

## Research Article

# TGF- $\beta$ prevents the denervation-induced reduction of bone formation and promotes the bone regeneration through inhibiting ubiquitin-proteasome pathway

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**Background:** Transforming growth factor beta (TGF- $\beta$ ) can stimulate osteogenesis as a multifunctional protein. The present study was to explore if TGF- $\beta$  can prevent denervation-induced reduction of bone formation.

**Materials & methods:** The 6-week-old male mice were treated with recombinant human TGF- $\beta$  1 (rhTGF- $\beta$  1). Bone formation, endochondral bone growth rates, and gene expression of osteoblast markers were measured in the skeletal tissue by real-time PCR.

**Results:** RhTGF- $\beta$  1 treatment prevented the denervation-induced decrease in bone formation rates, endochondral growth, and expression of Cbfa1/Runx2 (runt-related transcription factor 2), Osteocalcin (OC), and CollA1. TGF- $\beta$  1 partially inhibited the denervation-induced ubiquitination of Cbfa1/Runx2 in mouse cancellous bones via ubiquitin-proteasome pathway.

**Conclusion:** TGF- $\beta$  prevents denervation-induced reduction of bone formation and promotes the bone regeneration through inhibiting ubiquitin-proteasome pathway at least partially.

## Introduction

Denervation results in bone loss in human and animal models [1,2]. Clinical and experimental studies indicate that bone metabolism becomes abnormal following denervation including markedly diminished bone density [3,4] and linear growth velocity [5]. In previous attempts to improve growth velocity in denervated-mice, recombinant human growth hormone, insulin-like growth factor binding protein (IGFBP), and insulin-like growth factor (IGF) were administered during the initial treatment [6]. However, it rarely attracts attention that the effects of local and systemic regulators on bone metabolism after denervation, including the activin/TGF- $\beta$ /bone morphogenetic protein cytokine family which is important in stimulating bone regeneration.

TGF- $\beta$  belongs the family of molecules with multifarious actions relative to bone metabolism [7]. There are several evidences indicated TGF- $\beta$  1, 2, and 3 as having autocrine and/or paracrine roles in regeneration and remodeling of bone [8]. TGF- $\beta$  stimulates chondrogenic differentiation [9] and increases cell proliferation [10], and chondrocytes [11] *in vitro*. Also, TGF- $\beta$  promotes collagen forming [12] and collects osteoblast-like cells [13], and improves the wound of soft tissue after denervation [14] *in vivo*.

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Administration of TGF- $\beta$  promotes bone formation and osteogenesis at the injection site in experimental models. Local injections of TGF- $\beta$ 1 and TGF- $\beta$ 2 into the subperiosteal regions of either parietal or long bones resulted in stimulation of osteogenesis [15,16]. TGF- $\beta$  is continuously expressed during fracture repair, and exogenous TGF- $\beta$  affects dramatically on gene expression and differentiation of bone cells and cartilage [17]. Additionally, application of TGF- $\beta$  *in vivo* can lead to the callus formation in normal bone, rapid closure of skull defects [18], and improve bone regeneration and strength during rat tibiofibular fractures repair [19]. Recent studies demonstrated that hypertrophy of TGF- $\beta$  stimulates chondrocyte proliferation and differentiation [20]. In conclusion, TGF- $\beta$  can regulate osteoclast and osteoblast function, but the role of endogenous TGF- $\beta$  in bone formation and bone remodeling remains unclear. Therefore, the present study investigated the possibility that TGF- $\beta$  may prevent bone loss after denervation.

## Materials and methods

### Animals and denervation

All experiments were approved by the Nanfang Hospital Animal Ethics Committee Laboratory (Guangzhou, P.R. China) and conducted according to the guidelines of the National Health and Medical Research Council of China. All experimental procedures were performed in Nanfang Hospital Experimental Animal Center (Guangzhou, P.R. China). Eighty-four 6-week-old male C57/BL6 mice weighing 16–18 g were obtained from the Southern Medical University (Guangzhou, P.R. China). The mice were maintained on a 12 h light/12 h dark cycle. All mice were randomly divided into four groups (twenty-one/group): Sham + Veh, Sham + TGF, DNV + Veh, and DNV + TGF. The mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). For DNV + Veh and DNV + TGF mice, the left nerve was ligated at approximately 1 cm proximal to the nerve trifurcation and removed out about 0.5 cm to prevent spontaneous regeneration. We performed sham surgeries to the Sham + Veh and Sham + TGF mice.

### Recombinant human TGF- $\beta$ 1 treatment and sampling

After the surgery, the Sham + Veh and DNV + Veh mice were treated by subcutaneous injection with vehicle and the Sham + TGF and DNV + TGF mice were administered using osmotic minipumps (Alza Corp., Palo Alto, CA, U.S.A.) with recombinant human TGF- $\beta$ 1 (rhTGF- $\beta$ 1) (R&D Systems, Minneapolis, MN, U.S.A.) 100  $\mu$ m/kg daily at 2 h.

At week 1, 2, and 3, the mice were weighed and killed to sampling. Lumbar vertebral bodies and/or distal femurs were fixed in 70% ethanol and embedded in methyl methacrylate. A cross-section of the anterior portion of the vertebral body (15  $\mu$ m) and the distal femur (4 and 15  $\mu$ m) was prepared. The 4  $\mu$ m sections were stained with toluidine blue, von Kossa's silver nitrate, or modified Giemsa, while the 15  $\mu$ m sections were not stained to evaluate of the fluorochrome bone markers.

### Morphometry

We used fluorochrome-based histomorphometric measurements of cancellous bone to determine the cross-sections of the lumbar vertebral bodies and frontal sections of the distal femurs in mice. All experiments have been repeated for three times. All of the morphometric parameters were calculated according to standard methods and were expressed in two-dimensional units. Measurements were performed on a digitized plate connected with a computer and an epifluorescence microscope using the morphometry program called "Stereology" (KSS Computer Engineers, Magna, UT, U.S.A.).

### Quantitative real-time PCR

The samples were quickly placed in a mortar precooled with liquid nitrogen. Repeatedly ground to a powder form in liquid nitrogen, and then transferred to a precooled homogenizer. The 1 ml of Trizol reagent (Invitrogen) was added, thoroughly homogenized, and centrifuged at 4°C (12000 r/min, 15 min). The supernatant was centrifuged with chloroform to separate RNA from cellular DNA, proteins and other components to obtain total RNA. RNA was suspended in RNase/DNase-free water (Gibco/Invitrogen, Carlsbad, CA) and quantified using an Agilent 2100 Bioanalyzer according to the RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA). First-strand cDNA synthesis was performed using the SuperScript first-stand synthesis system for real-time PCR (Life Technologies, Rockville, MD) using oligo(dT) as a primer according to the manufacturer's protocol. As an additional quality control, Arabidopsis thaliana mRNA was added to each RNA sample prior to cDNA synthesis. Real-time PCR was performed in a Smart Cycler (Cepheid, Sunnyvale, CA) using the LightCycler DNA Master SYBR Green I dye intercalation assay (Roche Molecular Biochemicals, Indianapolis, IN). Expression levels were calculated according to the  $2^{-\Delta\Delta C_t}$  method. Primers were generated as following:

Cbfa1/Runx2 (runt-related transcription factor 2) (NM.009820), 5'-ATGCTTCATTCGCCTCACAAACAAC-3' (sense) and 5'-ATTAACCATTTAAACGCCAGAG-3' (antisense); Osteocalcin (OC) (X04142), 5'-AACAGACTCCGGCGCTACCTTG-3' (sense) and 5'-AGCTCGTCACAAGCAGGGTTAAG-3' (antisense); Col1A1(X54876), 5'-AGACGGGAGTTTCTCCTCGGGAC-3' (sense) and 5'-TGTAGACTCTTTGCG GCTGGGGTG-3' (antisense).

## Cell culture and Runx2 degradation assay

Mouse primary osteoblastic cell lines (MPOC) were cultured in RPMI-1640 medium (GIBCO, C11875500BT) supplemented with 10% fetal bovine serum (GIBCO, Cat. No. 10099-141) at 37°C and 5% CO<sub>2</sub>. For Runx2 degradation assay, MPOC were transiently transfected with pHA-Sunx2 expression vector by using Lipofectamine 2000 according to the instructions specified by the manufacturer (Invitrogen). The empty pcDNA3 vector was used as a negative control.

## Western blotting

Protein expression was detected by Western blotting according to the established protocols. The primary antibodies used were as follows: Anti-Runx2 (Oncogene, Cambridge, MA), anti-actin (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-ubiquitin (sc-8017); anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Ambion, Austin, TX). All images were captured and analyzed using a Tanon 5200 Chemiluminescent Imaging System (Tanon Science & Technology Co., Ltd, Shanghai, China), with densitometry analyses performed using ImageJ software (NIH, U.S.A.).

## Immunoprecipitation

Cancellous bone tissue lysates were centrifuged at 12000 × *g* for 10 min at 4°C. The resulting supernatants were collected for immunoprecipitation to assay the endogenous Runx2 degradation and *in vitro* Runx2 ubiquitination. The degradation assay was also performed in the presence of 1 × proteasome inhibitor mix (0.25 mM MG132 and 0.25 mM MG115; EMD Biosciences, San Diego, CA, U.S.A.).

## Statistical analysis

Statistical analyses were performed using SPSS 24.0 (IBM SPSS, Armonk, NY, U.S.A.) software. Comparisons between groups were assessed using Wilcoxon test. The  $P < 0.05$  was considered statistically significant. Results are presented as mean ± standard deviations.

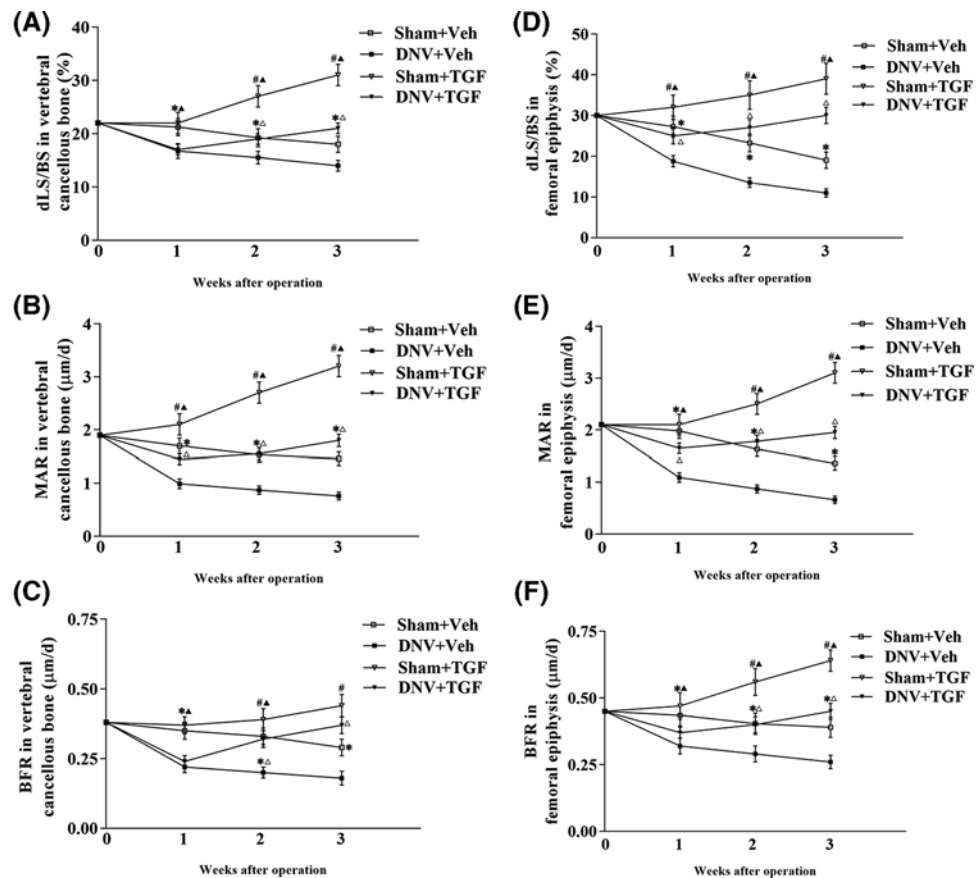
## Results

### rhTGF-β1 prevented denervation-induced reduction of bone formation in mice lumbar vertebral cancellous bone

Double-labeled surfaces (dLS) (Figure 1A), mineral apposition rate (MAR) (Figure 1B), and bone formation rate (BFR) (Figure 1C) in the lumbar vertebral cancellous bone were significantly lower in DNV groups than in Sham groups at 1 week and were much lower at 3 weeks, reflecting denervation-induced severe reduction of bone formation as early as 1 week and throughout the experimental period. The rhTGF-β1 treatment prevented all those changes as early as at 1 week after denervation for MAR and BFR, and all reductions of dLS, MAR, and BFR were recovered to normal level at 2 weeks after denervation.

### RhTGF-β1 prevented denervation-induced reduction of bone formation in mice femoral epiphysis

A modest yet significant (30%,  $P < 0.05$ ) reduction in the single-labeled surface and a substantial decrease (42%,  $P < 0.05$ ) in the femoral epiphysis dLS in DNV groups were observed compared with the Sham groups (Figure 1D). Mineralizing surface and corrected MAR (Figure 1E) as well as surface and area referent BFR (Figure 1F) was significantly decreased (41%,  $P < 0.05$ ) in DNV groups at 1 week and were much lower at 3 weeks. Similar to the lumbar vertebral cancellous bone, rhTGF-β1 treatment prevented all those changes from 1 week DNV mice and reached to normal levels at 3 weeks in femoral epiphysis.



**Figure 1. Effect of TGF- $\beta$ 1 on bone formation in lumbar vertebral and femoral epiphysis cancellous bone**

(A) Double-labeled surfaces/bone surface (dLS/BS), (B) Mineral apposition rate (MAR) and (C) Bone formation rate/bone surface (BFR/BS) in vertebral cancellous bone. (D) dLS/BS, (E) MAR, and (F) BFR/BS in femoral epiphysis. \*Sham + Veh vs DNV + Veh,  $P < 0.05$ ; #Sham + Veh vs Sham + TGF,  $P < 0.05$ . DNV + Veh vs DNV + TGF,  $P < 0.05$ ; Sham + TGF vs DNV + TGF,  $P < 0.05$ . For all time points per group,  $n = 7$ .

## RhTGF- $\beta$ 1 prevented denervation-induced reduction of osteoblast marker gene expression in mice femoral epiphyses

In femur, the expression of *Cbfa1/Runx2* mRNA was significantly decreased, from 22 to 43% at week 1 and 3 in DNV groups compared with Sham groups (Figure 3A). With the treatment of TGF- $\beta$ 1, the *Cbfa1/Runx2* mRNA level was elevated to as same as control group from 1 to 3 weeks (Figure 2A). Along with the change of *Cbfa1/Runx2* mRNA in long-bone metaphyseal osteoblasts, the levels of OC mRNA decreased by 23, 30, and 50%, respectively ( $P < 0.05$ ) at week 1, 2, and 3 of DNV compared with Sham group (Figure 2B). TGF- $\beta$ 1 treatment for 1 week or late treatment for 2–3 weeks completely corrected the abnormal OC expression in metaphyseal bone of DNV + TGF group (Figure 2B). As same to the effects of denervation on *Cbfa1/Runx2* mRNA and OC mRNA levels, the *Col1A1* mRNA levels were reduced by 25, 29, and 53%, respectively ( $P < 0.05$ ) at week 1, 2, and 3 in metaphyseal bone compared with Sham groups. TGF- $\beta$ 1 treatment increased the *Col1A1* mRNA levels to the normal as in Sham + TGF group at week 1–3 (Figure 2C). Meanwhile, TGF- $\beta$ 1 treatment increased the mRNA levels at week 2 and 3 for *Cbfa1/Runx2* and *Col1A1* and in week 3 for OC in Sham + TGF group. The results showed that exogenous TGF- $\beta$ 1 could restore the expression of osteoblast marker gene in the metaphyseal bone of DNV mice and promote normal bone formation.

## Effect of rhTGF- $\beta$ on denervation-induced elevation of bone resorption in mice

A modest (34–37%, at week 1–3,  $P < 0.05$ ) decrease in the osteoid surface of DNV compared with Sham groups. There were significant differences in the numbers of nuclei per osteoclast or the numbers of osteoclasts per millimeter

between Sham and DNV groups. However, the rhTGF- $\beta$ 1 treatment did not prevent denervation-induced increases in bone resorption although denervation-induced decrease in osteoid surface was prevented by rhTGF- $\beta$ 1 and even reached to the normal levels (Figure 3).

## RhTGF- $\beta$ prevented denervation-induced reduction of endochondral growth in distal femurs of mice

A significant decrease in endochondral growth (EcG) indices, including growth plate width (GPW), EcG, calculated rate of chondrocyte production (RCP) and hypertrophic cell size in DNV-mice compared with Sham-mice (Figure 3). These animals grew rapidly, with distal femoral endochondral bone elongation rates of 28  $\mu$ m/d for DNV-mice and 53  $\mu$ m/d for Sham-mice at 3 weeks. DNV-mice had final body weights of  $18 \pm 2$  g compared with  $27 \pm 3$  g for Sham group (Figure 4I). Interestingly, treatment with rhTGF- $\beta$ 1 resulted in increases significantly in longitudinal GPW and growth rate, and returned body weights in DNV group to the normal (Sham group) level. However, rhTGF- $\beta$ 1 treatment did not show any effects on RCP and hypertrophy cell size in both Sham and DNV groups. Examination of vertebrae examined under polarized light revealed that bone formation in the rhTGF- $\beta$ 1 treated group was layered rather than woven bone (Supplementary Materials).

## Denervation increased degradation of Runx2 in the cancellous bone

According to the *in vitro* degradation assay, degradation of Runx2 protein increased in the denervated cancellous bone, compared with the Sham group (Figure 4A). More than 70% of Runx2 protein vanished after a 16-h incubation (Figure 4B). In this case, only 15% of Runx2 protein disappeared in Sham groups. In addition, the results showed that proteasome inhibitors highly prevent denervation-induced Runx2 degradation in cancellous bone (Figure 4E). It suggested that denervation-induced the reduction of bone formation may through increasing proteasome-mediated degradation of Runx2 protein in the cancellous bone.

## Denervation promotes Runx2 ubiquitination in the cancellous bone

As shown in Figure 4C, for the cancellous bone, anti-ubiquitin antibodies detected multiple bands of Runx2 protein that were significantly poly-ubiquitinated in the reaction using homogenate (lanes 2–4). In contrast, only a fuzzy band was observed in the samples of the Sham groups (lane 1). Quantitative measurements showed that, at 3 weeks after denervation, the poly-ubiquitination Runx2 abundance of cancellous bone homogenate was five-times greater than that of the Sham group (Figure 4D), which suggested that ubiquitination activity for Runx2 protein is enhanced in the denervated cancellous bone.

## TGF- $\beta$ 1 prevents the Runx2 degradation and ubiquitination in denervated cancellous bone

We tested the Runx2 degradation and ubiquitination levels in sham and DNV cancellous bone with or without TGF- $\beta$ 1 treatment at 3 weeks. In the absence of TGF- $\beta$ 1 treatment DNV group, Runx2 abundance obviously reduced about 80% compared with sham (Figure 4F). However, TGF- $\beta$ 1 treatment markedly prevented Runx2 protein degradation in denervated cancellous bone. Similarly, TGF- $\beta$ 1 treatment largely prevented Runx2 ubiquitination in denervated cancellous bone (Figure 4G,H). It suggested that TGF- $\beta$ 1 prevents the denervation-induced the reduction of bone formation may be via inhibiting Runx2 protein degradation through ubiquitin-proteasome pathway.

## Discussion

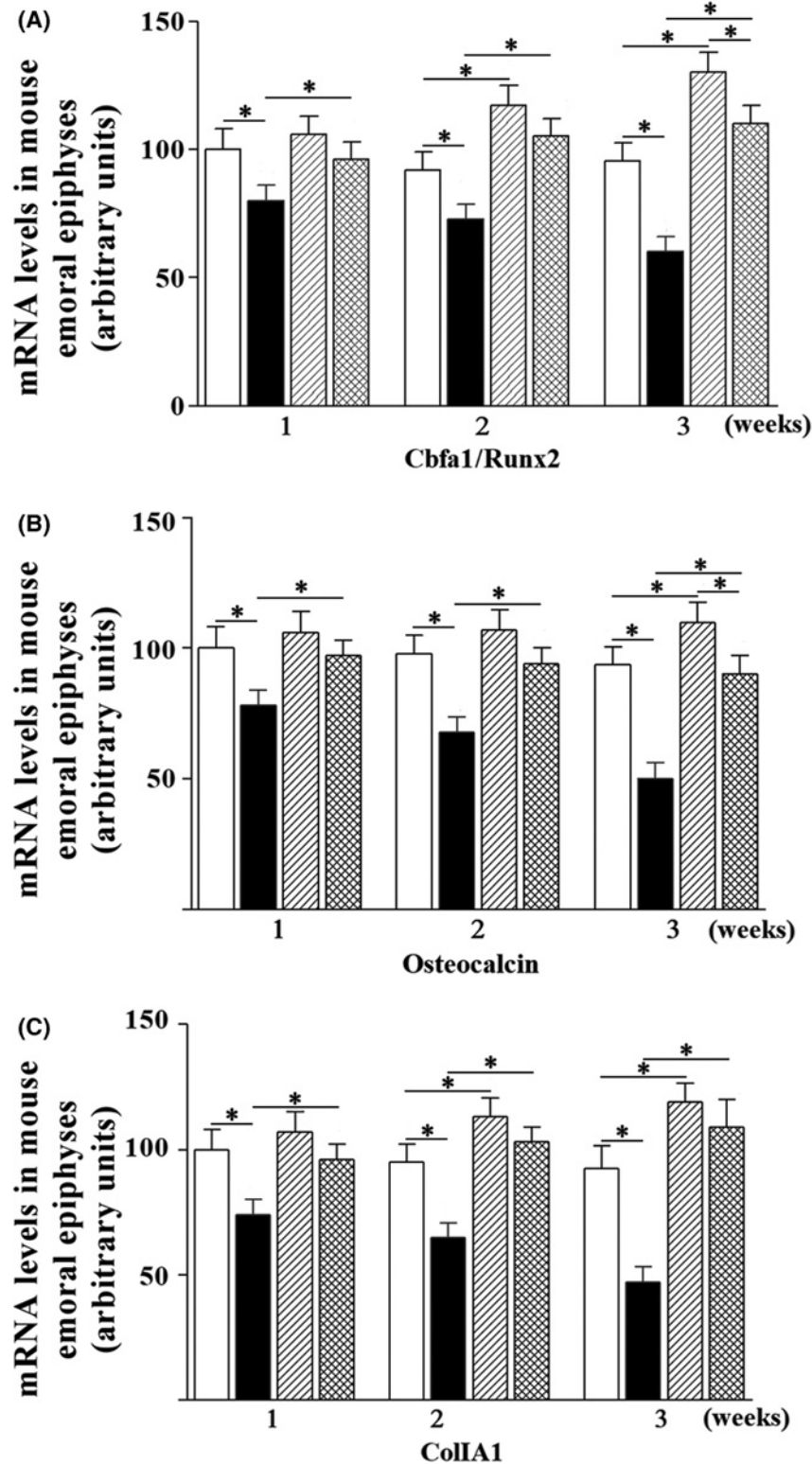
The use of growth factors to stimulate bone formation provides a possible treatment for patients with denervation. In clinical and experimental, most of the therapies are based on the stimulation of bone formation, but inhibition of bone resorption still needs further research. TGF- $\beta$  has a strong regulatory effect in both bone resorption and formation, and the sufficient evidences demonstrate that TGF- $\beta$  plays an important role in osteogenesis and chondrogenesis [7,10,12,15,18]. In the present study, we describe that TGF- $\beta$  prevents denervation-induced reduction of bone formation in mice over the short term.

The dramatic recovery of BFR observed following TGF- $\beta$  treatment in DNV-mice can be attributed to a significant inhibition of the reduction in mineral appositional rate, which means that rhTGF- $\beta$ 1 treatment stimulates osteoblasts produce bone matrix. Several *in vitro* studies have shown that TGF- $\beta$  can increase the collagen-forming ability of osteoblasts [10,12,15] and improve bone matrix attachment to bone organ culture [21].

In DNV groups, rhTGF- $\beta$ 1 treatment inhibited reduction the dLS, which suggested TGF- $\beta$  prevented a decrease in the number of active osteoblasts. @It is unknown that which TGF- $\beta$  exerts its action. However, according to

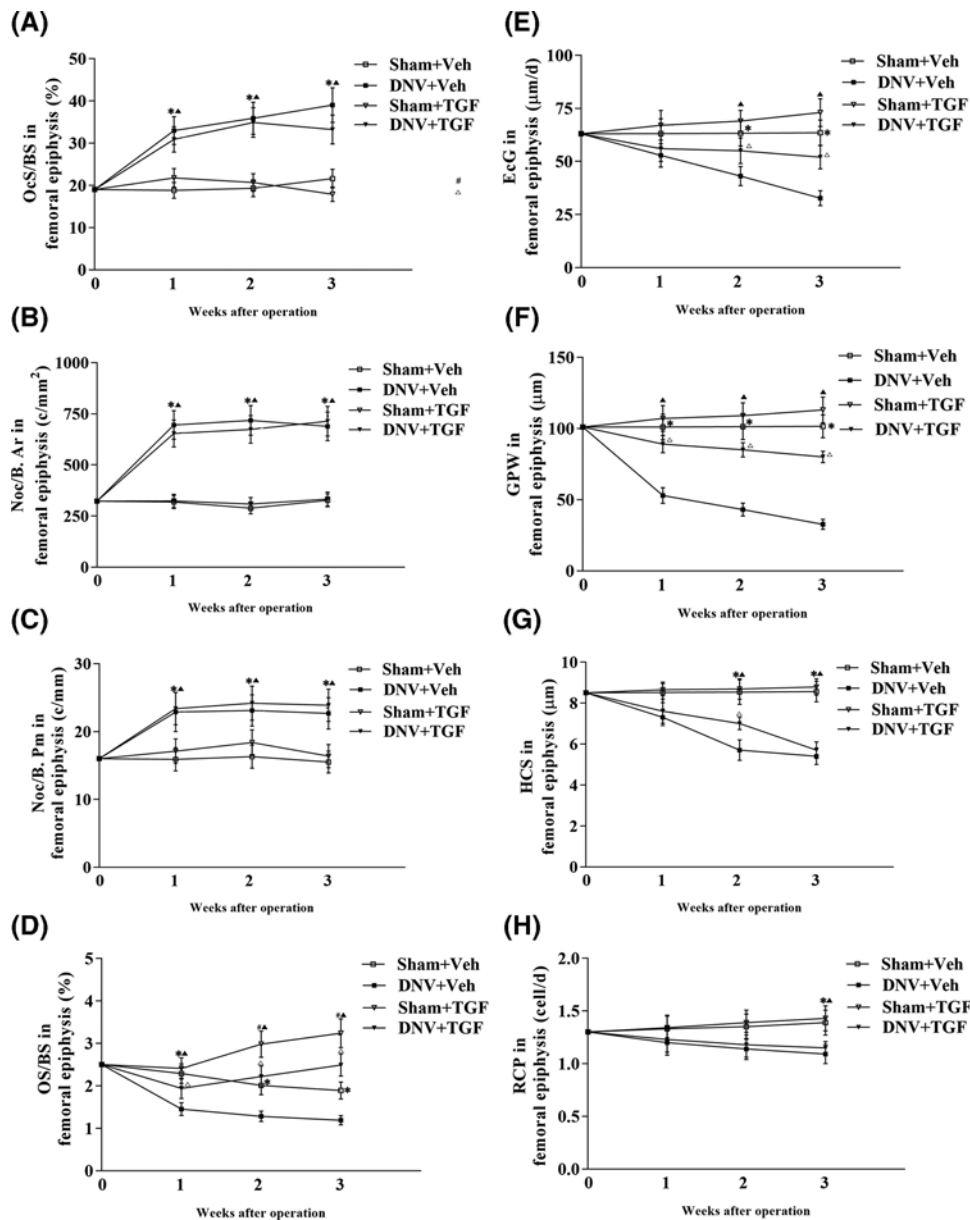


□ Sham+Veh ■ DNV+Veh ▨ Sham+TGF ▩ DNV+TGF



**Figure 2.** Effect of TGF- $\beta$ 1 on denervation-induced reduction of osteoblast marker gene expression in mice femoral epiphyses

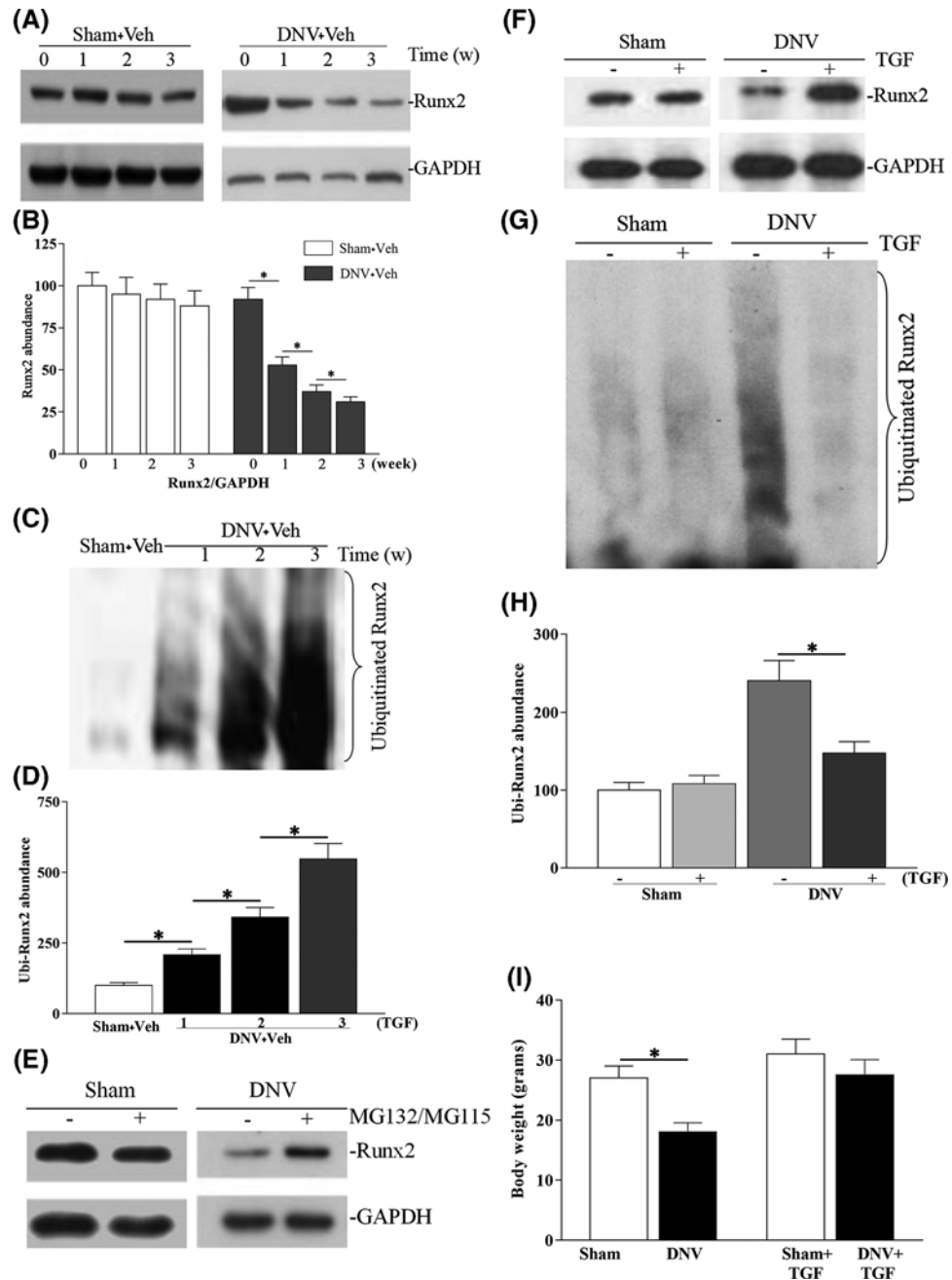
Quantitative analysis of mRNA expression for (A) Cbfa1/Runx2, (B) OC, and (C) Col1A1 gene in mice femoral epiphysis. \* $P < 0.05$ . For all time points per group,  $n = 7$ .



**Figure 3. Effect of TGF-β1 on bone resorption in the proximal femoral metaphysis and endochondral growth indices of the proximal femurs**

(A) Osteoclast surfaces/bone surface (OcS/BS), (B) Number of osteoclast profiles/mm<sup>2</sup> of cancellous bone (Noc/B. Ar), (C) Number of osteoclast profiles/mm of cancellous bone (Noc/B. Pm), (D) Osteoid surface/bone surface (OS/BS), (E) Endochondral growth (EcG), (F) Growth plate width (GPW), (G) Hypertrophic cell size (HCS) and (H) Rate of chondrocyte production (RCP) in femoral epiphysis. \*Sham + Veh vs DNV + Veh,  $P < 0.05$ ; #Sham + Veh vs Sham + TGF,  $P < 0.05$ . DNV + Veh vs DNV + TGF,  $P < 0.05$ ; Sham + TGF vs DNV + TGF,  $P < 0.05$ . For all time points per group,  $n = 7$ .

these rapid responses, the administration of rhTGF-β1 stimulated mitogenic differentiation of osteoblast progenitor cells or accelerated differentiation of already committed osteoblast progenitor cells. Due to the chemotactic response of TGF-β stimulated to osteoblast-like cells [13], the mitogenic responses to TGF-β may also be involved. Mitogenesis, accelerated differentiation of osteogenic precursors and chemotaxis of osteoblasts are all theoretical sequelae of rhTGF-β1. More research is needed to ascertain the responsible phenomena for the rapid recovery of BFR in denervated-mice after the initiation of TGF-β observed in the present study.



**Figure 4. Effect of TGF- $\beta$ 1 on degradation and ubiquitination of Runx2 in denervated cancellous bone**

(A) Western blotting of Runx2 protein from denervated-cancellous bone of Sham + Veh and DNV + Veh groups. (B) Quantitative determination of Runx2 protein in (A). (C) The immunoprecipitated of HA-Runx2 protein in Sham + Veh and DNV + Veh groups. (D) Quantitative determination of the polyubiquitinated Runx2 abundance in (C). (E) Blockade of Runx2 protein degradation by the proteasome inhibitors. (F) Western blotting of Runx2 protein in denervated-cancellous bone from Sham + Veh, Sham + TGF, DNV + Veh, and DNV + TGF groups at 3 weeks. (G) The immunoprecipitated of exogenous Runx2 in the denervated-cancellous bone from Sham + Veh, Sham + TGF, DNV + Veh, and DNV + TGF groups at 3 weeks. (H) Quantitative determination of the poly-ubiquitinated Runx2 abundance shown in (G). (I) Total body weight in four groups. \* $P < 0.05$ . For all time points per group,  $n = 7$ .



TGF- $\beta$  has been found in osteoclasts [22]. It has been reported that TGF- $\beta$  promotes resorption in cultured neonatal mouse calvarias by a prostaglandin-dependent mechanism [23]. However, evidence exists both for and against a role for prostaglandins in bone formation following denervation [23,24]. Thus, the effect of TGF- $\beta$  is complex on osteoclasts and best studied *in vivo*. The exact mechanism of TGF- $\beta$  activity is still unknown.

In recent years, research on TGF- $\beta$  and bone marrow stem cells (BMSCs) has been increasing. In fact, the TGF- $\beta$  related pathways could regulate almost all aspects of BMSCs function [25]. For example, MiR-663 inhibited the proliferation and migration of BMSCs by targeting TGF- $\beta$  1 [26], and MOTS-c promoted cell differentiation of BMSCs to osteoblasts via TGF- $\beta$ /Smad pathway [27]. Also study showed that HFD disrupted the TGF- $\beta$  receptor within lipid rafts, associated with impaired Smad2/3-dependent TGF- $\beta$  signaling [28]. Thus BMSCs might play an important role in the process of TGF- $\beta$  increasing bone regeneration, especially the cell differentiation of BMSCs to osteoblasts.

Several researches reported that the additional osteoclasts were collected to reshape the TGF- $\beta$  induced bone, which was not directly induced by TGF- $\beta$  [29,30]. With rhTGF- $\beta$  administration of short periods, as the present study, we did not observe a significant recovery in bone volume, nuclei per cell profiles, and the number of osteoclast per millimeter. When decreased BFR are sustained with longer periods of TGF- $\beta$  treatment, increased bone mass should be observed. While, with the significant recovered levels in BFR observed here, no corresponding recovery in bone mass was observed. This complicated issue needs further study.

Denervation-induced increases in bone resorption may contribute to increased production of endogenous corticosteroids [31], impairing osteoblast recruitment and function. Our results, however, did not demonstrate rhTGF- $\beta$  prevention of the denervation-induced increase in bone resorption, and no report exists indicating an interaction between glucocorticoids and rhTGF- $\beta$ .

Endochondral bone formation includes a cascade of cellular events. Prevention by rhTGF- $\beta$  1 of denervation-induced reduction of endochondral bone formation may stimulate chondrocyte proliferation and chondrogenic differentiation via a complex network of endogenous growth/differentiation signaling molecules, including the Indian hedgehog/parathyroid hormone-related peptide (Ihh/PTHrP) feed-loop pathway. There is evidence showing that TGF- $\beta$  up-regulates the Ihh and PTHrP gene expression in chondrocyte and both genes stimulate chondrocyte proliferation and chondrogenic differentiation [32]. The molecular mechanism by which TGF- $\beta$  prevents denervation-induced reduction of endochondral bone formation requires further exploration.

Runx2 has been found to play an important role in the regeneration of various tissues recently years [33–36]. Especially the role of Runx2 in bone tissue regeneration is particularly popular. Such as the high-fat and high-glucose microenvironment inhibited bone regeneration, which was related to the inhibition of Runx2 expression [37]. Also in the bone fractures and postmenopausal osteoporosis inhibiting bone regeneration, Runx2 expression was reduced [36,38]. In our study, we found the bone regeneration was inhibited in the DNV group, and also the expression of Runx2 was decreased. When treated with TGF- $\beta$ , the inhibiting of bone regeneration was decreased and the expression of Runx2 was increased. Thus the Runx2 might be a potential drug target, which could be used to increase the bone regeneration in multiple diseases clinically.

Denervation induced a significant decrease in the expression of Cbfa1 / Runx2 (a transcriptional activator of osteoblast differentiation), consistent with the reduction in bone formation. This is related to a decrease in the expression of the Col1A1 and OC genes known to be partially controlled by the Cbfa1/Runx2 in postnatal organisms [39]. Thus, the loss of Cbfa1/Runx2 expression may contribute to a decrease in Col1A1 and OC expression in denervated osteoblasts together with other transcriptional activators. Here, exogenous addition of TGF- $\beta$  1 into denervated-mice can reduce the formation of defective bone. TGF- $\beta$  1 treatment was related to the increase of Cbfa1/Runx2 expression, and the increase of Col1A1 and OC mRNA levels in denervated-mice, suggesting that TGF- $\beta$  1 acts by increasing these target genes expression in metaphyseal osteoblasts. While the correction of osteoblastic gene expression also may be affected by post-transcriptional effects of TGF- $\beta$  1 [40]. Our current study showed TGF- $\beta$  inhibited denervation-induced Runx2 protein degradation and ubiquitination, but whether denervation increases OC and Col1A1 protein degradation and/or ubiquitination; TGF- $\beta$  affects it; if so whether it is through runx2 regulation and/or through ubiquitin-proteasome pathway, further studies are necessary to clarify this point.

Protein degradation is essential for controlling many cellular processes. The ubiquitin-proteasome pathway consists of a highly organized cascade of enzymatic reactions and requires energy, which can sequence, label and destroy a variety of physiologically important proteins [41]. The explanation of protein substrates caused by ubiquitin-proteasome proteolytic pathway defects is associated with many devastating disorders [41–43]. Under both physiologic and pathologic conditions, the Smad signaling pathway including Runx2 undergo ubiquitin-proteasome mediated degradation. Increased proteasomal degradation and ubiquitination against Runx2 in denervated-cancellous bone means that Runx2 specifically targets ubiquitin-dependent destruction *in vivo* during bone-remodeling. Studies suggest

that TGF- $\beta$ 1 can inhibit Runx2 degradation in denervated cancellous bone (Figure 4). This is consistent with previous studies that TGF- $\beta$ 1 also is shown to reduce matrix degradation [44]. These observations suggest that changes in key signaling regulators in ubiquitin-proteasome degradation may be due to interference with important cellular signaling, which in turn leads to osteoblast dysfunction and contributes to the pathogenesis of bone formation. Functionally, stabilization of Runx2 by the TGF- $\beta$ 1 leads to an inhibition denervation-induced reduction of bone formation gene expression. Therefore, these findings establish that TGF- $\beta$  regulating protein degradation may be via ubiquitin-proteasome pathway and may play an imperative role in the pathogenesis of denervation-induced reduction of bone formation.

## Conclusion

In summary, we report that TGF- $\beta$  prevents denervation-induced reduction of bone formation for short periods of time by inhibiting its decreasing mRNA level of Cbfa1/Runx2, OC and Col1A1, and ubiquitination of Runx2. The precise role of the TGF- $\beta$  superfamily in injury-induced skeletal changes in denervated-mice is unknown. It is not certain whether rh TGF- $\beta$  acts directly on osteoblasts/chondrocytes or their progenitor cells. Other endocrine, paracrine and/or autocrine media may also be involved. We report here that TGF- $\beta$ 1 prevents the denervation-induced reduction of bone formation, suggesting a mechanism involving TGF- $\beta$ 1 in the altered differentiation of osteoblasts in denervated-mouse bone.

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## Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

## Author Contribution

Study design: Y.Z., W.M., L.T., and W.Y. Experiment performance: Y.Z., W.M. and L.Y. Data collection, analysis, and explanation: Y.Z., W.Y., C.Y., and W.Z. Manuscript drafting and revising: S.M., D.Z. and L.F.

## Abbreviations

BFR, bone formation rate; Col1A1, Collagen type I alpha1; DLS, Double-labeled surfaces; DNV, Denervation; EcG, endochondral growth; GPW, growth plate width; MAR, mineral apposition rate; MPOC, mouse primary osteoblastic cell lines; OC, Osteocalcin; rhTGF- $\beta$ 1, recombinant human TGF- $\beta$ 1; Runx2, runt-related transcription factor 2; TGF- $\beta$ , transforming growth factor beta.

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