NOTCH1 Regulates Osteoclastogenesis Directly in Osteoclast Precursors and Indirectly via Osteoblast Lineage Cells*

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Shuting Bai†, Raphael Kopan§, Wei Zou†, Matthew J. Hilton¶, Chin-tong Ong‡, Fanxin Long‡, F. Patrick Ross†, and Steven L. Teitelbaum††

From the Departments of ††Pathology and Immunology, §Molecular Biology and Pharmacology, and ‡Internal Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

NOTCH signaling is a key regulator of cell fate decisions in prenatal skeletal development and is active during adult tissue renewal. In addition, its association with neoplasia suggests that it is a candidate therapeutic target. We find that attenuated NOTCH signaling enhances osteoclastogenesis and bone resorption in vitro and in vivo by a combination of molecular mechanisms. First, deletion of Notch1–3 in bone marrow macrophages directly promotes their commitment to the osteoclast phenotype. These osteoclast precursors proliferate more rapidly than the wild type in response to macrophage colony-stimulating factor and are sensitized to RANKL and macrophage colony-stimulating factor, undergoing enhanced differentiation in response to low doses of either cytokine. Conforming with a role for NOTCH in this process, presentation of the NOTCH ligand Jagged1 blunts the capacity of wild-type bone marrow macrophages to become osteoclasts. Combined, these data establish that NOTCH suppresses osteoclastogenesis via ligand-mediated receptor activation. Although NOTCH1 and NOTCH3 collaborate in regulating osteoclast formation, NOTCH1 is the dominant paralog. In addition, NOTCH1 deficiency promotes osteoclastogenesis indirectly by enhancing the ability of osteoblast lineage cells to stimulate osteoclastogenesis. This is achieved by decreasing the osteoprotegerin/RANKL expression ratio. Thus, NOTCH1 acts as a net inhibitor of bone resorption, exerting its effect both directly in osteoclast precursors and indirectly via osteoblast lineage cells. These observations raise caution that therapeutic inhibition of NOTCH signaling may adversely accelerate bone loss in humans.

NOTCH signaling is an evolutionarily conserved pathway that profoundly impacts mammalian development by regulating survival, proliferation, and cell fate decision in a context-dependent manner. It contributes to tissue maintenance and/or renewal in the adult intestine (1), skin (2), hematopoietic system (3), mammary epithelium (4), and central nervous system (5) and can either promote (6–9) or suppress (10) cancer. There are four NOTCH receptors (NOTCH1–4) and at least seven NOTCH ligands (JAGGED1, JAGGED2, DLL1 (Delta-like1), DLL3, DLL4, and DNER (11) and contactin/F3/NB-3 (12)) in mice and humans. The receptors and ligands are single-pass transmembrane proteins expressed on the surface of adjacent cells. Activation of NOTCH signaling requires cell/cell contact because ligand binding to specific epidermal growth factor-like repeats in the extracellular domain of NOTCH receptors must induce a conformational change (13), most likely by trans-endocytosis (14), to expose the juxtamembrane region to cleavage by ADAM metalloproteases. The exposed N terminus is recognized by γ-secretase (15), which cleaves NOTCH again within its transmembrane domain. This cleavage releases the NOTCH intracellular domain (NICD), which translocates to the nucleus, where it associates with the DNA-binding protein CSL and other transcriptional coactivators. This complex is responsible for the transcription of NOTCH target genes, including those of Hes (hairy and enhancer of split) and the HES-related transcription factor Hey (16, 17).

NOTCH signaling plays a critical role in somitogenesis (18). For example, mice deficient in presinilin-1, the catalytic component of γ-secretase, develop deformed vertebrae (19). Moreover, deletion of the NOTCH ligand Jagged2 results in cleft palate and syndactyly in mice (20). More important, patients bearing mutations of the NOTCH ligand DLL3, the modifier Fng, or the target Mesp2 also have severe deformities of the axial skeleton (18, 21).

Due to the contribution of NOTCH signaling to somitogenesis and fetal skeletal development, attention has turned to skeletal cells (22–26). Regarding the impact of NOTCH on osteoblast differentiation, these efforts have yielded disparate results. Whereas some investigators report that NOTCH signaling dampens osteoblastogenesis (22, 24), others describe the opposite (25, 26). NOTCH signaling has also been reported to negatively impact osteoclastogenesis in vitro via a mechanism involving osteoclast precursors per se as well as regulatory stromal cells (23).

Although these efforts have been informative, genetic assessment of the role of NOTCH signaling in osteoblast and osteoclast formation and function is not in hand. Most important,
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no information exists regarding the role of NOTCH in maintaining the mature skeleton, a highly plastic tissue, in vivo and how it would respond to inhibition of NOTCH signaling in the adult.

Osteoclasts differentiate from bone marrow macrophages (BMMs) in response to RANKL (receptor activator of NF-κB ligand) and macrophage colony-stimulating factor (M-CSF) (27). Alternatively, recruitment of these resorptive polykaryons is physiologically restricted by osteoprotegerin (OPG) (27). Molecules that modulate the resorptive process often do so by regulating expression of these osteoblast-produced cytokines. Alternatively, osteoclastogenesis is also governed by direct targeting of osteoclast precursors by agents such as tumor necrosis factor-α (28).

In this study, we focused on mice with individual and combined deletions of Notch1–3 and found that the selective absence of Notch1 in either osteoclast or osteoblast lineage cells enhances osteoclastogenesis by different mechanisms, resulting in stimulated bone resorption. Thus, NOTCH1 signaling limits postnatal bone degradation. These observations identify osteoporosis as a potential complication of therapeutic inhibition of NOTCH activity in humans.

EXPERIMENTAL PROCEDURES

Mice—The generation of Notch1<sup>flox/flox</sup> Notch2<sup>flox/flox</sup>, and Notch3<sup>−/−</sup> B6.129P2-LysM<sup>M1cre</sup>/J (also known as LysMcre) were purchased from The Jackson Laboratory. Mice transgenic for Col1-cre, which expresses Cre under the control of a 2.3-kb promoter sequence of the murine Coll1(I) gene and which was shown previously to function effectively in more committed osteoblasts, were provided by Dr. Henry Kronenberg (Massachusetts General Hospital) (30). All mice were housed in the animal care unit of the Department of Pathology, Washington University School of Medicine, and were maintained according to guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experimentation was approved by the Animal Studies Committee of Washington University School of Medicine. 6–8-week-old mice were used in all circumstances.

Reagents—Recombinant murine M-CSF was obtained from R&D Systems (Minneapolis, MN), Glutathione S-transferase-RANKL was expressed in our laboratory as described (31). Anti-hemagglutinin (HA) antibody was obtained from Covance Research Products (Princeton, NJ). All other chemicals were obtained from Sigma. The plasmid transfection reagent FuGENE 6 was purchased from Roche Applied Science.

Plasmids and Transfection—Turbo-cre was cloned into the pMX vector to generate retroviruses. NICD1 was amplified from a mouse macrophage cDNA library by PCR and cloned into the pMX vector linked to a HA tag at the C terminus. Mutant Nicd1 (referred to as M2) (32) was cloned from a mouse full-length Notch1 plasmid by PCR into the pMX vector. For transfection, cells were plated into 100-mm tissue culture dishes for 24 h, and 8 µg of plasmid was transfected according to the protocol of Roche Applied Science.

Western Blotting—HA-tagged Nicd1 and M2 in the pMX vector were transfected into Plat-E cells to generate retrovirus. BMMs from C57B/6 mice were infected with the viruses and selected by blasticidin. Anti-HA antibody was used to detect the expression levels of these proteins. Anti-NOTCH1 and anti-NOTCH3 antibodies were obtained from Abcam (Cambridge, MA).

Osteoclast Lineage Cells—Macrophages/osteoclast precursors and osteoclasts were generated from bone marrow precursors as described previously (33).

Characterization of Osteoclasts—BMMs were cultured in 96-well cell culture dishes in the presence of M-CSF (10 ng/ml) and RANKL (100 ng/ml or the doses detailed in the figure legends). Media were changed on day 3. On day 5, osteoclast-like cells were characterized by staining for tartrate-resistant acid phosphatase (TRAP) activity. The number of osteoclasts was counted as described previously (33).

Primary Osteoblast and Macrophage Co-culture Assay—Primary osteoblasts were extracted from 3–5-day-old neonatal calvariae. Macrophages were extracted from bone marrow. The cells (5 × 10<sup>4</sup> of each type/well) were mixed and cultured in α-10 cell culture medium in 24-well plates with 1,25-dihydroxyvitamin D (10<sup>−8</sup> M) for 7 days. The osteoblasts were lifted by collagenase, and the remaining cells were stained for TRAP activity.

Bone Resorption Assay in Vitro—Osteoclasts were generated on whale dentin slices from BMMs in the presence of M-CSF and RANKL. On day 7, the cells were fixed in 4% paraformaldehyde and stained for TRAP activity. To visualize resorption lacunae, cells were removed from the dentin slices with 0.5 M ammonium hydroxide and mechanical agitation. Dentin slides were stained with toluidine blue.

Cell Stimulation and Immunoblotting—For M-CSF and RANKL/RANK signaling experiments, BMMs were starved for 6 h, following which RANKL (100 ng/ml) or M-CSF (50 ng/ml) was added to the media. The cells were lysed over time, and the lysate was subjected to immunoblotting.

Mineralized Bone Nodule Formation—Osteoblasts were cultured in osteoblast differentiation medium for 14 days (34). The cells were washed with phosphate-buffered saline (PBS) and fixed in ice-cold 70% ethanol for 1 h at 4 °C. The cells were washed with and incubated in 0.4% alizarin red S solution at room temperature for 10 min. The cells were then washed, dehydrated in ethanol, and air-dried.

Reverse Transcription-PCR—Total RNA was isolated using RNAesy kits (Qiagen, Valencia, CA). First-strand cDNA was generated from 1 µg of total RNA using the SuperScript first-strand synthesis system for reverse transcription-PCR (Invitrogen) as recommended by the manufacturer. PCR was performed with 1 µl of cDNA reaction mixture using Taq polymerase and appropriate primers in a volume of 50 µl. The genes to be tested were amplified in a PCR Express Thermal Cycler (Thermo Hybaid, Ulm, Germany). The cDNA was denatured at 94 °C for 5 min and subsequently subjected to various amplification cycles consisting of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The primers used were as follows: glyceraldehyde-3-phosphate dehydrogenase, 5′-ACTTTGTCAAGCTTATCCT3′; β-actin, 5′-ATGGATGACGATATCGT3′-3′; TGGTAGTCTGTCAGGT-3′; and 5′-TGGTAGTCTGTCAGGT-3′; M-CSF, 5′-GACTTCA7GCCAAGTTGCC-3′ and 5′-GGTGGCTTATAGGTACCC-3′.
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RESULTS

Notch1–3 Deletion Promotes Osteoclast Differentiation—To avoid ambiguity due to possible redundancy among NOTCH paralogs and to circumvent embryonic lethality associated with loss of Notch1 or Notch2 (35–38), we generated compound mice lacking all three receptors in osteoclast lineage cells. Thus, we mated Notch1\(^{flox/flox}\) Notch2\(^{flox/flox}\) Notch3\(^{-/-}\) mice with animals transgenic for Cre recombinase driven by the myeloid-specific lyz promoter, producing offspring bearing the LysMcre(+) Notch1\(^{flox/+}\) Notch2\(^{flox/+}\) Notch3\(^{-/-}\) genotype. These animals served as controls in all experiments relating to the absence of Notch1–3 in osteoclasts. They were also backcrossed with Notch1\(^{flox/flox}\) Notch2\(^{flox/flox}\) Notch3\(^{-/-}\) mice. One product of this mating, viz. LysMcre(+)

\(\text{Notch1}^{flox/flox} \text{Notch2}^{flox/flox} \text{Notch3}^{-/-}\) were then bred with

\(\text{Notch1}^{flox/flox} \text{Notch2}^{flox/flox} \text{Notch3}^{-/-}\) mice to generate LysMcre(+)

\(\text{Notch1}^{flox/flox} \text{Notch2}^{flox/flox} \text{Notch3}^{-/-}\) mice whose osteoclast lineage cells were deleted of all three Notch genes (hereafter referred to as Notch1,2,3\(^{0\text{C}^{-/-}}\)).

Using these compound mice, we investigated whether loss of NOTCH signaling in osteoclast lineage cells impacts their capacity to differentiate into mature resorptive polykaryons. Osteoclast precursors in the form of BMMs isolated from Notch1,2,3\(^{0\text{C}^{-/-}}\) and control BMMs were treated with 25 or 100 ng/ml M-CSF in the presence of low dose RANKL (25 ng/ml) (A) or with 25 or 100 ng/ml RANKL in the presence of low dose M-CSF (10 ng/ml) (B). After 5 days, the cultures were stained for TRAP activity. The number of osteoclasts/well were counted (C). * \(p < 0.05\); ** \(p < 0.001\).

FIGURE 1. NOTCH suppresses M-CSF- or RANKL-stimulated osteoclast differentiation in vitro. Notch1,2,3\(^{0\text{C}^{-/-}}\) and control BMMs were treated with 25 or 100 ng/ml M-CSF in the presence of low dose RANKL (25 ng/ml) (A) or with 25 or 100 ng/ml RANKL in the presence of low dose M-CSF (10 ng/ml) (B). After 5 days, the cultures were stained for TRAP activity. The number of osteoclasts/well were counted (C). * \(p < 0.05\); ** \(p < 0.001\).

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Notch1–3 Deletion Increases Osteoclast Resorptive Activity in Vitro and in Vivo—To determine whether the stimulated differentiation of Notch1,2,3OC−/− osteoclasts translates into increased bone resorption, we maintained BMMs on dentin in the presence of M-CSF and RANKL (100 ng/ml). Similar to plating on plastic, the abundance of dentin-residing Notch1,2,3OC−/− osteoclasts was substantially increased compared with controls (Fig. 2A). Consequent to their enhanced numbers, Notch1,2,3OC−/− cells generated greater numbers of resorption lacunae on dentin, identified by toluidine blue staining (Fig. 2B). The augmented levels of the resorption marker CTx in the medium conditioned by Notch1,2,3OC−/− osteoclasts on dentin slices confirmed that matrix degradation was enhanced (Fig. 2C).

To verify that global Notch deletion in osteoclast lineage cells also stimulates resorption in vivo, we prepared TRAP-stained histological sections of calvariae from Notch1,2,3OC−/− and control mice. Consistent with our in vitro findings, absence of Notch1–3 in osteoclast lineage cells promoted increased numbers of bone-resorptive cells in vivo (Fig. 2D). For a more quantitative assessment of global bone resorption, we injected control or Notch1,2,3OC−/− mice with RANKL (100 μg/day) for 7 days and measured serum CTx 24 h later. Again, in keeping with our in vitro observations, circulating CTx was increased by 2-fold in RANKL-treated Notch1,2,3OC−/− mice, demonstrating a sensitized response in vivo (Fig. 2E). The enhanced resorptive activity in RANKL-treated Notch1,2,3OC−/− mice resulted in accelerated skeletal loss as shown by μCT-determined trabecular bone volume (Fig. 2F).

Notch1–3 Deletion Stimulates Osteoclast Precursor Proliferation—Enhanced osteoclastogenesis of Notch1,2,3OC−/− BMMs may reflect accelerated proliferation in response to M-CSF in addition to their sensitized response to cytokine. To examine this possibility, we treated cytokine-starved Notch1,2,3OC−/− and control BMMs with 20 ng/ml M-CSF. We measured their proliferative rate after 24 h by BrdUrd incorporation. As shown in Fig. 3A, Notch1,2,3OC−/− BMMs incorporated 3-fold more BrdUrd than did their control counterparts, indicating a larger fraction of cells in S phase. This observation may be explained by the observation that Notch1,2,3OC−/− BMMs (lacking all three NOTCH paralogs) expressed increased amounts of c-Fms, the receptor of M-CSF (Fig. 3B). In contrast, there was no appreciable difference in RANK expression (data not shown). Thus, NOTCH signaling inhibits the response to M-CSF-induced precursor proliferation, which is likely to contribute to the stimulated osteoclastogenesis evident in Notch1,2,3OC−/− osteoclast lineage cells.

NOTCH Ligand Inhibits Osteoclastogenesis—The data presented thus far suggest that NOTCH signaling blunts osteoclastogenesis. To investigate whether NOTCH1 activation inhibits osteoclastogenesis, we retrovirally transduced wild-type (WT) BMMs with HA-tagged Nicd1 or the transcriptionally inactive Nicd1 mutant, M2 (Fig. 4A) (32). The infected cells were then exposed to M-CSF and RANKL for 5 days. As shown in Fig. 4B, empty vector-transduced BMMs, but not those expressing NICD1, readily differentiated into osteoclasts. Hence, NOTCH1 signaling suppresses osteoclast differentiation. Interestingly, M2-transfected BMMs produced more and larger osteoclasts than those bearing empty vector. Because M2 can bind to CSL but cannot assemble a transcription complex (39), its effect on osteoclast formation is consistent with a weak dominant-negative effect on endogenous NOTCH receptors.

To confirm that physiological activation of NOTCH receptors by their ligand inhibits osteoclast formation, we transduced Chinese hamster ovary cells stably expressing NOTCH2 with a luciferase-bearing Notch promoter–response element. We cultured these cells with native NIH3T3 cells or those stably expressing JAGGED1 for 24 h and measured the luciferase
activity. Relative to parental NIH3T3 cells, co-culture with those expressing JAGGED1 activated the NOTCH reporter construct in N2-CHO cells by 7-fold (Fig. 4C). Next, we co-cultured WT BMMs for 5 days with parental NIH3T3 cells or those overexpressing JAGGED1 in the presence of M-CSF and RANKL. JAGGED1-expressing NIH3T3 cells markedly reduced the differentiation of WT BMMs into osteoclasts (Fig. 4D), establishing that ligand-mediated NOTCH activation suppresses osteoclastogenesis.

**NOTCH1 and NOTCH3 Inhibit Osteoclastogenesis**—Having established that NOTCH signaling inhibits osteoclastogenesis, we turned to the specific role of individual NOTCH receptors in the differentiation of osteoclast lineage cells. First, we measured the abundance of each paralog throughout osteoclastogenesis. Immunoblot analysis showed that NOTCH1 and NOTCH3 proteins were expressed in BMMs and throughout the osteoclast differentiation process (Fig. 5, A and B). On the other hand, NOTCH2 protein was undetectable in osteoclasts and their precursors (data not shown).

We next transduced Notch1^{flox/flox} BMMs in vitro with Cre recombinase-expressing, blasticidin-selectable retrovirus or with empty vector controls. The infected cells were selected with blasticidin in the presence of M-CSF. These Notch1-deficient BMMs, their empty vector-containing counterparts, or those obtained from Notch3^{-/-} or WT mice were placed in M-CSF and RANKL for 5 days to determine their osteoclast-forming potential. Because NOTCH2 was not detected in osteoclast lineage cells, Cre recombinase-treated Notch2^{flox/flox} BMMs served as an additional control. Loss of NOTCH1 in osteoclast lineage cells greatly enhanced osteoclastogenesis (Fig. 5C). NOTCH3 deletion also modestly promoted osteoclast formation. As expected, removal of Notch2 had no impact on differentiation of the resorptive cell. Thus, both NOTCH1 and NOTCH3 were required to fully inhibit osteoclast formation in these assays, most likely by mutual inhibition, as osteoclastogenic macrophages express JAGGED1 and JAGGED2 (40). We conclude that NOTCH1 in BMMs plays a dominant role in suppressing their differentiation into osteoclasts.
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Absence of NOTCH1 in Osteoblasts Enhances Osteoclast Formation—Having established that NOTCH activation attenuates osteoclast differentiation and bone resorption, we assessed the impact of NOTCH signaling on the osteoblast, a cell that regulates osteoclast differentiation by expression of the positive regulators RANKL and M-CSF or the repressor OPG.

We first addressed this issue in vitro by infecting calvarial osteoblasts (isolated from 3–5-day-old Notch1flox/flox Notch2flox/flox Notch3−/− mice) with empty vector or Cre-bearing adenovirus. Green fluorescent protein-tagged adenovirus, serving as efficiency control, demonstrated that ~90% of the cells were infected (data not shown). We examined the effects of NOTCH loss on osteoblast function by culturing the Cre adenovirus-infected cells (Notch1,2,3OBC−/−) in osteoblastogenic medium and after 14 days assessed mineralized bone nodule formation. Similar to the effects on osteoclasts, the absence of the three NOTCH paralogs enhanced the osteogenic capacity of osteoblasts (Fig. 6A). To determine whether Notch1 is a functional receptor in osteoblast differentiation, we generated Col1-cre;Notch1flox/flox mice to yield animals lacking Notch1 in osteoblast lineage cells (Notch1OBC−/−). Similar to Notch1,2,3OBC−/− osteoclasts, calvarial cells lacking Notch1 underwent enhanced mineralized bone nodule formation (Fig. 6B), confirming a role for NOTCH1 in delaying osteoblast differentiation.

We next investigated whether deletion of Notch1 in osteoblasts impacts osteoclastogenesis. To this end, we cultured Notch1flox/flox or Notch1OBC−/− osteoblasts with WT BMMs for 5 days in the presence of 1,25-dihydroxyvitamin D, which promotes osteoclast differentiation in this co-culture system. The osteoblasts were then removed with collagenase, and osteoclasts were identified by TRAP staining. More mature osteoclasts were present in co-cultures containing Notch1-deficient osteoblasts compared with control osteoblasts (Fig. 6, C and D).

The data presented thus far have established that Notch deletion in either BMMs or osteoblast lineage cells enhances osteoclastogenesis. To establish whether osteoclast recruitment can be enhanced further with Notch deficiency in both cell types, we cultured various combinations of WT and Notch-deficient BMMs and osteoblasts in 1,25-dihydroxyvitamin D. Cultures containing osteoblasts and BMMs (both lacking NOTCH) were substantially more osteoclastogenic (Fig. 6E, panel 4) than those in which both cells were WT (panel 1). The absence of the receptor in only BMMs (panel 2) or osteoblast lineage cells (panel 3) yielded osteoclast cultures indistinguishable from those in which both cell types lacked Notch (Fig. 6F). Hence, Notch deficiencies in either osteoclast or osteoblast lineage cells optimally promote osteoclast formation.

Absence of Notch1 in Osteoblasts Suppresses OPG Expression and Enhances Osteoclast Resorptive Activity—To understand how Notch-deficient osteoblasts promote osteoclastogenesis, we measured the expression of RANKL and M-CSF mRNAs, the principal cytokines with which osteoblasts promote osteoclastogenesis, and that of OPG, a RANKL decoy receptor that suppresses osteoclast formation. Although the quantity of M-CSF mRNA was essentially unaltered in Notch1-deficient osteoblasts, RANKL mRNA was enhanced, and there was a profound decrease in OPG mRNA (Fig. 7A), resulting in a pronounced pro-osteoclastogenic shift in the cytokines produced.

Unlike osteoblast-produced RANKL, which is membrane-bound, OPG is secreted. Thus, one would expect medium con-
ditioned by Notch1-deficient osteoblasts, which produce a paucity of OPG, to be pro-osteoclastogenic. To determine whether this is so, we cultured WT BMMs in medium conditioned by Notch1-deficient or control osteoblasts to which we added RANKL (25 ng/ml) and M-CSF (10 ng/ml). Under both conditions, TRAP-expressing cells appeared at these relatively low concentrations of osteoclastogenic cytokines after 5 days (Fig. 7B). However, although those generated in WT conditioned medium were small and irregular, characteristically large, spread osteoclasts were abundant in wells containing medium conditioned by Notch1-deficient osteoblasts.

To test the physiological relevance of Notch1 deletion in osteoblasts on bone resorption, we administered PBS or PTH-(1–34), a bone resorption agonist exerting its osteoclast-stimulating effects indirectly by targeting osteoblast lineage cells, to control or Notch1-deficient mice four times a day. After 4 days, serum CTx levels revealed enhanced osteoclast activity in mice with Notch1 deficiency restricted to osteoblast lineage cells both in the basal state and after stimulation with PTH-(1–34) (Fig. 7C). Thus, Notch1 deficiency in either osteoclast or osteoblast lineage cells promotes osteoclastic bone resorption both in vitro and in vivo.

**DISCUSSION**

NOTCH activates a conserved signaling system that participates in elaboration of cell fate and patterning in all Metazoa. The pathway uniquely employs proteolytic cleavage of a ligand-activated transmembrane receptor to mobilize its intracellular domain, which enters the nucleus, where it joins a DNA-associated protein to engage in gene transcription. NOTCH signaling is active throughout life in many systems. Herein, we examined its role in the skeleton after birth.

Bone formation and resorption are modulated by the activities of osteoblasts and osteoclasts, respectively, and skeletal dynamics is the net result of the simultaneous activity of both cell types. Moreover, recruitment of osteoblasts to the bone-remodeling process induced by osteoclastic activity and the key extracellular signals regulating osteoclastogenesis (RANKL, M-CSF, and OPG) are the products of osteoblast lineage cells. Thus, osteoclast formation is controlled by directly regulating osteoclast precursors and indirectly by the activity of
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FIGURE 7. NOTCH deficiency modulates OPG and RANKL expression. A, RANKL, OPG, M-CSF, and NOTCH1 mRNA levels in Notch1<sup>flox</sup> and Notch<sup>1Ob</sup>−/− osteoblasts were measured by reverse transcription-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a loading control. B, WT BMMs were cultured in medium conditioned for 1 day by Notch1<sup>flox</sup> or Notch<sup>1Ob</sup>−/− osteoblasts in the presence of low dose RANKL (25 ng/ml) and M-CSF (10 ng/ml). 5 days later, the cells were stained for TRAP activity. C, Notch1<sup>flox</sup> and Notch<sup>1Ob</sup>−/− mice were injected with PBS or PTH (1–34) four times/day for 4 days, after which serum CTx levels were determined by enzyme-linked immunosorbent assay. *, p < 0.05; **, p < 0.01.

the osteoblast. We have demonstrated, for example, that tumor necrosis factor-α exerts its osteoclastogenic effects by stimulating both cell types (28). Hence, we investigated whether NOTCH signaling impacts osteoclast formation in vitro and in vivo and dissected the osteoclast-autonomous requirements as well as the role of NOTCH signaling in the supportive osteoblast lineage cells. Both contribute to bone resorption.

NOTCH1 and NOTCH3 are constitutively expressed throughout osteoclastogenesis, whereas NOTCH2 is undetectable. To determine whether NOTCH receptors impact differentiation of osteoclast precursors, we produced single or compound mutant mice lacking either one or all three NOTCH paralogs in this lineage. To avoid lethality associated with global deletion of Notch1 or Notch2, we generated Notch3<sup>−/−</sup> mice with conditional alleles for Notch1 and Notch2 and mated them with transgenic mice expressing Cre recombinase driven by the myeloid-specific lysozyme M-cre promoter. Our rationale for deleting the Notch2 gene in our compound knock-out mice was to avoid the possibility of compensatory NOTCH2 expression in the absence of the other two paralogs. Using this strategy, we discovered that although NOTCH1 is the dominant NOTCH receptor mediating osteoclastogenesis, NOTCH3 is also active in this regard.

M-CSF and RANKL are the key regulators of bone resorption and act directly on osteoclast precursors. We found that the absence of Notch1–3 in these cells lowers the threshold for either molecule, eventuating in enhanced bone resorption both in vitro and in vivo. This finding suggests that NOTCH negatively controls osteoclastogenesis at low doses of RANKL or M-CSF. We confirmed this hypothesis by demonstrating that JAGGED1-expressing cells inhibit differentiation of BMMs to the osteoclast phenotype in the presence of RANKL.

NOTCH signaling requires trans-endocytosis of receptor bound to ligand. We propose that NOTCH-mediated regulation of osteoclastogenesis involves physical interaction between BMMs or their interaction with ligand-expressing stromata. Because osteoclastogenic macrophages express NOTCH ligand and utilize NOTCH to regulate their differentiation, our data suggest that osteoclastogenesis is modulated by lateral or mutual inhibitory interactions between osteoclast precursors.

Osteoclast formation also reflects the number of progenitors, and others have shown that NOTCH activation prompts programmed death of osteoclast lineage cells (23, 41). Similarly, we found that the absence of NOTCH potentiates the proliferative response of BMMs to M-CSF most likely caused by enhanced expression of c-Fms, the cytokine receptor, by Notch-deficient osteoclast precursors.

NOTCH paralogs are similar in structure and ligand recognition, but each may regulate particular events in a cell-specific manner (42). NOTCH1 and NOTCH3 are constitutively expressed during osteoclastogenesis, and Notch1 deletion dramatically promotes osteoclast formation. However, NOTCH1 does not completely regulate osteoclastogenesis, as eliminating only NOTCH3 increases the number of bone-resorptive polykaryons. Although both paralogs have been considered by some to be antagonistic (43), this is the first example of a process controlled by each in a parallel fashion. Consistent with our failure to detect NOTCH2 protein in BMMs, Cre-mediated deletion of its gene in osteoclast lineage cells does not alter RANKL- and M-CSF-induced differentiation. In a separate study, however, we found that NOTCH2 plays a predominant role in bone marrow mesenchymal progenitors to control osteoblastogenesis.3

Having established that NOTCH signaling regulates osteoclastogenesis cell-autonomously, we investigated whether

3 Hilton, M. J., Tu, X., Bai, S., Zhao, J., Kobayashi, T., Kronenberg, H. M., Teitelbaum, S. L., Ross, F. P., Kopan, R., and Long, F. (2008) Nat. Med., in press.
NOTCH activity in accessory cells, viz. those of the osteoblast lineage, contributes to regulation of osteoclastogenesis. We herein report that Col1a-cre-mediated inactivation of only the Notch1 gene in osteoblasts enhances osteoclastogenesis in vitro and osteoclast activity in vivo. We favor the hypothesis that this is due to an increased ratio of RANKL/OPG expression because we observed a reduced ability of medium conditioned by Notch1-deficient osteoblasts to block osteoclastogenesis in response to low concentrations of M-CSF or RANKL. This most likely represents deficiency of the RANKL decoy receptor, given that RANKL is typically membrane-residing and OPG is secreted. This increase in both the osteogenic and osteoclastogenic capacity of Notch1-deficient osteoblasts provides a likely explanation for the normal skeletal mass of 2-month-old Notch1<sup>Col1a-cre<sup> mice. On the other hand, mice lacking both Notch1 and Notch2 in osteoblasts (by Col1-Cre) have a lower bone mass compared with controls by 5 months of age, indicating that suppressed bone formation ultimately dominates the skeletal phenotype.4

The increased bone resorption in mice lacking Notch1, specifically in osteoblast lineage cells, occurs under basal conditions and in mice treated with PTH-(1–34), a resorption-enhancing hormone that targets the osteoblast to promote its expression of the RANKL and to inhibit that of OPG. On the other hand, Calvi et al. (44, 45) have shown that PTH-(1–34) increases the number of hematopoietic stem cells by inducing JAGGED1 expression in osteoblasts. Given that PTH-(1–34) is proresorptive and that osteoclasts are of hematopoietic origin, these data are seemingly at odds with ours, which have established that Notch1 signaling specifically in osteoblast lineage cells, contributes to regulation of osteoclastogenesis. We here report that this signaling dampens osteoclast recruitment not by targeting primitive precursors, but by targeting those committed to macrophage differentiation.

Finally, these results have significant therapeutic implications. Because NOTCH signaling is implicated in a variety of hematological and solid malignancies, it is an attractive therapeutic target (46, 47). Our findings demonstrate that NOTCH inhibition in the adult stimulates bone resorption and raise caution that drugs inhibiting this pathway may accelerate bone loss and predispose patients to fracture.

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