 expression and indicated that cells in physiologic mechanical environments could be involved in the corresponding Adrb2-mediated biological responses, such as bone remodeling, through force-induced Adrb2. In this study we found the force-induced upregulation of Adrb2 in PDLs but found no significant increase of Adrb2 expression in OMCs, which suggests that PDLs-expressed Adrb2 may be more sensitive to mechanical stimulation than OMCs. During orthodontic treatment, mechanical force loaded on tooth is transduced to the PDL, and then PDLs respond to the stimuli and induce a chain of biological responses (Lecic and McCulloch, 1996). Generally,



**Figure 5.** Orthodontic force-induced osteoclast differentiation partially depends on  $\beta$ -2 adrenergic receptor (Adrb2). **(A)** Tartrate-resistant acid phosphatase (TRAP) staining for osteoclasts in rats with orthodontic force application for 7 d. Arrows indicate the direction of tooth movement. Large boxed areas show higher-magnification views of the small boxes. **(B)** Semiquantification of TRAP<sup>+</sup> multinucleated cells. The Force + ISO group presented the highest number of TRAP-positive multinucleated osteoclasts, followed by the Force and control groups.  $n = 5$ . \* $p < .05$  vs. the control group. \*\* $p < .01$  vs. the control group. # $p < .05$  vs. Force group. **(C)** TRAP staining for osteoclasts in mice with orthodontic force application for 7 d. **(D)** Semiquantification of TRAP<sup>+</sup> multinucleated cells. Orthodontic force-induced osteoclast formation was decreased in the Adrb1/2<sup>-/-</sup> group compared with that in the wild-type group.  $n = 5$ . \* $p < .05$ .

ankylosed teeth, in which the tooth root is directly connected with the alveolar bone, cannot be moved by orthodontic force because of the lack of PDL (Kanzaki *et al.*, 2002). Our results supported the significant role of PDLCs played in OTM. Different cell types, microenvironments, and sources of development may contribute to the different responses of PDLCs and OMCs to mechanical force. However, more evidence is needed to reveal how OMCs influence the SNS-regulated OTM.

$\beta$  adrenergic receptors play a major role in the regulation of cardiac function. Upon sympathetic stimulation, Adrb2 can stimulate cAMP production and enhance Ca<sup>2+</sup> channel activity in the heart (Fajardo *et al.*, 2013; Harvey and Hell, 2013). In this study, we found that force-induced upregulation of Adrb2 was eliminated by the intracellular Ca<sup>2+</sup> chelator BAPTA-AM, suggesting that force-induced Adrb2 in PDLCs partially depended on the increase in intracellular Ca<sup>2+</sup>. Intracellular Ca<sup>2+</sup> is essential in several signal pathways, such as MAPK, CREB, and NF- $\kappa$ B pathways, which are reported to be Adrb2-positive transcriptional regulators (Aksoy *et al.*, 2001; Yeh *et al.*, 2012). Ca<sup>2+</sup> induces the stimulation of adenylyl cyclase and the elevation of intracellular cAMP levels (Watson *et al.*, 2000). cAMP response element-binding protein can positively regulate Adrb2 expression (Collins *et al.*, 1990). The results may help understand the mechanism by which force-induced increase in intracellular

Ca<sup>2+</sup> concentration contributes to the upregulation of Adrb2.

In the downstream region of the PDLCs-expressed Adrb2, Adrb2 activation by ISO increased the RANKL/OPG ratio, which was decreased by Adrb2-selective antagonist ICI 118,551 and Adrb2 siRNA transfection. Our results are consistent with those of a previous study, which reported that Adrb2 favors bone resorption by increasing the expression of osteoclast differentiation factor RANKL in osteoblast progenitor cells (Elefteriou *et al.*, 2005). Mechanical stimulation also intensified the Adrb2-regulated upregulation of RANKL protein expression. The results of the cocultured PDLCs and peripheral blood mononuclear cells confirmed that the activation of Adrb2 in PDLCs promoted osteoclast formation. Adrb1 and Adrb2 signaling exert opposite effects on bone remodeling. Adrb2 promotes bone resorption, whereas Adrb1 contributes to bone formation. In the double-knockout animal model, the concomitant loss of Adrb1 may counteract the effect of Adrb2 deficiency on bone (Pierroz *et al.*, 2012). In OTM, the rate-limiting step is the compression-associated osteoclasts formation (Masella and Meister, 2006). We found that Adrb1/2<sup>-/-</sup> mice showed decreased osteoclast differentiation and OTM dis-

tance, suggesting that the Adrb2 deficiency-induced decrease of osteoclast formation plays a major role in this process. In addition, selective antagonist and siRNA of Adrb2 reduced osteoclastogenic response in PDLCs. This finding suggests that Adrb2 in PDLCs contributes to the compression-associated osteoclast formation in OTM. However, bone homeostasis is a dynamic change between bone resorption and formation, and bone apposition may present in the compression site. Whether PDLCs express Adrb1 and play osteoblastogenic function in OTM still needs further study.

In conclusion, mechanical force upregulated Adrb2 expression in PDLCs through the force-induced increase in the intracellular Ca<sup>2+</sup> concentration. The activation of Adrb2 in PDLCs contributed to osteoclast differentiation through the RANKL/OPG system and promoted SNS-regulated OTM. The results may help understand the mechanism by which mechanical force affects the alveolar bone remodeling of OTM.

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## REFERENCES

- Aitken SJ, Landao-Bassonga E, Ralston SH, Idris AI (2009). Beta2-adrenoreceptor ligands regulate osteoclast differentiation in vitro by direct and indirect mechanisms. *Arch Biochem Biophys* 482:96-103.
- Aksoy MO, Bin W, Yang Y, Yun-You D, Kelsen SG (2001). Nuclear factor-kappa B augments beta(2)-adrenergic receptor expression in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 281:L1271-L1278.
- Biewener AA (1985). Bone and its functions: the mechanical adaptations of bones. *Science* 227:629-630.
- Collins S, Altschmied J, Herbsman O, Caron MG, Mellon PL, Lefkowitz RJ (1990). A cAMP response element in the beta 2-adrenergic receptor gene confers transcriptional autoregulation by cAMP. *J Biol Chem* 265:19330-19335.
- Cooper SM, Sims MR (1989). Evidence of acute inflammation in the periodontal ligament subsequent to orthodontic tooth movement in rats. *Aust Orthod J* 11:107-109.
- Coura GS, Garcez RC, de Aguiar CB, Alvarez-Silva M, Magini RS, Trentin AG (2008). Human periodontal ligament: a niche of neural crest stem cells. *J Periodontol Res* 43:531-536.
- de Souza RL, Pitsillides AA, Lanyon LE, Skerry TM, Chenu C (2005). Sympathetic nervous system does not mediate the load-induced cortical new bone formation. *J Bone Miner Res* 20:2159-2168.
- Dunn MD, Park CH, Kostenuik PJ, Kapila S, Giannobile WV (2007). Local delivery of osteoprotegerin inhibits mechanically mediated bone modeling in orthodontic tooth movement. *Bone* 41:446-455.
- Dupin E, Coelho-Aguiar JM (2013). Isolation and differentiation properties of neural crest stem cells. *Cytometry A* 83:38-47.
- Dupin E, Sommer L (2012). Neural crest progenitors and stem cells: from early development to adulthood. *Dev Biol* 366:83-95.
- Elefteriou F, Ahn JD, Takeda S, Starbuck M, Yang X, Liu X, et al. (2005). Leptin regulation of bone resorption by the sympathetic nervous system and CART. *Nature* 434:514-520.
- Fajardo G, Zhao M, Urashima T, Farahani S, Hu DQ, Reddy S, et al. (2013). Deletion of the beta2-adrenergic receptor prevents the development of cardiomyopathy in mice. *J Mol Cell Cardiol* 63:155-164.
- Fortino VR, Chen RS, Pelaez D, Cheung HS (2014). Neurogenesis of neural crest-derived periodontal ligament stem cells by EGF and bFGF. *J Cell Physiol* 229:479-488.
- Garlet TP, Coelho U, Silva JS, Garlet GP (2007). Cytokine expression pattern in compression and tension sides of the periodontal ligament during orthodontic tooth movement in humans. *Eur J Oral Sci* 115:355-362.
- Harvey RD, Hell JW (2013). CaV1.2 signaling complexes in the heart. *J Mol Cell Cardiol* 58:143-152.
- Huiskes R, Ruimerman R, van Lenthe GH, Janssen JD (2000). Effects of mechanical forces on maintenance and adaptation of form in trabecular bone. *Nature* 405:704-706.
- Immermann EW, Compere EL (1954). Bone metastases of neurogenic tumors of the sympathetic nervous system in children. *Q Bull Northwest Univ Med Sch* 28:138-142.
- Iwasaki LR, Chandler JR, Marx DB, Pandey JP, Nickel JC (2009). IL-1 gene polymorphisms, secretion in gingival crevicular fluid, and speed of human orthodontic tooth movement. *Orthod Craniofac Res* 12:129-140.
- Kang YG, Nam JH, Kim KH, Lee KS (2010). FAK pathway regulates PGE production in compressed periodontal ligament cells. *J Dent Res* 89:1444-1449.
- Kanzaki H, Chiba M, Shimizu Y, Mitani H (2002). 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* 17:210-220.
- Kondo M, Kondo H, Miyazawa K, Goto S, Togari A (2013). Experimental tooth movement-induced osteoclast activation is regulated by sympathetic signaling. *Bone* 52:39-47.
- Lekic P, McCulloch CA (1996). Periodontal ligament cell population: the central role of fibroblasts in creating a unique tissue. *Anat Rec* 245:327-341.
- Ma Y, Nyman JS, Tao H, Moss HH, Yang X, Elefteriou F (2011). Beta2-adrenergic receptor signaling in osteoblasts contributes to the catabolic effect of glucocorticoids on bone. *Endocrinology* 152:1412-1422.
- Marenzana M, Chenu C (2008). Sympathetic nervous system and bone adaptive response to its mechanical environment. *J Musculoskeletal Neuronal Interact* 8:111-120.
- Marenzana M, De Souza RL, Chenu C (2007). Blockade of beta-adrenergic signaling does not influence the bone mechano-adaptive response in mice. *Bone* 41:206-215.
- Masella RS, Meister M (2006). Current concepts in the biology of orthodontic tooth movement. *Am J Orthod Dentofacial Orthop* 129:458-468.
- Matthews EK, Quilliam JP (1964). Effects of central depressant drugs upon acetylcholine release. *Br J Pharmacol Chemother* 22:415-440.
- Mitsui N, Suzuki N, Maeno M, Mayahara K, Yanagisawa M, Otsuka K, et al. (2005). Optimal compressive force induces bone formation via increasing bone sialoprotein and prostaglandin E(2) production appropriately. *Life Sci* 77:3168-3182.
- Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, et al. (1987). Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* 2:595-610.
- Passatore M, Roatta S (2007). Modulation operated by the sympathetic nervous system on jaw reflexes and masticatory movement. *Arch Oral Biol* 52:343-346.
- Pierroz DD, Bonnet N, Bianchi EN, Bouxsein ML, Baldock PA, Rizzoli R, et al. (2012). Deletion of beta-adrenergic receptor 1, 2, or both leads to different bone phenotypes and response to mechanical stimulation. *J Bone Miner Res* 27:1252-1262.
- Sato T, Miyazawa K, Suzuki Y, Mizutani Y, Uchibori S, Asaoka R, et al. (2014). Selective beta2-adrenergic antagonist butoxamine reduces orthodontic tooth movement. *J Dent Res* 93:807-812.
- Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahimi J, et al. (2004). Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 364:149-155.
- Takeda S, Elefteriou F, Lévassour R, Liu X, Zhao L, Parker KL, et al. (2002). Leptin regulates bone formation via the sympathetic nervous system. *Cell* 111:305-317.
- Watson EL, Jacobson KL, Singh JC, Idzerda R, Ott SM, DiJulio DH, et al. (2000). The type 8 adenylyl cyclase is critical for Ca2+ stimulation of cAMP accumulation in mouse parotid acini. *J Biol Chem* 275:14691-14699.
- Wong GL (1979). Induction of metabolic changes and down regulation of bovine parathyroid hormone-responsive adenylyl cyclase are dissociable in isolated osteoclastic and osteoblastic bone cells. *J Biol Chem* 254:34-37.
- Xiaojie L, Dapeng L, Ping G, Yan D, Gang S (2014). Biological behavior of neurally differentiated periodontal ligament stem cells on different titanium implant surfaces. *J Biomed Mater Res A* 102:2805-2812.
- Yamaza T, Ren G, Akiyama K, Chen C, Shi Y, Shi S (2011). Mouse mandible contains distinctive mesenchymal stem cells. *J Dent Res* 90:317-324.
- Yeh CK, Chandrasekar B, Lin AL, Dang H, Kamat A, Zhu B, et al. (2012). Cellular signals underlying beta-adrenergic receptor mediated salivary gland enlargement. *Differentiation* 83:68-76.