

Original Article

Intermittent activation of notch signaling promotes bone formation

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Abstract: Stimulatory and inhibitory effects of Notch signaling pathway on osteogenesis were both widely reported, questioning the effectiveness of small molecules targeting the Notch pathway for prevention or treatment of bone loss diseases. Here we showed that Notch signaling is activated in osteocytes embedded within the mineralized matrix and in late stages of bone marrow mesenchymal cell osteogenic cultures. Inhibition of Notch signaling markedly reduced mineralization activities of bone marrow mesenchymal cells and inhibited expressions of mineralization-associated genes when Notch ligand Jagged1 was conditionally deleted, confirming the essential roles of Notch signaling in mineralization stages of osteoblast differentiation. Moreover, intermittent activation of Notch signaling showed significant increases of bone formation in mice, rats and ovariectomized rats. A two-phase action model of Notch signaling in osteogenesis is proposed, where activation of Notch signaling in early stages of osteoblast differentiation results in proliferation of immature preosteoblast lineage cells and activation in late stages promotes differentiation of osteoblasts into osteocytes. Moreover, valproic acid is a strong activator of Notch signaling, and yearly administration of valproic acid daily showed little side effects, indicating that long term and intermittent activation of Notch signaling will be a safe and ideal way to promote anabolic bone formation for treatment of osteoporosis. Therefore, Notch signaling pathway is a good therapeutic target for bone loss diseases, and valproic acid, resveratrol and other Notch activators are promising therapeutic molecules for promoting anabolic bone formation when administered intermittently.

Keywords: Notch, osteogenesis, bone, anabolic, valproic acid, resveratrol

Introduction

Notch signaling pathway is an evolutionary conserved pathway regulating cell proliferation, differentiation, cell fate determination in both embryonic and adult organs [1-4]. In mammals, the Notch transmembrane receptor family consists of four members, Notch1-4. The Notch ligands identified, Jagged1/2 (Jag1/2) and delta-like 1/3/4 (Dll1/3/4), are type I transmembrane proteins [1-4]. Notch intracellular signaling is initiated upon ligand binding followed by two sequential proteolytic cleavage events mediated by tumor necrosis factor (TNF)- α converting enzyme (TACE) or γ -secretase, releasing the Notch extracellular domain and Notch intracellular domain (NICD). Subsequently NICD translocates into the nucleus, regulating expressions of target genes [1-4]. Deletion or mutation of any one or more of genes in Notch signaling pathway showed severe skeletal phe-

notypes in humans and in mouse models [1, 2, 5-7]. However, contradictory results were reported for roles of the Notch signaling pathway on skeletogenesis [1-4, 8-16]. In in vitro cell cultures, activation of Notch signaling was reported to either promote [8, 9] or inhibit [10, 11] osteoblast differentiation and mineralization, while inhibition of Notch signaling also showed to promote [11] or inhibit [12] differentiation and mineralization of osteoblasts. In animal models, loss of Notch function resulted in radiodense [13, 14] or osteoporotic [13-15] bones, while gain of Notch function was reported to have either osteoporotic [11, 16] or osteosclerotic [12, 15, 16] phenotypes. Therefore, it is difficult to interpret functions of Notch signaling pathway in osteogenesis using a single-action model.

Here we show that Notch signaling pathway is strongly activated in osteocytes and intermit-

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tent activation of Notch signaling showed significant increases of bone formation in mouse and rat models. A two-phase model was then proposed, explaining Notch actions on osteogenesis and therapeutic potentials of intermittent activation of Notch signaling for bone loss diseases.

Materials and methods

Animals

All mouse and rat experiments were approved by the Institutional Animal Care and Use Committees of Wuhan University, and all applicable institutional and/or national guidelines for the care and use of animals were followed. The Jag1^{fl/fl} mice [17], TNR mice [18] and Mx1Cre mice [19] were from The Jackson Laboratory. To induce the expression of Cre recombinase in Mx1Cre mice, female Jag1^{fl/fl}; Mx1-Cre (J1MX1) mice were treated with 250 µg of polyinosinic-polycytidylic acid (pIpC) intraperitoneally every other day for three times at six weeks of age and sacrificed at 8 weeks for analysis.

For in vivo study, mice, rats and ovariectomized rats were treated with valproic acid (VPA, 100 mg/kg of body weight dissolved in PBS) or vehicle control (PBS) intraperitoneally for three months. Four groups of studies were performed. Group A: Three-month-old female C57BL/6 mice (n=8 for each subgroup). A regimen of daily treatment of VPA was continued for 0, 2, 3, 4 or 7 days followed by 7, 5, 4, 3 or 0 days of control (PBS) treatment each week. This weeklong paradigm was repeated 13 times. Group B: Five-month-old female C57BL/6 mice (n=12 for each subgroup). A regimen of daily treatment of VPA was continued for 0, 2, 3, 4 or 5 days followed by 5, 3, 2, 1 or 0 days of control (PBS) treatment each week. This weeklong paradigm was repeated 13 times. Group C: Six-month-old female Sprague-Dawley rats (n=12 for each subgroup). A regimen of daily treatment of VPA was continued for 0, 2, 3, 4 or 5 days followed by 5, 3, 2, 1 or 0 days of control (PBS) treatment each week. Daily treatment of PTH (100 µg/kg of body weight) subcutaneously for 5 consecutive days each week was as a positive control. This weeklong paradigm was repeated 13 times. Group D: Four-month-old female Sprague-Dawley rats (n=12 for each subgroup) were bilaterally ovariectomized (OVX) or sham operated (SHAM).

Treatment regimen started at six months of age. A regimen of daily treatment of VPA in OVX rats was continued for 0, 2, 3, 4 or 5 days followed by 5, 3, 2, 1 or 0 days of control (PBS) treatment each week. Daily treatment of PTH (100 µg/kg of body weight) in OVX rats or PBS in SHAM rats for 5 consecutive days each week were as controls. This week long paradigm was repeated 13 times.

Mouse bone marrow mesenchymal cell culture

Primary BMSCs were obtained from mouse femurs or tibia and cultured as previously described [12]. Briefly, bone marrow was flushed from the femurs or tibia and cultured in basal medium (α -modified Eagle's minimal medium (α MEM) supplemented with 10% FBS and 1% penicillin/streptomycin) at a cell density of $1-2 \times 10^7$ /ml. For osteogenic differentiation, BMSCs were maintained in basal medium for 7 days, and changed to osteogenic medium (basal medium supplemented with 10^{-8} M dexamethasone, 8 mM β -glycerophosphate, and 50 µg/ml L-ascorbate) for additional 14 days. For DAPT treatment experiments, BMSCs were cultured in media supplied with various concentration of DAPT or DMSO as indicated. Media were replaced every three days.

Gene expression analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) followed by DNase treatment. First-strand cDNA was synthesized using oligo(dT) primer and Superscript II reverse transcriptase. Quantitative real-time PCR was performed in triplicate and the relative amount of mRNA was normalized to the expression of cyclophilin A.

Trabecular bone analysis

Femurs from mice and rats were scanned using μ CT50 as previously described [12]. Briefly, the bones were dissected, cleaned, fixed in 10% formalin, transferred to 75% ethanol, loaded into scanning tubes, and imaged with the following parameters: 70 kV, 114 µA, 0.5 mm Al filter, integration time 300 ms, 1000 projections/180 degree, resolution 6 µm for mouse samples and 15 µm for rat samples. Trabecular bones were extracted and analyzed using custom scripts. Data is available upon request and custom scripts can be downloaded from www.bomomics.com.

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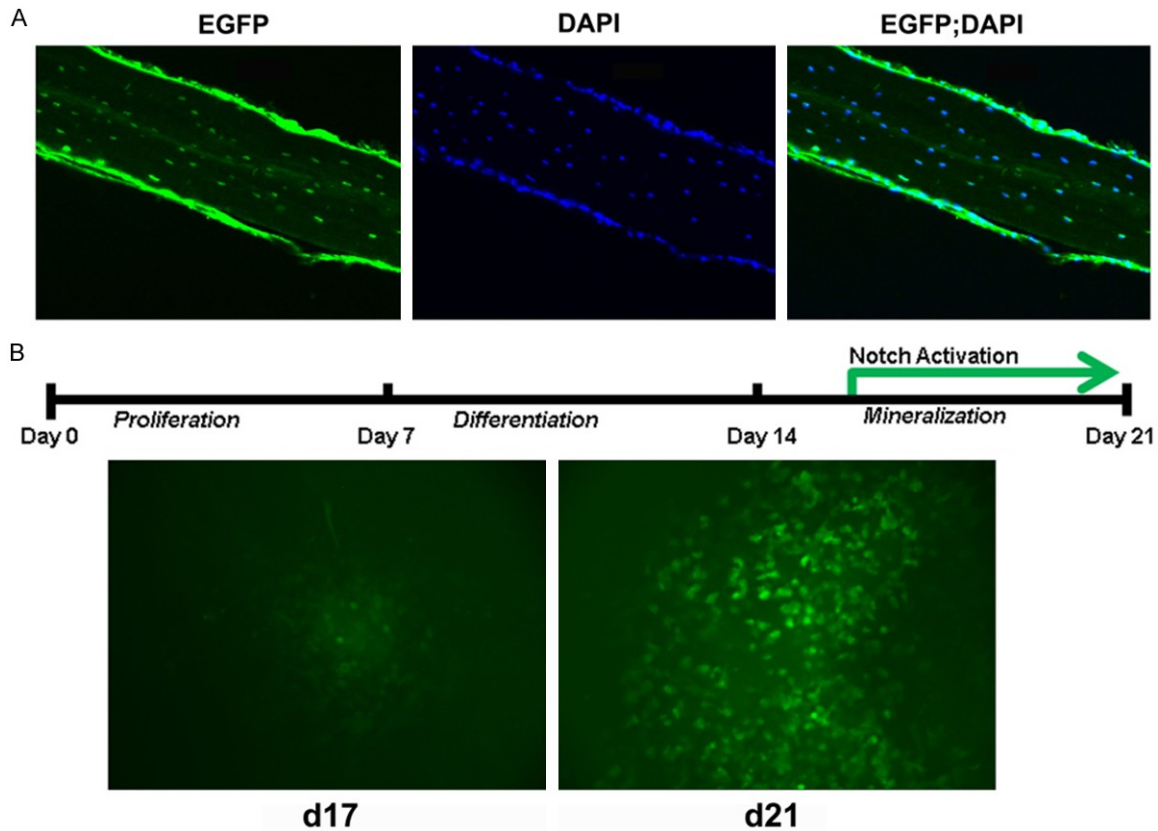


Figure 1. Notch signaling activation in late stages of osteoblast differentiation. A: Osteocytic expression of active Notch (EGFP) and DAPI staining of cell nuclei (DAPI) in calvaria from 8-week-old female TNR mice. B: Notch activation in late stages of in vitro osteoblast differentiation. Low level of GFP expression was detected at day 17 and strong GFP expression at day 21.

Statistical analysis

All values were reported as means \pm standard deviations (SD). Intergroup comparisons between paired control and experimental groups were analyzed using the Student's t-test. All data were analyzed using IBM SPSS 22.0 software.

Results

Activation of Notch signaling in late stages of osteoblastogenesis

Transgenic Notch reporter (TNR) mice were developed for monitoring Notch activities in neural [20] and hematopoietic cells [18], where activated Notch signaling is associated with strong green fluorescence. As reported, we observed activated Notch signaling in osteoblasts and osteocytes of trabecular and cortical bones from femurs and vertebrae of TNR mice [12]. Similarly, all nucleated cells in cal-

varia of TNR mice showed strong green fluorescence (**Figure 1A**), indicating that Notch signaling is activated in osteocytes embedded within bone matrices. During in vitro osteoblastic differentiation of bone marrow mesenchymal cells (BMSCs), BMSCs firstly undergo proliferation, then differentiate into osteoblasts and finally differentiate into osteocytes embedded in the mineralized matrix (**Figure 1B**). In vitro osteoblastic differentiation of BMSCs from TNR mice showed that green fluorescence was undetectable in early stages of cultures, but showed low level expression of green fluorescence at around day 17 and strong expression at around day 21 in cells within mineralized nodules (**Figure 1B**).

Inhibition of Notch signaling impairs in vitro mineralization

N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a γ -secretase inhibitor (GSI), blocks the intracellular proteo-

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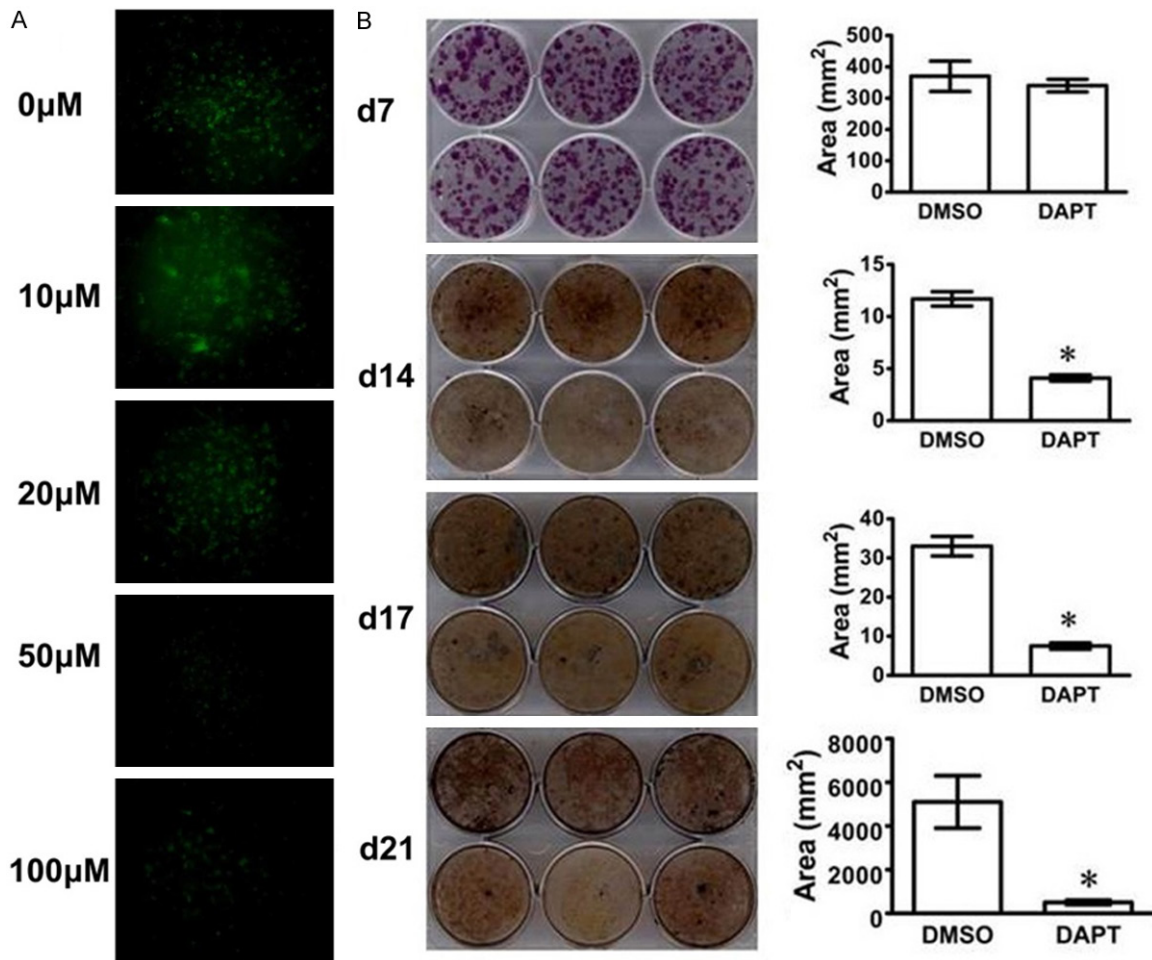


Figure 2. Inhibition of Notch Signaling impairs mineralizing activities of BMSCs. A: BMSCs from TNR mice were cultured in basal media for seven days and then changed to osteogenic media supplemented with various concentrations of γ -secretase inhibitor DAPT. GFP expression was monitored at day 21. B: BMSCs from C57BL/6 mice were cultured in basal media for seven days and then changed to osteogenic media supplemented with 50 μ M DAPT or DMSO for additional 14 days. ALP staining (d7) and von kossa stainings (d14, d17 and d21) were performed. Upper wells: DMSO (Vehicle control); lower wells: DAPT. Mineralization areas were quantified using ImageJ software and analyzed using unpaired t-test. *, $P < 0.05$.

lytic cleavage of Notch receptor and the release of NICD, efficiently preventing activation of Notch signaling pathway [12]. In osteogenic cultures of BMSCs from TNR mice (**Figure 2A**), low concentration (10 or 20 μ M) of DAPT showed little effects on the expression of green fluorescence, however, high concentration of DAPT (50 or 100 μ M) markedly inhibited the expression of green fluorescence, indicating efficient inactivation of Notch signaling pathway. Furthermore, no significant difference between treated and control groups was observed when BMSCs were cultured in proliferating media before day 7, however, mineralized nodular formation was greatly inhibited by DAPT in late stages of BMSC cultures starting from day 14

when cultured in osteogenic media (**Figure 2B**), indicating that notch inhibition by GSI impaired mineralization of BMSCs in osteogenic media. As reported, expressions of mineralization-associated genes, such as matrix extracellular phosphoglycoprotein (Mepe), dentin matrix protein-1 (Dmp1), sclerostin (Sost) and phosphate regulating endopeptidase homolog X-linked (Phex), were greatly inhibited by DAPT in late stages but not in early stages of BMSC osteogenic cultures [12].

Jag1 is an important Notch ligand playing essential roles in skeletogenesis, and conditional deletion of Jag1 impaired in vitro mineralization of BMSCs [12]. Similar to the effects of

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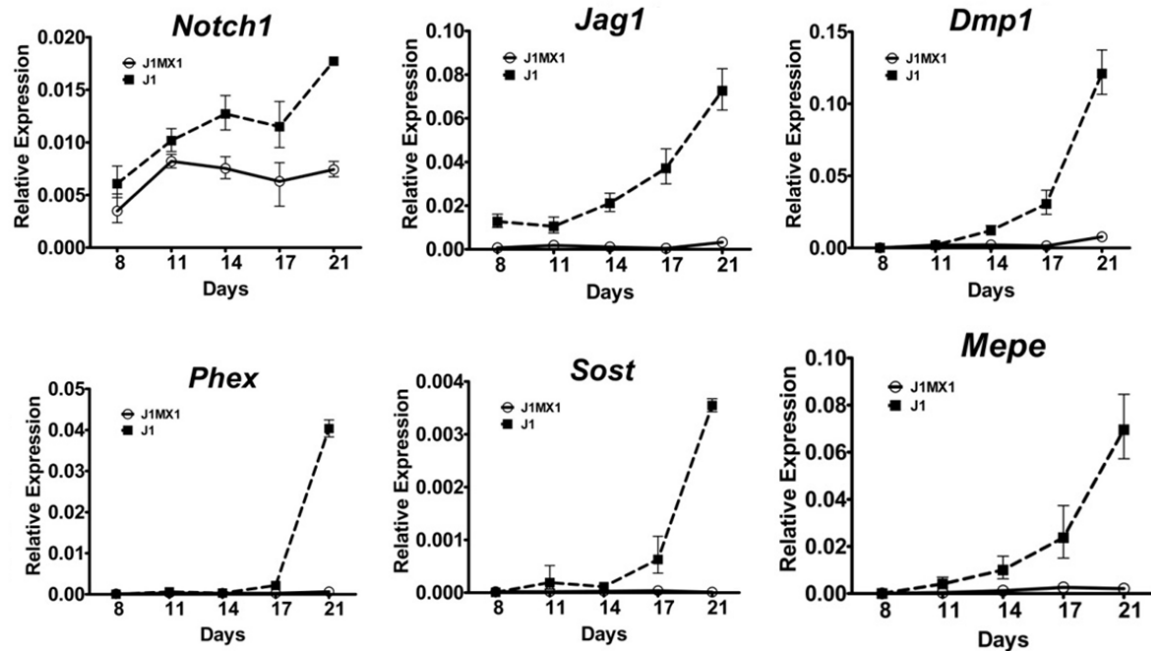


Figure 3. Impaired Notch signaling inhibited expressions of osteocytic mineralization-associated gene markers. Female *Jag1^{+/+}* (J1) and *Jag1^{+/+}; Mx1-Cre* (J1MX1) mice were treated with plpC at six weeks of age and sacrificed at 8 weeks. BMSCs from plpC treated mice were cultured in basal media for seven days and then in osteogenic media for additional 14 days. Total RNA was extracted at specific time point and quantitative PCR performed for expression of osteocytic mineralization markers (n=3 wells per group in quadruplicate).

Notch pathway inhibition by DAPT [12], conditional deletion of *Jag1* resulted in markedly reduced expression of mineralization-associated genes (Figure 3) in late stages of BMSC osteogenic cultures, indicating essential roles of Notch signaling pathway in regulating the mineralization of osteoblast differentiation.

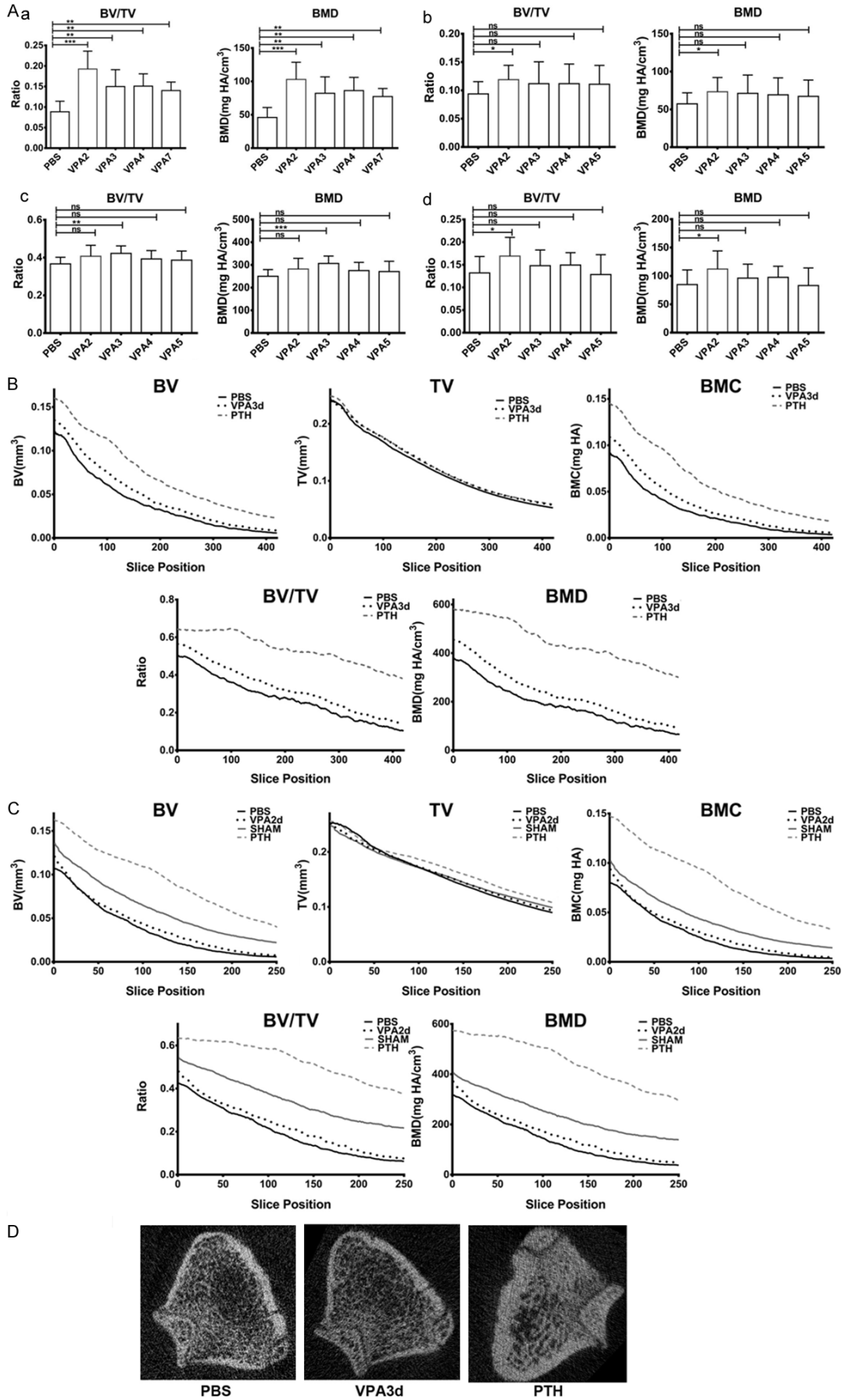
Intermittent activation of Notch signaling stimulates bone formation

As restricted activation of Notch signaling in osteocytes showed profound bone formation [12, 15, 16, 21], Notch signaling pathway is a potential therapeutic target for bone loss diseases. However, restricted activation of Notch signaling in osteocytes by small molecules is challenging. In addition, constitutive activation of Notch signaling displayed osteosclerosis by *Col3.2*, *Col2.3* or *Dmp1* promoters [12, 15, 16, 21], and osteoporotic phenotypes by *Col3.6* or Osteocalcin promoters [11, 16], while conditional inactivation of Notch signaling by *Prx1* or *Col2.3* promoters showed age-dependent bone loss in old mice [13-15], indicating that Notch activation has inhibitory roles at early stages of

osteoblast differentiation and stimulatory roles at late stages of osteoblast differentiation. Therefore, continuous activation or inactivation of Notch signaling pathway by small molecules is not optimal for stimulating bone formation, as such molecules tend to activate Notch signaling pathway in all cells of osteoblastic lineages instead of to restrict to osteocytes.

To evaluate the therapeutic potential of Notch signaling pathway on bone loss diseases, effects of intermittent activation of Notch signaling pathway on bone formation were tested using mouse and rat models. Valproic acid (VPA) is known to activate Notch signaling pathway in various tissues [22-25] and showed enhanced mineralization and ossification in *in vitro* cultures [26-31]. As shown, VPA strongly stimulated trabecular bone formation in mice, rats and ovariectomized rats (Figure 4A) only when administered intermittently, while long duration of VPA treatment showed little anabolic bone formation effects. Here, optimal stimulation of bone formation by VPA in mice and ovariectomized rats was the regimen of two-day consecutive VPA treatment followed by

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Figure 4. Intermittent activation of Notch signaling enhanced trabecular bone formation. A: Mice or rats were treated with saline (PBS) or VPA (VPA2-VPA7 or VPA2-VPA5) for three months, and trabecular bones from distal femurs were analyzed. Data were represented as a mean \pm SD. a. Three-month-old female C57BL/6 mice (n=8 for each group) was treated with VPA for 2, 3, 4 or 7 consecutive days or PBS each week. b. Five-month-old female C57BL/6 mice (n=12 for each group) was treated with VPA for 2, 3, 4 or 5 consecutive days or PBS each week. c. Six-month-old female Sprague-Dawley rats (n=12 for each group) was treated with VPA for 2, 3, 4 or 5 consecutive days or PBS each week. d. Female Sprague-Dawley rats were bilaterally ovariectomized (OVX) at four months of age and treatment started at six months of age. OVX rats (n=12 for each group) was treated with VPA for 2, 3, 4 or 5 consecutive days or PBS each week. Student's t-test was used for between group analyses. *P<0.05, **P<0.01, ***P<0.001. B: Layer-by-layer analysis of femurs from six-month-old rats treated with PBS, VPA (for 3 consecutive days each week) or PTH. Slice position is relative to the growth plate reference position of rat distal femurs. Data were represented as a mean at each layer. C: Layer-by-layer analysis of femurs from six-month-old OVX or SHAM rats treated with PBS, VPA (for 2 consecutive days each week) or PTH. Slice position is relative to the growth plate reference position of rat distal femurs. Data were represented as a mean at each layer. D: Representative images of rat femurs treated with PBS, VPA or PTH respectively 1.5 mm (100 layers) away from growth plate reference positions.

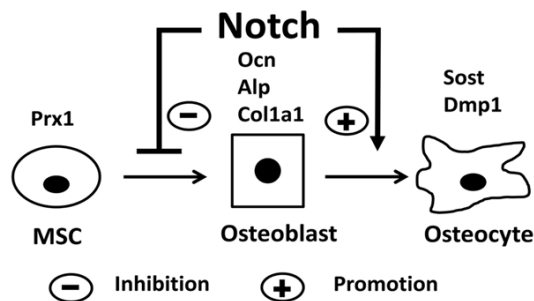


Figure 5. Model of notch signaling in osteoblast differentiation. Notch inhibits the differentiation of MSCs into osteoblasts and promotes the differentiation of osteoblasts into osteocytes.

five-day control treatment each week, while consecutive treatment of VPA more than 2 days each week showed less profound bone formation effects. In six-month-old SD rats, optimal treatment regimen was three-day consecutive treatment of VPA followed by four-day control treatment each week, indicating that intermittent rather than continuous activation of Notch signaling by VPA is essential to promote bone formation.

Layer-by-layer analysis showed that intermittent activation of Notch signaling by VPA promoted small but significant trabecular bone formation in SD rats and ovariectomized SD rats (**Figure 4B** and **4C**), and such an increase of bone formation showed no structural irregularities (**Figure 4D**), while profound bone formation by PTH administration was observed associated with significant changes of trabecular microarchitectures (**Figure 4D**), indicating that Notch signaling pathway is a good anabolic drug target for prevention or treatment of bone loss diseases.

Model of Notch signaling in osteoblast differentiation

In view of the fact that Notch signal pathway is not activated in early stages of BMSC osteogenic differentiation but strongly activated in late stages of differentiation, the following model of Notch's roles on osteoblastogenesis was proposed (**Figure 5**): Notch signaling is not activated during the differentiation of mesenchymal stem cells (MSCs) into osteoblasts, and activation of Notch signaling in this stage inhibits the differentiation of MSCs into osteoblasts, resulting in accumulation of immature pre-osteoblast lineage cells. Notch signaling is activated during the differentiation of osteoblasts into osteocytes and activation of Notch signaling in this stage promotes osteoblasts differentiation and mineralization into osteocytes. Moreover, inhibition of Notch signaling in early stages of MSC osteogenic differentiation promotes the differentiation of MSCs into osteoblasts resulting in accumulation of mature osteoblasts, while inhibition of Notch signaling pathway in late stages of osteoblast differentiation inhibits differentiation and mineralization of mature osteoblasts into osteocytes.

Discussion

During osteoblastogenesis, mesenchymal stem cells (MSCs) differentiate into functional osteoblasts. Subsequently, functional osteoblasts undergo matrix maturation and differentiate into osteocytes, which are embedded in the mineralized matrix. Here we reported that Notch signaling pathway is activated in osteocytes and inhibition of Notch signaling impairs in vitro mineralization of BMSCs. Enhanced bone formation by intermittent activation of

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Notch signaling was observed in mouse and rat models and a dimorphic Notch action model on osteoblastogenesis was then proposed.

Conflicting results of Notch effects on osteoblastogenesis were reported, however our dual action model of Notch signaling pathway in osteoblastogenesis could explain nearly all reported inconsistencies for in vivo and in vitro data. As reported, forced activation of Notch signaling in preosteoblasts resulted in accumulation of immature osteoblasts [32, 33], and Notch activation in early stages of osteoblast differentiation (Prx1Cre; Rosa-NICD^{f/+} or Col3.6-NICD) showed enhanced proliferation and suppressed differentiation of mesenchymal progenitor cells (MPC) in the developing limb [34] and features of osteopenia in adult mice [11], while Notch activation in late stages of osteoblast differentiation (Col2.3Cre; Rosa-NICD^{f/+}, Dmp1Cre; Rosa-NICD^{f/+}, Col3.2CreERT2; Rosa-NICD^{f/+} or Col2.3-NICD) resulted in increased bone formation [12, 15, 16]. Such gain of Notch function results in osteoblastogenesis can be well explained by our dimorphic Notch action model: continuous activation of Notch signaling in early stages of osteoblast differentiation inhibits the differentiation of MSCs (or MPCs) into osteocytes, resulting in proliferation of MPCs and decreasing of osteocyte numbers, thus features of osteopenia were observed in adult mice due to impaired differentiation of MSCs into osteocytes. However, continuous activation of Notch signaling in late stages of osteoblast differentiation promotes differentiation of osteoblasts into osteocytes, resulting in temporarily reduced osteoblast numbers and increased osteocyte numbers. To keep the balance between MSCs and osteoblasts, more MSCs were promoted to differentiate into more osteoblasts. Hence, the net outcome of continuous activation of Notch signaling in late stages of osteoblast differentiation is to promote more MSCs to differentiate into more osteocytes, resulting in increased bone formation. Similarly, Notch inactivation in both early and late stages of osteoblast differentiation (Prx1Cre; Notch1^{-f}Notch2^{f/f}, Prx1Cre; Notch2^{f/f}, Prx1-Cre; RBPjk^{f/f} or Col2.3Cre; Psen1^{f/f}Psen2^{-/-}) showed age-dependent bone loss [13-15], and marked increases of radiodensity and double-labeled surfaces within the trabecular bones were observed in young mice when Notch signaling was conditionally inactivated in early stages of osteoblast differentiation [13].

According to our Notch action model, inactivation of Notch signaling in early stages of osteoblast differentiation promotes the differentiation of MSCs into mature osteoblasts, but inhibits the differentiation and mineralization of mature osteoblasts into osteocytes due to the inactivation of Notch signaling, resulting in accumulation of osteoblasts, upregulation of osteoblast activities and reduction of osteocytes. Subsequently, proliferations and accumulations of osteoblasts resulted in marked increases of bone mass in young mice, possibly by induced ectopic mineralization of osteoblasts independent of Notch signaling pathway or incomplete inactivation of Notch signaling mediated by Cre recombinase. When Notch is inactivated in late stages of osteoblast differentiation, differentiation of osteoblasts into osteocytes was inhibited and severe age-related bone loss was observed in old mice as expected. Furthermore, results from in vitro loss of Notch function or gain of Notch function studies using primary bone marrow mesenchymal cells [8, 9, 12], osteoblastic cell lines [8] or cell lines derived from bone marrow mesenchymal cells [10] were all consistent with our proposed Notch action model. However, in the context of BMP-2 treatment, conflicting results were reported about Notch's roles on osteoblastogenesis [8, 35-38]. As BMP-2 has profound bone stimulation effects and such effects may be independent of Notch signaling pathway, the controversial results of Notch actions in osteoblastogenesis under BMP-2 treatment condition will not affect the validity of our proposed Notch action model.

Here, intermittent activation of Notch signaling by valproic acid (VPA) showed significant increases of trabecular bone formation in mice, rats or ovariectomized rats. Such observations can be explained by our model as well. During the phase of Notch activation by VPA, osteoblasts were promoted to differentiate into osteocytes, resulting in decreased osteoblast numbers and increased osteocyte numbers. In the meanwhile, osteoprogenitor cells fail to differentiate into osteoblasts when Notch is activated by VPA, leading to increased osteoprogenitor numbers and decreased osteoblast numbers. During the phase of normal Notch activity when No VPA is present, increased osteoprogenitors numbers and decreased osteoblast numbers promote more osteopro-

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genitors to differentiate into more osteoblasts. Hence, the net outcome of intermittent activation of Notch pathway by VPA is promoting more osteoprogenitors to differentiate into more osteocytes, leading to increased bone formation.

In theory, alternate activation and inhibition of Notch signaling will have profound bone formation effects as well, and such a view was supported by published report [39] indirectly. In the mouse model of systemic lupus erythematosus (MRL/lpr mice), high expressions of Notch1, Notch2, Jag1 and NICD proteins in BMSCs [39] and severe osteoporotic phenotype were observed due to continuous activation of Notch signaling in BMSCs. When Notch signaling in MRL/lpr mice was intermittently inhibited by DAPT, markedly increased bone formation was observed. In this treatment regimen, Notch inhibitor DAPT was administered subcutaneously on a daily basis for three consecutive days (Notch inhibition) to MRL/lpr mice followed by 4-days without treatment each week (Notch activation). Similar to the scenario of intermittent activation of Notch signaling, such a treatment regimen of alternating activation and inhibition of Notch signaling resulted in extensive bone formation as expected.

Osteoporosis is a metabolic bone disease with impaired bone strength and increased risk of fracture, affecting up to 50% postmenopausal women older than 50 years [40, 41]. Currently, most approved drugs for treatment of osteoporosis are antiresorptive agents with potential adverse effects on long time usage [40, 41], and PTH and anti-sclerostin antibodies (romosozumab and blosozumab) are the only approved anabolic agents for treatment of osteoporosis [40, 41], where PTH is restricted to a single 24 months regimen and long-term safety of anti-sclerostin antibodies are uncertain [40, 41]. Here we showed that intermittent activation of Notch signaling has strong anabolic effects on bone formation, indicating that Notch signaling pathway is a new identified target for the treatment of osteoporosis. Valproic acid (VPA) is a strong Notch activator and has been used to treat a variety of seizure and bipolar disorders for decades [42, 43]. Moreover, long term usage of VPA are not associated with severe side effects [42, 43], indicating that long term activation of Notch signaling is not

likely to be associated with severe side effects. Therefore, Notch signaling pathway is an ideal target for developing anabolic agents for the treatment of osteoporosis.

VPA and Resveratrol (RESV) both strongly activated Notch signaling in various cell lines [22-25, 44-48] and enhanced ossification and mineralization in osteogenic cultures [26-31, 49-54], consistent with their Notch activating activities in these cells. In humans, the half-life of VPA is 10-20 h, and about 6-9 h in children. Therefore, it is reasonable to see reported severe bone loss in patients with long-term use of VPA [42, 43], which can be well explained by continuous activation of Notch signaling, and reported cases of enhanced bone formations in young patients [55], possibly due to shorter half-lives or changed metabolic profiles of VPA in those patients. In addition, RESV was reported to significantly promote bone formation in animal models [56-58] and in a randomized placebo-controlled clinical trial [59]. Due to the rapid metabolism and clearance of RESV [56, 57, 60], daily administration of RESV in animal studies and clinical trials is similar to intermittent activation of Notch signaling, thus stimulatory effects of RESV on bones were reported. Therefore, VPA, RESV and their long half-life derivatives are in theory better molecules for the treatment of osteoporosis by promoting anabolic bone formations through intermittent activation of Notch signaling pathway.

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Disclosure of conflict of interest

A patent application has been filed relating to this work.

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