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ORIGINAL ARTICLE

Inhibition of mechanical stress-induced NF-κB promotes bone formation

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OBJECTIVE: Nuclear factor kappaB (NF-κB) plays an important role in osteogenesis. This study was performed to investigate the effects of mechanical force on NF-κB activity in osteoblast-like cells.

MATERIALS AND METHODS: U2OS cells were harvested at specific time points after mechanical loading. U2OS nuclear extracts were prepared for Western blot assay and electrophoretic mobility shift assay electrophoretic mobility shift assay (EMSA). Total RNA was isolated using TRIzol for real-time PCR.

RESULTS: P-p65 (Ser536) expression in the nucleus was significantly higher after loading force on U2OS cells. The amount of nuclear NF-κB also increased. Ammonium pyrrolidinedithiocarbamate(PDCT) inhibited compressive force-induced NF-κB activity in EMSA. Further, PDTC attenuated the transcriptional inhibition of alkaline phosphatase (ALP), RUNX-2 and Osteocalcin by down-regulating NF-κB activity.

CONCLUSIONS: Mechanical force enhances NF-κB activity in osteoblast-like cells, and compressive force affects the downstream bone marker genes through NF-κB.

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Keywords: nuclear factor kappaB; compressive force; osteoblast; bone formation

Introduction

Histological analyses shows that orthodontic tooth movement induces the aggregation of osteoclasts on the pressure side, while the tension side contains more osteoblasts after loading orthodontic force (van de Velde *et al*, 1988). The mechanisms of tooth movement and bone metabolism are related to osteoblast and osteoclast cells. There is substantial literature regarding osteoclastic bone resorption; however, the mechanism of osteoblast bone rebuilding under orthodontic stress is unclear.

Mechanical stimulation is essential in orthodontic tooth movement. It was revealed that on the pressure side bone absorption is enhanced, while bone formation is enhanced on the tension side. This makes the orthodontic teeth move toward the direction of force *in vivo* (Waldo and Rothblatt, 1954). *In vitro*, direct or indirect force loading on cells has been examined among all stimulation methods; pressure stimulation is a simple but effective model (Kanai *et al*, 1992).

The significant relationship between nuclear factor kappaB (NF-κB) and bone remodeling has attracted more and more attention in recent years. Specifically, research on the relationship between NF-κB and osteoporosis established the idea that inhibiting NF-κB for 2–4 weeks could promote osteoblast osteogenesis (Chang *et al*, 2009. NF-κB expression after compressive stimulation has been investigated (Liu *et al*, 2007). However, research on NF-κB activity shortly after pressure stimulation is limited, thus the control of downstream genes by NF-κB after pressure stimulation is unclear.

We hypothesized that *in vitro* compressive force stimulation on osteoblasts could affect NF-κB activity and thereby regulate the expression of downstream osteogenesis-related genes.

Materials and methods

Cell culture

Osteoblast-like cells U2OS were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA), containing 10% fetal bovine serum (FBS), in a humidified atmosphere of 5% CO_2 in air.

Applications of mechanical stress

A glass cylinder was placed over a confluent cell layer in one well of a six-well plate. The number of lead granules

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placed in the cylinder controlled the compressive force, as using the uniform compression method similar to that described previously (Kanai *et al*, 1992; Kanzaki *et al*, 2002). Briefly, thin glass plates were placed over the confluent cell layers in the culture dishes. The compressive force was adjusted by adding lead weight to the glass plates. The stainless wire bridge was built up on the glass plates, so as not to touch the weight to the culture medium. The cells were subjected to 1.0 g cm^{-2} compressive force for 1, 4, 8, 12, or 24 h. As the control, by thin glass plates the cells were covered without lead weight. Compression force of this condition was 0.1 g cm^{-2}.

Real-time polymerase chain reaction (real-time PCR)
Totol RNA was isolated by using the Trizol (Invitrogen, Grand Island, NY) and cDNA was prepared with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MA), in accordance with the protocol's instructions. The primers were used as followed. GAPDH 5′-TCCATGACAACTTTGGTATCG-3′, 5′-TGTAGCCAAATTCGTTGTCA-3′ (Lee *et al*, 2007). Alkaline phosphatase (ALP) 5′-AGCACTCCCACTTCATCTGGAA-3′ 5′-GAGACCCAATAGGTAGTCCACATTG-3′(Zhang *et al*, 2010) Osteocalcin (OCN): 5′-AGGGCAGCGAGGTAGTGA-3′ 5′-CCTGAAAGCCGATGTGGT-3′ (Fan *et al*, 2007)RUNX-2: 5′-AGGAATGCGCCCTAAATCACT-3′ 5′-ACCCAGAAGGCACAGACAGAAG-3′ BSP: 5′-GGCAGTAGTGACTCATCCG-3′ 5′-ATAGCCCAGTGTTGTAGCA-3′ IL-1β: 5′ -TGCGTGTTGAAAGATGATAAG-3′ 5′–TTGGGGAACTGGGCAGAC-3′(Chen *et al*, 2008) IL-6: 5′-CTCCAGAACAGATTTGAGAGTAGTG-3′ 5′-TTGTGGTTGGGTCAGGGGTG-3′ COX-2: 5′- CGCTCAGGAAATAGAAACC-3′ 5′-TCCGCCGTAGTCGGTGTACTCGTAG-3′.

Nuclear extraction of protein from cultured cells
U2OS were harvested at specific time points after the mechanical loading. Nuclear extracts from U2OS were prepared using a Nuclear-Cytosol Extraction Kit (Fermentas, USA). The extracts were kept at −80°C until use.

Immunofluorescence assay
Cells were grown on cover slips in six-well dishes. After loading the force, cells were treated for 10 min with 4% formalin in phosphate buffered saline (PBS) (keep wet) and washed in three changes of PBS. Sections were blocked with 10% bovine serum for 1 h at room temperature, and then incubated with antiphosphorylated NF-κB p65 antibody (1:100 diluted in PBS containing 1% bovine serum) at 4°C overnight. On the next day, they were washed with PBS, the sections were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (1:200). Nuclear DNA was stained with Hoechst 33342. The cover slips were mounted on microscope slides with Kaiser's glycerol gelatine and further analyzed using a laser scanning microscope (Olympus BH2-HLSH 100).

Western blotting analysis
Nuclear and cytoplasmic protein (20 μg) underwent electrophoresis in 10% or 15% polyacrylamide gel with SDS and were transferred to NC membranes (Pall Filtron, Northborough, MA). Membranes were probed with the primary antibody (1:1000; antibodies to NF-κB(p65) (sc-8008; Santa Cruz, Santa Cruz Biotechnology, CA), phosphorylated p65 (Ser 536) (Cell Signaling) or Actin overnight at 4°C). After incubation with a horseradish peroxidase-conjugated secondary antibody (1:4000, goat anti-mouse IgG or goat anti-rabbit IgG) for 1 h at room temperature, the immunoreactive bands were visualized by enhanced chemiluminescence (ECL). Relative quantitation of ECL signal was detected by Fujifilm LAS-3000 Imaging System (Tokyo, Japan).

Electrophoretic mobility shift assay (EMSA)
The NF-κB-binding motif (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was contained in the 3′ biotin-

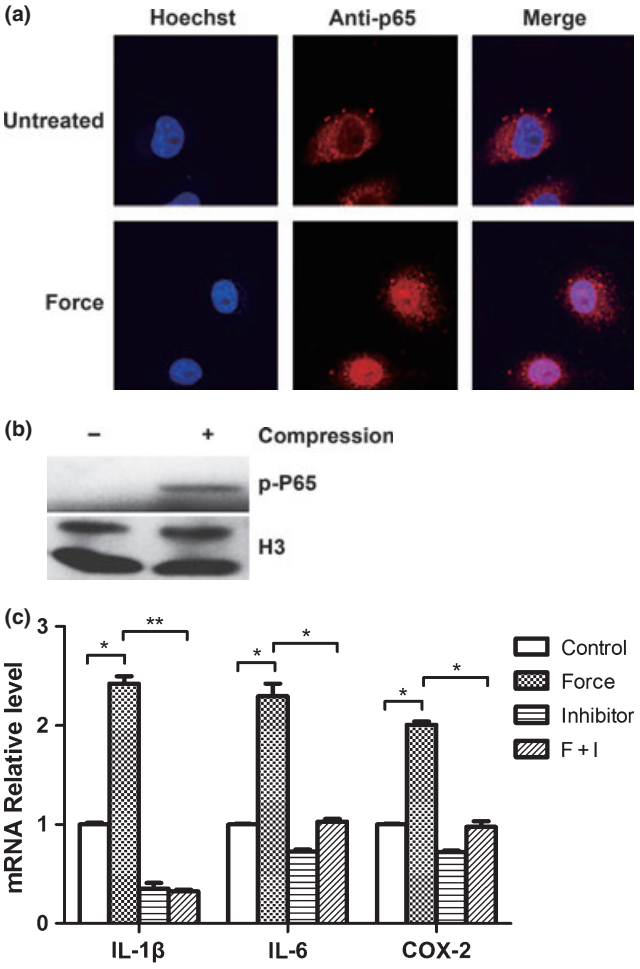

Figure 1 Effects of compressive force on nuclear factor kappaB (NF-κB) activity. (**a**) Phosphorylated p65 intercellular distribution was analyzed by immunofluorescence. After compression loading, p65 was translocated into the nucleus. (**b**) Western blot analysis of p65 and phosphorylated p65 from U2OS cell nuclear extracts treated with compressive force (1 g cm^{-2}) and harvested after 8 h. (**c**) NF-κB downstream cell signal factors IL-1β, IL-6, and COX-2 were determined by real-time PCR after loading force for 8 h. Statistical analysis was conducted using the Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$

labeled and unlabeled single-stranded oligonucleotides. Binding reaction was performed using the LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL). The DNA-protein complex samples were analyzed on a 6% polyacrylamide gel.

Alkaline phosphatase activity and mineralization assay
Alkaline phosphatase activity and alizarin red mineralization staining were performed as described previously. Briefly, alkaline phosphatase activity was determined by Alkaline Phosphatase kit (Jiancheng, Nanjing, China) according to the manufacture's instructions. After 7 days of osteogenic differentiation, alkaline phosphatase activity was determined in cell lysates. For alizarin red staining, cells were fixed in 4% formalin for 15 min, washed, and then stained with 1% alizarin red for 30 min (Behr *et al*, 2011). Then, we used Leica DM2500M materials analysis microscope and Leica DFC450 digital color microscope camera to record the stain (Kiuchi *et al*, 2012).

Cell proliferation assay
Cell proliferation assay was performed by CloneSlect Imager (Genetix, Sunnyvale CA, USA) as previously described (Marques-Gallego *et al*, 2010). Briefly, after the application of 8 h compressive force or PDTC, U2OS cells were seeded at a density of ~8000 cells per well in a 24-well plate. The plate was incubated for 72 h in complete DMEM with 10% FBS. And cells were observed every 24 h.

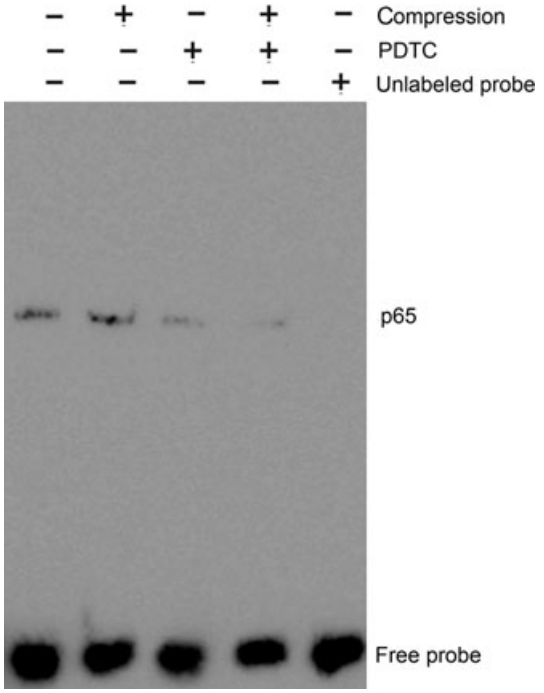

Figure 2 Effects of PDTC and compressive force (1 g cm^{-2}) on nuclear factor kappaB (NF-κB) DNA-binding activity. Electrophoretic mobility shift assay (EMSA) showed that DNA-binding activity of NF-κB in U2OS cells was potentiated by compressive force. NF-κB activation was examined with EMSA after the induction of force for 8 h. PDTC decreased NF-κB activity. Note: excessive unlabeled probes completely blocked the formation of DNA-binding complexes

Results

Effect of compressive force on NF-κB activity in osteoblast-like cells
Mechanical force promotes NF-κB expression in osteoblast-like cells (Kaneuji *et al*, 2011). Our first aim was to investigate NF-κB activity after compressive force loading on the osteoblast-like cells U2OS. In this study, we determined NF-κB nuclear translocation (Figure 1a) and the expression of NF-κB and phosphorylated p65. The results showed that after force loading, the amount of NF-κB located in the nucleus increased. Further, we determined phosphorylated p65 protein levels from nuclear U2OS extracts after 8 h force treatment (Figure 1b). Nuclear p-p65 expression was significantly higher after force loading; p65 was not significantly different between the two groups. And the NF-κB downstream cell signal reactions such as IL-1β, IL-6, and COX-2 were determined too. After stimulated by the force, the mRNA level of IL-1β, IL-6, and COX-2 were significantly increased. In contrast, after treating the U2OS cells with PDTC (ammonium pyrrolidinedithiocarbamate; 100 μM) for 1 h in advance, when loading the mechanical force, the mRNA level of IL-1β, IL-6, and COX-2 did not show any significant increase (Figure 1c).

PDTC inhibited compressive force-induced NF-κB activity
U2OS cells were incubated with PDTC (100 μM) for 1 h and then loaded with compressive force (Ozaki *et al*, 1997; Cui *et al*, 2010). As shown by the electrophoretic mobility shift assay (EMSA; Figure 2),

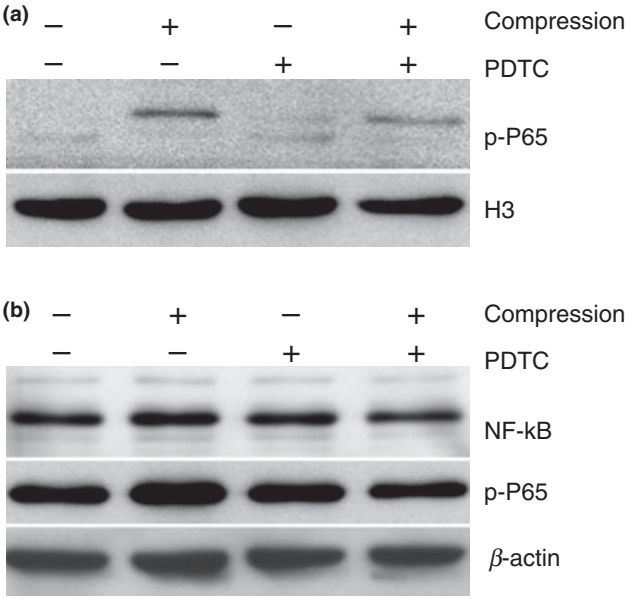

Figure 3 PDTC inhibited compressive force-induced nuclear factor kappaB (NF-κB) activity. (**a**) Western blot analysis for expression of p65 and phosphorylated p65 from U2OS cell nuclear extracts treated with PDTC for 1 h and then loaded with compressive force and harvested after 8 h. (**b**) Western blot analysis for expression of p65 and phosphorylated p65 from U2OS cell cytosolic extracts treated with PDTC for 1 h and then loaded with compressive force and harvested after 8 h

NF-κB DNA-binding activity in U2OS cells was potentiated by compressive force, and the inhibitor PDTC significantly decreased NF-κB DNA-binding activity. Moreover, phosphorylated p65 expression from nuclear extracts reconfirmed these results. However, the difference in p65 expression between groups was not remarkable (Figure 3a). Expression of p65 and phosphorylated p65 from cytosolic extracts was not markedly changed by compression or PDTC (Figure 3b).

Effects of compressive force and PDTC on U2OS cells

We first examined whether compressive force could suppress osteogenesis in U2OS cells. Therefore, alkaline phosphatase (ALP), OCN, RUNX-2, and BSP were determined to examine the osteogenesis activity. As shown in Figure 4a, ALP, OCN, and RUNX-2 transcription was significantly attenuated after loading force. But after NF-κB activity was inhibited by PDTC, the transcription of ALP, OCN, and RUNX-2 were rescued. The results showed that inhibiting NF-κB activity potentiated ALP, OCN, and RUNX-2 transcription (Figure 4b). We also examined osteogenesis by determining alkaline phosphatase activity and alizarin red staining. Figure 4c,d show that inhibiting NF-κB activity increased alkaline phosphatase activity and calcium deposition after force stimulation. Moreover, to verify whether compressive force or PDTC would affect the proliferation of U2OS cells, we observed four groups including 0, 24, 48, and 72 h after stimulating. Figure 4e shows that there were no significant differences in cell proliferation among the four groups, in which cells were treated differently (Control, Force, Inhibitor and Force + inhibitor).

Discussion

Bone remodeling is an essential process during orthodontic tooth movement. It ensures that the tooth remains stable after moving to a new position (King *et al*, 1997). Although it was reported that mechanical force mediated osteogenesis, the mechanism was unclear. In our study, we demonstrated that mechanical force promoted the expression, nuclear translocation, and DNA-binding activity of NF-kB (EMSA). These results indicate that mechanical force affected osteogenesis via the NF-kB pathway.

Nuclear factor kappaB is associated with osteoclast activity and bone absorption (Xiong *et al*, 2011). There is also a relationship between NF-κB and osteoblast activity. However, whether the loading of orthodontic force could activate NF-κB and affect osteogenesis accordingly is our area of interest. We verified that osteoblast activity was reduced after NF-κB activation.

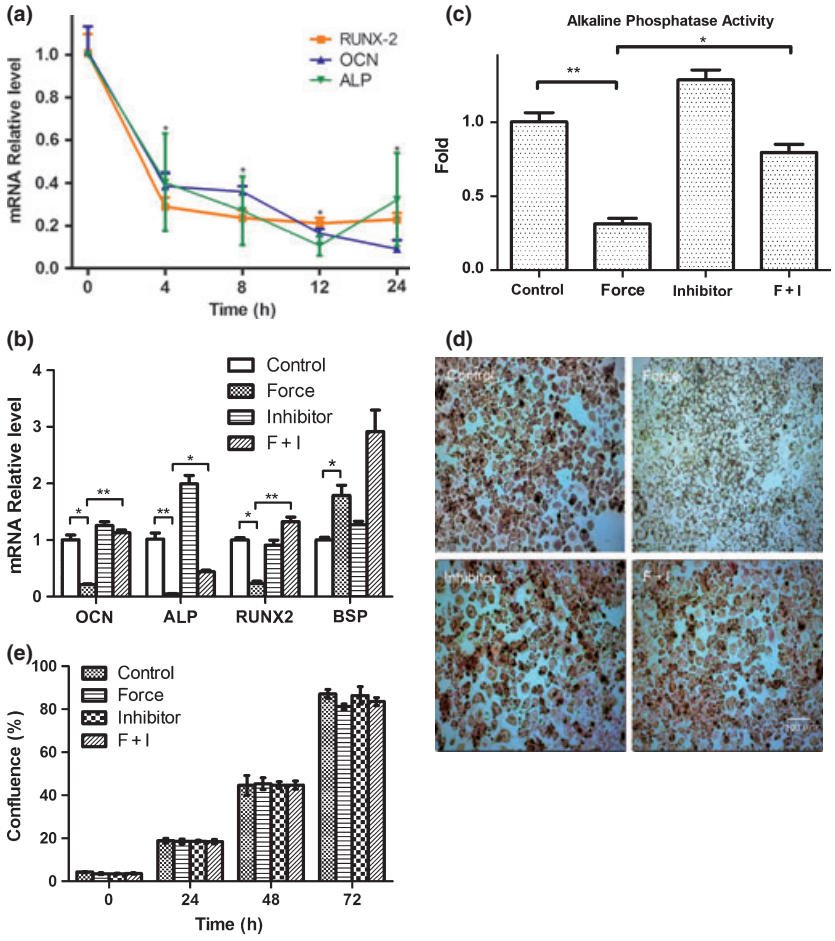

Figure 4 (**a**) Compressive force attenuated transcription of potential nuclear factor kappaB (NF-κB) target genes in U2OS cells. alkaline phosphatase (ALP), osteocalcin (OCN) and RUNX-2 transcriptions were examined with real-time PCR after force loading for 0, 4, 8, 12, or 24 h. The mRNA expression of these genes was significantly attenuated in the 4, 8, 12, and 24 h groups. $*P < 0.05$ *vs* control group, $**P < 0.01$ *vs* control group. (**b**) Inhibiting NF-κB activity potentiated transcription of ALP, OCN, and RUNX-2 as determined by real-time PCR after force loading for 8 h. $*P < 0.05$, $**P < 0.01$. (**c**) Inhibiting NF-κB activity increased alkaline phosphatase activity. $*P < 0.05$. (**d**) Leica DM2500M materials analysis microscope (10 × 10) and Leica DFC450 digital color microscope camera were used to record the stain, alizarin red staining revealed decreases on treating force to U2OS cells for 8 h. While after inhibiting NF-kB, no significant differences was found. (**e**) No significant differences in cell proliferation after compressive force loading or PDTC for 0, 24, 48, and 72 h

Nuclear factor kappaB is one of many nuclear transcription factors observed in osteoblasts after loading mechanical force stimulation. It was reported that, after loading mechanical force, RANK/RANKL (Fan *et al*, 2006; Rubin *et al*, 2006), RUNX-2 (Hesse *et al*, 2010), MAPK-ERK (Fan *et al*, 2006; Liu and Li, 2010), and other pathways could all directly or indirectly influence changes in osteoblast activity. There are cross talks between these pathways, and collectively they regulate osteogenesis activity (Baeza-Raja and Munoz-Canoves, 2004). Variation in NF-κB activity could lead to changes in osteogenesis activity. However, this experiment did not touch upon whether NF-κB activated the transcription and translation process of ALP directly or indirectly.

The process of *in vivo* force loading was simulated through mechanical force stimulation *in vitro* to investigate the variation in bone rebuilt after loading force. Researchers have applied static pressure, fluid shear stress (Young *et al*, 2010), distractive forces, vibration force (Lau *et al*, 2010), or combined forces (Sittichockechaiwut *et al*, 2009), with different levels and frequency of force; the corresponding bone rebuilding effects vary as well (Liu *et al*, 2007). In this study, we chose to apply a force loading method that was simple and inexpensive but effective, and whose force value could be adjusted randomly as the stimulating factor.

The phosphorylation of different serines could indicate different things. P65 (Ser536), which is phosphorylated on serine 536, can enhance p65 transactivation potential (Viatour, Merville *et al* 2005; Moreno, Sobotzik *et al* 2010). In this study, we detected a reaction of p65 (Ser536) to mechanical stimulation in osteoblast nuclear extracts (Figures 1b and 2b). This also demonstrated that p65 activity could be effectively changed after loading pressure stimulation on osteoblasts, thereby regulating upstream osteoblast function. While the changes of p65 in the cytosol extractions after stimulation were not obvious, the reason could be that the pressure stimulation and inhibition of PDTC inhibitor affect mainly the changes of NF-kB activity, instead of its expression.

Nuclear factor kappaB is associated with osteogenesis because of bone mass changes observed in 3-week-old NF-kB$^{-/-}$ mice(Chang *et al*, 2009). To determine whether short-term osteogenesis was affected by NF-kB, we examined a series of bone marker genes (i.e., ALP, OCN, RUNX-2, and BSP). We found that after loading force, all bone marker genes except BSP (i.e., ALP, OCN, and RUNX-2) were mediated by NF-kB. This indicated that NF-kB partly affected osteogenesis pathways and markers.

In addition, inflammatory factors directly related to NF-kB, such as IL-6 and IL-1 (Barnes and Karin, 1997) underwent significant changes, owing to mechanical force stimulation; these inflammatory factors could also be the major factors that caused osteogenetic changes.

Although we extensively examined downstream signals related to osteogenic changes caused by mechanical force on NF-kB, changes in upstream signaling owing to mechanical force on NF-kB are still unknown. It is also unclear whether NF-kB regulation on osteogenesis signals, such as ALP and RUNX-2, constitute an indirect transcriptional regulation achieved via inflammatory factors such as IL-6. These questions provide direction for our further investigations.

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Author contributions

J. Lin and F. Chen had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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