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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*$^{\Delta/\Delta}$;*Notch2*$^{\Delta/\Delta}$ 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*$^{\Delta/\Delta}$;*Notch2*$^{\Delta/\Delta}$ mice than in control littermates and the effect was more pronounced in female mice. One month old *Osx-Cre*$^{+/-}$;*Notch1*$^{\Delta/\Delta}$;*Notch2*$^{\Delta/\Delta}$ male mice transiently exhibited an increase in osteoblast number and a modest suppression in bone resorption. *Osx-Cre*$^{+/-}$;*Notch1*$^{\Delta/\Delta}$;*Notch2*$^{\Delta/\Delta}$ 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*$^{\Delta/\Delta}$;*Notch2*$^{\Delta/\Delta}$ 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.

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 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-κb 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

Abbreviations: 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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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 the 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 (μCT) and histomorphometric analysis of the femur.

Experimental procedures

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 (kb) 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 [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 $Osx\text{-}Cre^{+/-};Notch1^{loxP/wt};Notch2^{loxP/wt}$ mice, which were bred with $Notch1^{loxP/loxP};Notch2^{loxP/loxP}$ to obtain $Osx\text{-}Cre^{+/-};Notch1^{loxP/loxP};Notch2^{loxP/loxP}$ 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 ($Osx\text{-}Cre^{+/-};Notch1^{\Delta/\Delta};Notch2^{\Delta/\Delta}$), and littermate controls ($Notch1^{loxP/loxP};Notch2^{loxP/loxP}$), not carrying the *Osx-Cre* transgene. In parallel studies, we compared $Osx\text{-}Cre^{+/-}$ 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 the Saint Francis Hospital and Medical Center.

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 (*Fabp*)1 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 μCT 40 scanner (Scanco Medical AG, Bassersdorf, Switzerland) were used to measure femoral length.

Microcomputed tomography

Femurs were scanned in 70% ethanol at an energy level of 55 kVp, an intensity of 145 μA, and an integration time of 200 ms on a μCT 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 μm acquired at an isotropic voxel size of 216 μm^3, 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 middiaphysis 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].

Table 1
Primers used for allele identification by PCR.

Allele	Strand	Sequence 5′- to -3′	Amplicon size (base pairs)
Genotyping			
Fabp1	Forward	TGGACAGGACTGGACCTCTGCTTTCC	200
	Reverse	TAGAGCTTTGCCACATCACAGGTCAT	
$Notch1^{loxP}$	Forward	CTGACTTAGTAGGGGGAAAAC	$Notch1^{wt} = 300$
	Reverse	AGTGGTCCAGGGTGTGAGTGT	$Notch1^{loxP} = 350$
$Notch2^{loxP}$	Forward	GCTCAGCTAGAGTGTTGTTCTTG	$Notch2^{wt} = 400$
	Reverse	TTTGTGGCCGTAACTTTCTCATG	$Notch2^{loxP} = 500$
Osx-Cre	Forward	GCGGTCTGGCAGTAAAAACTATC	100
	Reverse	GTGAAACAGCATTGCTGTCACTT	
Recombination			
$Notch1^{\Delta}$	Forward	CTGACTTAGTAGGGGGAAAAC	370
	Reverse	TAAAAAGAGACAGCTGCGGAG	
$Notch2^{\Delta}$	Forward	GCTCAGCTAGAGTGTTGTTCTTG	450
	Reverse	ATAACGCTAAACGTGCACTGGAG	