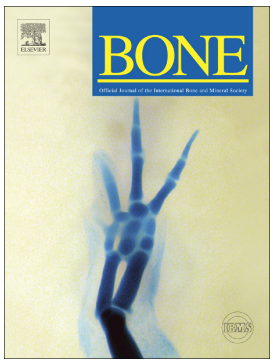

Bone resorption deficiency affects tooth root development in RANKL mutant mice due to attenuated IGF-1 signaling in radicular odontoblasts

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Bone resorption deficiency affects tooth root development in RANKL mutant mice due to attenuated IGF-1 signaling in radicular odontoblasts[1]

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Key Words: Cell differentiation; Growth factor(s); Odontoblast(s); Osteopetrosis; Tooth development; Signal transduction.

[1] Abbreviations: RANKL, receptor activator of NF-κB ligand; IGF-1, Insulin-like growth factor-1; Nfic, nuclear factor I C; DECM, dentin extracellular matrix; HERS, hertwig's epithelial root sheath; PCNA, proliferating cell nuclear antigen; BRCM, bone resorption-conditioned medium.s

Abstract

The tooth root is essential for normal tooth physiological function. Studies on mice with mutations or targeted gene deletions revealed that osteoclasts (OCs) play an important role in tooth root development. However, knowledge on the cellular and molecular mechanism underlying how OCs mediate root formation is limited. During bone formation, growth factors (e.g. Insulin-like growth factor-1, IGF-1) liberated from bone matrix by osteoclastic bone resorption stimulate osteoblast differentiation. Thus, we hypothesize that OC-osteoblast coupling may also apply to OC-odontoblast coupling; therefore OCs may have a direct impact on odontoblast differentiation through the release of growth factor(s) from bone matrix, and consequently regulate tooth root formation. To test this hypothesis, we used a receptor activator of NF-κB ligand (RANKL) knockout mouse model in which OC differentiation and function was entirely blocked. We found that molar root formation and tooth eruption were defective in *RANKL*$^{-/-}$ mice. Disrupted elongation and disorganization of Hertwig's epithelial root sheath (HERS) was observed in *RANKL*$^{-/-}$ mice. Reduced expression of nuclear factor I C (NFIC), osterix, and dentin sialoprotein, markers essential for radicular (root) odontogenic cell differentiation indicated that odontoblast differentiation was disrupted in RANKL deficient mice likely contributing to the defect in root formation. Moreover, down-regulation of IGF/AKT/mTOR activity in odontoblast indicated that IGF signaling transduction in odontoblasts of the mutant mice was impaired. Treating odontoblast cells *in vitro* with conditioned medium from *RANKL*$^{-/-}$ OCs cultured on bone slices resulted in inhibition of odontoblast differentiation. Moreover, depletion of IGF-1 in bone resorption-conditioned medium (BRCM) from wild-type (WT) OC significantly compromised the ability of WT osteoclastic BRCM to induce odontoblast differentiation while addition of IGF-1 into *RANKL*$^{-/-}$ osteoclastic BRCM rescued impaired odontoblast differentiation, confirming that root and eruption defect in RANKL deficiency mice may result from failure of releasing of IGF-1 from bone matrix through OC bone resorption. These results suggest that OCs are important for odontoblast differentiation and tooth root formation, possibly through IGF/AKT/mTOR signaling mediated by cell-bone matrix interaction. These findings provide significant insights into regulatory mechanism of tooth root development, and also lay the foundation for root regeneration studies.

1. Introduction

Bone homeostasis is maintained by a balance between the combined processes of osteoblast-mediated bone formation and osteoclast (OC)-mediated bone resorption. Several disorders, such as osteoporosis and osteoarthritis, result from a disruption of this delicate balance [1-4]. One disorder in particular, osteopetrosis, is caused by disruption of OCs and can lead to death due to ablation of the bone marrow. To discover new insights into the fundamental biology of OCs, various mutant models have been designed to determine the genes involved in early OC differentiation [5-8], terminal OC differentiation [9, 10], and bone resorption [11-14]. Receptor activator of nuclear factor-κB ligand (RANKL) is a member of the tumor necrosis factor receptor (TNFR) superfamily that competes with osteoprotegerin (OPG) for binding to its receptor RANK on the surface of OC precursors [15-17]. This dynamic interaction between RANK and RANKL orchestrates the cascade of osteoclastogenesis transcriptional factors, which subsequently drive OC differentiation, activation, and function [16, 17]. The human form of the RANKL gene has been found to be one of the genes for autosomal recessive osteopetrosis [18].

Studies on mice with mutations or targeted deletions of OC-related genes have revealed that OC loss or dysfunction is often associated with tooth root development defects, indicating that OCs play a critical role in tooth root development [6, 13, 19]. Currently, OCs are mainly considered to function in two coordinated processes: formation of the eruption pathway and the vertical movement of a developing tooth bud into the oral cavity [20, 21]. In order for the eruption pathway to form, numerous OCs must be recruited and the alveolar bone must be actively resorbed [22, 23]. Notably, tooth root development and tooth eruption are two separated processes which is involved with different regulatory mechanisms [24]. Many previous studies focused on the role of OCs in facilitating tooth eruption [23, 25, 26]. However, the role of OCs in root formation and the underlying cellular and molecular mechanisms still remain largely unknown.

Teeth are regarded as one of the best models for developmental biology and organ regeneration. Development of mammalian teeth is the result of reciprocal interactions between oral epithelium and underlying neural crest derived mesenchyme [27]. Tooth root development begins after the completion of crown formation at post-natal day 4 in mice and continues to elongate until mice are approximately 3 weeks old [28]. Hertwig's epithelial root sheath (HERS), the bilayer epithelial structure derived from the inner and outer enamel epithelia that fuse below the level of the crown cervical margin, is widely accepted as the main structure responsible for root formation. The apical tip of HERS bends inwards towards the pulp chamber at the early stage of root formation and controls cell proliferation resulting in root elongation. Further,

odontoblasts responsible for root formation (radicular odontoblasts) are neural crest-derived cells and their differentiation is controlled by the reciprocal interactions between HERS and dental pulp mesenchyme [29]. Thus, HERS plays an important role in root formation during tooth development. In addition, normal cytodifferentiation and maturation of odontoblasts is essential for root elongation. Several markers can be used to assess odontoblast differentiation and function status including Nuclear factor I C (Nfic), dentin sialophosphoprotein (Dspp), nestin, and osterix (Osx).

Previous studies have shown that regulatory factors such as growth hormones and insulin-like growth factors (IGFs) are associated with the secretion of dentin-related proteins and odontogenesis [30-32]. Immunoreactivity for IGF-I is observed in epithelial cells around the cervical loop of molar tooth germs [33] and in the entire HERS of molars on postnatal days 6–15 in rats [34], indicating the role of IGF-1 in regulating the proliferation and differentiation of dental epithelia. Conversely, one of the major mechanisms for osteoblast-OC coupling is through growth factors released from the bone matrix by osteoclastic bone resorption. IGF-1 is one of the most abundant bone matrix proteins, and is believed to be involved in maintenance of bone mass [35]. During the process of bone resorption, OCs release growth factors from the bone matrix, which induce stem cell migration and osteoblast differentiation [36, 37]. Thus, osteoblast differentiation for new bone formation often occurs at the osteoclastic bone resorption site [38]. And IGF-1 is identified as one such coupling growth factor [39]. Therefore, we have hypothesized that IGF-1 is involved in tooth root formation by acting as a coupling growth factor that is released through normal osteoclastic bone resorption.

In the current study, we sought to determine the role of OCs in tooth root formation using the *RANKL*$^{-/-}$ osteopetrosis mutant mouse model and multiple approaches, including X-ray, micro-CT, immunohistochemistry, immunofluorescence, quantitative real-time polymerase chain reaction (qRT-PCR), and antibody neutralization by immunoprecipitation. Hereafter, multiple markers, including *Nfic*, *Dspp*, *nestin*, *Col1a1* and *Osx*, were used to assess the differentiation status of root odontoblasts. We found that there was a complete absence of OCs in the mutant alveolar bone. More importantly, HERS elongation and radicular odontoblast differentiation were disrupted in RANKL-deficient mice. We found that IGF/AKT/mTOR signaling was attenuated in odontoblast of RANKL-deficient mice, possibly resulting from failure of releasing IGF-1 from bone matrix through osteoclastic bone resorption.

2. Methods

2.1 Animals. Male wild-type (WT) C57BL/6J mice (from Jackson Laboratory) were used for the study as the control group. *RANKL*$^{+/-}$ mice were kindly given by Yongwon Choi and Sandy C. Marks Jr. (University of Massachusetts Medical School) [40] and were crossed with C57BL/6J WT mice and then intercrossed to generate *RANKL*$^{-/-}$ mice. The animals were maintained in the University of Alabama at Birmingham (UAB) animal facility and were given distilled water and allowed ad libitum access to food. All experimental protocols were approved by the NIH and the Institutional Animal Care and Use Committee [12].

2.2 Harvest and preparation of samples. We used 20-30 *RANKL* knockout mice in Fig.1 for the rootless phenotype analysis. More than 6 WT or *RANKL* knockout mice samples were used in each group in Fig. 2-5 for histology and qPCR analysis. Animals were euthanized by CO_2 inhalation. The mandibles were removed and hemisected. The left side of the jaw samples were processed for X-ray and Micro-CT analysis, and the right side of the jaw samples were processed for paraffin embedding and histological analysis. After removal of soft tissue, jaw samples from the left side were fixed in 4% formaldehyde for 24 hours then stored in 70% ethanol prior to X-ray and Micro-CT analysis. The jaw samples from the right side were fixed in 4% paraformaldehyde and prepared for histological analyses. The right sections were prepared for histological analysis according to standard protocol for samples that were prepared for paraffin sectioning.

2.3 Histological Analyses. Histological analyses were performed as described [41]. Tartrate-resistant acid phosphatase (TRAP) stain was used as a marker for OCs using a commercial kit (Sigma) according to the manufacturer's instructions. Multinucleated TRAP-positive cells appeared as dark purple cells and were counted by light microscopy. Hematoxylin & eosin (H&E) staining was done as described previously [42, 43].

2.4 Radiographic procedures. X-ray analysis and microcomputed tomography (MicroCT) radiography were performed as previously described [42, 44, 45] at the University of Alabama at Birmingham Small Animal Bone Phenotyping Core associated with the Center for Metabolic Bone Disease.

2.5 Immunohistochemistry (IHC) analysis. We performed IHC staining using corresponding alveolar and femoral sections from WT and *RANKL*$^{-/-}$ mice as described [42, 44, 45]. Mandible and long bone samples of different groups were sectioned, then the slides were analyzed by

IHC for expression of proteins of the following markers: proliferating cell nuclear antigen (PCNA) (Goat polyclonal) (Santa Cruz, USA) (1:400), nestin (Chicken polyclonal) (aves LABS) (1:10000), dentin sialoprotein (DSP) (Rabbit polyclonal) (Santa Cruz, USA) (1:200), osterix (Osx) (Goat polyclonal) (Santa Cruz, USA) (1:200), IGF-1 (Rabbit polyclonal) (Santa Cruz, USA) (1:200), mTOR (Rabbit polyclonal) (Cell Signaling) (1:200), Phospho-Akt (Rabbit monoclonal) (Cell Signaling) (1:200), and Col1a1(Goat polyclonal) (Santa Cruz, USA) (1:50).

2.6 Preparation of bone resorption-conditioned medium (BRCM). For preparation of BRCM, we isolated OC precursors from the spleen of WT and *RANKL*$^{-/-}$ mice as described previously [41, 46]. Mouse calvaria bone slices were harvested from 4- to 6-week-old mice. Disk-like calvaria bone was harvested intact from the mouse heads followed by removal of the soft tissue including the periosteum. The average thickness of the calvaria bone was 0.5mm. Harvested calvaria bone slices were then washed three times in iced phosphate buffered saline (PBS) solution, and then snap-frozen with liquid nitrogen to kill living cells in the calvaria bone slices and to preserve bone matrix cytokines. Splenocytes were then plated on bone slice in 48-well tissue culture plates ($1x10^5$ cells per well) and cultured in α-modified MEM (GIBCO-BRL) with 10% (vol/vol) fetal bovine serum (FBS) (GIBCO-BRL) containing 20 ng/mL macrophage colony-stimulating factor (M-CSF; R&D Systems). After 24 hours, cells were cultured in the presence of 10 ng/mL receptor activator of NF-κB ligand (RANKL; R&D Systems) and 10 ng/mL M-CSF to induce OC formation. The conditioned media from OC-mediated bone resorption was harvested at days 8 to 10 after induction.

***2.7 In Vitro* differentiation of odontoblast cell line.** Odontoblast cell line A4 [47, 48], were plated into 12-well plates at a density of $4X10^5$ cells per well and cultured in the following media: osteogenic (mineralization) medium, Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 units/ml penicillin/streptomycin, 50 μg/ml ascorbic acid, and 10 mM sodium β-glycerophosphate (Sigma); WT OC conditioned medium: 20% conditioned media from WT OC culturing on plate (without bone slices) diluted with DMEM; WT OC + bone conditioned medium: 20% conditioned media from WT OC culturing on bone slice diluted with DMEM; *RANKL*$^{-/-}$ OC + bone conditioned medium: 20% conditioned media from *RANKL*$^{-/-}$ OC culturing on bone slice diluted with DMEM. Culture media were changed every 2 days and cells were harvested for IF and qPCR analysis at corresponding time points.

Antibody neutralization and immunoprecipitation were conducted to deplete IGF-1 from BRCM. 2ug/ml IGF-1 monoclonal antibody (Santa Cruz) was added to BRCM, incubated overnight in

4°C, and then absorpted to Protein G Magnetic Beads (NEB). BRCM added to mouse IgG (Jackson Immunology) or beads were used as controls. For *in vitro* rescuing assay, recombinant mouse IGF-1 (50 ng/mL; Cat#9897, Cell Signaling, USA) was added to *RANKL*$^{-/-}$ BRCM (*RANKL*$^{-/-}$ BRCM + IGF-1) to treat plated pre-odontoblast cell line.

2.8 RNA extraction and quantitative real-time PCR (qRT-PCR). qPCR analysis using tissue of the alveolar bone-root region, which included root, dental pulp and alveolar bone surrounding the root was conducted. We harvested the root samples cutting from the alveolar bone-root region of the WT mice mandible, which included root, dental pulp and alveolar bone surrounding the root and excluding the crown. As there is no tooth root for *RANKL*$^{-/-}$ mice, we harvested the root samples from the same area as from the WT mice in order to compare the gene expression level of the same alveolar bone-root area. For RNA extraction, the prepared samples were transferred to a tube prefilled with beads (Nextadvance Company, USA) and homogenized using a Bullet blender (Nextadvance Company, USA). The RNA extraction was performed using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. The extracted RNA was used for reverse transcription using the RevertAid Reverse Transcriptase kit (Thermo Scientific, Waltham, MA). Real-time quantitative PCR (qPCR) was performed as described previously (10, 17) using primers purchased from Invitrogen as listed (see Table 1). Briefly, cDNA fragments were amplified with Sybr green fast advanced master mix (Applied Biosystems, Foster City, CA) and detected by the Step-One real-time PCR system (Applied Biosystems). The mRNA expression level of the *Hprt1* housekeeping gene was used as an endogenous control and specific mRNA expression levels were calculated as a ratio to the *Hprt1* level.

Table 1. Primers used for qRT-PCR

Primers used for qRT-PCR		
Gene Symbol	Forward Primers (5'-3')	Reverse Primers (5'-3')
Nfic	GGAACCGGACCCAACTTCTC	CGTCCTCTTCCATCGAGCC
Dspp	AGTTCGATGACGAGTCC	GTCTTCTCCCGCATGT
Runx2	AGTGCTCTAACCACAGTCCATGCA	TACAAACCATACCCAAGTACCTGTTT
Osx	*CTGGGGAAAGGAGGCACAAAGAAG*	*GGGTTAAGGGGAGCAAAGTCAGAT*
Nestin	CACACCTCAAGATGTCCC	GAAAGCCAAGAGAAGCCT
IGF-1	*ACAGGCTATGGCTCCAGCA*	*GCACAGTACATCTCCAGTCTCCTC*
IGF-2	*TGTTGACACGCTTCAGTTTGTCTG*	*GAAGCAGCACTCTTCCACGATG*
IGF-1R	*CACGACGATGAGTGCATGC*	*GAGCAGAAGTCACCGAATCGA*
Hprt1	GGTGGAGATGATCTCTCAACTTTAA	GGGAAAGCAAAGTTTGCATTGTT

2.9 Statistical analysis and data quantification. Experimental data are reported as means ± SD. The figures are representative of the data (N=6). Data were analyzed with the two-tailed Student's t-test or ANOVA analysis, as indicated in the figure legend. P values <0.05 was considered significant. Data quantification analyses were performed using the NIH ImageJ Program as described [42, 43].

3. Results

3.1 Root formation is disrupted in *RANKL* knockout mice at 3 weeks. It was observed that the *RANKL*$^{-/-}$ mice were noticeably smaller in size with prevalent developmental and dental defects compared to WT (Fig. 1A). The photographic images and Hematoxylin and Eosin (H&E) staining of first mandibular molars demonstrated that the root formation in *RANKL*$^{-/-}$ mice group was completely impaired at 3 weeks (Fig. 1B, D). Interestingly, crown formation in the *RANKL*$^{-/-}$ mice group was unaffected. X-ray analysis of mandibles from *RANKL*$^{-/-}$ mice further showed that root formation was disrupted in the *RANKL*$^{-/-}$ mice compared to WT mice (Fig. 1C). Mice heterozygous for the *RANKL* mutation appeared phenotypically normal, with fully-formed tooth roots and normal body size as compared to the WT mice.

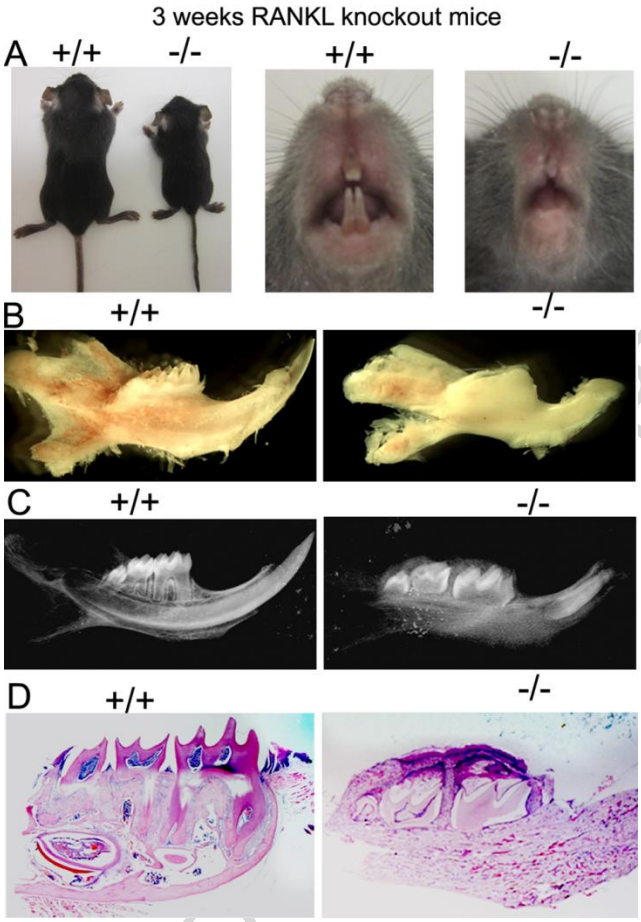

Fig. 1. 3-week-old RANKL knockout mice exhibit disrupted tooth root formation. (**A**) Representative photographic images of 3-week-old wild-type (WT, or +/+) and *RANKL*$^{-/-}$ mice. (**B**) Representative photographic images of first mandibular molar showed no teeth eruption and root development in *RANKL*$^{-/-}$ mice compared to WT mice **(C)** Representative radiographs showed disrupted root formation in *RANKL*$^{-/-}$ mice compared to WT. (**D**) Representative images of H&E stain of first mandibular molars showed normal tooth development in WT compared to impaired tooth development in *RANKL*$^{-/-}$ mice. n $\geqslant$ 6 in each group.

3.2 OCs are absent in alveolar bone of *RANKL* knockout mice. To characterize the OCs in the bone surrounding the tooth, we analyzed OC activity in *RANKL*$^{-/-}$ mice using H&E and TRAP staining of the bone surrounding the first mandibular molar at various stages of development (Supplemental Fig. 1). Root development was absent in the molars of *RANKL*$^{-/-}$ mice by 3 weeks after birth whereas root formation of teeth was well established in the control group at the same age (Supplemental Fig. 1A). TRAP staining showed that OCs were completely absent in the alveolar bone at the base of the teeth in the *RANKL*$^{-/-}$ mice group (Supplemental Fig. 1B). The results suggest that alterations in bone remodeling and poorly formed bone environment in RANKL knockout mice could play a role in mediating the root dysplasia phenotype observed.

3.3 HERS is disrupted in *RANKL* knockout mice. To explore the basis of the root phenotype, we compared H&E staining sections of the first molar from postnatal day 4 (PN4) and postnatal day 14 (PN14) WT and *RANKL*$^{-/-}$ mice. At PN4, the tooth crowns in WT and mutant mice were similar. In WT mice, the double layered-structure of the epithelial HERS was clear on the distal side of the tooth near the second molar and could be seen elongating downward and inward into the mesenchyme (Fig. 2C, arrow). In contrast, extension of the HERS was not detected in *RANKL*$^{-/-}$ mouse molars at this stage (Fig. 2F, arrow). At PN14, in the first molars of the WT mice, the root formation was well established (Fig. 2G-I). Radicular dentin was deposited by well-organized odontoblast in the root regions (Fig. 2I). However, the root of the first molar in the mutant mice did not develop (Fig. 2J-L); there was no evidence of root elongation and the HERS was missing or truncated. The histological morphology of the mutant teeth suggested that RANKL deficiency may alter the function of HERS and/or odontoblasts resulting in defective root development.

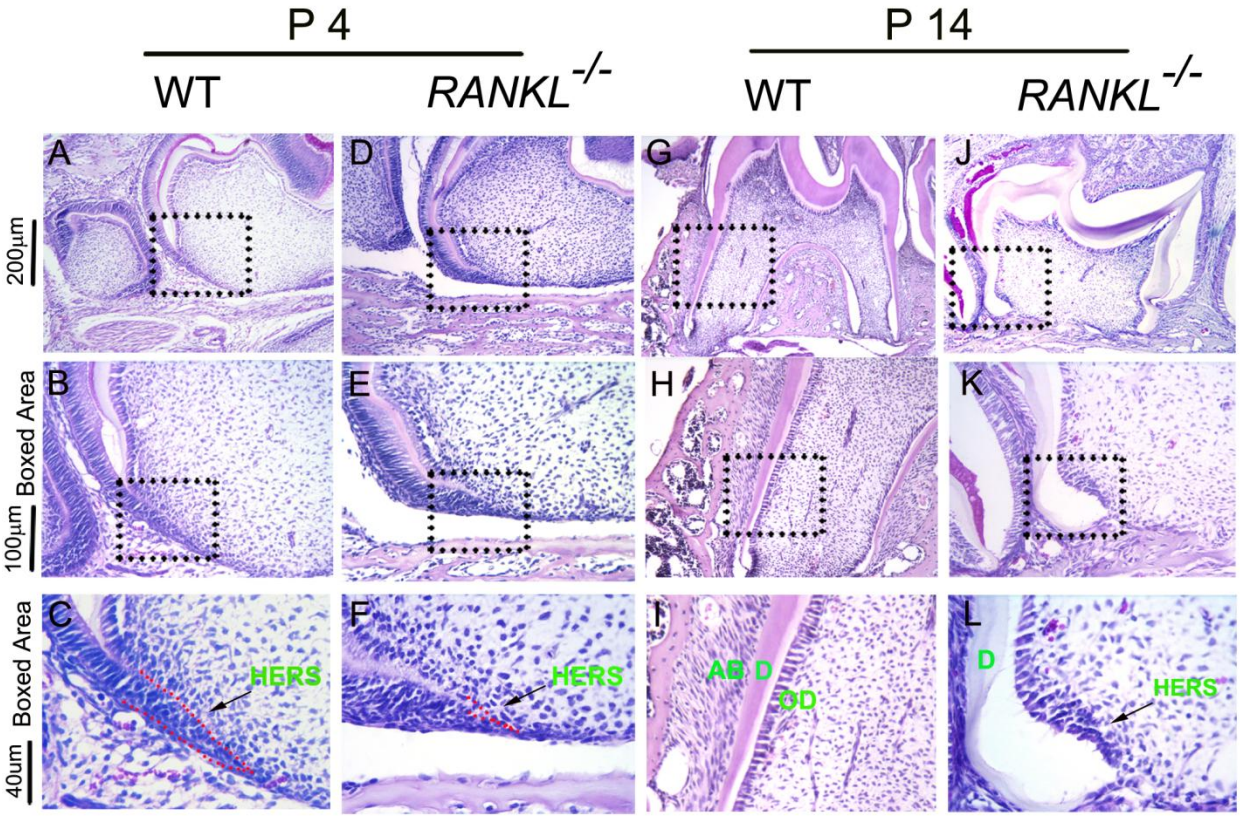

Fig. 2. Comparison of tooth histology. Representative images of H&E stained mandibular first molar from postnatal day 4 (PN4) WT (A-C) and *RANKL*$^{-/-}$ (D-F), and postnatal day 14 (PN14) WT (G-I) and *RANKL*$^{-/-}$ (J-L) mice. Sagittal images of tooth samples are shown. HERS-

Hertwig's epithelial root sheath; AB-ameloblasts; D-dentin; OD-odontoblast. *RANKL*$^{-/-}$ mice demonstrate alterations in the histology of HERS and odontoblasts. n $\geqslant$ 6 in each group.

3.4 Proliferation and differentiation of odontogenic cell is disrupted in RANKL deficient tooth roots. Apart from the discovery that the elongation of HERS was affected in RANKL-deficient mice, we sought to determine whether proliferation and differentiation of odontogenic cells, essential processes for root development, were affected. IHC staining of PCNA showed that in 4-day old first molar samples the proliferative activity of odontogenic cells of the RANKL deficient mice significantly decreased compared with that of WT mice (Fig. 3A). Nestin is a member of the intermediate filament family and is considered a specific marker for mature odontoblasts [49]. IHC staining of nestin demonstrated that the expression level of nestin decreased in the *RANKL*$^{-/-}$ mice group compared to the WT group at both PN4 and PN14 (Fig. 3B, green arrows). Dentin sialoprotein (DSP) is a dentin protein that has been shown to be tooth-specific and predominantly expressed by odontoblast cells. Osterix (Osx) has been found to play a critical role in root-polarized odontoblast formation [50]. Hence, we performed IF staining for DSP and Osx in WT and *RANKL*$^{-/-}$ mice at PN4 and found that the expression of DSP and Osx decreased significantly in RANKL deficient odontoblasts (Figs. 3C, D). Furthermore, qRT-PCR confirmed that the gene expression of *Nfic*, *Dspp*, *nestin*, and *Runx2*, markers essential for odontoblast differentiation, all decreased significantly in root samples of *RANKL*$^{-/-}$ mice at both 4-days and 2-weeks old as compared to WT mice (Fig. 3E). These results indicate that odontoblast differentiation and function is affected by ablating RANKL during tooth development.

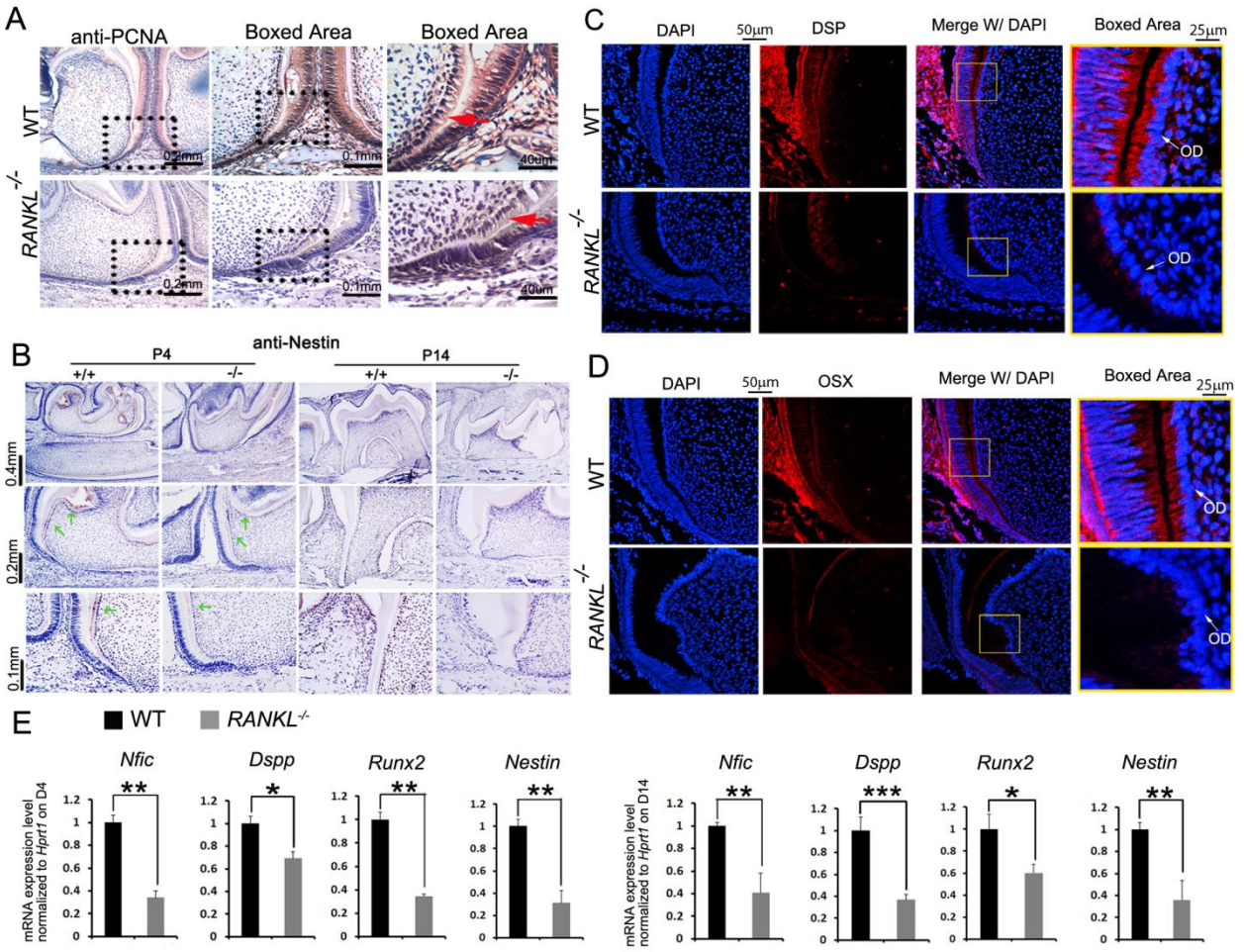

Fig. 3. RANKL deficiency affects odontogenic cell proliferation and odontoblast differentiation during tooth root development. (**A**) IHC staining of PCNA in WT and *RANKL*$^{-/-}$ mice at 4 days. (**B**) IHC staining of nestin in odontoblasts of WT and *RANKL*$^{-/-}$ mice groups at 4 days. (**C**) Immunofluorescent staining of DSP in odontoblasts of WT and *RANKL*$^{-/-}$ mice groups at 4 days. (**D**) Immunofluorescent staining of OSX in odontoblasts of WT and *RANKL*$^{-/-}$ mice groups at 4 days. (**E**) qRT-PCR analysis of the expression of odontoblast differentiation markers in WT and *RANKL*$^{-/-}$ mice root samples at 4 days and 14 days. *Hprt1* was used as an endogenous control. n $\geqslant$ 6 in each group; *, P<0.05; **, P<0.01; ***, *P*<0.001 (Student's t-test).

3.5 Knockout of RANKL decreases the activity of IGF-1/ATK/mTOR signaling in alveolar bone and root odontoblasts. IHC staining of IGF-1 demonstrated that the expression level of IGF-1 decreased in the *RANKL*$^{-/-}$ mice group compared to the WT group at both PN4 and PN14 (Fig. 4A, black arrows). qRT-PCR results confirmed that gene expression of IGF-1, IGF-2, and IGF-1R all decreased significantly in root samples of both 4-day old and 14-day old *RANKL*$^{-/-}$ mice as compared to WT mice (Fig. 4B). IHC staining of phospho-AKT showed a reduced expression level in the PN4 *RANKL*$^{-/-}$ mice (Fig. 5C,D) as compared to the WT mice (Fig. 5A,B).

At PN14, p-AKT was widely expressed in odontoblasts at the formed root region (Fig. 5E,F). However, the expression of p-AKT in the root region of the *RANKL*$^{-/-}$ mice was barely detectable (Fig. 5G,H). Expression level of mTOR in PN4 *RANKL*$^{-/-}$ group decreased slightly (Fig. 5K,L) as compared to that in the WT group (Fig. 5I,J). Notably, anti-mTOR staining in the PN14 mutant mice revealed a significant decrease in the number of positive cells in both the root region (Fig. 5Q) and the alveolar bone region under the tooth root (Fig. 5R) as compared to the same regions of the WT mice (Fig. N and O, respectively). These results indicate that RANKL deficient odontoblasts are not capable of differentiating to mature and functional cells and promoting root formation, mainly due to drastic reduction in cell growth and proliferation from the inhibition of IGF-1/p-ATK/mTOR signaling.

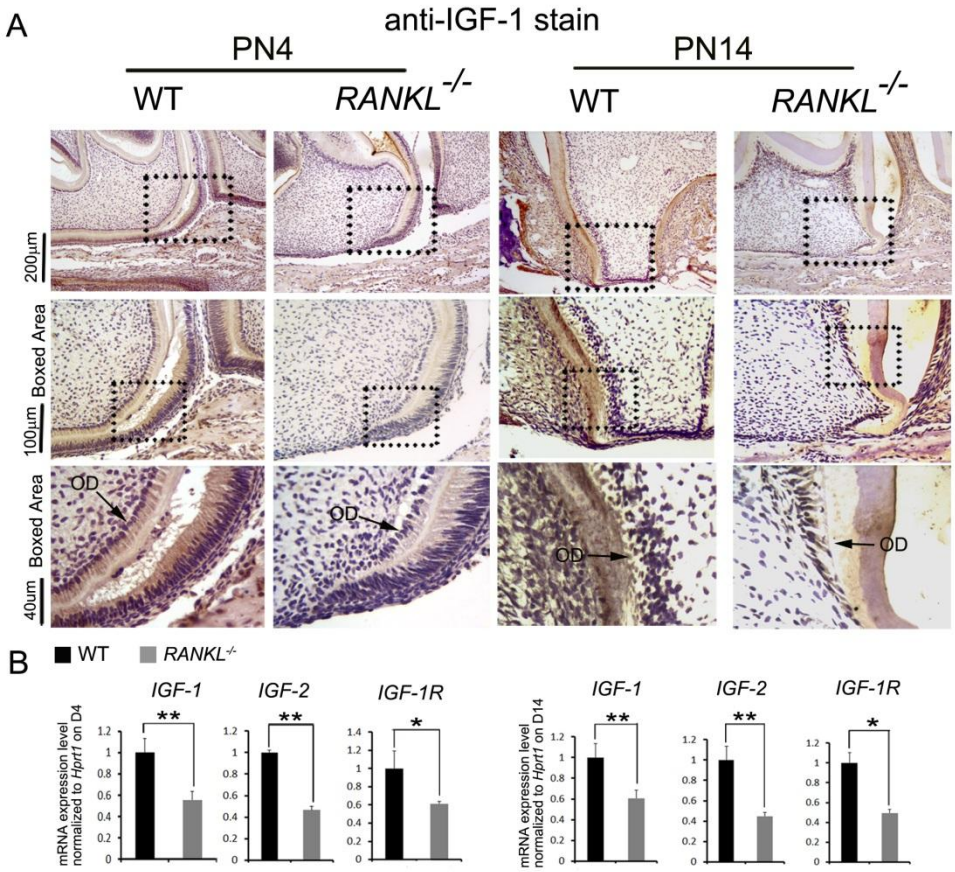

Fig. 4. IGF-1 expression in RANKL knockout mice significantly reduced at 4 days and 14 days compared with that in WT mice. (**A**) IHC staining of IGF-1 in odontoblasts and alveolar bone in WT and *RANKL*$^{-/-}$ mice group. n $\geqslant$ 6 in each group. (**B**) qRT-PCR analysis of the expression of *IGF-1*, *IGF-2* and *IGF-1R* in WT and *RANKL*$^{-/-}$ mice root samples at 4 days and 14 days. Hprt1 was used as an endogenous control. n $\geqslant$ 6 in each group; *, P<0.05; **, P<0.01 (Student's t-test).

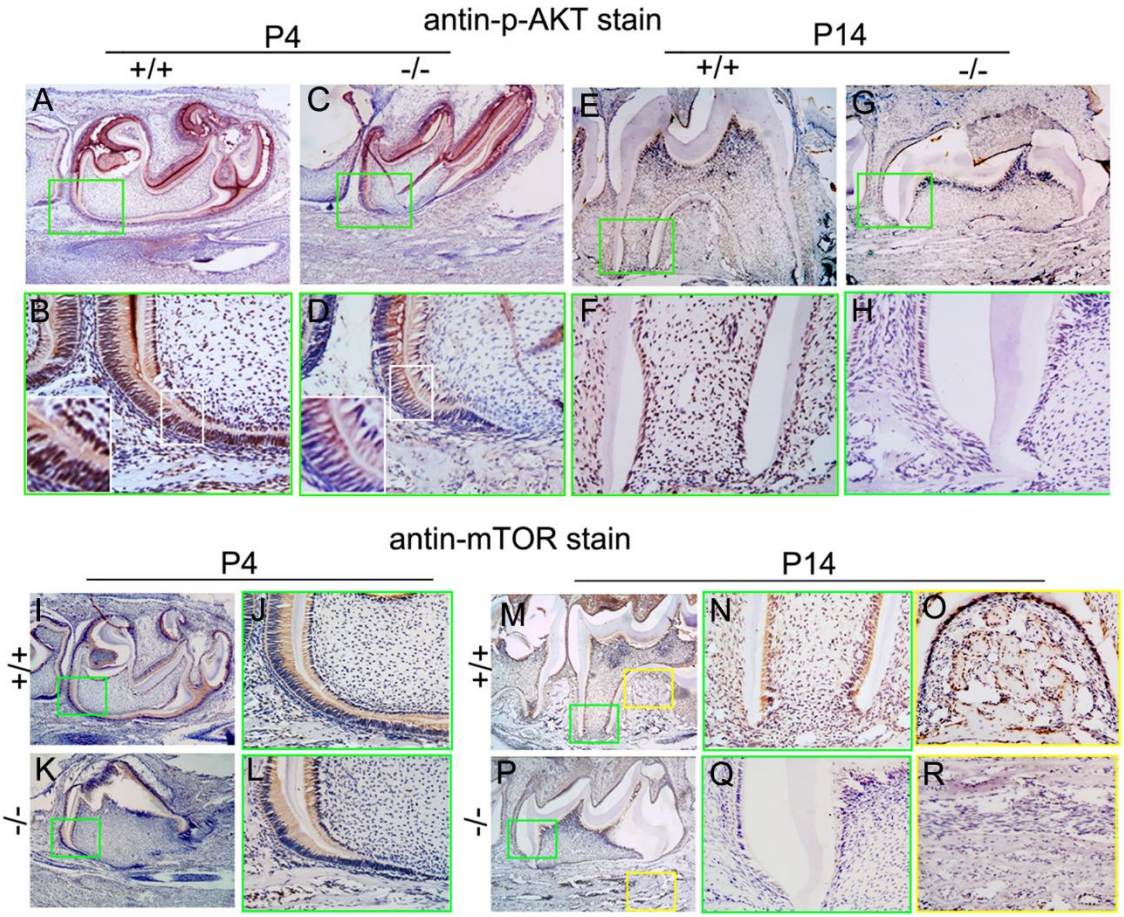

Fig. 5. Knockout of RANKL decreases the activity of p-ATK/mTOR signaling pathways in odontogenic cells and alveolar bone at 4 days and 14 days. (**A-H**) anti-p-ATK IHC staining of representative mandibles in WT and *RANKL*$^{-/-}$ mice group at day 4 and day 14. Sagittal images of tooth samples are shown. (B), (D), (F) and (H) are the magnified pictures of the green boxed areas of (A), (C), (E) and (G), respectively. Insets in (B) and (D) are the magnified images of the white boxed areas in the same figure. (**I-R**) anti-mTOR IHC staining of representative mandibles in WT and *RANKL*$^{-/-}$ mice group at day 4 and day 14. (J) and (L) are the magnified pictures of the green boxed areas of (I) and (K), respectively. (N) and (Q) are the magnified pictures of the green boxed areas of (M) and (P), respectively. (O) and (R) are the magnified pictures of the yellow boxed areas of (M) and (P), respectively. n $\geqslant$ 6 in each group.

3.6 RANKL deficient OC bone resorption-conditioned medium inhibits odontoblast differentiation *in vitro*

As IGF-1 signaling transduction was found disrupted in *in vivo* analyses of RANKL deficient mice and IGF-1 is one of the coupling growth factors that mediates OC-osteoblast communication, we hypothesized that lack of OCs in RANKL deficient alveolar bone leads to failure of IGF-1 release from the bone matrix by osteoclastic bone resorption, which results in inhibited odontogenic cell proliferation and differentiation. To test this hypothesis, we conducted

in vitro odontoblast differentiation assay using BRCM from WT and *RANKL*$^{-/-}$ OCs. TRAP staining of calvaria bone slices showed that WT splenocytes plated on bone slice were able to differentiate into mature and functional TRAP-positive OCs and create various eroded pits on the bone slice surface. In contrast, splenocytes from RANKL knockout mice were not able to differentiate into TRAP-positive OCs on calvaria bone slices (Supplemental Fig. 2). IF staining of the pre-odontoblast cell line showed that BRCM from WT OCs cultured on bone slices induced significantly more DSP and Col1a1 expression. BRCM from both WT OC cultured without bone slices and *RANKL*$^{-/-}$ OC cultured on bone slices could barely induce DSP and Col1a1 expression in odontoblasts (Figs. 6A, B). qRT-PCR analysis of the mRNA expression of odontoblast differentiation markers (*Nfic, Dspp, Osx*, and *Col1a1*) in the odontoblast cell line induced by *RANKL*$^{-/-}$ OC BRCM for 7 days was significantly lower than that by WT OC BRCM (Fig. 6C).

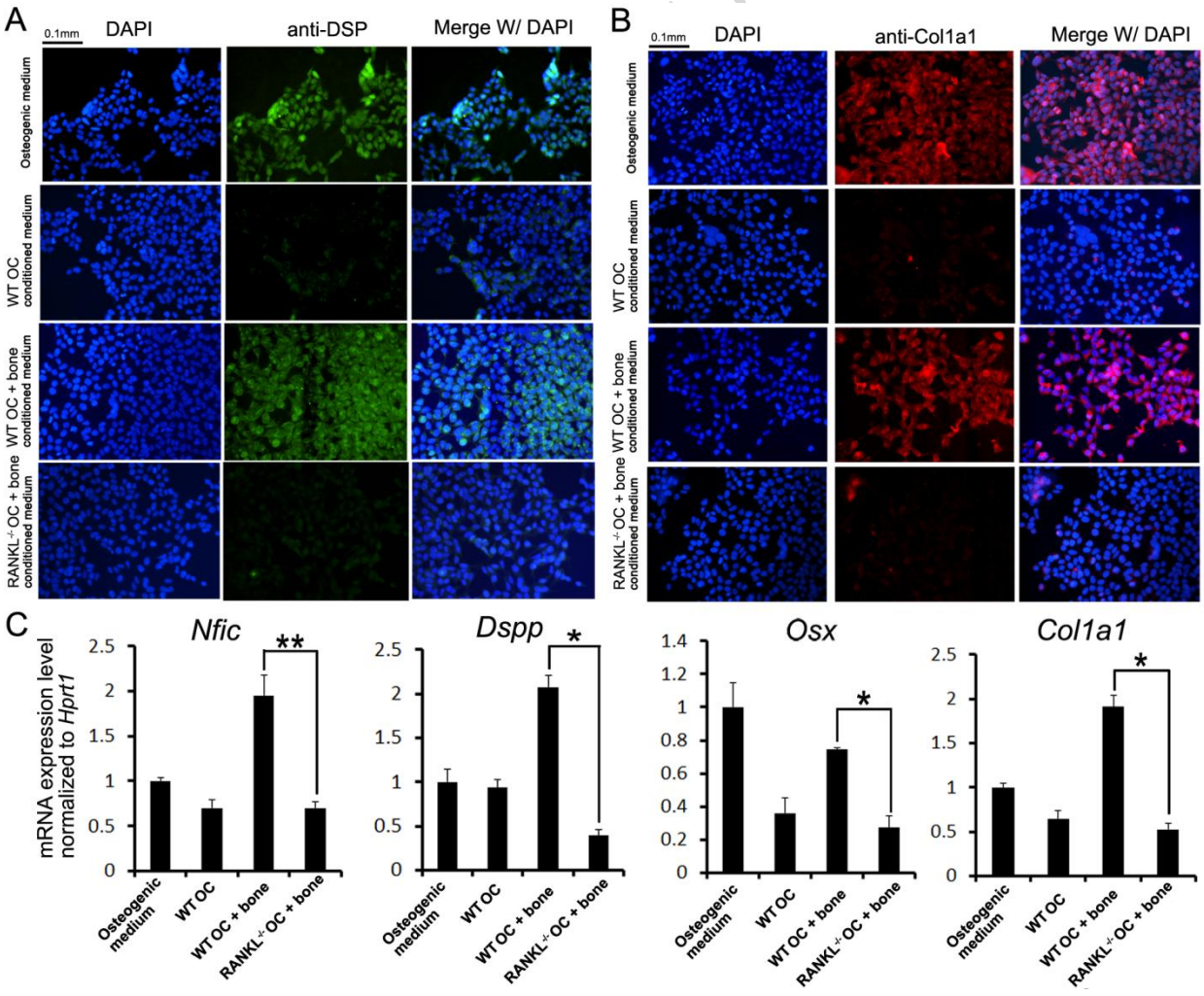

Fig. 6. Bone resorption-conditioned medium (BRCM) from RANKL deficient osteoclast inhibited odontoblast differentiation *in vitro*. (**A**) and (**B**) Immunofluorescent staining of DSP and Col1a1in odontoblast cell line induced by different cell culture media for 3 days. n $\geqslant$ 6 in each group. (**C**) qRT-PCR result of the expression of odontoblast differentiation markers in odontoblast cell line induced by different cell culture media for 7 days. Hprt1 was used as an endogenous control. WT OC, WT osteoclast culture medium; WT OC + bone, WT osteoclast cultured with bone; *RANKL* $^{-/-}$ OC + bone, *RANKL* $^{-/-}$ osteoclast cultured with bone. n $\geqslant$ 6 in each group; *, P<0.05; **, P<0.01 (ANOVA analysis).

To confirm the critical role of IGF-1 in inducing odontoblast differentiation in WT OC BRCM, we depleted IGF-1 in WT OC BRCM by addition of an antibody specific to IGF-1 to WT OC BRCM. We found that depletion of IGF-1 notably inhibited the expression of odontoblast differentiation markers (*Dspp, Nfic, Osx, Runx2,* and *Col1a1*) in pre-odontoblast cells induced for 7 days (Fig. 7A). In addition, we attempted to rescue impaired odontoblast differentiation by adding IGF-1 into *RANKL* $^{-/-}$ OC BRCM. We found that addition of IGF-1 promoted odontoblast differentiation, which was demonstrated by the significantly increased expression of odontoblast differentiation markers (*Nfic, Osx, Runx2,* and *Col1a1*) as compared to that induced by *RANKL* $^{-/-}$ OC BRCM (Fig. 7B). Taken together, these data illustrated that defective odontoblast differentiation and root formation in RANKL deficient mice may result from inhibited release of IGF-1 from bone matrix due to lack of OCs in RANKL deficient alveolar bone (Fig. 8).

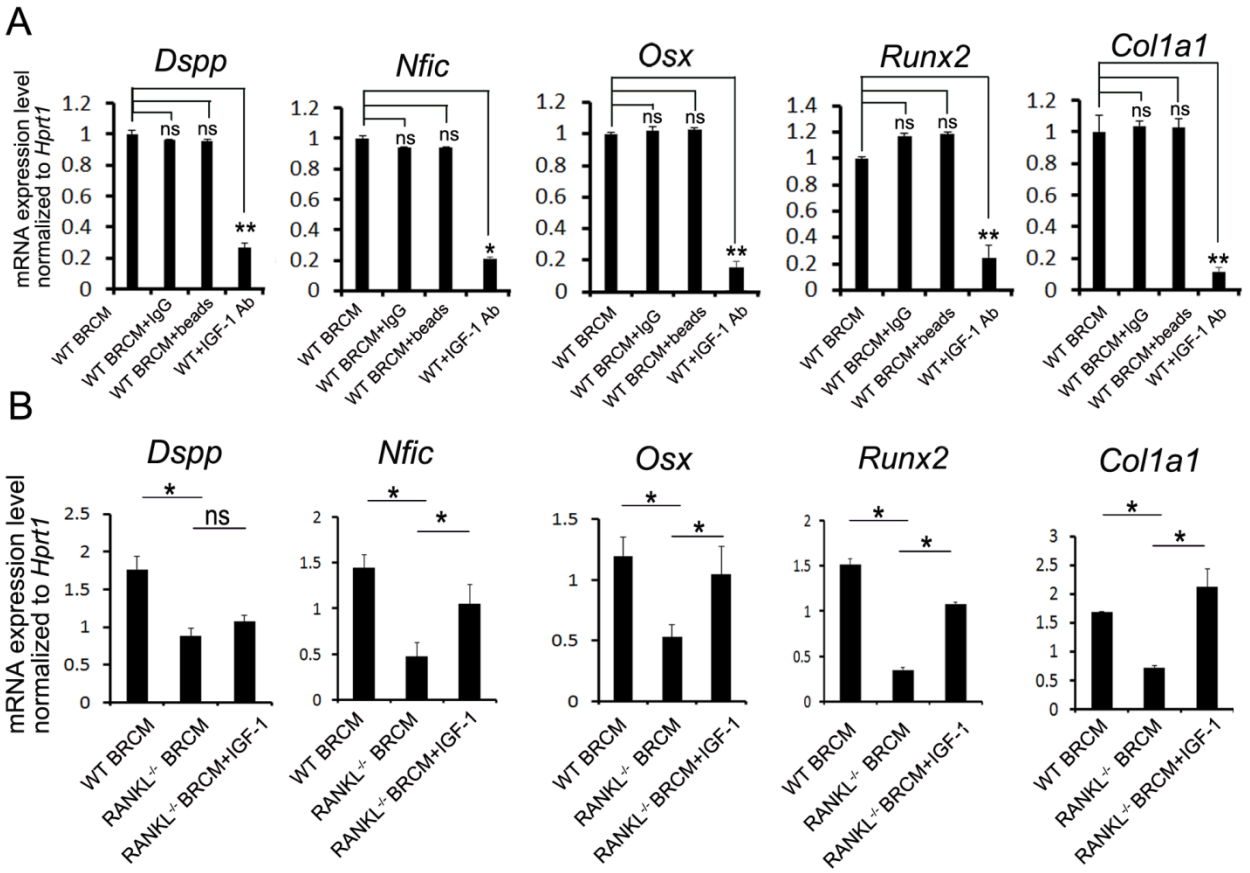

Fig. 7. Rescue of odontoblast differentiation *in vitro* with addition of IGF-1 into conditioned medium from *RANKL*$^{-/-}$ OC. (**A**) qRT-PCR result of the expression of odontoblast differentiation markers in odontoblast cell line induced by WT OC BRCM with addition of IGF-1 neutralization antibody (Ab) for 7 days. WT OC BRCM added with IgG or beads were used as the control groups. (**B**) qPCR result of the expression of odontoblast differentiation markers in odontoblast cell line induced by *RANKL*$^{-/-}$ OC BRCM with addition of IGF-1 for 7 days. WT BRCM, conditioned medium from WT OC cultured with bone; *RANKL*$^{-/-}$ BRCM, conditioned medium from *RANKL*$^{-/-}$ osteoclast cultured with bone. Hprt1 was used as an endogenous control. n $\geqslant$ 6 in each group; *, P<0.05; **, P<0.01; ns, not significant (ANOVA analysis).

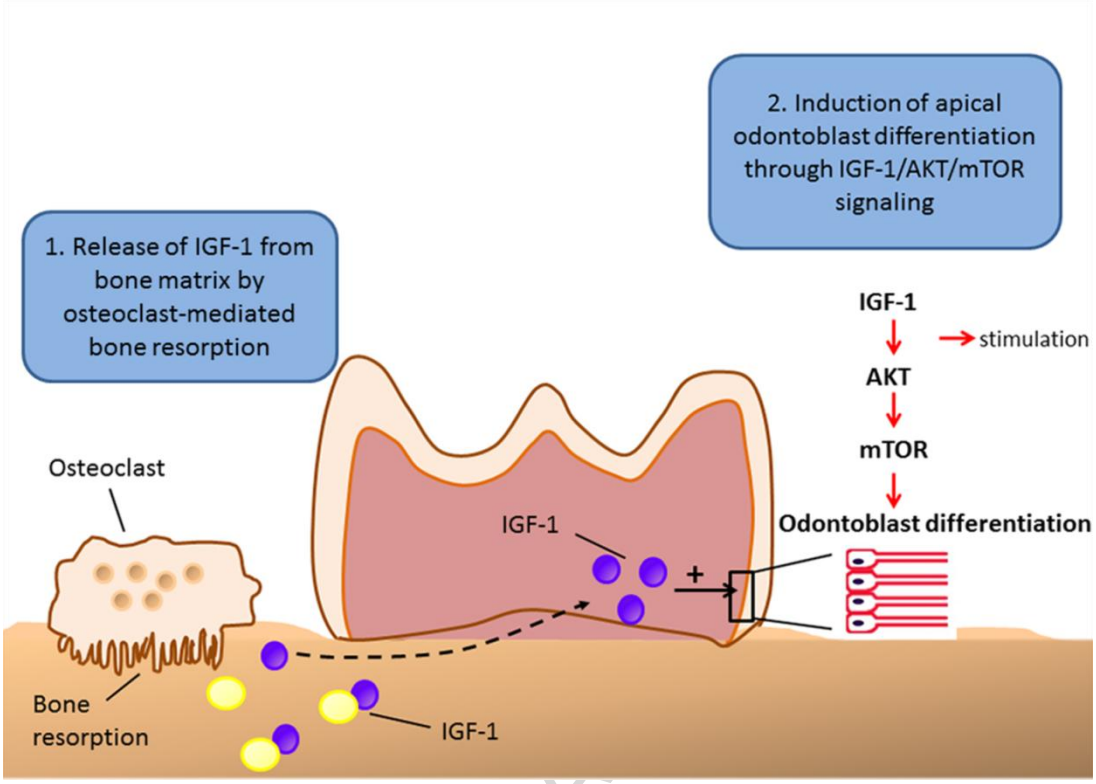

Fig. 8. Schematic diagram of the working hypothesis: IGF-1 is released from bone matrix by osteoclast-mediated bone resorption. IGF-1 promotes the differentiation of odontoblast responsible for tooth root development through IGF-1/AKT/mTOR signaling. Thus, RANKL deficiency affects root formation possibly due to the failure of liberating IGF-1 coupling growth factor by osteoclast-mediated bone resorption, leading to the defected radicular odontoblast maturation.

4. Discussion

The tooth is an ideal model to study developmental biology and organ regeneration. Multiple studies have addressed the process of tooth development and its reciprocal induction events [51-53]. Signals from members of the hedgehog (Hh) [54], Wnt [55], fibroblast growth factor (FGF) [56], transforming growth factor-β (TGF-β) superfamily [57], and IGF [58-60] are involved in the epithelial-mesenchymal interactions required for regulating tooth development. IGF-1 is a unique peptide that functions in an endocrine/paracrine and autocrine manner in most tissues. It is important for a number of different growth and differentiation processes across a wide variety of tissues. Notably, IGF-1 is an important regulator during craniofacial development, including tooth formation [61, 62]. IGF-1 functions in numerous ways as both a proliferative and differentiative factor. All cell lineages in the skeleton have been shown to not only require IGF-1 for normal development and function, but also to respond to IGF-1 via the IGF-1 receptor.

Ligand receptor activation leads to downstream signaling cascades, which have significant implications for both cell differentiation and protein synthesis [35]. With IGF-1 induction, initial activation of PI3K subsequently propagates into a cascade of p-ATK/mTOR signaling activity [63]. mTOR, which is a downstream target of AKT (also known as the mammalian target of rapamycin), is known to be the central element regulating cell growth and proliferation, particularly protein synthesis [64, 65]. In the present study, the possible roles of OC in regulating IGF-1 signaling pathway during tooth root development are explored and discussed in detail. Alveolar bone-root sample including root, dental pulp and alveolar bone surrounding the root were used for *in vivo* qRT-PCR analysis. The mixed tissues data may reflect mRNA expression from both root and periodontal bone.

RANKL is a component of the RANK/RANKL/OPG signaling axis, a central theme in OC differentiation, activation, and function [15-17]. Activation of the receptor activator of NF-kB (RANK) by its ligand, RANKL, is a crucial step in osteoclastogenesis. OCs are required to remodel bone, and defects in OC numbers or activity have been shown to affect root development as well as tooth eruption [6, 20, 66]. Furthermore, over-expression of RANK in the alveolar OCs resulted in early tooth eruption and accelerated root elongation [66]. Recent study also found that disrupted osteoclastogenesis in *c-Fos* mutant mice resulted in absence of tooth root formation and tooth eruption, similar to our discovery[67]. In our current study, RANKL knockout mice demonstrated an absence of TRAP positive cells in the bone beneath the tooth crown. These mice also demonstrated defects in tooth eruption and molar root formation which are similar to the disturbances observed in osteopetrosis patients [68-70]. The role of OC in tooth development is generally recognized as opening an avenue through the jaw bone for eruption of teeth [71]. However, inhibited odontoblast differentiation along with impaired IGF signaling transduction observed at as early as postnatal day 4 in the mutant mice indicates that OCs not only function in the process of tooth eruption of which the onset occurred around postnatal day 14, but also play critical roles in mediating HERS elongation and odontoblast differentiation through providing essential cytokines at the initiation stage of root formation.

Skeletal and dental development share similar biochemical and physiological properties. Communication between osteoblasts and OCs has been actively studied in recent years [36, 38, 72]. OCs regulate the differentiation and activity of osteoblasts via a number of potential mechanisms. One essential mechanism for osteoblast-OC coupling is through growth factors released by osteoclastic bone resorption. It has previously been shown that IGF-1 released from the bone matrix by bone resorption during bone remodeling stimulates osteoblastic differentiation, thus maintaining proper bone mass [39]. In the present study, the use of bone

resorption-conditioned medium to induce odontoblast differentiation has allowed the *in vitro* analysis of the coupling effect of IGF-1 on odontoblast differentiation. We found that IGF-1 released from bone matrix not only regulates root dentin formation through inducing proliferation of odontogenic precursor cells as shown by IHC staining of tooth root samples, but also directly promotes differentiation and terminal maturation of odontoblasts as displayed by the *in vitro* experiments. Thus, this indicates that, in addition to its coupling role by inducing osteoblast differentiation, IGF-1 functions on odontoblast differentiation in root development. In addition, besides the regulatory effect of OC on odontoblast through IGF-1 release, the secondary effect of the compact bone (due to absence of osteoclasts) on tooth root development due to mechanical disturbance should also be considered, which could also be linked to HERS disturbance as reported earlier [67, 73, 74].

Proper differentiation and maturation of root-odontoblasts is essential for elongation of HERS and formation of tooth roots. Interactions between HERS and dental pulp mesenchyme play an important role in promoting odontoblast differentiation. Previous studies revealed that HERS produce factors that act on dental pulp mesenchymal cells in the dental pulp apex region and thus induce the differentiation of odontoblasts that are responsible for root dentin formation. In turn, odontoblasts also regulate morphogenesis and elongation of the HERS [75, 76]. In the present study, the HERS is found severely truncated and there is no sign of root elongation in RANKL deficient mice, which could be the consequence of impaired odontoblast differentiation. Moreover, proliferation of odontogenic cells was significantly decreased in RANKL knockout mice relative to controls. In fact, it has been reported that IGF-1 affects cell proliferation in HERS and regulates tooth root elongation [58]. As IGF-1 levels are found decreased in the mutant mice, we propose that the failure of the growth of HERS was possible due to the alterations of IGF-1 signaling in the dental mesenchyme and odontogenic cells.

Nfic is a transcription factor which is a key regulator of root odontoblast differentiation and root formation [77, 78]. Nfic is required in the dental mesenchyme to promote radicular odontoblast differentiation independent from crown odontoblasts. Deficiency of Nfic resulted in disrupted root odontoblast differentiation and completely blocked root formation. But crown dentin formation is relatively normal [77] . Since the tooth roots of *RANKL*$^{-/-}$ mice were absent and alterations in root odontoblast differentiation were present, but the crown development was unaffected, we detected Nfic expression in RANKL deficient odontoblasts. Lower Nfic expression by IHC and mRNA expression by real time RT-PCR in isolated root sample were detected in *RANKL*$^{-/-}$ mice. This suggests that OC deficiency only affected root-specific molecular expression and radicular odontoblast differentiation, implying that the development of the tooth crown and root has

different signaling and molecular mechanisms. This is supported by the study of the role of Osx on root development which found that as one of the key downstream molecules of Nfic, Osx participates in the regulation of root, but not crown, formation [50]. However, the exact mechanisms underlying how and why odontoblasts within the same pulp but different anatomical areas are regulated differentially waits further investigation.

5. Conclusion

We determined that RANKL knockout *in vivo* deprives OCs in alveolar bone which resulted in an osteopetrosis-like phenotype and disrupted tooth root formation. Notably, RANKL deficiency affected HERS elongation and odontoblast differentiation, and also impaired IGF/AKT/mTOR signaling in root odontoblasts. Thus, this study presents important insights into the critical role of OC on regulating root odontoblast differentiation and thereby promoting tooth root formation, possibly through releasing of IGF-1 from bone matrix by bone resorption.

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Highlights:

- Knowledge on mechanisms underlying osteoclast mediated root formation is limited.
- We hypothesize that osteoclasts regulate odontoblast differentiation.
- A receptor activator of NF-κB ligand (RANKL) knockout mouse model were used.
- Root defect in RANKL deficiency mice may result from failure of IGF-1 releasing.
- Osteoclasts are important for odontoblast differentiation and tooth root formation.