Asperosaponin VI Injection Enhances Orthodontic Tooth Movement in Rats

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Background: This study was performed to investigate the effect of local injection of asperosaponin VI (ASA VI) on the orthodontic tooth movement in rats.

Material/Methods: A total of 64 healthy female Sprague-Dawley rats were selected and divided into 2 groups randomly: the ASA VI group and the control group. For the ASA VI group, 10 mg/kg ASA VI solution was injected into buccal submucoperiosteal of bilaterally first maxillary molars, and the same volume of normal saline was given to the control group. The orthodontic force was applied to the maxillary first molars. All rats were sacrificed on days 3, 7, or 14. Tooth movement effects on the periodontium were analyzed through hematoxylin and eosin (H&E) staining, tartrate-resistant acid phosphatase (TRAP) staining and immunohistochemistry analysis. Tooth movement measurements and alveolar bone volumetric changes were analyzed using a micro-computed tomography (CT) scan. Molecular changes were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot.

Results: The ASA VI group presented with a significant increase of tooth movement, osteoclast number, and the expression of osteoclast differentiation factor (ODF) compared with the control group. ASA VI also induced a significant decrease in bone volume and density and an increase in trabecular spacing and RANKL (receptor activator of nuclear factor kappa-B ligand) expression at the compression side. Furthermore, ASA VI stimulated bone formation on the tension side by enhancing OCN (osteocalcin) expression and RUNX2 (runt-related transcription factor 2) expression, increasing bone volume and density and decreasing in trabecular spacing.

Conclusions: Injection of ASA VI may accelerate tooth movement via increasing the activity of osteoclasts, stimulating bone resorption at the compression side. Furthermore, ASA VI has a positive effect on bone formation at the tension side.

MeSH Keywords: Bone Remodeling • Medicine, Chinese Traditional • Tooth Movement

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Background

Generally speaking, the duration of orthodontic treatment is 24 to 36 months [1]. Some complicated or adult cases may require an even longer time to achieve the desired results [1,2]. Prolonged treatment time may increase the risk of side effects such as dental decalcification, root resorption, gingivitis, and periodontitis [3,4]. Therefore, how to accelerate tooth movement is of great concern for orthodontists. Orthodontic tooth movement is achieved through bone resorption mediated by osteoclasts on the pressure side and new bone formation mediated by osteoblasts on the tension side [5]. Therefore, enhancing alveolar bone remodeling can accelerate orthodontic tooth movement. Many methods have been tried to shorten the treatment time, including physical therapy (such as laser [6] and low-intensity pulsed ultrasound [7]), drug therapy (such as local injection of 1,25-dihydroxy vitamin D and prostaglandin E$_2$ [PGE$_2$] [8]) and surgical therapy [9]. The laser and low-intensity pulsed ultrasound therapy are high cost. Local injection of 1,25-dihydroxy vitamin D and PGE$_2$ has systematic effects on body metabolism. Surgical therapy has a harmful effect on periodontal tissues and patient’s acceptance of this procedure is low. Hence the researchers are always looking for minimal side effects and low costing methods to enhance the orthodontic tooth movement.

Recently traditional Chinese medicine (TCM) has attracted widespread concern of scholars for its low price, little side effects and suitable for long time use. Dipsacus asper Wall (commonly known as Chuanxuduan) has been one of the most widely used traditional herbal medicine for the therapy of rheumatoid arthritis, low back pain, bone fractures and bone strengthening [10]. It has been reported that Dipsacus asper Wall extract could increase bone mineral density and ameliorate bone histomorphology in rats [11,12]. As the chemical constituents of Dipsacus asper Wall are complicated, the Dipsacus asper Wall may act on multi-targets in clinical treatment. It was difficult to detect the mechanism. Several chemical constituents have been isolated from Dipsacus asper Wall [13]. ASA VI (asperosaponin VI, C$_{47}$H$_{76}$O$_{18}$; molecular weight: 929.10; molecular structure seen in Figure 1) is the main active ingredient separated from Dipsacus asper Wall. In vitro studies revealed that ASA VI enhanced osteogenesis and bone formation [14]. However, the effect of ASA VI on orthodontic tooth movement has not been studied so far. The aim of this study was to investigate the effects and mechanisms of ASA VI on alveolar bone remodeling in orthodontic tooth movement and consequently to provide a theoretical basis for accelerating orthodontic tooth movement by application of TCM.

Material and Methods

Reagents

ASA VI was obtained from Shanghai Baoman Biotechnology Co., Ltd., purity (HPLC) 98% (China). The hematoxylin and eosin (H&E) staining kit and tartrate-resistant acid phosphatase (TRAP) staining kit were purchased from Solarbio Science Technology (China). Primary antibodies against ODF (osteoclast differentiation factor) and tissue total protein lysis buffer were obtained from Boster Biotech (China). RNA extract kit was purchased from Axygen Scientific Inc. (USA). Reverse Transcriptase kit and SYBR Green Premix Ex Taq were obtained from TaKaRa Biotech (Tokyo, Japan). Primary antibodies against OCN (osteocalcin) and RANKL (receptor activator of nuclear factor kappa-B ligand) were purchased from Abcam Inc. (Cambridge, UK). Primary antibodies against RUNX2 (runt-related transcription factor 2) and β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ASA VI solution was dissolved in 0.9% normal saline, stored at 4°C in the dark.

Experimental animals

All animal experiments were performed according to the guidelines for animal research of National Institutes of Health (NIH) and approved by the Shandong University Animal Care and Use Committee (201302065).

Figure 1. The molecular structure of asperosaponin VI.
Sixty-four healthy female 8-week-old Sprague-Dawley (SD) rats weighing 180 to 200 g were chosen from Experimental Animal Center of Shandong University. All rats were raised in individually ventilated cages (IVC), at 25°C, the humidity of 56%, with a 12-hour artificial light/dark cycle, regular ultraviolet (UV) disinfection and ventilation, indoor noise control below 60 dB. They were fed with sterilized solid diet (0.1% calcium, 0.4% phosphorus, 2000 IU/kg vitamin D) and sterilized water.

The rats were divided into 2 groups randomly after adaptive feed for 1 week, designated as the control group and ASA VI group. Rats in the ASA VI group were treated with 10 mg/kg ASA VI solution through injection into the buccal submucoperiosteal of bilaterally first maxillary molars every day during the orthodontic tooth movement period, the control group received the same volume of normal saline followed the same protocol.

Tooth movement

The animals were anesthetized by intraperitoneal injection of 10% chloral hydrate (3.3 mL/kg) and supplemented as needed. After anesthesia, the rats were immobilized on the operation table in the supine position. Head and extremities were fixed, respectively. A nickel titanium (Ni-Ti) closed-coil spring (0.012 inch in diameter and 4 mm in length) was set between both sides of the first maxillary molar and the maxillary incisors to move the first molar mesially. The maxillary incisors on both sides of all animals were connected together with a self-made individual band as a whole to strengthen the anchorage force and stabilize orthodontic force direction. The Ni-Ti coil was fixed with 0.20 mm stainless steel wire ligature, one head was attached around the first maxillary molar, and the other head was fixed to the incisors band. The coil spring produced a force of 40 g on the molar teeth after activation. (Figure 2 shows the orthodontic appliance employed in this study.) The force magnitude was measured three times with an orthodontic stress and tension gauge (3M 807-006 model, USA, accurate to g) in the mouth. Since the appliances were activated, the food was replaced with powder in order to reduce the drop-off rate of orthodontic appliances. The appliances were checked every day and re-secured if needed.

Micro computed tomography (micro-CT) scanning

Four rats were selected randomly from each group on days 0, 3, 7, and 14 of tooth movement. The rats were anesthetized with excess 10% chloral hydrate (4 mL/kg) and transcardially perfused with 200 mL 0.9% normal saline followed by 300 mL 4% paraformaldehyde. The soft tissue and muscle surrounding the maxillae were removed. Subsequently, the isolated maxillae containing teeth were fixed in 4% paraformaldehyde and stored in 70% alcohol solution at room temperature. These samples were scanned using a micro computed tomography (micro-CT) scanner (Quantum GX2 micro-CT, PerkinElmer, USA). The scanning condition was 70 kV, 142 μA, the layer thickness was 18 μm. Reconstruction of the raw image data was done using the built-in software. The measured parameters contain the amount of tooth movement, bone mineral density (BMD), bone volume/total volume (BV/TV) and trabecular separation (Tb.Sp), according to previous studies [15,16]. The nearest distance between the first molar crown and the second molar crown was measured in a 2-dimensional transverse section as the amount of tooth movement (Figure 3A). We selected 2 rectangular volumes 200×400×1400 μm (width×thickness×height) of the alveolar bone adjacent to the mesiobuccal and distobuccal roots as the region of interest (ROI, Figure 3B, 3C) [17]. The measured parameters in the tension sites and compres-sion sites were measured, respectively. During the analysis, all samples were randomly named and assigned to ensure the blindness of the experiment.

Structural and morphometric analysis

After micro-CT scanning, the 8 maxillae of each group on days 3, 7, and 14 were then decalcified in 10% EDTA solution for 3 months, washed in buffer, dehydrated in a series of different ethanol concentrations, embedded in paraffin molds. Slices of 5 μm thickness were cut parallel to the first maxillary molar axis in the sagittal direction. Hematoxylin and eosin (H&E) staining, tartrate-resistant acid phosphatase (TRAP) staining and immunohistochemical analysis were performed on each sectioned slice and were observed under a light microscope. Images were taken and the number of osteoclasts was observed.

Real-time polymerase chain reaction (RT-PCR) and western blot analysis

Six rats in each group were sacrificed by cervical dislocation for quantitative real-time polymerase chain reaction (qRT-PCR) and western blot detection on days 3, 7, and 14. Bone and root tissue removed from the mesial and distal aspect of the
first maxillary molars were taken as the compression side and tension side. Then tissues were washed in pre-chilled normal saline and crushed in liquid nitrogen.

Total RNA was isolated from tissues using RNA extract kit (Axygen Scientific Inc., USA), quantified at 260 nm by the NanoDrop (Thermo Scientific, USA) and reversed into cDNA using reverse transcriptase kit (TaKaRa, Japan), then quantitative qRT-PCR was performed using SYBR Green Premix Ex Taq (TaKaRa, Japan). GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as an internal control, $2^{-\Delta\Delta Ct}$ method was used for data processed. The primer sequences for the RT-PCR are listed in Table 1.

Total protein was extracted using lysis buffer (Boster Biotech, China), supplemented with a phosphatase inhibitor cocktail kit and a protease inhibitor cocktail kit. Protein concentration determination by the bicinchoninic acid (BCA) method. The protein extracts were size-fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene fluoride (PVDF) membrane. After blocking, the PVDF membrane was incubated with the primary antibodies against OCN, RANKL (Abcam, USA) and RUNX2 (Santa Cruz, CA, USA) and rabbit anti-β-actin (Proteintech, China), followed by horseradish peroxidase-conjugated anti-rabbit secondary antibodies. Enhanced chemiluminescence (ECL) technique was performed to identify protein expression. Densitometry analysis of western blots was performed using the NIH-ImageJ program (NIH, USA).

**Results**

There was no difference in the body weight among the experiment group and the control group, and there were no adverse reactions observed during the experiment period.

**Tooth movement measurements (Figure 4)**

The ASA VI group and the control group exhibited similar tooth movement. A phase of rapid tooth movement was detected during the first 3 days, and then tooth movement decreased from day 3 to 7, followed by a linear phase of tooth movement after day 7. However, the amount of tooth movement in the ASA VI group was greater than the control group from the beginning to 14 days, which was approximately 1.2 times greater.
greater at day 3, 1.44 times greater at day 7, and 1.54 times greater at day 14. A significant difference was found between the ASA VI group and the control group on day 7 and day 14 ($P<0.05$). These results indicated that ASA VI is an effective drug for promoting tooth movement.

**H&E staining**

Light microscopic findings of the periodontal tissue showed no pathologic alveolar bone resorption or necrosis associated with tooth movement was found in each group. On the tension side, the periodontal ligament width increased, telangiectasia, osteoblast appearance, and new bone formed. Besides, considerable new bone formation in the ASA VI group compared with the control group. On the compression side, the periodontal ligament width decreased, absorption of the alveolar bone and hyaline degeneration were confirmed, more evident bone resorption lacunae on the alveolar bone surface of the compression side in the experimental group than in the control group in Figure 5. Furthermore, we observed increased dilatation of the blood vessels and proliferation of the fibroblasts in the periodontal ligament in the ASA VI group than the control group.

**Quantification of TRAP-positive cells**

Changes in TRAP positive osteoclasts of the periodontal ligament in the compression side are shown in Figure 6A. TRAP positive osteoclasts identified as red-stained cells along the loaded root surface were observed. We next investigated the number of osteoclasts. Comparisons in the number of osteoclasts are shown in Figure 6B. From day 3, osteoclasts emerged along the border of alveolar bone in all groups. The number of osteoclasts gradually increased with the extension of time and peaked on day 14. From the beginning to 14 days, the ASA VI group had significantly more TRAP-positive osteoclasts on the compression side compared with the control group ($P<0.05$).

**Immunohistochemical staining**

ODF is a ligand for osteoclastogenesis-inhibitory factor, which is an import signal for osteoclast progenitors differentiate into osteoclasts. In order to quantitatively determine bone resorption on the compression side, we performed immunohistochemical staining to observe the expression of ODF (Figure 7A). The expression of ODF gradually increased with the extension of time, peaked on day 14 (Figure 7B). The expression of ODF in the experimental group was significantly higher than the control group on day 7 and day 14 ($P<0.05$).

**Micro-CT analysis**

Evaluation of trabecular bone in the compression side and tension side at different time points are showed in Figure 8. On the compression side, the bone mineral density (BMD) in the ASA VI group was significantly lower than the control group on day 7 and day 14 ($P<0.05$). The ASA VI group also showed lower bone volume to total volume ratio (BV/TV) compared to the control group on day 7 ($P<0.05$). However, there was no significant difference in BV/TV between the groups on day 14. Increased trabecular separation (Tb.Sp) was observed in the ASA VI group at day 7 and day 14 ($P<0.05$). Greater osteoclast activity was observed in the compressed side of ASA VI after day 7.

On the tension side, BMD in the ASA VI group was greater than the control group at day 7 and day 14 ($P<0.05$). BV/TV ratio was significantly higher in the ASA VI group at day 7 and day 14 ($P<0.05$). The ASA VI group showed less trabecular separation and larger trabecular thickness at day 7 and day 14 ($P<0.05$). No significant difference was found for BMD, BV/TV and Tb.Sp between the 2 groups on day 3. ASA VI group showed significantly increased BMD and BV/TV but significantly decreased Tb.Sp at the tension side of alveolar bone than the control at day 7 and day 14.

**ASA VI increased RANKL expression on the compression side**

At the mesial aspect of the first maxillary molar, the qRT-PCR (Figure 9A) and western blot data (Figure 9B–9D) showed that ASA VI significantly increased RANKL expression on days 3, 7, and 14 compared to the control group. And the expression...
of RANKL mRNA was upregulated at all time points and increased with treatment time with the highest level occurred on day 14. (The original western blot images are shown in Supplementary Figure 1A–1C).

ASA VI increased OCN, RUNX2 expression on the tension side

At the distal aspect of the first maxillary molar, the qRT-PCR (Figure 10A, 10B) and western blot data (Figure 10C–10E) showed that ASA VI significantly increased OCN and RUNX2 expression.
Figure 6. (A) Tartrate-resistant acid phosphatase (TRAP) staining at the mesial periodontium of the first maxillary molar (400×). a – alveolar bone; p – periodontal space; r – root. Black arrows indicate TRAP-positive osteoclasts. Bar=20 μm. (B) The number of TRAP-positive osteoclasts on the compression side. Data are expressed as mean±standard deviation (SD); * P<0.05.

Figure 7. (A) Photomicrography of the osteoclast differentiation factor (ODF) immunostaining at the mesial periodontium of the first maxillary molar (400×). p – periodontal space; r – root. Bar=20 μm. (B) Graphical representation of the ODF expression on the compression side of all studied groups. Data are expressed as mean±standard deviations (SD); * P<0.05.
expression on days 3, 7, and 14 compared to the control group. And the expression of OCN mRNA and RUNX2 mRNA of the ASA VI group were upregulated at all time points and increased with treatment time with the highest level occurred on day 14. (The original western blot images are shown in Supplementary Figure 1D–1F).

Figure 8. BMD (bone mineral density), BV/TV (bone volume and total volume ratio), Tb.Sp (trabecular separation) at the compression side and tension side. Data are expressed as mean±standard deviation (SD); * P<0.05.

Discussion

Dipsacus asper Wall has long been used as an anti-osteoporosis, analgesic, and anti-aging agent for the therapy of rheumatic arthritis, low back pain, traumatic hematoma, and bone fractures [10]. The chemical constituents of Dipsacus asper Wall are complicated. According to its chemical structures, it can be classified into 5 categories. The representative compound of Dipsacus asper Wall is ASA VI which enhanced osteogenesis and bone formation [14]. However, the effect and mechanism of ASA VI promoting orthodontic tooth movement is
unknown. In the present study, we investigated the effects of ASA VI on orthodontic tooth movement in rats and explored the possible mechanism involved. The therapy dose we chose was 10 mg/kg, a dose proven efficient in protecting the heart from ischemia injury [18].

Experimental tooth movement was achieved according to the methods of previous studies, which were proven to be feasible and effective [19–21]. Our appliance differed from prior studies in that the maxillary incisors were connected together with a self-made individual band as a whole, to strengthen the anchorage force and stabilize orthodontic force direction. By using this procedure, a fully controlled and reproducible force can be applied. A mesial directed force of 40 g to the first maxillary molar was applied in our experiment based on previous studies [22, 23].

Our results showed the amount of tooth movement in the ASA VI group was significantly greater than the control group on day 7 (1.44-fold) and day 14 (1.54-fold). Thus, ASA VI had an effective role to play in promoting tooth movement. Orthodontic tooth movement velocity depends on the ability of bone remodeling, i.e., bone absorptive capacity of osteoclasts on the compression side and osteogenic capacity of osteoblasts on the tension side. The first step in bone remodeling is resorption by osteoclasts [24]. In addition, the rate of a tooth moves through the bone is primarily restricted by the number and activity of the osteoclasts [25]. TRAP is usually found in osteoclasts, and has been regarded as a marker of bone remodeling because it can promote intracellular collagen degradation in osteoclasts, and accelerate the adhesion and migration of osteoclasts when secreted in the extracellular matrix [26]. TRAP staining has been used as histochemical labeling for osteoclasts. ODF, a ligand for osteoclastogenesis-inhibitory factor, is an important signal for osteoclast progenitors differentiate into osteoclasts [27]. In order to further elucidate the effect of ASA VI on osteoclastogenesis during orthodontic tooth movement, we used immunohistochemistry analysis to examine the expression of ODF. The number of TRAP-positive cells and the expression of ODF in the ASA VI group was significantly higher compared to the control group from day 7 to day 14. The observed significant increase of the orthodontic tooth movement rate in the ASA VI group corresponded to the influence on the increased osteoclast number and osteoclast differentiation.

Considering that orthodontic tooth movement creates both a tension side and a compression side in the periodontium,
micro-CT analysis, mRNA expression, protein expression, bone resorption, and deposition markers were analyzed separately. Micro-CT analysis showed that on the compression side BMD and BV/TV decreased with the increase of tooth movement while Tb.Sp increased in the 2 groups. BMD and BV/TV were lower while Tb.Sp was higher in the ASA VI group compared to the control group on day 7 and day 14. These results suggested that in the ASA VI group greater osteoclast activity enhanced bone metabolism, reducing the bone density of the compression side and promoting tooth movement. This hypothesis was also confirmed by the increase of RANKL expression at the compression side for the ASA VI group on day 7 and day 14. RANKL is an essential cytokine for osteoclast differentiation [28]. The role of RANKL is to promote the activation, differentiation, and formation of osteoclasts, thus increasing bone absorption. On the tension side, BMD and BV/TV were higher while Tb.Sp was lower in the ASA VI group compared to the control group on day 7 and day 14, indicating stimulation of osteoanabolic activity and increased new bone formation, which was in accordance with higher expression of OCN and RUNX2. As an important transcription factor, RUNX2 has a profound impact on the differentiation of osteoblasts in the bone microenvironment [29]. The expression of RUNX2 increased with the degree of osteogenic differentiation. OCN is considered to be a terminal marker of bone regeneration and plays a very important role in bone mineralization [30,31]. It is present study, the expression of RUNX2 and OCN in the tension side increased with the increase of tooth movement, and the expression level was also significantly higher than that in the control group. These results indicated that ASA VI can modulate the bone metabolism state in rats, leading to the appearance of osteoblast markers, increasing osteoblast activity, and activating osteogenesis.

Figure 10. (A) mRNA expression levels of RUNX2 (runt-related transcription factor 2) at different time points in the tension side. (B) mRNA expression levels of OCN (osteocalcin) at different time points in the tension side. (C) Protein expression levels of RUNX2 and OCN in the tension side on day 3. (D) Protein expression levels of RUNX2 and OCN in the tension side on day 7. (E) Protein expression levels of RUNX2 and OCN in the tension side on day 14. Data are expressed as mean±standard deviation (SD) * P<0.05.
In this study, it was found that local injection of ASA VI increased osteoclastogenesis on the compression side and osteoblast activity on the tension side during orthodontic tooth movement. A previous study reported ASA VI could promote angiogenesis and accelerate wound healing by upregulating the HIF-1α/VEGF pathway in rats [32]. Park et al. showed that overexpression of HIF-1α significantly increased the activity of RANKL promoter and enhanced osteoclastogenesis in the compression side of orthodontic tooth movement [33]. Vascular endothelial growth factor (VEGF) is the primary mediator of angiogenesis, which can increase vascular permeability, plays an important role in periodontal ligament remodeling and participates in bone absorption and formation during orthodontic tooth movement [34]. Several studies reported that VEGF was involved in the recruitment of osteoclasts and bone resorption in the compression side during orthodontic tooth movement [35–37]. In the presence of osteoblasts, hypoxia induces PBMNC to differentiate into osteoclasts via upregulated HIF-1α, which will occur on the compression side under orthodontic loading [38]. VEGF expression was also detected in osteoblasts on the tension side during orthodontic tooth movement [37]. Under mechanical stress, osteoblasts released VEGF, which stimulated bone remodeling, and osteogenesis [39]. The present study showed that a significant increase of RANKL expression was seen at the compression side and a significant increase of RUNX2 and OCN expression were seen at the tension side. We hypothesize that ASA VI increases levels of HIF-1α and VEGF and that increases RANKL expression, osteoclast numbers, osteoclastogenesis in the compression side and osteogenesis in the tension side. On the other hand, the upregulating of VEGF activates endothelial cells, increases vascular permeability. Interestingly, increased dilatation of the blood vessels and proliferation of the fibroblasts in the periodontal ligament in the ASA VI group than the control group was observed in this study. The increased angiogenesis brings bone-forming progenitors, osteoclast progenitors as well as oxygen, nutrition, and minerals necessary for mineralization and absorption. In addition, osteogenic factors such as BMP2 and osteoclast differentiation factors such as RANKL, released from blood vessels, promote osteoclastogenesis in the compression side and osteogenesis in the tension side.

Conclusions

Injection of ASA VI accelerated orthodontic tooth movement in our experiment. ASA VI induced a significant decrease in bone volume and density and an increase in trabecular spacing and RANKL expression at the compression side. Furthermore, ASA VI stimulated bone formation on the tension side by enhancing OCN and RUNX2 expression, increasing bone volume and density and decreasing in trabecular spacing. Taken together, our results suggest that ASA VI can accelerate tooth movement via increasing the activity of osteoclasts, stimulating bone resorption at the compression side. Furthermore, ASA VI has a positive effect on bone formation at the tension side. The limitation of our study was that we only studied the short-term effect of ASA VI on orthodontic tooth movement in rats. But orthodontic tooth movement is a complex process. The long-term effect of ASA VI on orthodontic tooth movement needs further evaluation.

Conflict of interest

None.
Supplementary Data

**A** Compression side

|         | Control | ASA VI |
|---------|---------|--------|
| RANKL   | 35 kDa  | 40 kDa |
| β-actin | 40 kDa  | 35 kDa |

**B** Control ASA VI

| RANKL   | 35 kDa  |
| β-actin | 40 kDa  |

**C** Control ASA VI

| RANKL   | 35 kDa  |
| β-actin | 40 kDa  |

**D** Tension side

|         | Control | ASA VI |
|---------|---------|--------|
| RUNX2   | 55 kDa  |        |
| OCN     | 15 kDa  | 40 kDa |
| β-actin | 40 kDa  | 15 kDa |

**E** Control ASA VI

| RUNX2   | 55 kDa  |
| OCN     | 15 kDa  |
| β-actin | 40 kDa  |

**F** Control ASA VI

| RUNX2   | 55 kDa  |
| OCN     | 15 kDa  |
| β-actin | 40 kDa  |

Supplementary Figure 1. (A) Original western images of the compression side on day 3. (B) Original western images of the compression side on day 7. (C) Original western images of the compression side on day 14. (D) Original western images of the tension side on day 3. (E) Original western images of the tension side on day 7. (F) Original western images of the tension side on day 14.

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