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From Cell Biology to Tissue Engineering

Sympathetic nervous system contributes to orthodontic tooth movement by central neural regulation from hypothalamus

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Summary. Orthodontic tooth movement (OTM) is a specific treatment of malocclusion, whose regulation mechanism is still not clear. This study aimed to reveal the relationship between the sympathetic nervous system (SNS) and OTM through the construction of an OTM rat model through the utilization of orthodontic nickeltitanium coiled springs. The results indicated that the stimulation of SNS by dopamine significantly promote the OTM process represented by the much larger distance between the first and second molar compared with mere exertion of orthodontic force. Superior cervical ganglionectomy (SCGx) can alleviate this promotion effect, further proving the role of SNS in the process of OTM. Subsequently, the ability of orthodontic force to stimulate the center of the SNS was visualized by the tyrosin hydroxylase (TH) staining of neurons in ventromedial hypothalamic nucleus (VMH) and arcuate nucleus (ARC) of the hypothalamus, as well as the up-regulated expression of norepinephrine in local alveolar bone. Moreover, we also elucidated that the stimulation of SNS can promote osteoclast differentiation in periodontal ligament cells (PDLCs) and bone marrow-derived cells (BMCs) through regulation of receptor activator of nuclear factor-κB ligand (RANKL)/osteoprotegerin (OPG) system, thus promoting the OTM process. In conclusion, this study provided the first evidence for the involvement of the hypothalamus in the promotion effect of SNS on OTM. This work could provide a novel theoretical and experimental basis for further understanding of the molecular mechanism of OTM.

Key words: Orthodontic tooth movement, Sympathetic nervous system, Dopamine, Periodontal ligament cells, Hypothalamus

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Introduction

Orthodontic tooth movement (OTM) is a process of dynamic remodeling of local periodontal tissue under continuous mechanical force (Li et al., 2018), which has been used as a specific treatment of malocclusion (Sá-Pinto et al., 2018). Although the molecular mechanism of OTM is still not clear, it is well known that mechanical force induced OTM involves the continuous compressive force-associated osteoclast formation in the direction of tooth movement, and bone formation in the opposite direction, which is facilitated by the tissue microenvironment provided by periodontal ligament (PDL) (Fitri et al., 2018). Accumulating evidence has proved that the altered activity of osteoblast and osteoclast in PDL represented by the receptor activator of nuclear factor-κB ligand (RANKL)/osteoprotegerin (OPG) system plays a critical role in OTM (Toygar et

al., 2008; Yamaguchi, 2009; Grant et al., 2013; Enhos et al., 2013; Macari et al., 2018). For example, a recent mechanistic study indicated that inhibition of GLUT1 can disturb the RANKL/OPG system by alleviating compressive force-mediated RANKL upregulation, thus promoting OTM (Wang et al., 2018). However, the underlying regulation mechanism of RANKL/OPG system as well as OTM by the compression force is still not clear.

Notably, our previous work (Cao et al., 2014) revealed the up-regulated expression of β-2 adrenergic receptor (Adrb2), one postsynaptic β adrenergic receptor in sympathetic nervous system (SNS), in periodontal ligament cells (PDLCs) by the exertion of mechanical force, which increased the RANKL/OPG ratio and promoted OTM, indicating the probable involvement of SNS in OTM regulation (Reid et al., 2005; Bonnet et al., 2008). Although it was traditionally considered that bone metabolism is mainly mediated by local autocrine, paracrine cytokines and hormones in bone tissue, the participation of the sympathetic nervous system (SNS) in bone metabolism has been established (Haug et al., 2003; Chenu and Marenzana, 2005). Karsenty et al. indicated that leptin can simultaneously regulate the process of bone formation and bone absorption through SNS (Ducy et al., 2000). Moreover, Adrb2 was also identified as a key mediator in the leptin regulation of bone resorption in an animal model, in which Adrb2 knockout induced 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). Despite all this, the direct relationship between SNS and OTM is still rarely reported and not established.

Further taking the association of PDLCs and nervous system into account (Li et al., 2014; Fortino et al., 2014), this study aimed to provide more direct evidence of the participation of SNS in orthodontic force induced OTM. Therefore, an OTM rat model was constructed to investigate the effects of superior cervical ganglionectomy (SCGx) and stimulation of ventromedial hypothalamic nucleus (VMH) in the hypothalamus by dopamine (DA) on OTM. Moreover, immunofluorescence was used to evaluate the orthodontic force induced excitation of hypothalamic functional nuclei associated with SNS activity. The expression levels of sympathetic hormone such as norepinephrine in local alveolar bone tissue and RANKL/OPG in PDLCs were detected for further verification.

Materials and methods

OTM animal model

Sprague-Dawley rats (male, 8 week-old, Beijing Vital River Laboratory Animal Technology Co., Ltd) were used to set up the OTM animal model. All the animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Peking University (LA2013-92).

Rats were anesthetized with an intraperitoneal injection of 1% sodium pentobarbital (40 mg/kg body weight) for the operation (n=5 for each group). 0.20 mm orthodontic ligatures were passed between the right maxillary first molar (M1) and second molar (M2) (Dunn et al., 2007). A nickel-titanium tension spring (0.2 mm in thickness, 1 mm in diameter, 5 mm in length; Smart Technology, Beijing, China) was ligated with a ligaturing wire, and one end of the tension spring was brought into close contact with the M1 mesial tooth surface. Two ligatures fixed grooves were grinded in the distal tooth surfaces of the rat's two upper maxillary incisors close to the gingiva. A 0.25 mm ligature with an orthodontic force of 60 g was fixed in the two fixing grooves for seven days. A model with a teeth gap between M1 and M2 without alveolar bone broken after modeling for 7 days was considered successfully. The health and behavior of animals were monitored twice every day. Rats were sacrificed at the 7th day of executing OTM through injection of 1% sodium pentobarbital (120 mg/kg body weight). The data were used for statistical analysis.

OTM distance measurement

After the experiment, rats were sacrificed by 1% sodium pentobarbital overdose (120 mg/kg body weight), and the maxilla was placed in a neutral formalin fixed solution for Micro CT broom. The broom parameters were: 80 kV, 500 μA, 9.08 μm effective pixels and 1500 ms exposure time. After three-dimensional reconstruction, picture was taken for obsevationg. Before orthodontic force application, the middle point of the M1 distal edge and the midpoint of the M2 mid-edge edge were fitted closely. After intercepting the picture, OTM distance was the distance of the two points.

Rat superior cervical ganglionectomy (SCGx)

The rats were anesthetized by intraperitoneal injection of 1% pentobarbital sodium for the operation (40 mg/kg) and sacrificed by injection of pentobarbital sodium (120 mg/kg body weight) after experiments. A Cut was made at approximately 2 cm in the ventral midline of the neck and fully exposed the common carotid artery and vagus nerve. The superior cervical sympathetic ganglion can be seen on the dorsal side of the bifurcation of the common carotid artery. The ganglion was carefully separated to its proximal and distal ends by 3 mm, and then removed and the incision was stitched. Unilateral removal was performed. The sham group was sham-operated and the ganglia were isolated but not removed. The modeling with symptoms of ganglion resection of ipsilateral superior ptosis appeared to be successful when the rats woke.

PDLCs and BMCs culture

All protocols used to obtain human tissue samples were approved by the Ethical Guidelines of Peking University (V160229001). The protocols were performed with appropriate informed consent (PKUSSIRB-201311103). Human PDLCs were isolated from PDL of normal orthodontic extracted bicuspids. PDLCs isolated from 3 different individuals were pooled together and used in this study. Human socket bone marrow-derived cells (BMCs) were isolated from 12-18 years old patients with healthy periodontium who had their wisdom teeth extracted. The location of bone removal is shown in Fig. 1.

Cell culture media were configured including α-MEM, 15% FBS, 100 μmol/L L-ascorbic acid-2-phosphate Trisodium acid, 2 mmol/L glutamine, 1% double antibody. After tissues were digested and centrifuged, PDLCs and BMCs suspension were collected for culture. After 7 days of culture, cell clones could be seen and at about 10-14 days the cell density was 80%-90% passaged. 3-4 generations of cells were used for experiments. The cellular experiments were performed in 3 independent experiments to avoid individual differences.

Hypothalamic VMH nuclear stereotaxy buried tube

300-320 g SD male rats were randomly selected and 1% pentobarbital sodium was intraperitoneally injected (40 mg/kg). With the brain stereotaxic apparatus, the rat skull was parallel to the bottom of the locator. About 1.5 cm was cut along the middle of the parietal bone, and the anatomical landmarks such as sagittal suture, coronal fontanelle, anterior fontanelle, and posterior fontanelle were exposed. Double catheters (1.5 mm apart) were positioned on the positioning rod of the stereotaxic apparatus. The left and right catheters were each 0.75 mm apart from the sagittal suture. Evans blue was used to mark the position of the lower end of the left and right catheters. After drilling at the marked point, catheters were vertically inserted 7.5 mm (the injection site was actually 9.5 mm below the skull). Holes were drilled on both sides of the sagittal suture and about 5 mm left of the posterior fontanelle. Self-curing plastic was applied around the catheters and the base of the catheter and screws were wrapped. Catheter caps were inserted to prevent plugging after the operation. After experiments, rats were sacrificed by injection of sodium pentobarbital (120 mg/kg body weight).

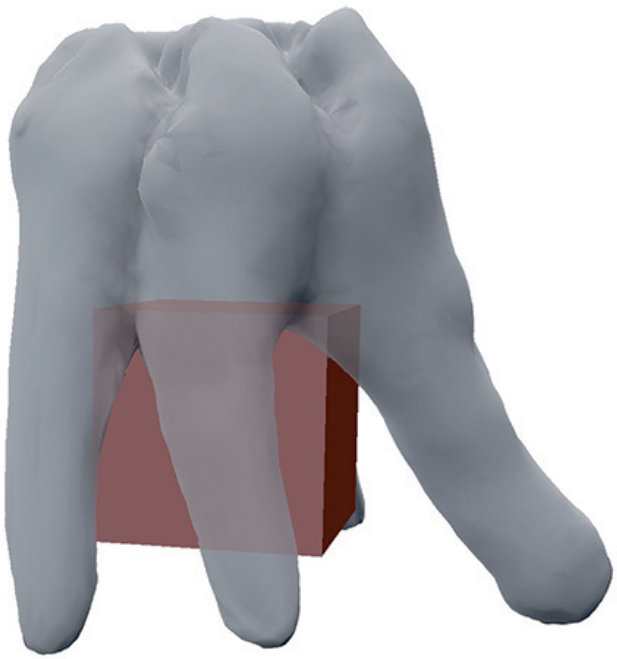

Fig. 1. The location of bone removal in this study.

Immunofluorescence

Frozen sections were placed in a refrigerator at 4°C and thawed at room temperature for 30 min and then baked at 37°C for 1 h. The sections were incubated with PBS with 0.3% Triton-X-100 for 2 h and then blocked by 10% goat serum for 1 h. The sections were incubated with the primary antibody rabbit anti-TH polyclonal antibody (1:150 dilution) (# LS-B3443, LSBio., US) overnight at 4°C, then washed with PBS for 10 min repeated 3 times. FITC-labeled mouse anti-rabbit fluorescent secondary antibody (1:200 dilution) (Beijing Zhongshan Jinqiao, China) was added for incubating for 1 h at room temperature in the dark. PBS washing for 10 min, repeated 3 times. Mounting medium containing DAPI (Beijing Zhongshan Jinqiao, China) was added. Observations were made with a laser confocal microscope at 488 nm (FITC) and 405 nm (DAPI) wavelengths to obtain green (FITC) and blue fluorescence (DAPI) signals and photos were taken.

ELISA for norepinephrine measurement

The supernatants of cultured local alveolar bone were collected after compressive force application, and the concentration of norepinephrine was quantified with the ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

RANKL, OPG, GAPDH and Adrb2 mRNA expression by RT-PCR

Total RNA was extracted from the PDLCs and BMCs using Trizol reagent (Invitrogen, US) according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA using reverse transcriptase. Quantitative real-time PCR analysis was performed with One Step SYBR PrimeScriptTM PLUS RT-PCR kit (Takara) using the ABI 7500 Real-Time PCR System

(Applied Biosystems). GAPDH was used as internal control. The relative transcript levels of target genes were calculated by $2^{-\Delta\Delta Ct}$. Nanodrop 2000/2000C spectrophotometric were used to analyze the relative levels of mRNAs. PCR primers are shown in Table 1.

Statistical analyses

All experiments were performed in triplicate and data are shown as mean ± SD. The statistical analysis was performed using the two-tailed Student's t test or

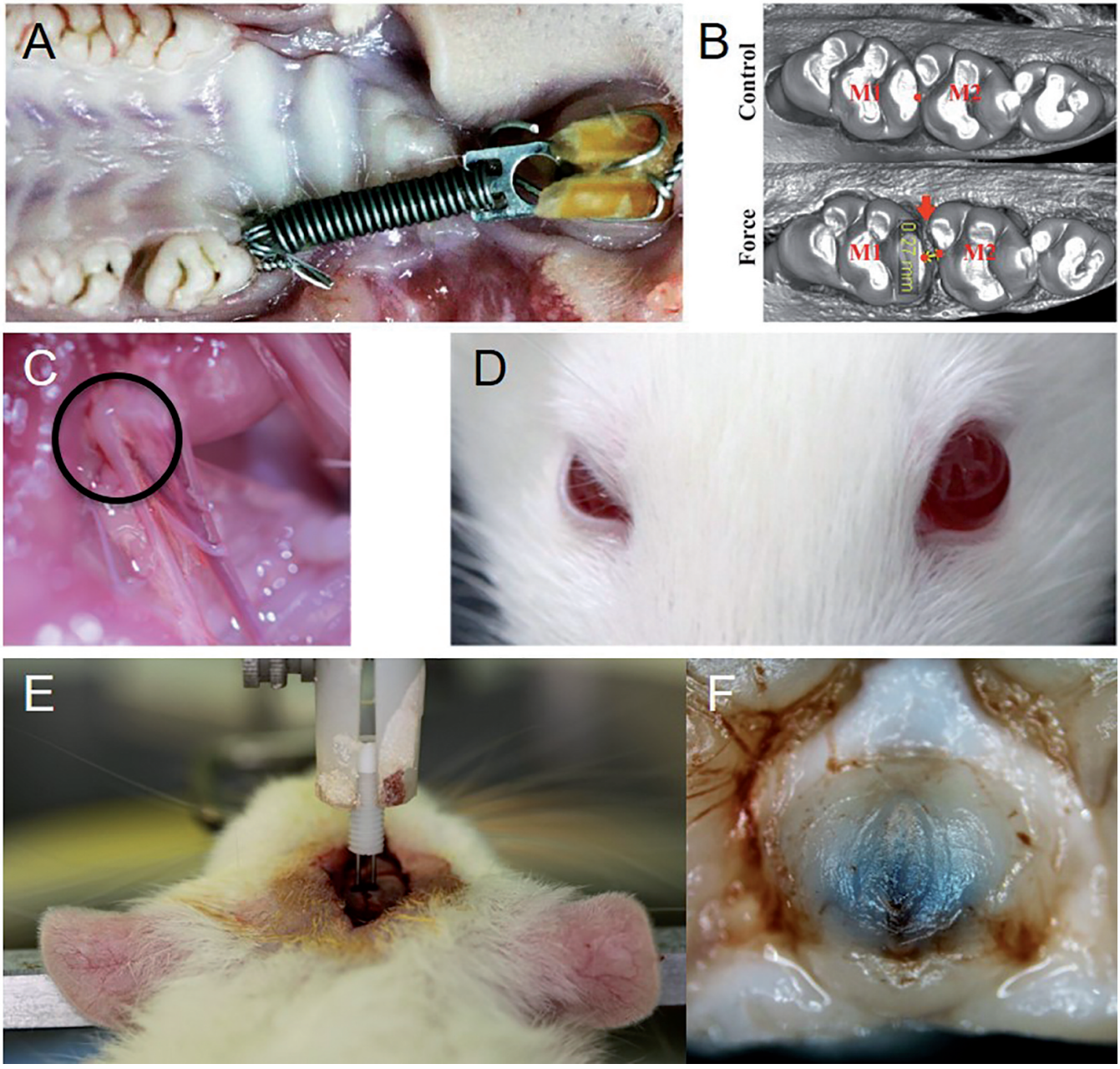

Fig. 2. Construction of rat OTM and SCGx models and stereotaxic buried tube of VMH nucleus in hypothalamus. **A.** The construction of rat OTM model. **B.** The method for measuring OTM distance. **C.** Anatomical position of the superior cervical ganglion in rats. **D.** The ipsilateral ptosis with resected superior cervical ganglion showed the validity of SCGx. **E.** Stereotaxic brain localization in rats. **F.** Confirmation of injection point in hypothalamus by Evans blue.

One-way ANOVA followed by Tukey's HSD post hoc test. Statistical significance (P value) was calculated by SPSS 18.0 (IBM, SPSS, Chicago, IL, USA) and P value <0.05 was considered statistically significant.

Results

Construction of rat models for OTM, SCGx and location of hypothalamus

In order to construct the OTM animal model, orthodontic nickeltitanium coiled springs (0.2 mm in thickness, 1 mm in diameter, 5 mm in length) were ligated between the right maxillary first molar and the incisors of the rats (Fig. 2A) (Dunn et al., 2007). After 7 days of exerting orthodontic force, if a gap appeared between the first and second molar and the alveolar bone was not broken, it was regarded as successful modeling, otherwise, the modeling was considered a failure. The method for measuring distance of OTM is shown in Fig. 2B, it is represented by the distance between the indicated point on the first and second molars. On the other hand, superior cervical sympathetic ganglions of some rats were isolated and removed through middle ventral surgery of the neck. The symptom of ptosis ipsilateral with ganglion resection in the rats indicated successful construction of rat SCGx model (Fig. 2C,D). Moreover, the intramuscular injection point in the hypothalamus was confirmed through stereotaxic buried tube of VMH nucleus in hypothalamus and microinjection of Evans blue solution (Fig. 2E,F).

Stimulation of SNS promoted process of OTM

After the successful construction of the various rat models, the effects of SNS excitation or SCGx on OTM

Table 1. PCR primers.

Target gene		Primers
RANKL	Forward	5'-AGA GCG CAG ATG GAT CCTAA-3'
	Reverse	5'-TTC CTT TTG CAC AGC TCC TT-3'
OPG	Forward	5'-GGAACC CCA GAG CGA AAT ACA-3'
	Reverse	5'-CCT GAA GAA TGC CTC CTC ACA-3'
GAPDH	Forward	5'-ATG GGG AAG GTG AAG GTC G-3'
	Reverse	5'-ATGGGG AAG GTG AAG GTC G-3'
Adrb2	Forward	5'-CGC TAC TTT GCC ATT AC-3'
	Reverse	5'-CAT AGG CTT GGT TCG T-3'

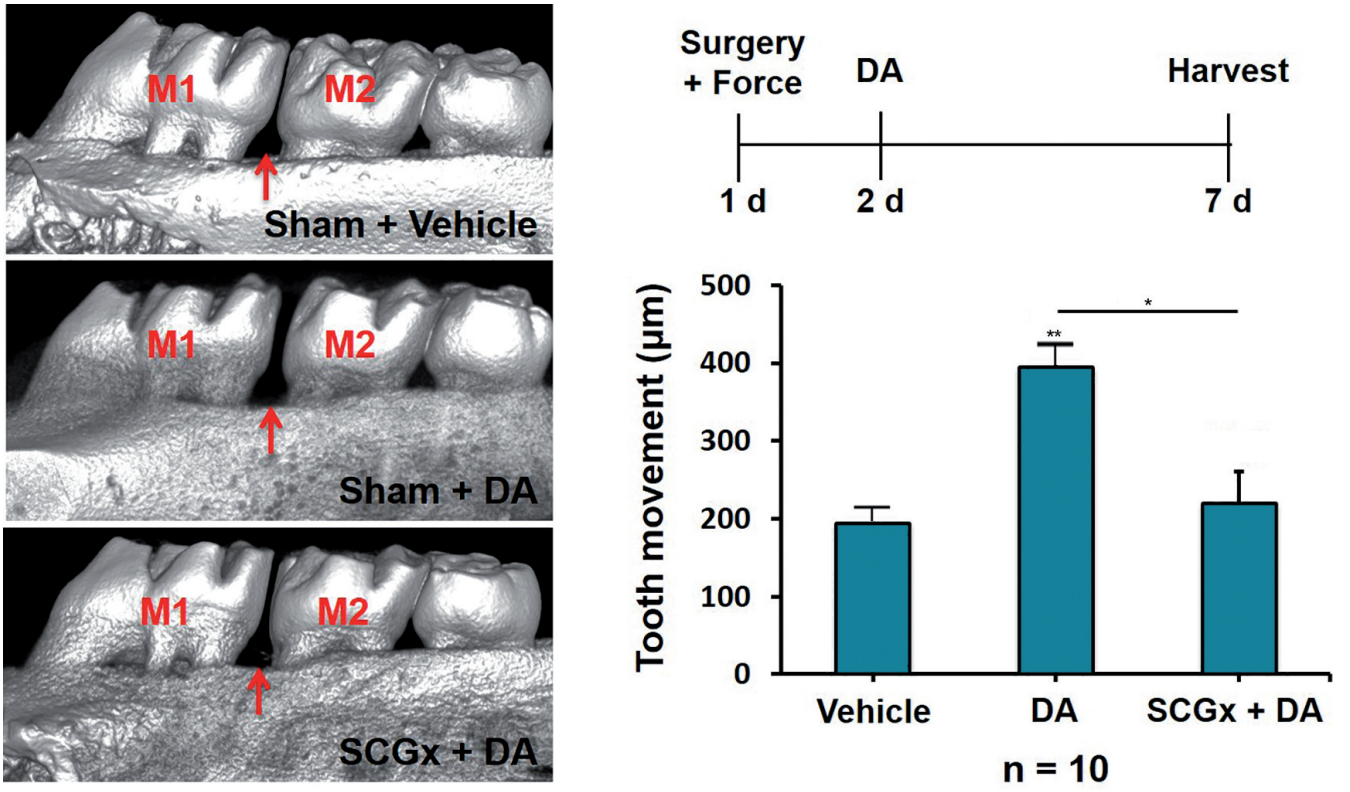

Fig. 3. Stimulation of SNS by DA promoted OTM. The injection of DA to hypothalamus promoted the distance of OTM in rats without SCGx. The injection of DA to hypothalamus of rats with SCGx showed little promotional effect of OTM. Analysis of the OTM distance was performed by micro-computed tomography scanning. The data are expressed as mean ± SD, P value less than 0.05 was considered statistically significant, *P<0.05, **P<0.01, ***P<0.001.

should be evaluated. Therefore, the injection of dopamine (DA), which is a commonly used agent for stimulating SNS, through the buried tube in VMH nucleus of hypothalamus based on both OTM and SCGx rat models were used to investigate the effects of neural stimulation and inhibition of SNS on OTM, respectively. The micro CT results shown in Fig. 3 demonstrate that, compared with the distance (~194 μm) between the first and second molar induced by merely exerting orthodontic force, the stimulation of SNS by DA significantly promoted the OTM process, represented by a distance of ~395 μm. Moreover, the promotional effect of DA stimulation was largely inhibited by SCGx, in which group the distance (~219 μm) was only slightly larger than the SCGx+vehicle group. The above results provided clear evidence that the stimulation of SNS was able topromote the process of OTM, indicating the involvement of SNS in OTM.

Orthodontic force stimulated the up-regulation of tyrosin hydroxylase in the hypothalamus

In order to provide direct evidence of the excitability of SNS upon the stimulation of orthodontic force, the expression of tyrosine hydroxylase (TH), which is a marker of sympathetic excitation in the hypothalamus, was detected through immunofluorescence. As shown in Fig. 4, it took only 2 h of orthodontic force stimulation for the number of TH positive neurons in ventromedial hypothalamic nucleus (VMH) and arcuate nucleus (ARC) of hypothalamus to show an apparent increase. Along with the lengthening time of orthodontic force stimulation, the TH positive neuron number kept increasing and remained at a relatively high level even at 7 days after the application of force. Conclusions could be made that the application of orthodontic force might stimulate the center of SNS in rat models.

Orthodontic force induced up-regulation of norepinephrine in local alveolar bone

Considering that the stimulation of SNS by orthodontic force application and the promotional effect of SNS excitation on OTM have both been proved, the underlying pathway should be further explored. Therefore, ELISA was used to detect the expression of norepinephrine, which is a representation of the excitability of SNS, in local alveolar bone. As shown in Fig. 5, the expression of norepinephrine in alveolar bone was distinctly up-regulated by the exertion of orthodontic force for 24 h. This result indicated that the effect of orthodontic force is not confined to alveolar bone, which could also stimulate the SNS.

ISO treatment promoted osteoclast differentiation in PDLCs and BMCs

Given the above results and our previously reported role of Adrb2 in the regulation of OTM process, isopropyl norepinephrine (ISO), an analogue of norepinephrine, was used to simulate the SNS stimulation induced microenvironment to investigate the effect of SNS stimulation on osteoclast differentiation in human primary culture PDLCs from PDL and bone

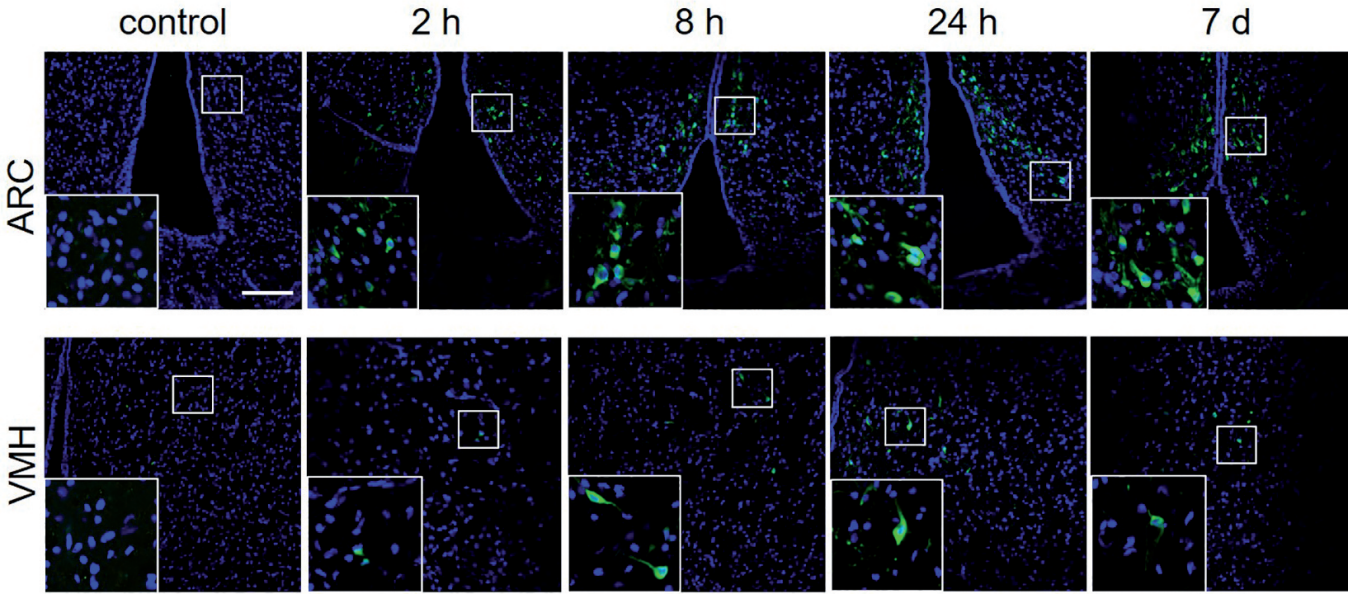

Fig. 4. Orthodontic force stimulated the up-regulation of tyrosin hydroxylase in hypothalamus. Immunofluorescence was used to detect the number of TH positive neurons in ARC and VMH nucleus of hypothalamus. TH was stained by FITC (green fluorescence) and cell nucleus was stained by DAPI (blue fluorescence). Scale bars: 100 μm.

marrow cells (BMCs) from tooth socket bone. As shown in Fig. 6, results of qRT-PCR showed the mRNA expression level of receptor activator of nuclear factor-κ ligand (RANKL), which is also known as osteoclast differentiation factor (ODF), was significantly up-regulated upon the treatment of ISO (0.1 μM) for 6 h in both PDLCs and BMCs. Conversely, the inhibition of the mRNA expression of osteoprotegerin (OPG), a competitive antagonistic factor of RANKL, was observed simultaneously. Accordingly, the ratio of RANKL/OPG increased by over 25-fold, which strongly verified the promoted osteoclast differentiation induced by the stimulation of ISO. Notably, all the effects of ISO on both PDLCs and BMCs might have been erased by further treatment of ICI-118551 (0.1 μM) for 6 h, which is a highly selective β-2 adrenergic receptor antagonist. Collectively, these results elucidated that the stimulation of SNS was able to promote osteoclast differentiation of PDLCs and BMCs through regulation of RANKL/OPG ratio, thus promoting the OTM process.

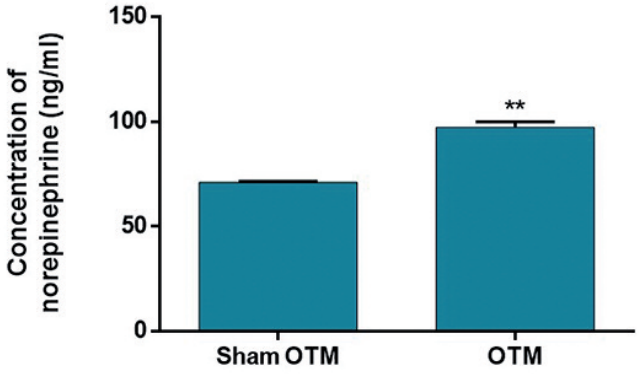

Fig. 5. Application of orthodontic force up-regulated expression of norepinephrine in local alveolar bone. ELISA was utilized to detect the differential expression of sympathetic hormone norepinephrine in local alveolar bone of sham group and OTM group after the application of orthodontic force for 24 h. The data are expressed as mean ± SD, P value less than 0.05 was considered statistically significant, *P<0.05, **P<0.01, ***P<0.001.

Discussion

Until now, although the involvement of osteoblast and osteoclast activity has been proved (Garlet et al., 2008), the molecular mechanism of the orthodontic force induced remodeling of alveolar bone is still largely unknown. It has been revealed that, in orthodontic tooth movement,

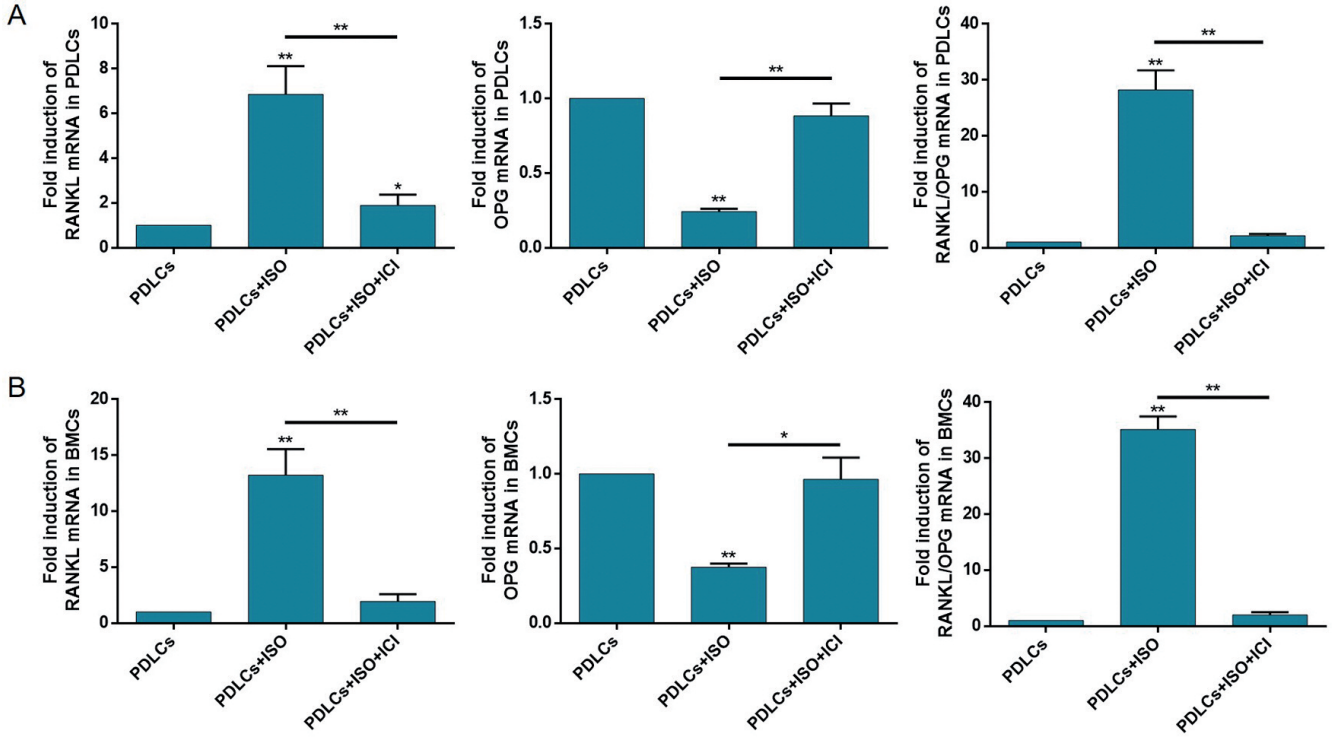

Fig. 6. Treatment of PDLCs and BMCs with ISO up-regulated the mRNA expression of RANKL and the RANKL/OPG ratio. **A.** qRT-PCR results showed the up-regulated mRNA level of RANKL, down-regulated mRNA level of OPG and the increased RANKL/OPG ratio by treatment of ISO (0.1 μM) for 6 h in PDLCs which could be reversed by treatment of ICI-118551 (0.1 μM) for 6 h. **B.** The up-regulated mRNA level of RANKL, down-regulated mRNA level of OPG and the increased RANKL/OPG ratio by treatment of ISO (0.1 μM) for 6 h in BMCs which could be reversed by treatment of ICI-118551 (0.1 μM) for 6 h. The data are expressed as mean ± SD, P value less than 0.05 was considered statistically significant, *P<0.05, **P<0.01, ***P<0.001.

immune responses under compression force promote the generation of inflammatory cytokines, thus affecting the consequent alveolar bone resorption. For example, Karacay et al. reported that heavy interrupted force can cause a rapid release of TNF-α, which can continue for a long time period (Karacay et al., 2007). IL-6, which possesses an autocrine/paracrine activity that stimulates osteoclast formation and bone-resorbing activity, was also proved to be highly expressed during orthodontic process and tightly related to the osteoclast activity of alveolar bone (van Gastel et al., 2011; Kikuta et al., 2016). Except for the immune response, the recently proposed concept of nervous system mediated bone metabolism expanded the understanding of bone remodeling. More importantly, along with the first report of regulation of bone formation by SNS upon the interaction of leptin, which provided direct evidence of the participation of SNS in bone metabolism, the regulatory effect of SNS on bone metabolism has become a focus in the research field of bone metabolism (Ducy et al., 2000; Elefteriou et al., 2005; Masella and Meister, 2006), which was also proved by a series of follow-up studies (Elefteriou et al., 2005). A piece of research reporting the bone loss induced by depression through stimulation of SNS also verified this novel conception (Yirmiya et al., 2006). Moreover, it was also reported that dopamine β-hydroxylase (DBH) knockout mice showed high bone density phenotype compared with wild type mice (Takeda et al., 2002). Considering the possible relationship between PDLCs and nervous system, all these results suggested the potential role of SNS in orthodontic force induced remodeling of alveolar bone through regulation of bone metabolism, thus in the process of OTM.

However, detailed research of the relationship between SNS and OTM was still rarely reported. Importantly, in our previous work, the potential involvement of SNS in the OTM process has been indirectly proved by the up-regulated expression of Adrb2, which is an important receptor in SNS, in PDLCs by exerting orthodontic force (Cao et al., 2014). Moreover, we also found that the stimulation of peripheral nervous system of SNS by ISO can promote OTM which was consistent with another study (Sato et al., 2014). Besides, we were still wondering whether the regulation of the central system of SNS, in which the hypothalamus plays a very important role by secreting catecholamine, could affect OTM. Therefore, in this study, we demonstrated for the first time that the injection of DA, which is a precursor of norepinephrine, into the hypothalamus, as well as the stimulation of SNS, could promote the OTM process. Moreover, the promotional effect can be reversed by SCGx (Kondo et al., 2013), which is a common method to construct sympathetic innervated rats in bone metabolism related studies (Ladizesky et al., 2000; Kim et al., 2009). More directly, the stimulation effect of orthodontic force to the central nervous system of SNS was further proved by the increased number of TH positive neurons in ARC and VMH nucleus of the hypothalamus after the application of orthodontic force. Subsequently, the underlying pathway of SNS to promote OTM was further verified by the up-regulation of expression of norepinephrine in local alveolar bone. Collectively, based on our previous work, all the above results further proved that SNS can be stimulated by the application of orthodontic force and promote OTM.

In the process of OTM, osteoclasts are differentiated and activated induced by osteoblasts and PDLCs, attached to the bone lacunae on the surface of the alveolar bone and realize remodeling of alveolar bone through production of acid and release of enzymes (Li et al., 2016). Actually, osteoclast differentiation related factors play a more direct role in the process of OTM. A large number of studies have shown that the expression of RANKL in PDL increased by orthodontic force, and the expression of osteoprotegerin (OPG) in the distraction side decreased (Nishijima et al., 2006; Andrade et al., 2009). The up-regulation of RANKL/OPG ratio can affect the activation of osteoclast function (Tan et al., 2009). Similarly, in gingival crevicular fluid, the ratio of concentration of RANKL to that of OPG was reported to be significantly higher than in control sites (Nishijima et al., 2006). Moreover, *in vivo* studies showed the presence of RANKL in periodontal tissues during experimental tooth movement of rat models, and that PDLCs under mechanical stress may induce osteoclastogenesis through up-regulation of RANKL expression during OTM (Yamaguchi, 2009). However, there are few reports concerning the effect of SNS stimulation on the expression of RANKL and OPG, and thus osteoclast differentiation. In this study, ISO was used to simulate the SNS stimulation induced microenvironment in PDLCs and BMCs to investigate the effect of SNS excitation on osteoclast differentiation. As expected, the treatment of both PDLCs and BMCs with ISO was able to significantly up-regulate the expression of RANKL and down-regulate that of OPG simultaneously, thus increasing the RANKL/OPG ratio by more than 25-fold.

In summary, this study demonstrated that the application of orthodontic force can lead to the excitation of SNS, which up-regulated the expression of norepinephrine in local alveolar bone, increased the RANKL/OPG expression ratio, thus promoting the process of OTM. This work provides a novel theoretical and experimental basis for further understanding of the molecular mechanism of OTM.

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Conflicts of interest. These authors disclose no conflicts of interest.

Author contributions. BF, XW and YZ made substantial contributions to the concept and design of the present study. HC conducted the experiments. HC conducted data analysis. HC produced the manuscript. All authors read and approved the final manuscript.

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