Original Article

Osteoblast lineage cells and periodontal ligament fibroblasts regulate orthodontic tooth movement that is dependent on Nuclear Factor-kappa B (NF-kB) activation

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ABSTRACT

Objectives: To investigate the role of NF-κB in osteoblast lineage cells and periodontal ligament (PDL) fibroblasts during orthodontic tooth movement (OTM).

Materials and Methods: Transgenic mice that expressed a dominant negative mutant of the inhibitor of kB kinase (IKK-DN) with lineage specific expression in osteoblastic cells and PDL fibroblasts driven by a response element in the collagen1α1 promoter and matched wild-type (WT) mice were examined. A 10-12 g force was applied by a NiTi coil and maintained for 5 or 12 days. OTM distance, PDL width, and bone volume fraction were measured using micro computed tomography. Osteoclast numbers were counted in tartrate-resistant acid phosphatase-stained sections. Activation of nuclear factor kappa B (NF-kB) was assessed by nuclear localization of p65, and the receptor activator of nuclear factor-κB ligand (RANKL) was measured by immunofluorescence and compared to control specimens with no orthodontic force.

Results: OTM-induced NF-kB activation (p65 nuclear localization) in WT mice was largely blocked in transgenic (TG) mice. OTM was significantly reduced in the TG mice compared to WT mice along with reduced osteoclastogenesis, narrower PDL width, higher bone volume fraction, and reduced RANKL expression.

Conclusions: Osteoblast lineage cells and PDL fibroblasts are key contributors to alveolar bone remodeling in OTM through IKKβ dependent NF-κB activation. (*Angle Orthod.* 2021;91:664–671.)

KEY WORDS: Osteoblast lineage cells; Periodontal ligament (PDL); Fibroblast; Nuclear Factor-kappa B (NF-κB); Orthodontic tooth movement; Mechanical force

INTRODUCTION

Bone remodeling in orthodontic tooth movement (OTM) is a highly regulated process that coordinates bone resorption by osteoclasts and new bone formation by osteoblasts.[1] OTM has three phases: (1) initial tooth tipping within periodontal ligament (PDL), (2) a lag phase, where the hyalinization area is observed on the compression side and osteoclasts mediate bone resorption, and (3) the post-lag phase, where rapid tooth movement occurs.[2] Osteoclasts are essential in

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Accepted: February 2021. Submitted: March 2020.
Published Online: April 14, 2021

bone maintenance, repair, and remodeling of bones. On mechanical loading, the cells around the tooth induce osteoclastogenesis through upregulation of the receptor activator of nuclear factor-κB ligand (RANKL).[3] When osteoclasts resorb alveolar bone next to the hyalinized tissue, PDL spaces are widened, and the tooth starts to move in the direction of the mechanical force. To date, the underlying mechanisms of main cell types in OTM and how they regulate this process remain unclear.

OTM involves the close interaction of several cell types that produce RANKL to induce osteoclastogenesis.[4] Osteoblast lineage cells including osteoblasts and osteocytes stimulate bone resorption by expression of RANKL and contribute to bone formation.[5] Osteocytes are the major mechanosensor in bone and play an essential role in inducing osteoclastogenesis during OTM.[6] PDL is the fibrous tissue between cementum and alveolar bone that maintains a tooth in the bony socket and PDL fibroblasts transmit mechanical signals in the PDL.[7] Recently, it was reported that RANKL from PDL fibroblasts and bone lining cells were essential in OTM and osteoclastogenesis in response to mechanical loading.[4] Interestingly, PDL fibroblasts have several characteristics similar to those of osteoblasts and exhibit promoter activity regulated by a 2.3 kb response element in the Col1α1 promoter, exhibit high alkaline phosphatase activity and have other bone-associated markers.[8] The 2.3 kb Col1α1-Cre transgenic mice are widely used in mechanistic studies to examine promoter activity and gene expression in osteoblasts, osteocytes, and PDL fibroblasts.[9–11] In previous studies, this regulatory element was used to generate transgenic mice that expressed a lineage specific dominant negative mutant of an inhibitor of kB kinase (IKK-DN) in osteoblast lineage cells and PDL fibroblasts.[1,9,11–14]

NF-kB is a master regulator of inflammation, induces osteoclastogenesis and inhibits new bone formation.[15,16] The members of NF-κB are RelA (p65), p50, p52, RelB and c-Rel. The p65/p50 heterodimeric complex, canonical NF-κB, is the most common isoform of NF-kB in mammalian cells. The canonical NF-κB pathway is activated by pro-inflammatory stimuli such as tumor necrosis factor (TNF) and IL-1, while the alternative NF-κB pathway is activated by a small subset of TNF family members. When NF-κB is inactive, it is combined with inhibitors of NF-κB (IκB) and exists in the cytoplasm. When the inhibitor of kB kinase (IKK) is activated, it causes IκB degradation and NF-κB translocates to the nucleus, stimulating pro-inflammatory gene transcription. Conversely, inhibition or deletion of IKK prevents activation NF-κB.[9,11,17–19] Previous studies reported that NF-κB affected bone remodeling predominantly by inducing osteoclast formation and activity.[20–22] Although the role of IKK/NF-κB signaling in periodontitis is well established,[9,11] its role in the mechanical force-induced bone remodeling has not been reported.

The goal of this study was to examine the effect of IKK/NF-kB signaling in osteoblast lineage cells and PDL fibroblasts during OTM, examining transgenic mice with a dominant negative inhibitor of NF-κB under the control of a 2.3 kb collagen 1α1 promoter. It was found that OTM was significantly reduced with less osteoclastogenesis, narrower PDL space, higher bone volume fraction, and less RANKL expression in experimental IKK-DN mice. This study demonstrated the importance of NF-κB activation in osteoblast lineage cells and PDL fibroblasts in transducing mechanical forces during OTM.

MATERIALS AND METHODS

Animal Model

The mouse experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Transgenic IKK-DN mice (TG) were generated that expressed a dominant negative IKK under the control of a 2.3 kb element of the collagen 1α1 promoter that restricts activation of NF-κB to osteoblast lineage cells and PDL fibroblasts.[1,9,12,21] Experiments were performed with adult mice 12–13 weeks old ($n = 8$ per group). C57BL/6 mice were used as a control group (wild-type, age and gender matched). Two to five mice were housed per cage under standard conditions with a 14-hour light/10-hour dark cycle. All the animals were closely monitored and fed a diet of powdered food, DietGel (ClearH_2O, Westbrook, ME) and water ad libitum throughout the experimental period.

Application of Orthodontic Force

OTM experiments were performed as previously described.[4] Briefly, mice were anesthetized by intraperitoneal administration of ketamine (80 mg/kg), xylazine (5 mg/kg), and acepromazine (1 mg/kg). Orthodontic force of 10-12 g was applied to move the upper right first molar for 5 and 12 days with a 0.006 × 0.030-inch NiTi coil spring (Ultimate Wireforms, Inc., Bristol, CT). OTM mouse models typically apply forces between 10 g and 50 g.[4,23–25] It has been reported that the higher level of force causes minor tissue damage and root resorption was observed with 0.50 N (51 g).[23] Therefore, lighter forces (10-12 g) were used, which have been successfully tested in several publications.[4,24,25] Self-etching primer and light-cured dental adhesive resin (Transbond XT; 3M Unitek, Monrovia, CA) were applied on maxillary incisors to prevent

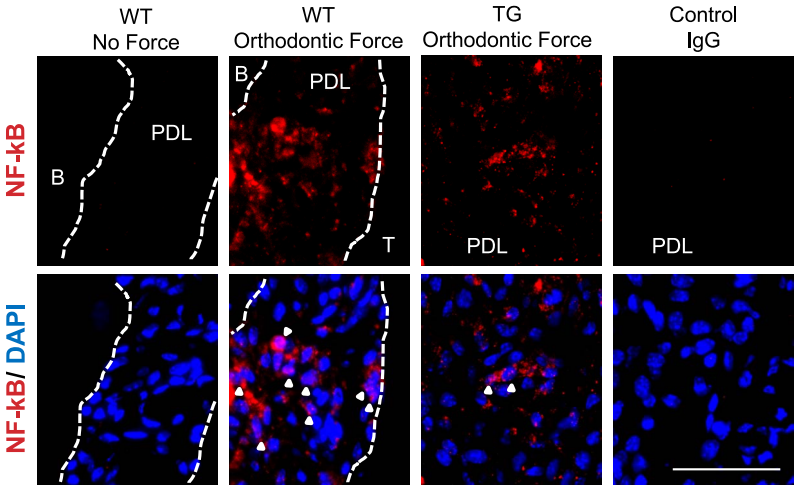

Figure 1. Nuclear NF-κB expression was significantly increased on the compression side in WT mice but this increase was blocked in TG mice. Sagittal sections from wild-type (WT) and transgenic (TG) mice were treated by immunofluorescence stain with anti-NF-κB p65 antibody. Nuclear translocation of NF-kB p65 (arrowheads), indicative of NF-kB activation, was examined on the compression side. Bar = 50 μm. B indicates alveolar bone; PDL, periodontal ligament; T, tooth.

detachment of a spring. The left side was used as a control. Mice were checked every day to assess their general conditions and euthanized on days 5 and 12 after orthodontic force loading.

Micro-CT

Samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 24 hours and then scanned using a micro-CT (MicroCT35; SCANCO Medical, Bassersdorf, Switzerland) at 55 kVp and 145 μA intensity with an integration time 200 ms. Maxillary molar areas were scanned at a 20 μm isotropic voxel size. After reconstruction, all images were converted to Digital Imaging and Communications in Medicine files and then imported to OsiriX (Pixmeo SARL, Bernex, Switzerland) for analysis. The OTM distance was measured as the minimum distance between the maxillary right first and second molar crowns in relation to the same measurements for the unloaded left side. Bone density was examined by assessing bone volume/total volume (BV/TV) without or with orthodontic forces as previously described.[26] A 250 × 250 × 250 μm^3 cube on the compression side of the coronal one-third of the distobuccal root of the maxillary first molar was selected for analysis. The average PDL width was measured from the coronal to the apical portion of the PDL on the compression side of the distobuccal root of the maxillary first molar as previously described.[4] OsiriX (Pixmeo SARL, Bernex, Switzerland) and Image Pro Plus (Media Cybernetics, Inc., Rockville, MD) software was used for 3D image reconstruction and quantitative image analysis.

Histology and TRAP stain

Specimens were decalcified in 10% ethylenediamine tetraacetic acid for 5 weeks, paraffin-embedded, and sectioned with 4-μm thickness. Tartrate-resistant acid phosphatase (TRAP) staining with hematoxylin counterstaining was performed according to the manufacturer's instructions (Sigma-Aldrich, Saint Louis, MO). TRAP-positive multinucleated cells were counted on the alveolar bone surfaces on the compression sides of the distobuccal root of the maxillary first molar under 10× and 20× objectives. Image analysis of TRAP-stained sections was performed with NIS-elements image analysis software (Nikon, Melville, NY).

Immunofluorescence stain

Immunofluorescence was performed as previously described with the following primary antibodies:[4] RANKL (ab216484; Abcam, Cambridge, MA) and NF-κB p65 (ab16502; Abcam) and compared to matched control antibody. Image analysis was performed at 40× magnification using NIS-Element software (Nikon) to examine the NF-kB p65 nuclear localization and measure the number of RANKL positive cells on the compression side of the distobuccal root of the maxillary first molar.

Statistics

Statistical analysis between WT and TG mice was performed using two-tailed Student's *t*-test and differences among multiple groups was established by analysis of variance with Scheffé's post-hoc test. Results were expressed as the mean ± SEM. $P < .05$ was considered statistically significant.

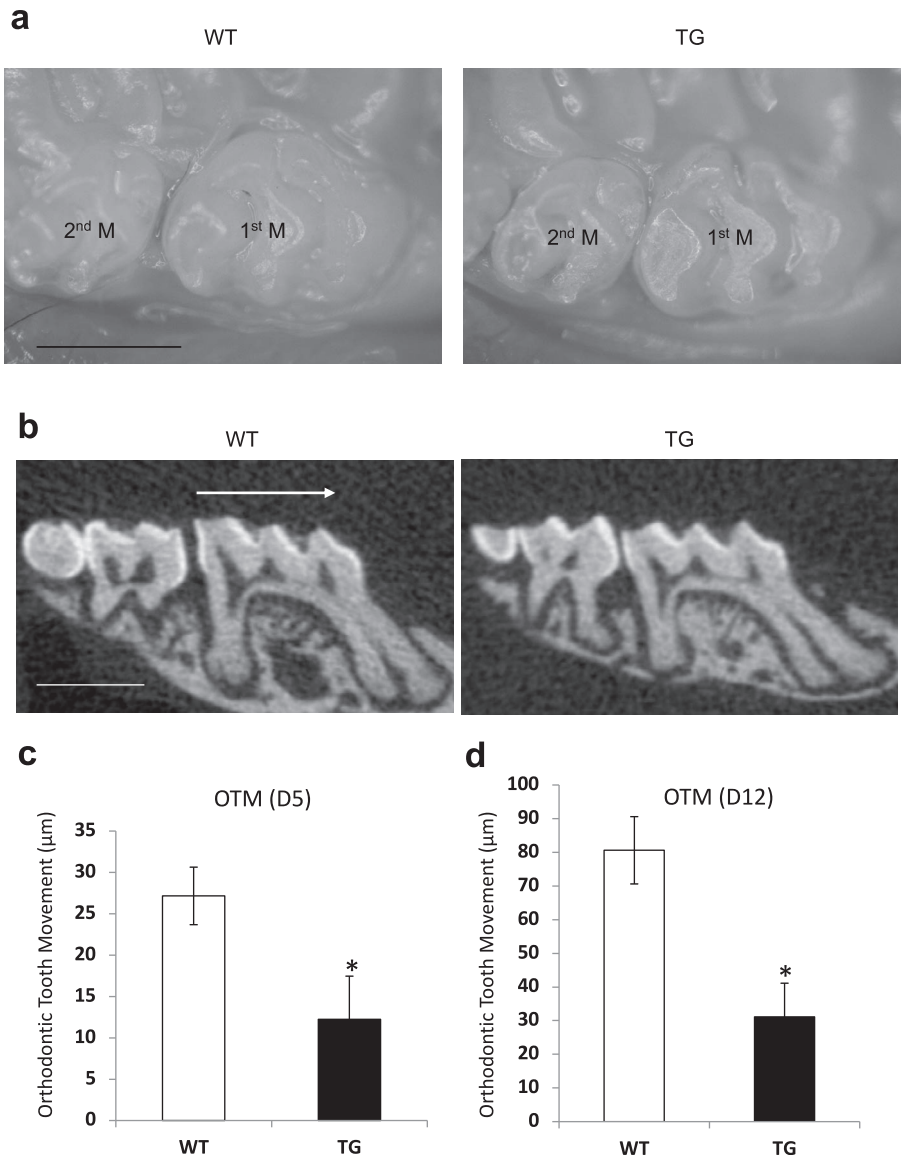

Figure 2. NF-κB inhibition in osteoblast lineage cells and PDL fibroblasts decreased orthodontic tooth movement. (a) Digital photos. Bar, 1 mm. (b) Orthodontic tooth movement was measured as the minimum distance between the most distal point of the maxillary right first molar crown and the most mesial point of the maxillary right second molar crown. Bar, 1 mm. (c) The amount of tooth movement on days 5 and 12. Each in vivo value is the mean ± SEM for n = 8 mice per group. * $P < .05$ vs WT mice group.

RESULTS

Application of orthodontic force increased NF-kB activation in wild-type but not experimental mice. NF-κB activation was assessed by p65 nuclear localization by immunofluorescence. Nuclear p65 immunopositive cells were substantially increased on the compression side in WT mice compared to the control side with no mechanical loading. The nuclear localization of p65 was largely blocked in experimental mice (Figure 1).

Inhibition of NF-κB in osteoblast lineage cells and PDL fibroblasts prevented OTM. Orthodontic tooth movement was examined in WT and experimental TG mice by measuring the minimum distance between the maxillary right first and second molar crowns (Figure 2a,b). On day 5, the teeth in the WT group moved 27.16 ± 3.48 μm and, on day 12, moved 80.6 ± 18.57 μm ($P < .05$, Figure 1c,d). The OTM amount decreased by 55% in the TG (12.24 ± 5.22 μm) on day 5 ($P < .05$) and, by 61% in the TG (31.13 ± 13.34 μm) compared with WT mice on day 12 ($P < .05$). There was no significant difference between groups on the unloaded control side ($P > .05$, data not shown).

Inhibition of NF-κB in osteoblast lineage cells and PDL fibroblasts showed higher bone volume fraction compared with WT mice after orthodontic force application. The bone volume fraction (BV/TV) was examined on the compression side of the coronal one-

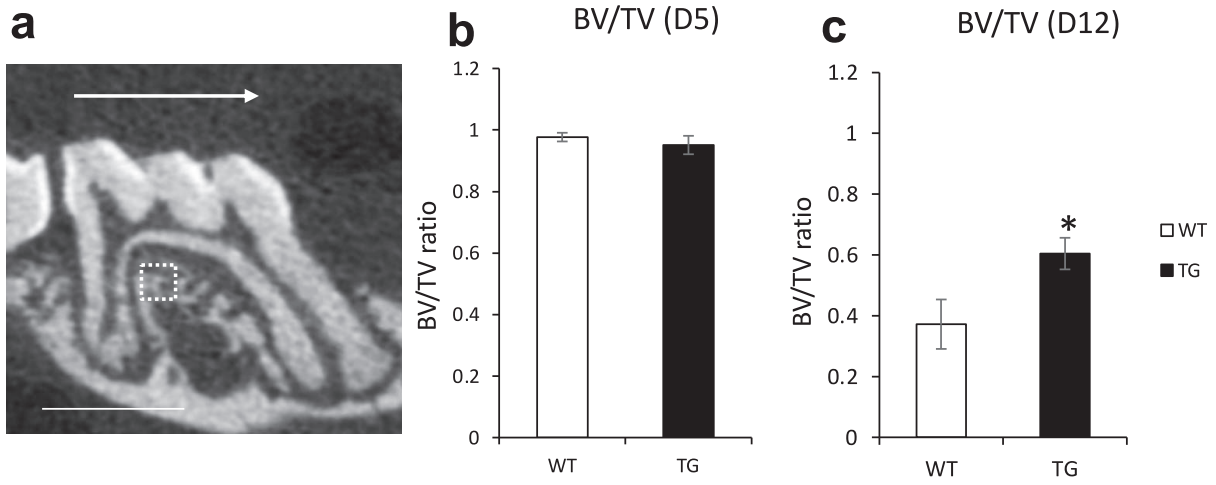

Figure 3. Inhibition of NF-κB in osteoblast lineage cells and PDL fibroblasts showed higher bone volume fraction after orthodontic force loading. (a) ROI: a 250 × 250 × 250 μm³ cube on the compression side of the coronal one-third of the distobuccal root of the maxillary first molar. Bar, 1 mm. (b and c) bone volume fraction (BV/TV) on days 5 and 12. Each in vivo value is the mean ± SEM for n = 8 mice per group. * $P < .05$ vs WT mice group.

third of the distobuccal root of the maxillary first molar (Figure 3a). MicroCT analysis revealed similar values on day 5 in the WT and experimental groups ($P > .05$, Figure 3b). On day 12, WT mice had a 63% decrease (0.37 ± 0.08) in bone volume fraction with orthodontic force compared to the unloaded side, which was almost twice the loss of bone density seen in TG mice ($P < .05$, Figure 3c).

Inhibition of NF-κB in osteoblast lineage cells and PDL fibroblasts showed narrower PDL width after orthodontic force application. The average PDL width on the compression side of the distobuccal root of the maxillary first molar was examined with microCT. As the bone on the compression side was resorbed by osteoclasts, the PDL space was widened. The PDL width increased by 200% in WT mice after orthodontic force loading (60.13 ± 7.46 μm) compared to the unloaded control side (29.88 ± 1.45 μm) (Figure 4, $P < .05$). Conversely, there was no increase on the compression side in the TG mice (36.88 ± 5.31 μm). The PDL width in the TG mice after mechanical force loading was significantly less than the WT mice ($P < .05$).

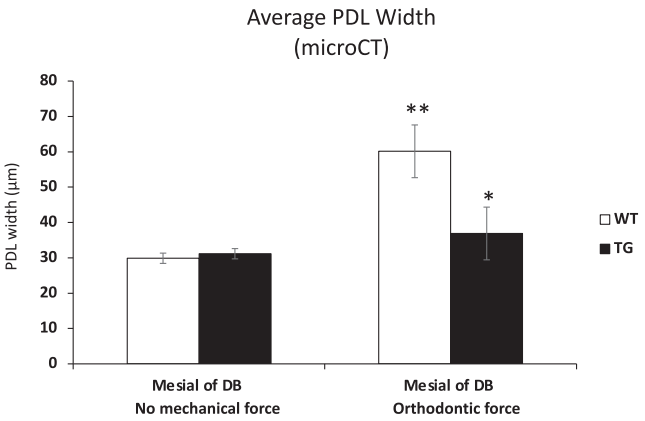

Figure 4. Inhibition of NF-κB in osteoblast lineage cells and PDL fibroblasts showed narrower PDL width after orthodontic force application (microCT). The average PDL width was measured on a sagittal section on the compression side of the distobuccal root of the maxillary right first molars. Each in vivo value is the mean ± SEM for n = 8 mice per group. * $P < .05$ vs WT mice group. ** $P < .05$ vs no mechanical force-matched mice group.

Inhibition of NF-κB in osteoblast lineage cells and PDL fibroblasts showed less osteoclast numbers and RANKL expression after orthodontic force application. Osteoclast formation was examined along the bone surface on the compression side of the distobuccal root of the maxillary first molar using the TRAP-stained sections. On mechanical force loading, the number of osteoclasts greatly increased in the WT mice and less in the TG mice (Figure 5a). On day 5, the osteoclast number was 86% less in the TG mice (1.34 ± 0.41/mm) compared to the WT mice (9.42 ± 1.85/mm) ($P < .05$; Figure 5b). There was no difference between groups on the unloaded control side ($P > .05$). On day 12, TG mice (7.18 ± 1.84/mm) had half the number of osteoclasts compared to the WT mice (14.56 ± 2.67/mm) ($P < .05$; Figure 5c).

To further investigate the potential mechanisms, RANKL expression was examined on the compression side by immunofluorescence. Mechanical loading caused an 8.7-fold increase in RANKL expression in WT mice (440.74 ± 163.79/mm²) compared with the control side (50.49 ± 26.10/mm²) on day 5, whereas this increase was significantly reduced in experimental TG mice (31.98 ± 20.99/mm²) ($P < .05$; Figure 6).

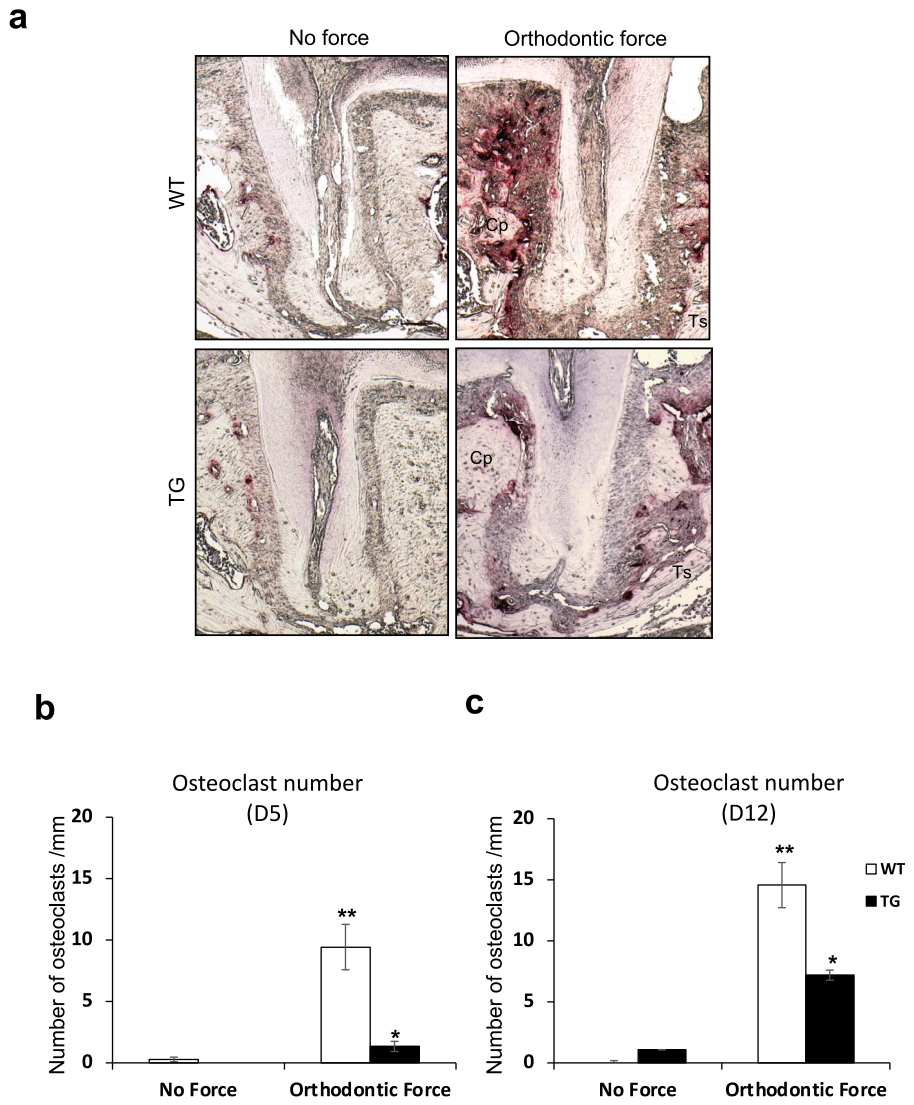

Figure 5. NF-κB inhibition in osteoblast lineage cells and PDL fibroblasts decreased osteoclast formation on the compression side of orthodontic force (TRAP stain). (a) Representative images of TRAP-stained sections (100× original magnification, distobuccal root). Bar, 100 μm. Cp, compression side; Ts: tension side. (b and c) Quantification of osteoclasts on days 5 and 12. Each in vivo value is the mean ± SEM for n = 7-8 mice per group. * $P < .05$ vs WT mice group. ** $P < .05$ vs no mechanical force matched mice group.

DISCUSSION

This study demonstrated for the first time that inhibition of NF-κB activation in osteoblast lineage cells and PDL fibroblasts significantly reduced OTM. This finding was further supported by less osteoclast formation, narrower PDL width, higher bone volume fraction, and reduced RANKL expression in experimental TG mice compared to WT mice. The results established the importance of osteoblast lineage cells and PDL fibroblasts in OTM in response to orthodontic forces mediated by NF-κB regulation in these cells.

The NF-κB signaling pathway plays important roles in physiological and pathological bone remodeling.[27,28] Zuo et al. reported that NF-κB was produced rapidly, mainly in osteoclasts, in response to orthodontic force and NF-κB activation was essential in RANKL-induced osteoclast differentiation in OTM.[29] A recent study demonstrated that vibration combined with orthodontic force increased the NF-kB activation and RANKL expression in osteocytes in vivo and in vitro.[30] Similarly, continuous orthodontic force with high-frequency vibration increased osteoclastogenesis and accelerated OTM via NF-kB activation in osteoblasts, osteocytes, and osteoclasts.[31] However, this was the first study to examine the effect of NF-κB on mechanical force-induced bone remodeling using lineage specific deletion models. NF-kB activation was observed on the compression side in WT mice, which was considerably less in experimental TG mice, leading to significantly reduced OTM.

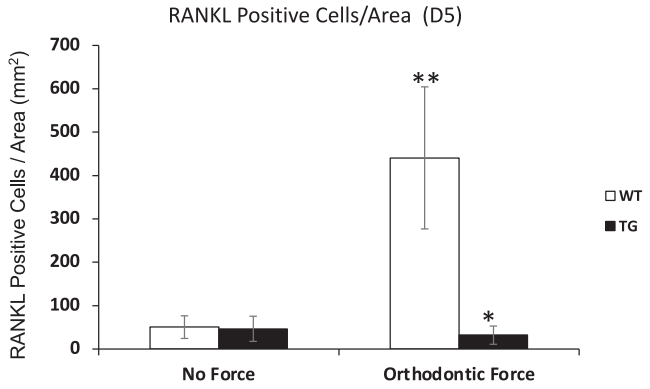

Figure 6. NF-κB inhibition in osteoblast lineage cells and PDL fibroblasts decreased RANKL expression on the compression side of orthodontic force. Sagittal sections from wild-type (WT) and transgenic (TG) mice were treated by immunofluorescence stain with anti-RANKL antibody. The number of RANKL immunopositive cells per area was counted on the compression side. Each in vivo value is the mean ± SEM for n = 5-6 mice per group. * $P < .05$ vs WT mice group. ** $P < .05$ vs no mechanical force matched mice group.

The roles of NF-κB on RANKL-induced osteoclastogenesis are well understood.[20,22,32] Inhibition of NF-κB prevents precursor cell differentiation into osteoclasts by TNF and RANKL, clearly demonstrating that NF-κB pathways are necessary for osteoclast differentiation and activity.[33] NF-κB p50 and p52 double deficient mice demonstrate lack of osteoclasts.[34] Inversely, Otero et al. found that constitutive activation of NF-κB was capable of inducing osteoclast differentiation in the absence of RANK or RANKL.[35] In the current study, both osteoclast formation and RANKL expression were found to be much less in TG mice with the NF-kB inhibition in osteoblast lineage cells and PDL fibroblasts, compared with WT mice. The same transgenic mice were previously examined in a periodontitis model, demonstrating that nuclear localization of the NF-κB, indicative of NF-κB activation, in osteoblast lineage cells and PDL fibroblasts, was suppressed in experimental TG mice and that inhibition of NF-κB activation blocked bone loss in normoglycemic and diabetic mice.[9,11] Thus, osteoblastic cells and PDL fibroblasts activated by NF-kB play a key role in bone remodeling in inflammatory periodontitis and in response to mechanical orthodontic forces.

The large number of PDL fibroblasts and osteoblast lineage cells, and their close proximity to alveolar bone, are consistent with their essential role in transducing mechanical forces that lead to bone resorption in response to orthodontic force. PDL fibroblasts constitute 50% to 60% of the total PDL cellularity,[36] are an important source of RANKL,[4,9] and have been shown to stimulate osteoclast progenitor cells.[37] In addition, NF-κB activation in osteoblasts is induced by mechanical force[38] and the inhibition of NF-κB in osteoblast lineage cells reduces bone resorption and enhances coupled bone formation to reduce periodontal bone loss.[11] Osteocytes, comprising >90% of all bone cells, show high levels of RANKL expression and support osteoclastogenesis in vitro[39] and in alveolar bone remodeling during OTM.[6]

In summary, it was demonstrated for the first time that osteoblast lineage cells and PDL fibroblasts play an essential role in OTM through NF-κB regulation. Blocking NF-κB activation in those cells significantly reduced osteoclastogenesis and RANKL expression in response to mechanical force and prevented tooth movement, indicating that the IKK/NF-kB signaling pathway in these cells is needed to mediate OTM. These studies provide new understanding about the biologic response to mechanical force and suggest the clinical benefits of using NF-κB activation/inhibition in clinical orthodontics.

CONCLUSIONS

- Inhibition of NF-κB in osteoblast lineage cells and PDL fibroblasts significantly blocked OTM with significantly less osteoclast formation and PDL width, higher bone volume fraction, and less RANKL expression on the compression side in TG mice compared to WT mice, demonstrating the importance of osteoblast lineage cells and PDL fibroblasts in osteoclastogenesis during OTM via NF-κB regulation.

ACKNOWLEDGMENTS

We thank Jiahui Madelaine Li for her help with sample staining procedure. This work was supported by NIDCR grants R01DE017732 and R01 AR0600 (DTG), the Schoenleber Pilot Grant from the University of Pennsylvania School of Dental Medicine (CHC) and the Orthodontic Faculty Development Fellowship Award from the American Association of Orthodontists Foundation (HHJ).

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