Published in final edited form as:

Bone. 2014 May ; 62: 22–28. doi:10.1016/j.bone.2014.01.023.

Notch1 and Notch2 Expression in Osteoblast Precursors Regulates Femoral Microarchitecture

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Abstract

Notch receptors regulate cell differentiation and function. *Notch1* and *Notch2* inactivation in osteoblasts and osteocytes increases cancellous bone volume, but the function of Notch signaling in osteoblast precursors is unknown. To inactivate Notch signaling in immature osteoblastic cells, mice homozygous for conditional *Notch1* and *Notch2* alleles (*Notch1*^{loxP/loxP}; *Notch2*^{loxP/loxP}) were crossed with mice where the osterix (*Osx*) promoter, regulated by a Tet-Off cassette, governs Cre expression (*Osx-Cre*). *Notch1*^{loxP/loxP}; *Notch2*^{loxP/loxP} control and *Osx-Cre*^{+/-}; *Notch1*^{Δ/Δ}; *Notch2*^{Δ/Δ} experimental littermate cohorts were obtained. To prevent the effects of embryonic *Osx-Cre* expression, doxycycline was administered to pregnant dams, but not to newborns. Recombination of conditional alleles was documented in calvarial DNA extracts from 1 month old mice. *Notch1* and *Notch2* inactivation did not affect femoral microarchitecture at 1 month of age. Cancellous bone volume was higher and structure model index was lower in 3 and 6 month old *Osx-Cre*^{+/-}; *Notch1*^{Δ/Δ}; *Notch2*^{Δ/Δ} mice than in control littermates and the effect was more pronounced in female mice. One month old *Osx-Cre*^{+/-}; *Notch1*^{Δ/Δ}; *Notch2*^{Δ/Δ} male mice transiently exhibited an increase in osteoblast number and a modest suppression in bone resorption. *Osx-Cre*^{+/-}; *Notch1*^{Δ/Δ}; *Notch2*^{Δ/Δ} female mice displayed a tendency toward increased bone formation at 3 months of age, although bone remodeling was suppressed in 6 month old *Osx-Cre*^{+/-}; *Notch1*^{Δ/Δ}; *Notch2*^{Δ/Δ} female mice. *Notch1* and *Notch2* inactivation increased porosity and reduced thickness of cortical bone. These effects were modest and more evident in 3 and 6 month old female than in male mice of the same age. In conclusion, *Notch1* and *Notch2* expression in osteoblast precursors regulates cancellous bone volume and microarchitecture.

Keywords

Notch1; Notch2; osteoblasts; cortical bone

1. INTRODUCTION

The microarchitecture of cancellous and cortical bone is determined by bone remodeling, the process of tissue renewal that is carried out by the concerted actions of osteoblasts, which form bone, and osteoclasts, the bone resorbing cells [1, 2]. Osteoblasts derive from

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mesenchymal stem cells residing in the bone marrow and the commitment of precursor cells to the osteoblastic lineage is governed by multiple factors, including molecules that regulate the activity of bone morphogenetic protein, Wnt and Notch receptors [3-6]. Expression of osterix (*Osx*) characterizes a population of osteoblast precursors that progresses to the mature osteoblastic phenotype and has the potential of further differentiation into endosteal lining cells and osteocytes [3, 7, 8]. Osteoclasts are multinucleated cells generated by the fusion of hematopoietic mononuclear precursors; interactions of the receptor of nuclear factor- κ B ligand (Rankl) with the Rank receptor induce osteoclastogenesis and osteoclast activity [9].

Notch signaling regulates cell lineage commitment and contributes to the replacement of aging cells in multiple tissues, including bone [10-12]. Interactions of Notch receptors with cognate ligands result in the proteolytic cleavage of the Notch intracellular domain, which translocates to the nucleus to activate transcription [13, 14]. Notch target genes include those encoding for the transcriptional repressors hairy enhancer of split (*Hes*) and *Hes*-related with YRPW motif (*Hey*) [15].

Notch1 and *Notch2* are expressed by skeletal cells and appear to mediate the effects of Notch signaling on bone microarchitecture and homeostasis, although skeletal cells also express low levels of *Notch3* transcripts [16-22]. Work from our laboratory and from others has established that the effects of Notch signaling in osteoblastic cells are in part determined by the degree of cellular maturity. Activation of Notch in the early phases of osteoblastic differentiation and in osteoblasts reduces cancellous bone volume and causes deposition of disorganized woven bone [19, 23-25]. Inactivation of Notch signaling in osteoblasts increases trabecular bone volume due to enhanced osteoblastogenesis followed by increased osteoclast number and bone resorption [19]. Accordingly, induction of Notch signaling in osteoblasts and osteocytes increases bone mass by suppressing bone resorption [22, 25]. Studies of Notch misexpression in osteoblasts and osteocytes revealed that Notch regulates bone resorption by inducing expression of osteoprotegerin, which is a soluble Rankl decoy receptor [18, 21, 22, 26]. Recently, we demonstrated that Notch induction in cells of the osteoblastic lineage and in osteocytes results in cortical bone that is either absent, porous or assumes the appearance of trabecular bone, revealing a novel role of Notch signaling as a determinant of cortical bone structure [25].

Dual conditional inactivation of *Notch1* and *Notch2* in the limb bud causes a lengthening of the growth plate and an increase in cancellous bone volume [21]. Similarly, conditional inactivation of *Hes1* in the developing limb increases trabecular bone volume [27]. However, interpretation of these findings is confounded by the consequences of Notch signaling inhibition during the early phases of skeletal development [28]. Inactivation of the Notch target *Hey2* in osteoblast precursors phenocopies the effects of Notch inactivation in the developing limb, although the effect is mild, suggesting genetic compensation from other *Hey* genes [29].

To determine the consequences of Notch signaling inhibition in osteoblast precursors, *Notch1* and *Notch2* were inactivated in post-natal life in cells expressing *Osx*. To this end, the skeletal phenotype of male and female mice where *Notch1* and *Notch2* were inactivated in cells expressing Cre under the control of the *Osx* promoter was investigated by microcomputed tomography (μ CT) and histomorphometric analysis of the femur.

2. EXPERIMENTAL PROCEDURES

2.1 Conditional Inactivation of Notch1 and Notch2 in Osteoblast Precursors

To express Cre recombinase in osteoblast precursors, we obtained C57BL/6 mice where the Cre coding sequence is cloned downstream of an Osterix (*Osx*) promoter (*Osx-Cre*) (Jackson Laboratory) [30]. In these mice, a Tet-Off cassette suppresses the activity of the *Osx* promoter in the presence of tetracycline [31]. For the conditional inactivation of *Notch1*, mice where the 3.5 kilobase upstream of the putative transcriptional start site and the first exon of the *Notch1* locus are flanked by *loxP* sequences (*Notch1^{loxP}*), were obtained from F. Radtke (Ludwig Institute for Cancer Research, University of Lausanne, Switzerland). In these mice, Cre recombination brings about the excision of DNA sequences that encode for the Notch1 signal peptide, precluding expression of a functional Notch receptor [32]. For the conditional inactivation of *Notch2*, mice where exon 3 of *Notch2* is flanked by *loxP* sequences (*Notch2^{loxP}*) were provided by T. Gridley (Jackson Laboratory, Bar Harbor, ME). In these mice, removal of the *loxP*-flanked DNA sequences by Cre recombination leads to a frame shift mutation and to the expression of a truncated and inactive Notch2 protein [33].

Notch1 and *Notch2* conditional mice, both provided in a 129SvJ/C57BL/6 background, were crossed to generate dual *Notch1^{loxP/loxP}* and *Notch2^{loxP/loxP}* mice (*Notch1^{loxP/loxP};Notch2^{loxP/loxP}*). *Osx-Cre* transgenics were crossed with *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* mice to create *Osx-Cre^{+/-};Notch1^{loxP/wt};Notch2^{loxP/wt}* mice, which were bred with *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* to obtain *Osx-Cre^{+/-};Notch1^{loxP/loxP};Notch2^{loxP/loxP}* mice. The latter were crossed with *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* to generate an experimental cohort, in which Cre excises the *loxP*-flanked sequences from the *Notch1^{loxP}* and *Notch2^{loxP}* alleles (*Osx-Cre^{+/-};Notch1^{ΔΔ};Notch2^{ΔΔ}*), and littermate controls (*Notch1^{loxP/loxP};Notch2^{loxP/loxP}*), not carrying the *Osx-Cre* transgene. In parallel studies, we compared *Osx-Cre^{+/-}* male mice to wild type littermate controls of the same sex. To suppress *Osx-Cre* expression during embryonic development, pregnant dams were administered chow containing approximately 0.55 g/kg doxycycline (Harlan Laboratories, Indianapolis, IN) from the time of conception to delivery, resulting in an estimated daily doxycycline dose of 2 to 3 mg. All animal experiments were approved by the Animal Care and Use Committee of Saint Francis Hospital and Medical Center.

2.2 Genotyping, Recombination of Conditional Alleles, Body Weight and Femoral Length

The presence of the *Osx-Cre* transgene and of the *Notch1^{loxP}* and *Notch2^{loxP}* alleles was determined by polymerase chain reaction (PCR) in tail DNA extracts from adult mice, and primers specific for fatty acid-binding protein 1 (*Fabp1*) were used as positive controls in the PCR reactions (Table 1). Recombination of conditional alleles was assessed by PCR in DNA extracts from the parietal bone of 1 month old mice, using primers specific for the *Notch1* and *Notch2* deletion (Table 1). Body weight was determined at the time of sacrifice. Femurs were dissected from surrounding tissues and fixed in 70 % ethanol, and images obtained on a μ CT 40 scanner (Scanco Medical AG, Bassersdorf, Switzerland) were used to measure femoral length.

2.3 Microcomputed Tomography

Femurs were scanned in 70 % ethanol at an energy level of 55 kVp, an intensity of 145 μ A, and an integration time of 200 ms on a μ CT 40 scanner. Trabecular bone volume fraction and microarchitecture were evaluated starting approximately 1.0 mm proximal to the femoral condyles. A total of 160 consecutive slices at a thickness of 6 μ m acquired at an isotropic voxel size of 216 μ m³, were chosen for analysis. Contours were manually drawn every 10 slices a few voxels away from the endocortical boundary to define the region of

interest for analysis, whereas the contours of the remaining slices were iterated automatically. Bone volume fraction, trabecular thickness, number and separation, connectivity density, and structure model index (SMI) were measured in the trabecular region using a Gaussian filter ($\sigma = 0.8$, support = 1) and a user defined threshold [34]. For the cortical region, a total of 100 slices at a thickness of 6 μm were measured at the femoral mid-diaphysis with an isotropic voxel size of 216 μm^3 . For determination of cortical microarchitecture, contours were iterated across all slices along the cortical shell and the bone marrow cavity was excluded. Cortical bone volume fraction, porosity and thickness, total area, bone area, periosteal and endocortical perimeters and density of material were determined using a Gaussian filter ($\sigma = 0.8$, support = 1) and a user defined threshold. Terminology and units used were those suggested by the Journal of Bone and Mineral Research [34].

2.4 Bone Histomorphometry

Cancellous bone at the distal femur was analyzed by static and dynamic histomorphometry after injection with calcein 20 mg/kg, and demeclocycline 50 mg/kg, at an interval of 2 days for 1 month old, and 7 days for 3 and 6 month old mice. Euthanasia was carried out by CO₂ inhalation 2 days after administration of demeclocycline. Femurs were sectioned on a microtome at a thickness of 5 μm (Microm, Richards-Allan Scientific, Kalamazoo, MI) and stained with 0.1 % toluidine blue. Static parameters of bone formation and resorption were measured with an OsteoMeasure morphometry system (Osteometrics, Atlanta, GA) in a defined area between 360 μm and 2160 μm from the proximal end of the distal growth plate [35]. Mineralizing surface per bone surface and mineral apposition rate were measured on unstained sections under ultraviolet light, using a triple diamino-2-phenylindole fluorescein set long pass filter, and bone formation rate was calculated. Terminology and units used were those recommended in the 2012 update of the American Society for Bone and Mineral Research Histomorphometry Nomenclature Committee [36].

2.5 Statistical Analysis

Data are expressed as means $\pm$ SEM. Statistical differences were determined by Student's *t*-test [37].

3. RESULTS

3.1 General Characteristics of Mice Harboring Notch1 and Notch2 Inactivation in Osteoblast Precursors

To inactivate *Notch1* and *Notch2* in osteoblast precursors, female *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* mice were bred with male *Osx-Cre^{+/-};Notch1^{loxP/loxP};Notch2^{loxP/loxP}* mice, so that the progeny was evenly distributed between *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* control and *Osx-Cre^{+/-};Notch1^{ΔΔ};Notch2^{ΔΔ}* experimental cohorts. In previous studies, we established that *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* do not display a skeletal phenotype or changes in weight and general appearance when compared to wild-type C57BL/6 mice [22]. Expression of *Osx-Cre* during embryonic development is reported to cause a modest and transient thinning of cortical bone and reduced body weight at 8 weeks of age [30, 38-40]. To ensure that the *Osx-Cre* transgene did not cause a skeletal phenotype, pregnant dams were administered doxycycline to suppress expression of Cre, and the phenotype of 1 month old *Osx-Cre^{+/-}* male mice and wild type littermates of the same sex was characterized. Body weight, femoral length and microarchitecture of cancellous bone were not different between *Osx-Cre^{+/-}* male mice and controls. The *Osx-Cre* transgene caused a modest reduction in total area, but not in cortical thickness or cortical area (Table 2). These findings confirm that in

the context of doxycycline exposure during embryonic development the *Osx-Cre* transgene does not cause an obvious skeletal phenotype [25].

To test for recombination of the *Notch1* and *Notch2* conditional alleles, DNA was extracted from parietal bones of 1 month old *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* and *Osx-Cre^{+/-};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* male and female mice. PCR products for the *Notch1* and *Notch2* recombined alleles were observed in DNA from *Osx-Cre^{+/-};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* mice and not from *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* mice (Figure 1A). Body weight increased as mice matured, but weight gain was less pronounced in *Osx-Cre^{+/-};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* male mice, so that at 6 months of age experimental male mice were smaller than control male littermates. Femoral length increased with age but the effect was less pronounced in *Osx-Cre^{+/-};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* female mice, so that femurs of 3 and 6 month old *Osx-Cre^{+/-};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* female mice were shorter than femurs of female littermate controls (Figure 1B).

3.2 Notch1 and Notch2 Inactivation in Osteoblast Precursors Increases Cancellous Bone Volume

Inactivation of *Notch1* and *Notch2* in osteoblast precursors did not affect cancellous bone microarchitecture in male mice at 1 month of age but caused an increase in trabecular bone volume in 3 month old mice. This effect was associated with an increase in trabecular number and in connectivity density, although trabeculae were thinner. A less pronounced phenotype was observed in 6 month old *Osx-Cre^{+/-};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* male mice (Table 3, Figure 2). One month old conditional *Notch1* and *Notch2* null male mice exhibited a higher number of osteoblasts and a greater osteoid surface coupled to a lower number of osteoclasts and decreased eroded surface than *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* littermates of the same sex. Despite a modest suppression in mineral apposition rate, bone formation was not affected by the inactivation of *Notch1* and *Notch2*. Although these effects may explain the increased bone volume observed in older animals, they were transient and *Osx-Cre^{+/-};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* male mice had no cellular phenotype at 3 and 6 months of age (Table 3).

Trabecular microarchitecture was not different between 1 month old *Osx-Cre^{+/-};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* female mice and *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* littermates of the same sex. At 3 and 6 months of age, trabecular bone volume, number and connectivity density were higher, although trabeculae were thinner in *Osx-Cre^{+/-};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* female mice than in *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* female littermates (Table 4, Figure 2). SMI was lower in *Osx-Cre^{+/-};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* female than in *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* female littermates at 3 and 6 months of age, indicating a higher ratio of plate-like over rod-like trabeculae (Table 4). *Notch1* and *Notch2* inactivation in female mice had no effect on osteoblast and osteoclast number or on bone formation and resorption at 1 month of age, although a tendency toward increased mineralizing surface was noticed at 3 months. At 6 months of age, the phenotype evolved and rates of bone formation and resorption were lower in *Osx-Cre^{+/-};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* female mice than in control female littermates (Table 4).

3.3 Notch1 and Notch2 Inactivation in Osteoblast Precursors Affects Cortical Bone Structure

In previous work, we reported that cortical bone is absent in the context of Notch activation in osteoblast precursors, and we tested whether conditional inactivation of *Notch1* and *Notch2* in these cells has an impact on cortical bone microarchitecture [25]. Three and 6 month old *Osx-Cre^{+/-};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* male mice exhibited higher cortical porosity than *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* male littermates of the same age, although the effect

was modest. Cortical bone was thinner in 1 month old *Osx-Cre^{+/-};Notch1^{ΔΔ};Notch2^{ΔΔ}* male mice than in control male littermates, but not in 3 and 6 month old male mice. *Notch1* and *Notch2* inactivation caused an increase in total and bone area at 6 months of age (Table 5, Figure 3).

Three and 6 month old *Osx-Cre^{+/-};Notch1^{ΔΔ};Notch2^{ΔΔ}* female mice exhibited higher cortical porosity than *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* female littermates. Cortical bone was not affected by *Notch1* and *Notch2* inactivation in 1 month old female mice but was thinner in 3 month old *Osx-Cre^{+/-};Notch1^{ΔΔ};Notch2^{ΔΔ}* female mice than in control littermates of the same sex. Total area was higher in the context of *Notch1* and *Notch2* inactivation in female mice, although at 3 months of age the effect was not statistically significant only at 1 and 6 months of age. Periosteal and endocortical perimeters were greater in 6 month old *Osx-Cre^{+/-};Notch1^{ΔΔ};Notch2^{ΔΔ}* female mice than in control female littermates. A modest but statistically significant reduction of density of material was observed in 3 and 6 month old *Osx-Cre^{+/-};Notch1^{ΔΔ};Notch2^{ΔΔ}* female mice in comparison to littermate female controls (Table 5, Figure 3).

4. DISCUSSION

In the present study, we report that inactivation of *Notch1* and *Notch2* in osteoblast precursors increases cancellous bone volume, providing further support to the notion that *Notch1* and *Notch2* are responsible for the effects of Notch in osteoblastic cells [6]. Presence of the *Osx-Cre* transgene in the male progeny of pregnant dams administered doxycycline did not modify trabecular microarchitecture and caused a small decrease in cross sectional area without affecting thickness of cortical bone. These changes were modest and do not phenocopy the effects of *Notch1* and *Notch2* inactivation in osteoblast precursors, indicating that the skeletal phenotype of *Osx-Cre^{+/-};Notch1^{ΔΔ};Notch2^{ΔΔ}* male mice is secondary to inactivation of *Notch1* and *Notch2* and not due to the *Osx-Cre* transgene [30, 38-40]. *Osx-Cre^{+/-}* female mice were not studied and it cannot be excluded with certainty that the transgene contributed to aspects of the skeletal phenotype of conditional *Notch1* and *Notch2* null female mice. *Hes1* and *Hey2* are induced by Notch in skeletal cells and the phenotype of *Osx-Cre^{+/-};Notch1^{ΔΔ};Notch2^{ΔΔ}* mice was reminiscent of the one observed in the context of *Hes1* inactivation in osteoblasts and of *Hey2* inactivation in osteoblast precursors of male mice [25, 27, 29]. However, the skeletal consequences of the conditional *Notch1* and *Notch2* inactivation in osteoblast precursors were more pronounced and observed in mice of both sexes, and inactivation of *Notch* did not affect expression of *Hes1* or *Hey2* in parietal bones of mice of both sexes (data not shown). These findings could suggest that *Hes1* and *Hey2* are not required for the effects of Notch signaling in osteoblast precursors, or that other cellular signals contribute to their expression [41, 42].

Conditional inactivation of *Notch1* and *Notch2* was associated with reduced body weight in male mice and shorter femurs in female mice, although systemic effects secondary to the inhibition of Notch signaling in osteoblastic cells were not reported in previous studies [6, 22]. Notch signaling is required for elongation of the hypertrophic chondrocyte zone, a process that determines the length of long bones and contributes to body size [43]. The *Osx-Cre* transgene is expressed in differentiating chondrocytes and post-natal inactivation of *Notch1* and *Notch2* in these cells might affect elongation of long bones and explain the reduced growth of *Osx-Cre^{+/-};Notch1^{ΔΔ};Notch2^{ΔΔ}* mice [44].

In male mice, *Notch* inactivation increased the number of osteoblasts at 1 month of age, confirming that *Notch1* and *Notch2* mediate the inhibitory effect of Notch on osteoblast differentiation [19, 21, 23]. A decrease in the activity of the *Osx-Cre* transgene as male mice

matured might explain why the cellular phenotype was transient. Notch inactivation did not result in a cellular phenotype in 1 month old female mice, although in agreement with the inhibitory effects of Notch activation on osteoblastogenesis, *Osx-Cre^{+/-};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* female mice exhibited a transient increase in mineralizing surface at 3 months of age [25]. This would indicate that a larger proportion of osteoblasts were actively making bone. Although the cellular phenotype was modest, *Notch1* and *Notch2* inactivation caused a sustained increase in cancellous bone in 3 and 6 month old female mice. Bone remodeling was suppressed in 6 month old *Osx-Cre^{+/-};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* female mice, possibly contributing to the sustained increase in cancellous bone. These findings are consistent with previous reports indicating that the effects of Notch misexpression in osteoblastic cells depend on the stage of cell maturation [19, 25].

Recent work from our laboratory demonstrated that osteoblast surface and number are higher in C57BL/6 female mice than in male littermates at 1 month of age [45, 46]. In the present manuscript, *Notch1* and *Notch2* inactivation in osteoblast precursors increased osteoblast surface and number in male but not in female mice, so that differences between sexes were no longer detectable, suggesting that *Notch1* and *Notch2* could mediate the effects of sex in osteoblasts. Notch signaling suppresses osteoblastogenesis and might be less active in female than in male mice, so that *Notch1* and *Notch2* inactivation in osteoblast precursors could affect male to a greater extent than female mice, providing a possible explanation for the sexually dimorphic phenotype at 1 month of age [21, 23, 25]. However, there is no apparent explanation for the sexually dimorphic effects of *Notch1* and *Notch2* inactivation on osteoclast number and bone resorption. Sex might have an impact on the function of *Notch1* and *Notch2*, and this would be consistent with previous studies, which reported differences related to sex in the cellular phenotype of mice where components of the Notch signaling pathway were inactivated in skeletal cells [27, 29]. The results reported in this study further illustrate the importance of performing analysis of the effects of gene misexpression in mice of both sexes.

Work from our laboratory and others demonstrated that *Notch* inactivation in osteoblasts and osteocytes causes an increase in osteoclast number and activity [21, 22]. Conversely, *Notch1* and *Notch2* inactivation in osteoblast precursors did not cause an increase in bone resorption. It is possible that when *Notch* is inactivated during the early stages of osteoblast differentiation, the effects on osteoblast number and function prevail over the effects on osteoclast number and bone resorption. These observations provide further support to the notion that the function of Notch signaling in osteoblastic cells is determined by the cellular context [25].

We reported that *Osx-Cre^{+/-};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* mice exhibit a modest degree of cortical porosity and thinning of cortical bone, an effect that is similar to the one observed following the inactivation of *Notch1* and *Notch2* in osteocytes [22, 25]. Selected cells expressing *Osx* differentiate into osteocytes and it is plausible that osteocytes derived from osteoblast precursors harboring the *Notch1* and *Notch2* deletion are responsible for the cortical bone phenotype observed [8]. In previous work we demonstrated that the effects of Notch induction under the control of the *Osx* promoter on cortical bone microarchitecture are similar to those of *Notch1* and *Notch2* inactivation governed by the same promoter [25]. These observations indicate that basal expression and activity of Notch receptors in osteoblast precursors is required for cortical bone integrity and that either an excess or a deficit of Notch signaling have an impact cortical bone structure.

In conclusion, *Notch1* and *Notch2* expression in osteoblast precursors regulates trabecular bone microarchitecture and osteoblastic differentiation and function.

Acknowledgments

The authors thank Drs. F. Radtke for *Notch1^{loxP/loxP}* and T. Gridley for *Notch2^{loxP/loxP}* mice. The authors thank A. Kent and L. Kranz for technical support and M. Yurczak for secretarial assistance.

This work was supported by grant AR063049 from the National Institute of Arthritis and Musculoskeletal and Skin Disorders (E.C.). Sponsors of this study had no role in the study design, collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

The abbreviations used are

Fabp	fatty acid-binding protein
Hes	Hairy Enhancer of Split
Hey	Hes-related with YRPW motif
HeyL	Hey-like
kb	kilobase
μCT	microcomputed tomography
Osx	osterix
Rank	receptor activator of NF-kappa-B
Rankl	Rank ligand
SEM	standard error of the mean
SMI	structure model index

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Highlights

- Inactivation of *Notch1* and *Notch2* in osteoblast precursors increases cancellous bone volume
- *Notch1* and *Notch2* mediate the effects of Notch signaling in osteoblastic cells
- Inactivation of *Notch1* and *Notch2* in osteoblast precursors increases porosity and reduces thickness of cortical bone

Figure 1. *Notch1* and *Notch2* inactivation in osteoblast precursors

One to 6 month old male or female *Osx-Cre*^{+/-};*Notch1* Δ/Δ ;*Notch2* Δ/Δ mice (*Notch1/2* Null, empty circles) or littermate *Notch1*^{loxP/loxP};*Notch2*^{loxP/loxP} controls of the same sex (Control, full circles), were studied. In panel A, the presence of the *Notch1* Δ and *Notch2* Δ alleles was determined by PCR in DNA extracted from parietal bones of 1 month old mice. Control reactions conducted in DNA from *Notch1*^{loxP/loxP};*Notch2*^{loxP/loxP} mice, or in water, were performed to confirm specificity of the primer pairs. Amplified DNA was separated by electrophoresis, and results from representative mice are shown. In panel B, body weight was determined at sacrifice (left) and length of dissected femurs was measured in radiographic images obtained by μ CT (right). Values are means $\pm$ SEM, n = 5-10. * Significantly different between *Notch1/2* Null and Control, $p < 0.05$.

Figure 2. *Notch1* and *Notch2* inactivation in osteoblast precursors increases cancellous bone volume

μCT images of proximal trabecular bone of femurs from representative 1 to 6 month old male or female *Osx-Cre*^{+/-};*Notch1*^{Δ/Δ};*Notch2*^{Δ/Δ} mice (*Notch1/2* Null) or littermate *Notch1*^{loxP/loxP};*Notch2*^{loxP/loxP} controls of the same sex (Control).

Figure 3. *Notch1* and *Notch2* inactivation in osteoblast precursors reduces cortical thickness and increases cortical porosity

μCT images of cortical bone at the femoral midshaft from representative 1 to 6 month old male or female *Osx-Cre*^{+/-}; *Notch1*^{Δ/Δ}; *Notch2*^{Δ/Δ} mice (*Notch1/2* Null) or *Notch1*^{loxP/loxP}; *Notch2*^{loxP/loxP} littermate controls of the same sex (Control).

Table 1

Primers used for allele identification by PCR.

Genotyping			
Allele	Strand	Sequence 5'- to -3'	Amplicon Size (base pairs)
<i>Fabp1</i>	Forward	TGGACAGGACTGGACCTCTGCTTTCC	200
	Reverse	TAGAGCTTTGCCACATCACAGGTCAT	
<i>Notch1^{loxP}</i>	Forward	CTGACTTAGTAGGGGGAAAAC	<i>Notch1^{wt}</i> = 300 <i>Notch1^{loxP}</i> = 350
	Reverse	AGTGGTCCAGGGTGTGAGTGT	
<i>Notch2^{loxP}</i>	Forward	GCTCAGCTAGAGTGTTGTTCTTG	<i>Notch2^{wt}</i> = 400 <i>Notch2^{loxP}</i> = 500
	Reverse	TTTGTGGCCGTAACTTTCTCATG	
<i>Osx-Cre</i>	Forward	GCGGTCTGGCAGTAAAACTATC	100 <i>Notch2^{loxP}</i> = 500
	Reverse	GTGAAACAGCATTGCTGTCACTT	
Recombination			
<i>Notch1^Δ</i>	Forward	CTGACTTAGTAGGGGGAAAAC	370
	Reverse	TAAAAAGAGACAGCTGCGGAG	
<i>Notch2^Δ</i>	Forward	GCTCAGCTAGAGTGTTGTTCTTG	450
	Reverse	ATAACGCTAAACGTGCACTGGAG	

Table 2

Body weight, femoral length and μ CT analysis of femoral microarchitecture of 1 month old *Osx-Cre^{+/-}* male mice or wild type littermate controls of the same sex.

	Wild type	<i>Osx-Cre^{+/-}</i>
Body weight (g)	16.3 $\pm$ 0.7	14.5 $\pm$ 0.7
Femoral length (mm)	12.3 $\pm$ 0.3	11.7 $\pm$ 0.2
Distal femoral cancellous bone	Wild type	<i>Osx-Cre^{+/-}</i>
Bone Volume/Total Volume (%)	2.8 $\pm$ 0.3	3.4 $\pm$ 0.4
Trabecular Separation (μ m)	290 $\pm$ 8	270 $\pm$ 12
Trabecular Number (mm^{-1})	3.48 $\pm$ 0.10	3.79 $\pm$ 0.17
Trabecular Thickness (μ m)	23 $\pm$ 1	22 $\pm$ 1
Connectivity Density (mm^{-3})	29 $\pm$ 17	80 $\pm$ 22
Structure Model Index (SMI)	3.23 $\pm$ 0.10	3.10 $\pm$ 0.08
Cortical bone at the femoral midshaft	Wild type	<i>Osx-Cre^{+/-}</i>
Bone Volume/Total Volume (%)	89.2 $\pm$ 0.6	89.2 $\pm$ 0.5
Cortical Porosity (%)	10.8 $\pm$ 0.6	10.8 $\pm$ 0.5
Cortical Thickness (mm)	98 $\pm$ 3	93 $\pm$ 3
Total Area (mm^2)	1.61 $\pm$ 0.05	1.42 $\pm$ 0.04*
Bone Area (mm^2)	0.50 $\pm$ 0.02	0.43 $\pm$ 0.02
Periosteal Perimeter (mm)	4.50 $\pm$ 0.07	4.22 $\pm$ 0.06*
Endocortical Perimeter (mm)	3.73 $\pm$ 0.06	3.52 $\pm$ 0.04*
Density of Material (mg HA/ cm^3)	931 $\pm$ 9	918 $\pm$ 11

Values are means $\pm$ SEM; n = 5 - 10.

* Significantly different between wild type and *Osx-Cre^{+/-}*, $p < 0.05$.

Table 3

μ CT and histomorphometry of distal femoral cancellous bone. One, 3 and 6 month old *Osx-Cre^{+/+};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* male mice (*Notch1/2* Null) or littermate *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* controls of the same sex (Control).

Male Mice	1 Month		3 Month		6 Month	
	Control	<i>Notch1/2</i> Null	Control	<i>Notch1/2</i> Null	Control	<i>Notch1/2</i> Null
μ CT						
Bone Volume/Total Volume (%)	4.6 ± 0.7	4.4 ± 0.5	4.6 ± 0.8	6.7 ± 0.5*	5.5 ± 0.8	7.0 ± 0.5
Trabecular Separation (μ m)	236 ± 12	239 ± 9	262 ± 6	190 ± 2*	266 ± 9	235 ± 13
Trabecular Number (mm^{-1})	4.31 ± 0.22	4.26 ± 0.17	3.85 ± 0.87	5.25 ± 0.54*	3.75 ± 0.11	4.32 ± 0.21*
Trabecular Thickness (μ m)	23.9 ± 0.8	25.1 ± 1.4	32.4 ± 1.5	28.4 ± 1.0*	33.3 ± 1.4	30.3 ± 2.9
Connectivity Density (mm^{-3})	137 ± 53	147 ± 26	86 ± 26	209 ± 18*	90 ± 15	231 ± 30*
Structure Model Index (SMI)	3.06 ± 0.10	3.08 ± 0.07	2.86 ± 0.13	2.64 ± 0.06	2.52 ± 0.15	2.17 ± 0.10 ⁺
Histomorphometry						
Osteoblast Surface/Bone Surface (%)	19.7 ± 2.1	30.9 ± 1.5*	19.3 ± 1.9	23.7 ± 1.7	19.1 ± 1.9	17.8 ± 2.3
Osteoblasts/Bone Perimeter (mm^{-1})	16.7 ± 1.7	24.4 ± 0.9*	16.6 ± 1.7	19.7 ± 1.3	18.1 ± 1.7	16.2 ± 1.8
Osteoid Surface/Bone Surface (%)	3.0 ± 0.5	5.1 ± 0.6*	3.8 ± 0.7	3.3 ± 0.3	2.5 ± 0.6	2.6 ± 0.7
Osteoclast Surface/Bone Surface (%)	16.9 ± 1.0	13.9 ± 0.9*	13.5 ± 0.8	15.8 ± 1.0	11.0 ± 1.1	11.0 ± 0.5
Osteoclasts/Bone Perimeter (mm^{-1})	8.9 ± 0.5	7.3 ± 0.5*	5.8 ± 0.4	6.8 ± 0.5	5.6 ± 0.8	5.3 ± 0.3
Eroded Surface/Bone Surface (%)	21.5 ± 1.2	18.2 ± 0.8*	13.6 ± 0.4	15.2 ± 0.9	12.0 ± 2.0	11.5 ± 0.8
Mineral Apposition Rate ($\mu\text{m day}^{-1}$)	2.68 ± 0.10	2.24 ± 0.06*	1.85 ± 0.16	1.58 ± 0.08	1.32 ± 0.16	1.52 ± 0.12
Mineralizing Surface/Bone Surface (%)	8.6 ± 1.9	10.3 ± 1.2	6.8 ± 1.3	6.2 ± 0.9	2.9 ± 0.7	4.9 ± 0.8
Bone Formation Rate ($\mu\text{m}^3 \mu\text{m}^{-2} \text{day}^{-1}$)	0.23 ± 0.05	0.23 ± 0.03	0.13 ± 0.03	0.10 ± 0.02	0.04 ± 0.01	0.08 ± 0.01

Values are means ± SEM; n = 4 - 10.

* Significantly different between *Notch1/2* Null and Control, $p < 0.05$.

Table 4

μ CT and histomorphometry of distal femoral cancellous bone. One, 3 and 6 month old *Osx-Cre^{+/+};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* female mice (*Notch1/2* Null) or littermate *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* controls of the same sex (Control).

Female Mice	1 Month		3 Month		6 Month	
	Control	<i>Notch1/2</i> Null	Control	<i>Notch1/2</i> Null	Control	<i>Notch1/2</i> Null
μCT						
Bone Volume/Total Volume (%)	3.4 ± 0.3	3.2 ± 0.2	1.8 ± 0.2	4.5 ± 0.4*	2.0 ± 0.3	5.5 ± 0.8*
Trabecular Separation (μ m)	289 ± 11	266 ± 10	379 ± 23	242 ± 15*	411 ± 21	291 ± 14*
Trabecular Number (mm^{-1})	3.52 ± 0.13	3.80 ± 0.14	2.70 ± 0.17	4.20 ± 0.21*	2.46 ± 0.12	3.48 ± 0.17*
Trabecular Thickness (μ m)	23.8 ± 0.7	22.2 ± 0.3	33.8 ± 0.7	29.0 ± 1.5*	36.7 ± 0.2	29.0 ± 1.0*
Connectivity Density (mm^{-3})	66 ± 18	61 ± 6	21 ± 3	94 ± 13*	20 ± 8	175 ± 39*
Structure Model Index (SMI)	3.00 ± 0.07	3.19 ± 0.13*	3.56 ± 0.05	2.79 ± 0.06*	3.30 ± 0.16	2.06 ± 0.17*
Histomorphometry						
Osteoblast Surface/Bone Surface (%)	27.3 ± 1.7	30.9 ± 1.8	33.7 ± 3.6	29.2 ± 3.6	22.5 ± 1.4	18.3 ± 2.4
Osteoblasts/Bone Perimeter (mm^{-1})	22.4 ± 1.3	25.8 ± 1.8	25.4 ± 2.6	23.6 ± 3.3	25.7 ± 1.5	20.7 ± 2.6
Osteoid Surface/Bone Surface (%)	5.7 ± 0.6	4.2 ± 0.7	4.5 ± 2.3	2.5 ± 0.7	5.0 ± 1.4	4.6 ± 1.0
Osteoclast Surface/Bone Surface (%)	16.5 ± 0.8	17.0 ± 1.1	13.8 ± 1.1	14.5 ± 0.7	11.6 ± 0.8	8.3 ± 1.0*
Osteoclasts/Bone Perimeter (mm^{-1})	8.2 ± 0.4	8.5 ± 0.5	6.0 ± 0.5	6.4 ± 0.3	7.9 ± 0.5	5.4 ± 0.6*
Eroded Surface/Bone Surface (%)	21.8 ± 1.0	21.2 ± 1.3	14.1 ± 1.2	14.8 ± 0.8	18.5 ± 1.2	14.5 ± 1.6
Mineral Apposition Rate ($\mu\text{m day}^{-1}$)	2.42 ± 0.11	2.17 ± 0.09	2.41 ± 0.39	2.35 ± 0.12	1.57 ± 0.08	1.43 ± 0.11
Mineralizing Surface/Bone Surface (%)	8.6 ± 0.8	6.8 ± 0.5	3.2 ± 0.9	5.6 ± 0.9*	23.5 ± 3.0	13.9 ± 1.0*
Bone Formation Rate ($\mu\text{m}^3 \mu\text{m}^{-2} \text{day}^{-1}$)	0.21 ± 0.03	0.15 ± 0.01	0.08 ± 0.04	0.13 ± 0.02	0.37 ± 0.06	0.20 ± 0.03*

Values are means ± SEM; n = 4 - 8.

* Significantly different between *Notch1/2* Null and Control, $p < 0.05$.

Table 5

μ CT of cortical bone at the femoral midshaft. One, 3 and 6 month old *Osx-Cre^{+/-};Notch1^{Δ/Δ};Notch2^{Δ/Δ}* mice (*Notch1/2* Null) or littermate *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* controls of the same sex (Control).

	1 Month		3 Month		6 Month	
	Control	<i>Notch1/2</i> Null	Control	<i>Notch1/2</i> Null	Control	<i>Notch1/2</i> Null
Male Mice						
Bone Volume/Total Volume (%)	89.3 ± 0.4	88.0 ± 0.5	93.0 ± 0.3	92.1 ± 0.3 *	93.1 ± 0.4	91.3 ± 0.7
Cortical Porosity (%)	10.7 ± 0.4	12.0 ± 0.5	7.0 ± 0.3	7.9 ± 0.3 *	6.9 ± 0.4	8.8 ± 0.7
Cortical Thickness (mm)	106 ± 2	99 ± 2 *	167 ± 7	154 ± 4	174 ± 6	154 ± 11
Total Area (mm ²)	1.53 ± 0.02	1.49 ± 0.06	1.77 ± 0.14	1.96 ± 0.07	1.75 ± 0.06	2.00 ± 0.11
Bone Area (mm ²)	0.52 ± 0.01	0.50 ± 0.02	0.80 ± 0.05	0.86 ± 0.04	0.82 ± 0.03	0.95 ± 0.05 *
Periosteal Perimeter (mm)	4.39 ± 0.03	4.33 ± 0.08	4.70 ± 0.18	4.95 ± 0.09	4.68 ± 0.08	5.01 ± 0.13
Endocortical Perimeter (mm)	3.57 ± 0.03	3.53 ± 0.07	3.48 ± 0.16	3.71 ± 0.07	3.41 ± 0.08	3.62 ± 0.15
Density of Material (mg HA/cm ³)	956 ± 6	947 ± 6	1108 ± 8	1086 ± 7	1159 ± 10	1146 ± 14
Female Mice						
Bone Volume/Total Volume (%)	87.9 ± 0.2	86.9 ± 0.4	92.8 ± 0.1	91.8 ± 0.3 *	93.8 ± 0.2	92.8 ± 0.1 *
Cortical Porosity (%)	12.1 ± 0.2	13.1 ± 0.4	7.2 ± 0.1	8.2 ± 0.3 *	6.2 ± 0.2	7.2 ± 0.1 *
Cortical Thickness (mm)	97 ± 2	92 ± 4	160 ± 3	146 ± 4 *	183 ± 7	168 ± 2
Total Area (mm ²)	1.42 ± 0.04	1.53 ± 0.06 *	1.50 ± 0.06	1.61 ± 0.06	1.42 ± 0.06	1.66 ± 0.05 *
Bone Area (mm ²)	0.47 ± 0.01	0.51 ± 0.02	0.67 ± 0.02	0.69 ± 0.02	0.73 ± 0.03	0.83 ± 0.04
Periosteal Perimeter (mm)	4.22 ± 0.05	4.37 ± 0.08	4.34 ± 0.08	4.49 ± 0.08	4.21 ± 0.08	4.56 ± 0.07 *
Endocortical Perimeter (mm)	3.45 ± 0.05	3.56 ± 0.08	3.19 ± 0.07	3.38 ± 0.08	2.93 ± 0.07	3.24 ± 0.04 *
Density of Material (mg HA/cm ³)	934 ± 5	927 ± 7	1122 ± 9	1097 ± 14 *	1205 ± 8	1168 ± 6 *

Values are means ± SEM; n = 5 - 10.

* Significantly different between *Notch1/2* Null and Control, $p < 0.05$.