Abaloparatide and teriparatide enhance mandibular growth in adolescent rats with site-specific effects

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Research Article

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Abstract

Aims

Teriparatide (TPTD) and abaloparatide (ABL) are two osteoanabolic drugs targeting PTH1R signaling. This study aimed to investigate the effects of TPTD and ABL on adolescent mandibular growth.

Method

Seventy 4-week-old male SD rats were randomly divided into 14 groups, treated with intermittent TPDT or ABL at various doses, accompanied by mandibular advancement (MA) or not. 3D printing was used to fabricate an innovative splint for MA. After 4-week treatment, morphological measurement, histological and immunohistochemical analysis were performed. Mandibular condylar chondrocytes (MCCs) were treated with TPTD or ABL, followed by CCK-8 assay, alcian blue staining, RT-PCR and immunofluorescent staining.

Result

In vivo, TPTD or ABL alone increased the condylar length and cartilage thickness, with up-regulated SOX9 and COL II, whilst down-regulated COL X; however, when combined with MA, the promotive effects were attenuated. TPTD or ABL alone increased the mandibular body height and mandibular angle width, whilst increased the mandibular body length and alveolar bone width when combined with MA. In vitro, TPTD or ABL enhanced the MCC proliferation, glycosaminoglycan synthesis, COL II and SOX9 expression, whilst down-regulated COL X, Ihh and PTH1R expression.

Conclusion

Abaloparatide or teriparatide alone increases the mandibular condylar length and cartilage thickness, mandibular body height and mandibular angle width. When combined with MA, they also increase the mandibular body length and alveolar bone width. In general, ABL seems more potent than TPTD.

Clinical Relevance:

Abaloparatide or teriparatide may be potential drugs for growth modification of the mandible, combined with functional appliance treatment or not.

Introduction
Class II malocclusion is a common dentofacial malocclusion, and among the class II patients seeking for orthognathic surgery treatment, as high as 89% are inflicted with mandibular retrusion [1, 2]. Mandibular hypoplasia (MH) not only affects the patient's facial appearance and occlusal function, but also increases the risk of obstructive sleep apnea-hypopnea syndrome (OSAHS) [3], which grievously harms health and growth. Orthognathic surgery can ameliorate severe MH in adulthood; however, before that the patient has to suffer from its detriment both physically and psychologically. Therefore, if MH could be improved, more or less, during the growing period, it would be considerably beneficial for the patient's occlusal function, respiratory capacity, facial appearance and mental health, with the difficulty of surgery reduced as well. For adolescent MH patients with growth potential, functional appliances are recommended by some orthodontists[4]. However, a wide range of clinical studies so far shed light on the frustrating conclusion that functional orthopedic treatment alone has only quite limited effect on promoting real skeletal mandibular growth[5, 6].

The growth sites of mandible involve the condyle, the posterior border of mandibular body, the sigmoid notch, and the alveolar crest[7], among which, intramembranous ossification of the mandibular main body and endochondral ossification of the condyle act as two major growth patterns [8, 9]. The condyle for particular plays an essential role in augmenting mandibular length and height[10] During endochondral ossification of the mandibular condylar cartilages, the differentiation of osteochondral progenitors into chondrocytes is regulated by SRY-related high mobility group-box gene 9 (SOX9)[11]. SOX9 directly triggers the expression of type II collagen (COL II)[12], which is produced by chondrocytes as a major collagen component of cartilage matrix[13]. Another cartilage matrix component, type X collagen (COL X), is synthesized by hypertrophic chondrocytes and its expression indicates termination of chondrogenesis with subsequent initiation of endochondral ossification[14, 15]. The growth and endochondral ossification of mandibular condyle in adolescence could be modulated by a variety of external signals, including mechanical stresses[16] and biomolecules like IGF-1[17], PTH[18], PTHrP[19], Ihh[20], etc. Since mechanical stimulation with functional appliances alone could not greatly increase mandibular growth, it is attractive to attempt the strategy of combining functional orthopedic treatment with medications that have pro-chondrogenic and/or osteoanabolic potentials.

Parathyroid hormone (PTH) is known as a regulator of calcium homeostasis[21]. Its paradoxical dual effects on bone metabolism have been widely studied, that is, continuous administration causes catabolic effects while intermittent dosing is anabolic[22]. Based on the role of PTH in promoting bone anabolism, teriparatide (TPTD), a peptide that is structurally identical to human PTH (1–34), has been approved by FDA as the first osteoanabolic drug for treating osteoporosis[23]. On the other hand, parathyroid hormone related protein (PTHrP), which is originally found in human malignant hypercalcemia, has similar osteoanabolic effects and shares the same type 1 PTH/PTHrP receptor (PTH1R) with PTH[24]. However, the short half-life of PTHrP makes it easy to degrade and therefore difficult to take effect in systemic administration[25]. In order to exploit the function of PTHrP and overcome its unstableness, abaloparatide (ABL), a synthetic analogue of PTHrP (1–34), has been developed and also approved for the treatment of osteoporosis[26]. Although TPTD (or PTH (1–34)) and ABL seem to have similar osteoanabolic effects, their osteolytic potential may discriminate due to
different relative affinities to the RG and R0 conformations of PTH1R, resulting in less bone-resorptive effects of ABL compared to TPTD[24]. In gross, ABL is believed to overcome the disadvantage of easy decomposition of PTHrP (1–34) and attenuate the bone metabolic window loss caused by TPTD.

Regarding cartilage, both PTH and PTHrP exert regulatory effects on mandibular condylar chondrocytes. PTH enhances the chondrocyte proliferation and increase the expression of COL II with a concomitant decrease in COL X and RUNX2, indicating promotion of chondrogenesis and suppression of terminal chondrocyte differentiation[18, 27]. PTHrP plays an essential role in the prenatal and postnatal growth of the condyle[8, 28]. The PTHrP-deficient mice show deformities of the ramus and mandibular body, indicating that PTHrP disruption may impair both intramembranous and endochondral osteogenesis of the mandible[8]. PTHrP may regulate the proliferation, chondrogenic and osteogenic differentiation of chondrocyte by up-regulating the expression of SOX9 and COLII, inhibiting the expression of RUNX2, COLX and ALP[29–31]. In the long bones of limbs, which are predominant with endochondral ossification, the negative feedback loop formed with PTHrP and Indian hedgehog (Ihh) has long been recognized to regulate the lengthening of growth plate. Notably, recent finding revealed skeletal stem cells among PTHrP-positive chondrocytes within the resting zone of the postnatal growth plate.[32] PTHrP might also interplay with Ihh to maintain homeostasis of the mandibular condylar cartilage[33]. Both in vivo and in vitro studies have demonstrated force-induced expressions of Ihh and PTHrP in the mandibular condylar cartilage, suggesting them as mechanotransduction mediators[29, 34].

In brief, PTH and PTHrP are both involved in the regulation of mandibular growth, and their derivative osteoanabolic drugs, TPTD and ABL respectively, may well have the potential to promote growth of the mandible in adolescents. Thus, this study was aimed to investigate the effects of intermittent TPTD or ABL on the mandible growth, with focus on the mandibular condylar cartilage, and furthermore, whether there are additive or synergistic effects when the drugs are combined with mandibular advancement (MA) mechanical stimulation.

Materials And Methods

Animal model and experimental design

The study was approved by the Animal Care and Ethics Committee of the State Key Laboratory of Oral Disease, West China School of Stomatology, Sichuan University, China (No. WCHSIRB-D-2017-259). All the experimental procedures followed the guidelines for animal experimentation of the university/the Animal Research N3CRs guidelines for Reporting of In vivo Experiments (ARRIVE) guidelines.

Seventy male Sprague-Dawley rats (4-week-old, body weight 105g±10g, provided by West China Animal Center, Sichuan University, China) were randomly allocated into 14 groups (n=5 in each), including one control group, one MA group, 6 drug groups and 6 drug+MA groups. The control group received daily subcutaneous injection of normal saline (NS), whilst the MA group received a functional appliance for mandibular advancement with daily subcutaneous injection of NS. The 6 drug groups include the high-
dose TPTD (TPTD\textsuperscript{high}, 8mg/kg), mid-dose TPTD (TPTD\textsuperscript{mid}, 800\μg/kg), low-dose TPTD (TPTD\textsuperscript{low}, 80\μg/kg), high-dose ABL (ABL\textsuperscript{high}, 8mg/kg), mid-dose ABL (ABL\textsuperscript{mid}, 800\μg/kg), and low-dose ABL (ABL\textsuperscript{low}, 80\μg/kg) group, which received daily subcutaneous injection of TPTD or ABL at the corresponding dose level. The 6 drug+MA groups include the TPTD\textsuperscript{high}+MA, TPTD\textsuperscript{mid}+MA, TPTD\textsuperscript{low}+MA, ABL\textsuperscript{high}+MA, ABL\textsuperscript{mid}+MA and ABL\textsuperscript{low}+MA group, which received both mandibular advancement and daily subcutaneous injections of TPTD or ABL at the corresponding dose level (Fig 1). All the subcutaneous injections were given at the back of the neck. All the rats were housed in a constant temperature of 25°C, 12-hour light/12-hour dark environment, and fed with standard soft diet and water ad libitum.

**Design and fabrication of the mandibular advancement appliance**

It is noteworthy that the appliance for mandibular advancement was developed via digital design and 3D printing to optimize the accuracy of mandibular repositioning in the rat model. Firstly, the digital model of an inclined bite splint was designed on Tinkercad software (https://www.tinkercad.com), and then the solid male mold was made through 3D printing. The bite splint is an isosceles trapezoid with a length of 4mm at the top, 8mm at the bottom and a height of 7mm. From side view, it is a straight angle trapezoid with a thickness of 2mm (Fig 2a, b). Innovatively, a smooth groove was designed on the occlusal surface of the guide plate to prevent mandibular deviation during MA (Fig 2a arrow). The male mold was placed in liquid silica gel and then solidified to obtain the template of the bite splint (Fig 2c). Then, resin was filled in the template and bonded on rat upper incisors under anesthesia with 70mg/kg ketamine (Ketavet 100, Intervet Productions Srl, Aprilia, Italy) and 5mg/kg xylazine (Sedaxylan, Eurovet Animals Health B.V., Bladel, Netherlands), making sure that the lingual surface of the upper incisor was in contact with the back wall of the template without left-right or forward-backward deflection (Fig 2d).

**Morphological measurements**

The rats in each group were euthanized using excessive anesthetic injection after 4-week treatment. The rat mandibles were dissected mid-sagittally and divided into left and right halves without muscle tissue attached to the bone surface. The mandibular morphology was measured referring to the reported method[35]. Briefly, pictures of the mandibles were taken using a digital single lens reflex camera (Nikon D700, Japan), which was mounted at 90° angle and at a fixed distance from the samples. The mandibular length and height in these photos were measured with Image-Pro plus (version 6.0, Media Cybernetics, Rockville, MD, USA), while the width of mandible was measured with vernier calipers. Measurement items included the mandibular condylar length, the mandibular body length, the mandibular body height, the mandibular incisal alveolar bone width, and the mandibular angle width, with the length and height calculated as ratio to the whole body length. The selected landmarks and measurement items were shown in Table 1 and Fig 3m.

**Histology and immunohistochemistry assays**
The condyles were harvested after digital pictures taken. The specimens of condyles were fixed in 4% paraformaldehyde for 24 hours and subsequently decalcified in 10% ethylene diamine tetraacetic (EDTA) for 8 weeks at 37°C. Subsequently, gradient-dehydrated, dipping wax, and paraffin-embedding were performed before sectioning condyles at 5 μm thickness to obtain serial sagittal slices.

These slices were stained with safranin O/fast green, or hematoxylin and eosin (HE), for cartilage demarcation and morphological measurement. The condylar cartilage was divided into 4 layers, i.e., the fibrous, proliferative, chondrogenic and hypertrophic layer[10, 36] (Fig 4m). The posterior region of the condyle was selected as region of interest (ROI) [11, 36]. Images of the samples were obtained using a light microscope (Leica DM 2500, Wetzlar, Germany), and thickness of each layer and the whole cartilage were measured using Image-Pro plus6.0.

For immunohistochemistry assay, the slices were incubated with primary antibodies (all from Abcam, 1:200) against SOX9, COL II, and COL X overnight at 4°C. Mean optical density (MOD) in the ROI of each sample was calculated with Image-Pro plus6.0 for the semi-quantitative analysis.

**Mandibular condylar chondrocyte isolation and culture**

Mandibular condylar cartilage was dissected from SD rats within 24 hours of birth. In brief, the mandibular condyles of newborn rats were harvested in penicillin-streptomycin (PS, Hyclone, Wuhan, China) and PBS solution. Subsequently, the condylar cartilages were dissected with microforceps after removing the soft tissue on their surface under a stereomicroscope. Then, cartilages were cut into small pieces followed by sequential digestion with trypsin (0.25%, Gibco, USA) for 20 minutes and type II collagenase (0.2%, Solarbio, Beijing, China) for 1 hour at 37°C. After resuspension and filtration, the chondrocytes were dispersed into single cells and cultured in DMEM/F12 supplemented with 10% FBS (Gibco, USA), and 1% PS under a humidified atmosphere of 5% CO2 at 37°C. Upon confluence, the primary cells were further sub-cultured to passage 1 (P1) for *in vitro* experiments. Alcian blue and safranine O staining were used to identify the mandibular condylar chondrocytes (MCCs).

**CCK-8 assay**

The cell counting Kit-8 (CCK-8) assay (Beyotime, Shanghai, China) was carried out to investigate the effects of TPTD and ABL on MCC proliferation. MCCs (5 × 10^3 cells/well) were seeded in 96-well cell plates and treated with 100 nmol/mL TPTD or ABL, and the media were changed every 24 hours for 3 days. Standard curves were constructed by measuring optical density (OD) value at 450 nm.

**Alcian Blue Staining**

P1 MCCs (5 × 10^4 cells/well) were seeded in 24-well cell plates and treated with 100 nmol/mL PBS, TPTD or ABL for 48 hours. The glycosaminoglycan (GAG) content of MCCs was identified using a standard alcian blue staining kit (Solarbio, Beijing, China). The cells were washed 3 times with PBS, fixed with 4% paraformaldehyde at room temperature for 20 min, again washed 3 times with PBS (3 min each
time), stained with alcian staining solution for 30 minutes, and washed 3 times with PBS. The images were acquired using a stereomicroscope with consistent photographic parameters.

**Total RNA extraction and quantitative real-time PCR analysis**

The P1 MCCs (15 × 10^4 cells/well) were seeded in 6-well cell plates and incubated with 100 nmol/mL PBS, TPTD or ABL for 24 hours. Total RNA was isolated from MCCs using RNA-Quick purification kit (ES Science, Shanghai, China) according to manufacturer's instructions, and PrimeScript RT reagent kit (TaKaRa, Japan) was used for reversed transcription of RNA for cDNA. Quantitative real-time PCR was performed using a SYBER Green qPCR kit (Bimake, USA), and carried on in StepOne Real-Time PCR System (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as an internal control gene to normalize the expression of other genes, including Col2a1, Col10a1, Sox9, Ihh, Pthlh, Pth1r. The results were calculated using the 2−ΔΔCT method and presented as fold increases relative to the negative control. The primer sequences are described in Table 2.

**Immunofluorescence staining**

P1 MCCs (3 × 10^4 cells/well) were seeded on 48-well plates, and incubated with 100 nmol/mL PBS, TPTD or ABL for 24 hours. Then MCCs were washed by PBS and fixed in 4% paraformaldehyde for 30 min, permeabilized in 0.1% Triton X-100 at room temperature for 10 minutes (for COL II, COL X and Ihh ) or 0.5% Triton X-100 on ice for 20 minutes (for SOX9 and PTHrP) and then blocked with 3% BSA at room temperature for 30 minutes, before incubated overnight with rabbit anti-rat COL II (1:200 dilution; Abcam 222 Biochemicals, UK), - COL X (1:200 dilution; Abcam 222 Biochemicals, UK), -SOX 9 (1:200 dilution; Abcam Biochemicals, UK), -Ihh (1:200 dilution; Affinity, China), -PTHrP (1:200 dilution; Affinity, China), and -PTH1R (1:200 dilution; Affinity, China) antibody at 4 °C. The cells were incubated with goat anti-rabbit secondary antibody 225 (1:200, ZSGB-Bio, China) and phalloidine (1:800, Solarbio, Beijing, China)) for 1hour, then washed twice with PBST, and incubated with DAPI for 10min. The MCCs were finally washed with PBS 3 times and analyzed with Leica fluorescence microscope (Leica, Wetzlar, Germany).

**Statistical Analysis**

Data analysis was performed using SPSS version 25.0 (Statistical Package for the Social Sciences, SPSS, Chicago, IL, USA). One-way analysis of variance (one-way ANOVA) with post hoc contrasts by LSD-T test was used to detect differences among the groups. Statistical significance was set as $P< 0.05$. All quantitative data were presented as mean ± standard deviation (SD).

**Results**

ABL and TPTD had synergistic effects with MA mechanical loading to enhance mandibular growth
Morphological measurement showed that the condylar length (Co-Mf) in the TPTD\textsuperscript{high} and ABL\textsuperscript{high} groups were significantly greater than that in the control group, whilst mid- or low-dose TPTD or ABL had no effects on the condylar growth (Fig. 3c, g).

TPTD and ABL also promoted growth of the mandibular body, including its height and length. All doses of ABL, with or without MA, significantly increased the mandibular body height (Mf-MP) (Fig. 3h). In contrast, TPTD could function independently only at high dose, however effective at mid- and low-doses only when combined with MA (Fig. 3d). Moreover, high-dose ABL was more effective than high-dose TPTD in mandibular body height increment. Interestingly, although TPTD, ABL or MA alone had no significant effects on the mandibular body length (Mf-L1), when high- or mid-dose TPTD or ABL was combined with MA, a significant increase in Mf-L1 was observed (Fig. 3e, i), indicating a synergistic effect of the drugs and MA mechanical loading on the growth of mandibular body.

Regarding width, TPTD or MA alone did not elicit significant changes in the lower anterior alveolar bone width (K-K'). However, when TPTD was combined with MA, regardless of the drug dose, the alveolar bone width was significantly increased compared to that of the control or MA group. In contrast to TPTD, high- or mid-dose ABL alone significantly increased the lower anterior alveolar bone width, and when combined with MA, the increase became significantly larger compared to ABL alone (Fig. 3f, j). In addition, when the two drugs were compared, the increase of alveolar bone width in the ABL\textsuperscript{high} group was significantly larger than that in the TPTD\textsuperscript{high} group. Furthermore, high- or mid-dose TPTD or ABL, whether combined with MA or not, significantly increased the mandibular angle width compared with the control (Fig. 3k, l).

Taken together, both ABL and TPTD enhanced the mandibular growth with effects varying with different sites. Of note, synergistic effects of the drugs and MA mechanical loading were shown at the mandibular body and the alveolar bone. In general, ABL demonstrated bone-augmenting effects more powerful than TPTD in the present context.

**Abl Increased The Condylar Cartilage Thickness Even More Than Tptd, Which Was Attenuated By Ma**

HE staining showed that MA, but not TPTD or ABL, significantly increased thickness of the fibrous layer, indicating that thickening of this layer was an adaptive response to the mechanical stimulation of MA (Fig. 4c, i). As for the proliferative layer, only the TPTD\textsuperscript{high} group had a significant decrease in the proliferative layer thickness, while the other experimental groups didn’t differ with the control group (Fig. 4d, j). Moreover, the chondrogenic layer, hypertrophic layer and the total cartilage were all significantly increased in thickness only when high-dose TPTD or ABL was used (Fig. 4e-f, k-l, n-o), and the increase was significant larger in the ABL\textsuperscript{high} group than TPTD\textsuperscript{high} group. However, when high-dose TPTD or ABL was combined with MA, thickness of the chondrogenic layer, hypertrophic layer and total cartilage was lower than that of the drug alone groups (still higher than the control group) (Fig. 4e-f, k-l, n-o).
Collectively, both high-dose ABL or TPTD alone could increase the condylar cartilage thickness, with ABL more effective than TPTD; however, the combination of the drugs with MA attenuated the promotive effects.

**ABL and TPTD promoted chondrogenesis and inhibited hypertrophy of the condylar cartilage, which was partially counteracted by MA**

Immunohistochemistry staining showed that the expression of SOX9 was significantly up-regulated by MA, high-dose TPTD, or ABL of any doses, and the combination of MA and drugs had an additive effect (Fig. 5a-d). However, the COL II expression was significantly up-regulated only in the TPTD<sup>high</sup> and ABL<sup>high</sup> groups, but not in the TPTD<sup>high</sup>+MA or ABL<sup>high</sup>+MA groups (Fig. 5e-h). In addition, the expression of COL X was significantly attenuated in the TPTD<sup>high</sup>, TPTD<sup>mid</sup> and ABL<sup>mid</sup> groups, but not in the corresponding drug + MA groups. (Fig. 5i-l).

Comparing TPTD and ABL, high-dose ABL exerted stronger up-regulation of SOX9 than high-dose TPTD with or without MA; nevertheless, they showed no significant difference in the regulation for COL II or COL X expression.

In summary, ABL and TPTD promoted chondrogenesis and inhibited hypertrophy of the mandibular condylar cartilage, with ABL more effective than TPTD to up-regulating the SOX9 expression; however, these effects were partially counteracted by MA, in terms of the COL II and COL X expression.

**ABL and TPTD enhanced proliferation and chondrogenic differentiation whilst inhibited hypertrophy of MCCs in vitro**

CCK-8 assay showed that both ABL and TPTD significantly enhanced the cell viability of MCCs compared with the control at 48h and 72h, with no significant difference between the two drugs (Fig. 6b). After incubated with ABL or TPTD for 24h, real-time PCR (Fig. 6c-h) showed up-regulated SOX9 and COL II, down-regulated COL X, Ihh and PTH1R, whilst unchanged PTHrP. When comparing the two drugs, ABL was stronger at up-regulating SOX9, while TPTD was better at down-regulating Ihh. The immunofluorescence staining results (Fig. 7) were consistent with the PCR. In addition, alcian blue staining showed significantly increased GAG in the experimental groups compared with the control at 48h (Fig. 6i).

Together, ABL and TPTD enhanced proliferation and chondrogenic differentiation, whilst inhibited hypertrophy of MCCs in vitro, with ABL more effective than TPTD to up-regulating the SOX9 expression.

**Discussion**

For long, functional appliance treatment has been the only clinical approach to promote mandibular growth in adolescence, with quite limited real skeletal effect. It is thus of great importance to pursue for other biotherapies that could also play the role. For instance, growth hormones, sex hormones, insulin,
and insulin-like growth factor-1 have been reported to improve the effects of functional appliance treatment[37]; physical stimulations including low intensity pulsed ultrasound (LIPUS)[38] and light-emitting diode (LED)[39] have been shown to enhance growth or repair of the mandibular condylar cartilage. In the present study, we observed and compared the effects of the two FDA approved osteoanabolic drugs, TPTD and ABL, on promoting mandibular growth in adolescent rats, with or without MA mechanical loading.

The mandible is a composite bone formed via both endochondral ossification at the mandibular condylar cartilage to increase the mandibular length and height, and intramembranous ossification at the mandibular body to increase the mandibular length, height and width[40]. As an essential growth site to increase the mandibular length and height, the condyle was paid special attention to. Morphological analysis showed that TPTD or ABL alone significantly enhanced the condylar growth. Accordingly, the total thickness of the condylar cartilage was significantly increased only when high-dose TPTD or ABL was used, and the increase was significantly larger in the ABL high group than TPTD high group, indicating ABL might be more effective to promote the condylar thickening compared with TPTD. Notwithstanding, the functional appliance MA did not significantly increase the cartilage thickness except that of the brous layer. The finding was largely similar with some previous studies[10], however inconsistent with others[41–43]. Furthermore, combination with MA even attenuated the effects of the two drugs on cartilage thickening. The MA splint used in the present study was made via digital design and 3D printing, which should be more accurate than the previous counterparts. The controversial results among the relevant studies might be due to different designs or regimes of the appliances, as well as different observation time points in each study, which is worth noting in future research. In addition, only ABL and TPTD of high-dose showed effects of condylar cartilage thickening, possibly due to the fact that the cartilage can only absorb limited drugs penetrating into the articular fluid from the blood vessels. Therefore, in future relevant studies, intraarticular injection of the ABL or TPTD could be attempted.

On the other hand, the various effects of the two drugs on different sites of the mandibular body were most interesting. Low-dose ABL alone could increase the mandibular body height, whilst TPTD required combination with MA to play this role; neither ABL nor TPTD could increase the mandibular body length, unless combined with MA; only high- and mid-dose ABL, but not TPTD, increased the mandibular anterior alveolar bone width; high- and mid-dose TPTD or ABL alone increased the mandibular angle width. The increment in mandibular body height, manifested by the accentuated antegonial notch depth (Fig. 3a, b), results in the increased posterior facial height, which might be beneficial to promote counterclockwise rotation of the mandibular growth. Although TPTD or ABL alone could not increase the mandibular body length through intramembrane ossification, they did show an emerging role in increasing the mandibular width. This is in line with the previous observation on long bones, in which the diameter of the femora was improved by PTH while the shaft length was not[44]. In the present study, the mandibular angle width was increased as long as TPTD or ABL was injected, whilst TPTD had no effect on alveolar bone width unless combined with MA. This difference could be explained by the different mechanical environment at the two sites. The lower anterior alveolar bone was covered by mucosal tissue and lacked tendon
attachment. In contrast, there were strong masticatory muscles attaching to the inner and outer sides of the mandibular angle, providing a more active mechanical environment, thus TPTD and ABL could herein promote intramembranous osteogenesis even without additional MA stimulation. The interactions between mechanical stimulation and PTH or PTHrP have been investigated in osteoporosis studies[45–47], which claimed that PTH or PTHrP could function without requiring extra mechanical loading, and the latter just had an additive effect. This might be attributed to gravity and the muscles attached to long bones, which have already established a sufficient mechanical environment, just like the mandibular angle. Taken together, the osteoanabolic effects of TPTD and ABL in the mandibular body seem to be synergistic with mechanical loading, and ABL seems to be more effective than TPTD to augment the mandibular size through intramembranous osteogenesis, especially at the alveolar bone.

Looking back at the condylar cartilage, a wide range of evidences indicate that PTH and PTHrP can enhance the chondrocyte proliferation[18, 19, 27, 36]; however, the HE staining showed unchanged or even decreased thickness of proliferative layers in the present study, which is somewhat counterintuitive. Condylar cartilage is a secondary cartilage, which is distinguished from primary cartilage, such as epiphyseal cartilage, in the modes of cell proliferation[48]. In primary cartilage, cell proliferation occurs in all the non-hypertrophied chondrocytes throughout the cartilage, while cell division only takes place in the prechondroblasts in the superficial layer of the secondary cartilage. The differentiated chondrocytes in condylar cartilage no longer exhibit division ability, which means that all chondroblasts and hypertrophic chondrocytes derive from the differentiation of the upper cells rather than their own replication[49]. In addition, BrdU labeling also demonstrated the cell migration and differentiation from proliferative layer to chondrogenic and hypertrophic layer in the mandibular condylar cartilage[29]. Therefore, although the proliferative layer thickness showed no increase after 4-week TPTD or ABL treatment in this study, significant thickening of chondrogenic and hypertrophic layer suggest the temporarily enhanced expansion of the proliferative layer, which may well have faded at the sacrifice time point (8 weeks of age). In addition, the CCK-8 results in the present in vitro study also supports that TPTD and ABL promote proliferation of the MCCs.

Regarding molecular mechanisms, the immunohistochemical results showed that the expression of SOX9 was up-regulated by TPTD, ABL or MA alone, among which ABL exerted the strongest effect, and when TPTD or ABL was combined with MA, the SOX9 expression was further enhanced. Both TPTD and ABL up-regulated COL II, whilst down-regulated COL X, suggesting that TPTD and ABL could promote chondrogenesis and inhibit chondrocyte hypertrophy, maintaining the young state of the chondrocytes to allow for lengthening the cartilage template for ossification. However, when combined with MA, the TPTD/ABL-induced up-regulation of COL II and down-regulation of COL X were at least partially offset, which was in accordance with the results that TPTD/ABL combined with MA could not increase the cartilage thickness, nor the condylar length. As stated above, the weakening effects of MA to TPTD or ABL on enhancing mandibular condylar growth remain to be expatiated in future.

The findings in vitro were consistent with in vivo, that is, CCK8, real-time PCR and immunofluorescence confirmed that ABL and TPTD could promote proliferation and chondrogenesis, while inhibit hypertrophy
of the MCCs. Moreover, it has been reported that hypertrophic chondrocytes secrete Ihh to trigger the expression of PTHrP in the proliferative and chondrogenic layer; PTHrP acts on cells expressing PTH1R in the superficial layer to keep them in a proliferative state and delay terminal differentiation; simultaneously, Ihh expression is inhibited by PTHrP, thus forming the Ihh-PTHrP negative feedback loop to maintain homeostasis of the condylar cartilage\cite{50, 51}. In the present study, exogenous TPTD and ABL reduced the expression of Ihh, which could promote hypertrophy of MCCs; the endogenous PTHrP expression was not changed, whilst the PTH1R expression of MCCs was suppressed by either TPTD or ABL. PTH (1–34) and PTHrP (1–40) were reported to inhibit the PTH1R expression in normal fetal bovine chondrocytes\cite{52}, and the down-regulation of PTH1R by PTHrP was also observed in normal human knee cartilage\cite{53} and the mouse chondrogenic ATDC5 cell line\cite{54}. The present result was consistent with the previous studies above, and the down-regulation of PTH1R may attenuate the effects of exogenous TPTD and ABL to some extent.

Conclusions

Intermittent ABL or TPTD promotes mandibular growth in adolescent rats, which is site-specific (Fig. 8). At the endochondral ossification site, they increase the mandibular condylar length and cartilage thickness via promoting chondrogenesis. At the intramembranous ossification sites, they enhance the mandibular body growth most evidently at the mandibular angle, which is mechanically hyperactive, with increase of the mandibular body height and angle width. When combined with MA mechanical loading, they also increase the mandibular body length and mandibular anterior alveolar bone width. Regarding the effects mentioned above, ABL seems more potent than TPTD in general.

Declarations

Author contribution Conceptualization and design: Ruyi Wang and Yu Li; methodology: Ruyi Wang, Hui Qiao and Yuran Qian; writing — original draft preparation: Ruyi Wang; writing — review and editing: Yu Li and Gehua Zhen. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Ethical approval All applicable international and institutional guidelines for care and use of animals were followed and approved by the Ethics Committee (No. WCHSIRB-D-2017-259).

Conflict of interest The authors declare no competing interests.

References


31. Jiang J, Leong NL, Mung JC, Hidaka C and Lu HH (2008) Interaction between zonal populations of articular chondrocytes suppresses chondrocyte mineralization and this process is mediated by PTHrP. Osteoarthritis Cartilage 16:70–82 https://doi.org/10.1016/j.joca.2007.05.014


Tables
Table 1
Definition of landmarks and measurement items

<table>
<thead>
<tr>
<th>Landmarks</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>The most anterior point of the lingual alveolar bone of lower anterior teeth</td>
</tr>
<tr>
<td>Mf</td>
<td>The midpoint of mandibular foramen</td>
</tr>
<tr>
<td>A</td>
<td>The most anterior point of the condylar</td>
</tr>
<tr>
<td>B</td>
<td>The most posterior point of the condyle</td>
</tr>
<tr>
<td>C</td>
<td>The midpoint between A and B</td>
</tr>
<tr>
<td>Co</td>
<td>The intersection point of the extension line of Mf-C and outer contour of the condyle</td>
</tr>
<tr>
<td>Me</td>
<td>The most posterior point where the digastric muscle attaches to the lower margin of the mandible</td>
</tr>
<tr>
<td>K</td>
<td>The most prominent point of the alveolar bone of the lower anterior teeth</td>
</tr>
<tr>
<td>K'</td>
<td>The projection of point K on the inner side of the mandible</td>
</tr>
<tr>
<td>M</td>
<td>The most posterior point of angular process</td>
</tr>
<tr>
<td>M'</td>
<td>The projection of point M on the inner side of the mandible</td>
</tr>
<tr>
<td>MP plane</td>
<td>The plane tangent to the mandibular lower margin through Me</td>
</tr>
</tbody>
</table>

Measurements

<table>
<thead>
<tr>
<th>Length</th>
<th>Co-Mf (mm)</th>
<th>The condylar length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mf-L1 (mm)</td>
<td>The mandibular body length</td>
</tr>
<tr>
<td>Height</td>
<td>Mf-MP (mm)</td>
<td>The distance from Mf to MP plane represents the mandibular body height</td>
</tr>
<tr>
<td>Width</td>
<td>K-K' (mm)</td>
<td>The mandibular incisal alveolar bone width</td>
</tr>
<tr>
<td></td>
<td>M-M' (mm)</td>
<td>The mandibular angle width</td>
</tr>
</tbody>
</table>
Table 2
The nucleotide sequences of primers for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5′–3′)</th>
<th>Reverse (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col2a1</td>
<td>CACGCTCAAGTCGCTGAACAA</td>
<td>TCAATCCAGTAGTCTCCGCTCT</td>
</tr>
<tr>
<td>Col10a1</td>
<td>ACAAGAGCGGACAGAGACC</td>
<td>AGAAGGACGAGTGGACATAC</td>
</tr>
<tr>
<td>Sox9</td>
<td>TCCAGCAAGAACAAGCCACA</td>
<td>TGCCCATTCTTCACCAGCCT</td>
</tr>
<tr>
<td>Ihh</td>
<td>AAACCTCGTCCTCTTGCTTA</td>
<td>AAACCTCGTCCTCTTGCTTA</td>
</tr>
<tr>
<td>Pthlh</td>
<td>GCTTGGTGCAGGCTAAACAC</td>
<td>TTTTGTTTGGAGGACAGGTT</td>
</tr>
<tr>
<td>Pth1r</td>
<td>TAAGCTTCGGGACCAATGC</td>
<td>AGCGGCACAGAGCAACACT</td>
</tr>
</tbody>
</table>

Figures

Figure 1
The study flow chart. Briefly, after 7-day acclimation to the laboratory environment, seventy 4-week-old SD rats were randomly divided into 14 groups, and the experimental groups were treated with TPDT or ABL alone at different doses, mandibular advancement (MA) alone, or combination of the drug and MA.

**Figure 2**

The design, fabrication and wearing of the innovative mandibular advancement (MA) appliance. a The digital design of the inclined bite splint, with a central groove (orange arrows) to prevent mandibular deviation during advancement. b The 3D-printed male mold. c The template made of silica gel. d The frontal and lateral views of the bite splint in the rat mouth. e Three-dimensional reconstruction of CBCT showed mandibular advancement with the bite splint.
Figure 3

Morphological measurement and analysis of the mandible. **a, b** Photographs show the mandible morphology of the TPTD-treated (**a**) and ABL-treated (**b**) groups. **c, g** The condylar length (Co-Mf). **d, h** The mandibular body height (Mf-MP). **e, i** The mandibular body length (Mf-L1). **f, j** The mandibular lower anterior alveolar bone width (K-K’). **k, l** The mandibular angle width (M-M’). **m** Illustration of landmarks and measurement items (see Table 1 for definitions).
Figure 4

Histological staining and thickness measurement of the mandibular condylar cartilage. HE and saffron O staining of the TPTD-treated groups (a-b) and the ABL-treated groups (g-h). Thickness analysis of the fibrous layer (c, i), proliferative layer (d, j), chondrogenic layer (e, k), hypertrophic layer (f, l), and total cartilage (n, o). m Schematic diagram of the various layers of condylar cartilage. (*Significant difference of $p<0.05$, scale bar=200μm).
Figure 5

Immunohistochemical staining and semi-quantitative analysis. a-d SOX9, e-h COL II, and i-l COL X expression after TPTD or ABL treatment with or without MA. (*Significant difference of $p \leq 0.05$, scale bar=200μm).
Figure 6

The proliferation and mRNA expression of the mandibular condylar chondrocytes (MCCs). **a** Morphology of the primary MCCs and identification with alcian blue and saffron O staining. **b** Cell viability of the MCCs at 24, 48, and 72 hours. mRNA expressions of **c** SOX9, **d** COL II, **e** COL X, **f** Ihh, **g** PTHrP, and **h** PTH1R of the MCCs after 24-hour TPTD or ABL treatment. **i** Alcian blue staining of the MCCs treated with TPTD or ABL for 48 hours. (*Significant difference of $p$ < 0.05).
Figure 7

Immunofluorescent staining for **a** SOX 9, **b** COL II, **c** COL X, **d** Ihh, **e** PTHrP and **f** PTH1R of the MCCs after 24-hour TPTD or ABL treatment.
Figure 8

Summary for the growth promotive effects of intermittent ABL or TPTD at various sites of the mandible in adolescent rats, accompanied with MA mechanical loading or not.