Published in final edited form as:

Nat Med. 2008 March ; 14(3): 299–305. doi:10.1038/nm1712.

DIMORPHIC EFFECTS OF NOTCH SIGNALING IN BONE HOMEOSTASIS

Feyza Engin¹, Zhenqiang Yao², Tao Yang¹, Guang Zhou¹, Terry Bertin¹, Ming Ming Jiang^{1,3}, Yuqing Chen^{1,3}, Lisa Wang³, Hui Zheng¹, Richard E. Sutton⁵, Brendan F. Boyce², and Brendan Lee^{1,2}

¹ Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Tx

² Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, Rochester, NY

³ Howard Hughes Medical Institute, Houston, Tx

⁴ Department of Pediatrics, Baylor College of Medicine, Houston, Tx

⁵ Department of Molecular Virology and Microbiology, Baylor College of Medicine, Tx

Abstract

Notch signaling is a central mechanism for controlling embryogenesis. However, its *in vivo* function during mesenchymal cell differentiation, and specifically, in bone homeostasis remains largely unknown. Here, we show that osteoblast-specific gain of Notch function causes severe osteosclerosis due to increased proliferation of immature osteoblasts. Under these pathological conditions, Notch stimulates early osteoblastic proliferation by up-regulating *Cyclin D*, *Cyclin E*, and *Osterix*. Notch also regulates terminal osteoblastic differentiation by directly binding *Runx2* and repressing its transactivation function. In contrast, loss of all physiologic Notch signaling in osteoblasts, generated by deletion of *Presenilin 1* and 2 in bone, is associated with late onset, age-related osteoporosis resulting from increased osteoblast-dependent osteoclastic activity due to decreased production of Osteoprotegerin. Together, these findings highlight the potential dimorphic effects of Notch signaling in bone homeostasis and may provide direction for novel therapeutic applications.

Evolutionarily conserved Notch signaling plays a critical role in cell fate determination, and various developmental processes by translating cell-cell interactions into specific transcriptional programs^{1, 2}. Temporal and spatial modulation of this pathway can significantly affect proliferation, differentiation and apoptotic events³. Moreover, the timing of Notch signaling can lead to diverse effects within the same cell lineage^{4, 5}. In mammals, activation of up to four Notch receptors by membrane-bound ligands initiates a process leading to presenilin-mediated cleavage and release of the Notch intracellular domain (NICD) from the membrane that then traffics to the nucleus. NICD subsequently regulates the expression of genes in cooperation with the transcription factor RBP-Jκ and Mastermind-like proteins.

The observation that mutations in the Notch ligand Delta homologue-3 (Dll-3) and γ-secretase Presenilin1 both cause axial skeletal phenotypes originally linked Notch signaling with skeletal development^{6, 7}. Recently, several *in vitro* studies with conflicting results implicated the Notch pathway in the regulation of osteoblast differentiation, but the *in vivo* role of Notch signaling in bone homeostasis still remains unknown^{8–12}.

In this study, we investigate the tissue, cellular, and molecular consequences of both gain and loss of function of Notch signaling in committed osteoblasts.

RESULTS

Gain of function of Notch signaling results in severe osteosclerosis

To determine the pathological consequences of *in vivo* gain of Notch function during bone formation and homeostasis, we generated transgenic mice expressing the Notch1 intracellular domain (N1ICD) under the control of the type I collagen (*Col1a1*) promoter (Suppl. Fig. 1a,b). Here, gain of Notch function would occur in committed osteoblastic cells since this marker is both an early and late marker of the osteoblastic lineage. Interestingly, founder mice expressing high levels of the transgene, were small at birth and showed progressive growth retardation. Analysis of three established lines showed increased bone mass on radiographs at 4 weeks and a thickened, osteosclerotic appearance following skeletal preparations (Fig. 1a and Suppl. Fig. 1c). Histologically, marrow spaces in 4 week-old transgenic mice were largely filled with trabecular bone composed predominantly of immature woven rather than lamellar bone and surrounded by fibrotic marrow containing cells with morphologic features of early osteoblastic precursors, suggesting increased proliferation of these cells (Fig. 1b). The cortices of the bones were also composed of woven bone, and this phenotype was also present in 11 week-old mice. Toluidine blue staining of 11 week-old transgenic mice indicated increased number of osteoblasts (Fig. 1c). Quantitative histomorphometry of an established mouse line confirmed the significant increase in trabecular bone volume and osteoblast surface, consistent with the high bone mass being due to increased osteoblastic activity (Fig. 1d). This increased osteoblastic activity led to increased production of osteoid (Fig. 1d) and bone formation (Suppl. Fig. 1d).

Since bone formation and resorption are coupled *in vivo*, we analyzed the status of osteoclasts by staining for tartrate-resistant acid phosphatase (TRAP) activity in osteoclasts from bone sections of 4 week-old mice. Although total TRAP staining was qualitatively increased in the limb sections of transgenic mice (Suppl. Fig. 1e), consistent with increased bone mass and remodeling, the osteoclastic parameters normalized to bone surface, i.e., osteoclast number per millimeter of bone surface and osteoclast surface, were significantly decreased in trabecular bone of transgenic mice (Suppl. Fig. 1e). Together, these data support that gain of Notch function in committed osteoblastic lineage cells stimulates the proliferation of early osteoblastic precursors that differentiate into immature osteoblasts producing increased amounts of immature woven bone. While osteoclastic activity was secondarily stimulated by this massive osteoblastic proliferation, bone formation much greatly outweighed bone resorption leading to an osteosclerotic phenotype.

To determine the underlying cellular and molecular mechanism for the increase in early osteoblastic precursors in transgenic bone, we cultured P6 calvarial osteoblasts and found significantly increased numbers of BrdU-positive cells, consistent with increased cellular proliferation (Fig. 1e). Quantitative real time RT-PCR (Q-RT-PCR) of 1 month-old calvarial RNA showed an increased abundance of early osteoblastic differentiation markers, including *Osterix (Osx)*, *Alkaline Phosphatase (Alp)* and *Bone Sialoprotein (Bsp)*. In contrast, later markers of osteoblast differentiation, including osteocalcin were down-regulated (Fig. 1f). To exclude that the increased bone mass was due to decreased osteoclastic activity, we assessed the expression of markers that regulate macrophage differentiation along the osteoclastic lineage in the forelimbs of 4 weeks-old transgenic mice. RANK Ligand (*Rankl*), Osteoprotegerin (*Opg*) and Macrophage Colony Stimulation Factor (*M-CSf*) were all highly expressed suggesting that the hyper-proliferation of the early osteoblastic pool was associated with increased production of both pro- (*Rankl* and *M-CSF*) and anti-osteoclastic (*Opg*) differentiation factors by osteoblasts (Fig. 1g). Hence, on both histological and molecular

levels, gain of Notch signaling in committed osteoblastic precursors resulted in their proliferation as well as secondary stimulation of differentiation of the monocytic lineage of osteoclastic precursors, but far fewer than the magnitude osteoblast proliferation. Thus, the net effect is an increase of immature woven bone formation resulting in severe clinical osteosclerosis.

Notch signaling regulates key osteoblast transcription factors and cell cycle proteins

How Notch signaling regulates these processes on a biochemical level is unknown. Osteoblast differentiation from mesenchymal stem cells and subsequent maturation steps require the function of the runt domain transcription factor *Runx2* and the zinc finger transcription factor *Osterix*. *Runx2* is required for commitment of mesenchymal osteochondroprogenitors to the osteoblastic lineage, differentiation into mature osteoblasts, and terminal differentiation into osteocytes. In contrast, *Osx* is important in expansion of the early osteoblastic pool¹⁹. While *Bsp* and *Alp* are markers of early osteoblasts, Osteocalcin is a marker of later, mature osteoblasts. To determine the mechanistic basis of Notch action in this context, we tested the effects of Notch expression on these key transcriptional regulators of osteoblast differentiation and maturation. Notch1 ICD alone was able to directly bind Runx2 and repress its transactivation of a reporter Osteocalcin enhancer *in vitro* (in Cos7 and in rat osteosarcoma Ros17/2.8 cells) (Fig. 2a–c, Suppl. Fig. 1f). Electrophoretic mobility shift assays (EMSA) showed that NICD could inhibit RUNX2 binding to a target cis element in the Type X collagen promoter (Suppl. Fig. 1g). Interestingly, there was significant down-regulation of Runx2 protein in P2 calvaria of transgenic mice (Fig. 2d). Hence, the down-regulation of Osteocalcin and the delay in late osteoblast differentiation *in vivo* is likely due in part, to direct repression of Runx2 by Notch at the protein level. At the same time, we observed up-regulation of *Osx* expression in the P2 calvaria of transgenic mice. Moreover, Notch1 ICD activated the *Osx* promoter in transient transfection studies in C2C12 cells that were induced to differentiate into osteoblasts with BMP2 treatment (Fig. 2e). These data suggest that Notch can induce proliferation of committed osteoblast precursors by directly up-regulating transcription of *Osx*, while it inhibits their maturation by repressing the function of Runx2.

To further understand the biochemical basis of Notch on osteoblastic proliferation, we analyzed the expression of cell cycle markers and detected increased RNA expression of *Cyclin D* and *Cyclin A* by Q-RT-PCR in osteoblasts over-expressing *Notch1 ICD* (Fig. 2f). This correlated with increased Cyclin D and Cyclin E expression at the protein levels (Fig. 2g). We did not, however, observe significant differences in the levels of two other important cell cycle regulators implicated in bone homeostasis, p53, and Rb. Interestingly, it has been shown with *in vitro* and *ex vivo* studies that Runx2 can suppress osteoblast proliferation, and promote osteoblast maturation by supporting exit from the cell cycle^{20, 21}. Moreover, CyclinD1-Cdk4 can induce Runx2 ubiquitination and degradation, thus Runx2 activity can be regulated by the cell cycle machinery²². Hence, gain of Notch can inhibit osteoblast maturation by direct repression of Runx2 function as well as by repressing Runx2's anti-proliferative effects via *Cyclin D1* up-regulation.

Loss of function of Notch signaling leads to age-related osteoporosis by inducing the osteoclastogenic pool

To determine if the pathological effects of gain of Notch signaling reflect a physiological function during bone homeostasis, we generated a tissue-specific model of loss of Notch signaling in osteoblasts. Because all Notch receptors are expressed in osteoblasts (data not shown), we abolished Notch signaling by generating null mice for both *Presenilin1* (*Ps1*) and *Presenilin 2* (*Ps2*). Since *Ps2* null mice are viable and fertile, we generated double homozygotes for the *Ps2* null allele and the *Ps1* floxed allele, but heterozygous for the type I collagen Cre recombinase transgene (*Ps1*^{f/f}; *Ps2*^{-/-}; *Col1a1*^{Cre/+} or DKO). DKO mice were compared to

their *Ps1*^{fl/fl}; *Ps2*^{-/-} littermates as controls and efficient deletion of the *Ps1*^{fl/fl} allele (approximately 92%) was confirmed by RT-PCR for Presenilin RNA expression and genomic PCR for DNA recombination in calvarial osteoblasts and tail DNA, respectively (Suppl. Fig. 2a,b). Moreover, we confirmed that this led to decreased Notch1 ICD processing on western analysis (Suppl. Fig. 2c). Histomorphometric analyses of 6 month-old, but not 3 month-old, DKO mice showed they were osteoporotic, a decreased tissue bone mass phenotype opposite that of the osteosclerotic tissue phenotype in gain of Notch function transgenic mice (Fig. 3a–c). Bone formation rates (BFR), osteoblast surfaces (Ob.S/BS), and mineralized surfaces (MS/BS) in vertebrae and long bones in the DKO mice were similar to those in control mice (Suppl. Fig. 3). However, osteoclast numbers, osteoclast surfaces, and eroded surfaces were increased in DKO vertebrae and long bones at only 6 months but not at 3 months (Fig. 3d,e; Suppl. Fig. 3). These findings suggest that loss of *Ps1* and *Ps2*, and hence, all Notch signaling in osteoblasts, led to osteoporosis through activation of osteoclastogenesis, resulting in increased bone resorption over bone formation with age-related penetrance.

Activated osteoblasts support osteoclast formation and differentiation from osteoclast precursors (OCPs) by expressing M-CSF and RANKL, but they also inhibit this process through Osteoprotegerin (*Opg*), which binds to and inactivates RANKL. To further examine the effects of loss of *Ps1/Ps2* in osteoblasts on osteoclastogenesis, we performed osteoblast/osteoclastic precursor (OCP) co-culture studies. In this *ex vivo* assay, P7 DKO calvarial osteoblasts stimulated formation of more osteoclasts from wild type spleen-derived OCPs than did wild type osteoblasts, suggesting that *Ps1/Ps2* deletions can affect osteoclastogenesis in a non-cell-autonomous fashion (Fig. 4a). To determine whether this effect was specific for Notch signaling, we tested whether heterologous expression of Notch1 ICD after lentiviral transduction of DKO osteoblasts could suppress osteoclastogenesis in co-cultures studies (Fig. 4b, Suppl. Fig. 4a). Compared to control vector expressing EGFP, Notch1 ICD lentiviral transduction of *Ps1/Ps2* mutant osteoblasts was able to suppress osteoclastogenesis suggesting that the DKO phenotype was due primarily to Presenilin activation of Notch signaling.

The *in vivo* relevance of this was confirmed by flow-cytometric marker analysis of bone marrow cells from 3 month-old DKO mice. This showed increased staining of early OCPs in total cells (cFMS⁺) and in more differentiated OCPs (CD11b⁺/Gr-1^{-lo}) compared to controls, indicating an expansion of the OCP pool in DKO mice (Fig. 4c). To determine if this increase of osteoclast differentiation was due to an imbalance of osteoblastic inductive (*Rankl* and *M-csf*) vs. suppressive signals (*Opg*), we analyzed their RNA expression in DKO vs. control bone at P4. We found comparable expression of *Rankl* in DKO mice, but expression of *Opg* was significantly decreased (Fig. 4 d). Similarly, we found decreased *Opg* production cultured DKO vs. WT calvarial osteoblasts (Suppl. Fig 4b). Hence, under physiological conditions, Notch signaling enabled by *Ps1/Ps2* function in osteoblasts represses osteoclast differentiation by regulating *Opg* expression.

Together, these *in vivo* gain and loss of function studies support for the first time a central role of Notch and Presenilin signaling in regulating both osteoclastogenesis and immature osteoblastic proliferation during bone homeostasis (Fig. 5).

Discussion

Until now, few primary signaling mechanisms regulating osteoblast differentiation and function during bone homeostasis have been identified *in vivo* by genetic and biochemical studies. Wnt signaling via LRP5/6 co-receptors and canonical β -catenin activity are required for osteoblast lineage commitment and function^{23–25}. Activation of this pathway leads to high bone mass^{26, 27}. Activating mutations in TGF β in humans is associated with increased bone formation and inhibition of bone resorption²⁸. However, not unexpectedly, apparently

discrepant results *in vivo* have been observed depending on the timing of gain vs. loss of TGF β function. Similarly, Notch signaling likely exhibits temporal and spatial dependence.

In bone, our data suggest that Notch and Presenilin signaling may be important in the physiological regulation of osteoclastogenesis by osteoblasts. Moreover, it raises the question of whether loss of Notch signaling contributes to age-related osteoporosis, since this type of osteoporosis is associated with increased resorption over bone formation as is seen in our DKO model²⁹. We discovered that the one function of Notch in committed osteoblasts is to regulate osteoclastogenesis via regulation of Opg production. The magnitude of Opg dysregulation and the age-related penetrance of the osteoporosis in the loss of function mouse phenotype correlate well with epidemiological data in humans where age-related osteoporosis has been associated with changes in OPG production^{30–33}. Furthermore, the report that heterozygote *Opg* mutant mice exhibit an age-related osteoporotic phenotype suggests that this mechanism is sufficient for disease pathogenesis³⁴. What is unclear is whether Opg dysregulation is due to direct regulation by Notch1 ICD or by its target transcription factors, given the still poorly characterized *Opg* regulatory region. Further studies showing chromatin immunoprecipitation (ChIP) analysis on a well defined functional OPG promoter with Notch1 ICD or with its target genes would help to address this issue. Similarly, our studies do not address the potential role of Notch signaling prior to osteoblastic commitment in the mesenchymal stem cell (**Fig. 6**). Here, *Runx2* plays the central role in osteoblastic commitment. Our data on the Notch-Runx2 interaction suggest that early loss of function of Notch would actually lead to increase commitment to the osteoblastic lineage and perhaps depletion of the mesenchymal stem cell compartment.

In a pathological disease context, our findings show that activation of Notch signaling in the committed osteoblastic lineage leads to expansion of an immature osteoblast pool. The primary mode of action is transcriptional up regulation of the early osteoblast transcription factor *Osterix*, and increase of Cyclin D and E proteins. These data raise the question of the potential contribution of activation of Notch signaling in human diseases related to osteoblastic proliferation such as in bone pathologies like human osteosarcomas. The significant up-regulation of *Cyclin D1* in the transgenic mice correlates with the observation in humans where 10% of osteosarcomas show amplification of the chromosomal region encoding *Cyclin D1*³⁵. While our data suggest that Notch can directly interact with Runx2 to inhibit its binding to target cis elements and its pro-differentiation function, this is not likely the main determinant of the gain of function phenotype in mice.

Finally, our data have important therapeutic implications. There are few anabolic bone agents for the treatment of osteoporosis, with most therapies targeted at inhibition of bone resorption. Up-regulation of Notch signaling may represent a potential approach for increasing bone formation over bone resorption as well as for inhibiting osteoclastogenesis. However, it is clear that temporal effects of Notch on other cellular compartments such as the mesenchymal stem cell pool would have to be considered, i.e., Notch inhibition of Runx2 function could inhibit mesenchymal stem cell commitment to the osteoblastic lineage. Second, in opposing fashion, inhibition of Notch signaling may be a therapeutic option to investigate for the treatment of proliferative disorders of the osteoblast such as in osteosclerotic diseases or bone cancers.

From a mechanistic perspective, the function of Notch signaling in bone constitutes a rare example of a signaling pathway capable of regulating both osteoblastic and osteoclastic lineages but differently when considering gain of function vs. loss of function scenarios. The other *in vivo* example for this is Ephrin B2 signaling where reverse signaling through ephrin B2 ligand expressed by osteoclasts suppress osteoclast precursors, whereas forward signaling through EphB4 receptor expressed by osteoblasts enhances osteoblast formation^{38,39}. Together, our data point to a dimorphic role for Notch signaling in osteoblast biology, i.e., the

stimulation of osteoblastic precursors in a pathological context, and the inhibition of osteoclastogenesis in the physiological regulation of bone mass and homeostasis.

METHODS

Mice

Myc-His tagged Notch1 ICD including amino acid position 1760–2556 (gift of Tom Kadesch) was cloned under the control of 2.3 kb osteoblast specific *Colla1* promoter in a coat color vector containing tyrosinase minigene and the WPRE posttranscriptional sequences⁴⁰. Transgenic founders were generated by pronuclear injections according to standard techniques. All transgenic lines were maintained on a FVB/N background. The transgenic mice were identified at birth by eye pigmentation and confirmed by PCR using primers specific for the WPRE. Previously published *Ps1^{fl/f}* and *Ps2^{-/-}* mice were crossed with *Colla1-Cre* mice (Gerard Karsenty), to generate osteoblast specific *Ps1/Ps2* double knock out (DKO) mice.

Skeletal analyses, histology and bone histomorphometry

We cleared and stained skeletons from 1 month-old mice with alcian blue for cartilage and alizarin red for bone as described⁴¹. Mice were sacrificed, and the whole skeleton was fixed in 10% neutral-buffered formalin for 18 hours. For radiographic analyses, the skeletons were analyzed by contact radiography with a Faxitron X ray cabinet (Faxitron Xray Corp., Wheeling, IL). Paraffin embedded tissues were sectioned at 4–7 μ m thickness, and stained with hematoxylin and eosin. Toluidine blue, Von Kossa and Goldner's stains were performed on 5–7 micron undecalcified lumbar vertebral plastic sections by using standard protocols. All static and dynamic histomorphometry analyses were performed according to standard protocols using the OsteoMeasure histomorphometry system (Osteometrics Inc. GA). Histomorphometric analyses were performed on 4 week-old transgenic mice and 6 month-old knockout mice with n=3 and n=5–7, respectively in each group. Micro-CT scanning of the trabecular bone of the distal femur was analyzed by the micro-CT system (μ CT-40; Scanco Medical, Bassersdorf, Switzerland).

Plasmids

Osterix-luciferase was a gift of Mark S. Nanes. For lentivirus vector production, plasmids pHIV-N1-IRES-eYFP was constructed by inserting a FLAG-tagged version of intracellular activated form of Notch1 (N1), just upstream of the 1.4 kb IRES-eYFP cassette of pHIV-IRES-eYFP⁴². For the primary osteoblasts, lentiviral vectors used were self-inactivating and had the 0.5 kb mouse phosphoglycerate kinase (PGK) promoter inserted upstream of either N1-IRES-eYFP or IRES-eYFP⁴³. VSV G-pseudotyped vector supernatants were produced as previously described⁴⁴. After 72 hours, cell culture supernatants were harvested and clarified. Typical titers after concentration by ultracentrifugation were in excess of 10^8 IU/ml, for the two SIN vectors as assessed on HOS cells by epifluorescence microscopy. Titers of unconcentrated non-SIN vectors were in excess of 10^7 IU/ml.

Co-culture studies

Calvarial osteoblasts from one week-old mice (n=3) were co-cultured with spleen cells at a number of 5×10^3 and 5×10^4 per well respectively in 96-well plates for 7 days in the presence 10^{-8} M of VitD₃. The cells were then stained for TRAP activity, and counted as described previously⁴⁵. For the lentiviral rescue experiment 5×10^3 osteoblasts isolated from calvaria of 10 day-old Ps1/Ps2 DKO mice were cultured in a 96-well plate for 2 days. The cells were then either infected with 5 μ l Notch1 ICD lentivirus or the YFP- lentiviral vector supernatant for 24 hours in 100 μ l α -MEM containing 10 % FBS and 8 μ g polybrene/ml. The infected cells

were then co-cultured with 5×10^4 spleen cells from 10 day-old WT mouse for 7 days in the presence of 10^{-8} M vitamin D₃.

Fluorescence-activated cell sorting (FACS) and cell sorting analyses

After lysis of erythrocytes with ammonium chloride solution, 2×10^6 cells of bone marrow or spleen were incubated 5 minutes with anti-murine CD16/32 to block Fc receptor-mediated antibody binding followed by triple staining of anti-mouse CD11b-APC, and Gr-1-FITC and c-Fms-PE antibodies for 30 minutes. The cells were then subjected to FACS to analyze the CD11b⁺/Gr-1^{-/lo} cells that contain osteoclast precursors and c-Fms⁺ cells in both total gated and CD11b⁺/Gr-1^{-/lo} population.

BrdU Incorporation

Osteoblasts from calvaria of P6 transgenic mice and wild type littermates (n=5 each group) were isolated as previously described⁴¹. 48 hours after the initial culture, cells were re-plated and expanded an additional day. Cells were treated with BrdU labeling reagent according to manufacturer's instructions for 6 hours, washed with PBS, and fixed with 70% ethanol for 25 minutes at 4°C (Zymed). Three to five areas for each genotype (n=3 slides) were counted by two independent observers blinded to genotype. BrdU positive cells over total cells were scored visually and with Automeasure software (Zeiss Axiovision).

Western Blot Analysis

Proteins were extracted from P2 mice by homogenizing the calvaria (n=3 each group) in a buffer containing 5% SDS, and 0.0625 M Tris.HCl. Western blot analyses were performed by using, anti-P53 antibody (gift of Larry Donehower), anti-Runx2 PEBP2αA (M70) polyclonal antibody (Santa Cruz Biotechnology), anti-CyclinD1 antibody H-2953 (Santa Cruz Biotechnology), anti-cyclin E antibody ab-7959 (Abcam). Protein content was normalized with anti-γ-tubulin mouse monoclonal antibody (Sigma).

GST pull down

Glutathione-S-transferase (GST), GST-NICDTAD, and GST-NICDRA (gift of Tom Kadesch) were expressed in the BL21 strain of *Escherichia coli* (Stratagene). GST proteins were induced with 0.2 mM isopropyl-b-d-thiogalactopyranoside (IPTG) (Promega), and the bacteria were allowed to grow an additional 4 to 5 hours. Following induction, cells were lysed by sonication. GST proteins were bound to glutathione resin (Amersham Biosciences, NJ). 625μM Methionine-labeled flag tagged Runx2 proteins were generated by a T7 *in vitro* transcription/translation kit (Novagen) and incubated with GST or GST-NICDTAD, GST-NICDRA immobilized on glutathione-Sepharose beads at 4°C for 2 hours. The beads were then washed five times with TNN buffer containing 1% NP40, boiled in 2×SDS sample loading buffer, and separated by SDS-PAGE. Western blot was performed to detect the Flag- tagged Runx2 protein, by using anti-flag M2 monoclonal antibody (Sigma).

RNA extraction and Q-RT-PCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) from calvaria and forelimbs of P4 and 4 week-old mice (n=5 and n=3 each group, respectively). cDNAs were synthesized from extracted RNA by using Superscript III First Strand RT-PCR kit (Invitrogen). Real-time quantitative PCR amplifications were performed on LightCycler (Roche) and with TaqMan assay (Applied Biosystems probe HS00172878-M1). β-actin and β2-microglobulin genes were used as internal controls for the quantity and quality of the cDNAs in real time PCR assays.

DNA Transfection

Cos7 and Ros17/2.8 cells were transfected with the 6XOSE2-luc reporter gene by using Lipofectamine Plus according to manufacturer's recommendations (Invitrogen). Luciferase and β -galactosidase activities were assayed 48 hrs after transfection. C2C12 cells were transfected with -1269/91 Osx-p-luc (gift of Mark S. Nanes) by using Fugene6 according to manufacturer's instruction (Roche). 24 hrs after the transfection, the cells were induced with 300ng/ml recombinant human BMP-2 (R&D Systems), and cells were harvested and assayed the following day. All transfections were performed in triplicates with pSV2 β gal as an internal control for transfection efficiency.

Statistical Analyses

Data are expressed as mean values $\pm$ standard deviation (SD). Statistical significance was computed using Student's paired *t* test. A *P* value < 0.05 was considered statistically significant.

SUPPLEMENTAL METHODS

Semi Quantitative RT-PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen) from P7 osteoblast cultures. cDNAs were synthesized by using Superscript III First Strand RT-PCR kit (Invitrogen). Primers specific to *Ps1* exon 4 (5'CTTGACAACCCTGAGCCAAT3') and exon7 (5'GAAATCACAGCCAAGATGAGC3') were used to amplify the *Ps1* floxed allele (422bp). The β -actin gene was used as an internal control of the quantity and quality of the cDNAs.

Western Blot Analysis and EMSA

Proteins were extracted from P2 transgenic mice or P4 *Ps1* DKO control mice by homogenizing the calvaria ($n=3$ each group) in a buffer containing 5% SDS, and 0.0625 M Tris-HCl. Western blot analyses were performed by using anti-Notch1 ICD antibody Val 1744 (Cell Signaling). Protein content was normalized with anti- γ -tubulin mouse monoclonal antibody (Sigma). For EMSA, labeling of oligonucleotide probes, incubation of *in vitro* translated proteins, and EMSA were performed as previously described⁴⁰.

Immunoprecipitation

HeLa cells (6×10^6 cells/dish) were plated in 10 cm dishes in DMEM+10%FBS for transient transfection. Forty-eight hours later, cells were harvested and lysed in lysis buffer (20 mM Tris pH 8.0, 200 mM NaCl, 0.5% Triton X-100) supplemented with protease inhibitors. Lysates were subjected to immunoprecipitation with anti-FLAG (Sigma), anti-Myc (Invitrogen), anti-Mouse IgG (Santa Cruz) and protein G agarose at 4°C overnight. Immunoprecipitates were then washed three times in lysis buffer and subjected to SDS-PAGE followed by western blot analysis for anti-FLAG (Sigma) or anti-Myc (Invitrogen) antibodies.

Skeletal Analysis, Histomorphometry, and Calcein labeling

Skeletons from 2-day old mice were prepared as described and stained with alcian blue 8GX for cartilage and alizarin red S for bone⁴¹. Toluidine blue and TRAP staining were performed on 5–7 micron undecalcified lumbar vertebral plastic sections by using standard protocols. All static and dynamic histomorphometric analyses were performed according to standard protocols using the OsteoMeasure histomorphometry system (Osteometrics Inc. GA). Double labeling was performed by intraperitoneal calcein (Sigma) injection twice with an interval of 7 days. Mice were sacrificed 2 days after the last injection. Calcein labeling was assessed in the vertebrae using formalin fixed undecalcified 5–7 micron-thick plastic sections. For ELISA, the co-culture medium was collected at day 2 and day 4 and analyzed according manufacturer's protocol (R&D System).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Melih Acar and Olga Sirin for technical assistance. This work was supported by NIH grants ES11253 (B. Lee), HD22657 (B. Lee), DE016990 (B. Lee), AR43510 (B. Boyce).

References

1. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science* 1999;284:770–6. [PubMed: 10221902]
2. Bray SJ. Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* 2006;7:678–89. [PubMed: 16921404]
3. Weinmaster G. The ins and outs of notch signaling. *Mol Cell Neurosci* 1997;9:91–102. [PubMed: 9245493]
4. Daudet N, Lewis J. Two contrasting roles for Notch activity in chick inner ear development: specification of prosensory patches and lateral inhibition of hair-cell differentiation. *Development* 2005;132:541–51. [PubMed: 15634704]
5. Brennan CA, Moses K. Determination of Drosophila photoreceptors: timing is everything. *Cell Mol Life Sci* 2000;57:195–214. [PubMed: 10766017]
6. Bulman MP, et al. Mutations in the human delta homologue, DLL3, cause axial skeletal defects in spondylocostal dysostosis. *Nat Genet* 2000;24:438–41. [PubMed: 10742114]
7. Shen J, et al. Skeletal and CNS defects in Presenilin-1-deficient mice. *Cell* 1997;89:629–39. [PubMed: 9160754]
8. Deregowski V, Gaggero E, Priest L, Rydziel S, Canalis E. Notch 1 overexpression inhibits osteoblastogenesis by suppressing Wnt/beta-catenin but not bone morphogenetic protein signaling. *J Biol Chem* 2006;281:6203–10. [PubMed: 16407293]
9. Sakamoto K, Chao WS, Katsube K, Yamaguchi A. Distinct roles of EGF repeats for the Notch signaling system. *Exp Cell Res* 2005;302:281–91. [PubMed: 15561108]
10. Sciaudone M, Gaggero E, Priest L, Delany AM, Canalis E. Notch 1 impairs osteoblastic cell differentiation. *Endocrinology* 2003;144:5631–9. [PubMed: 12960086]
11. Tezuka K, et al. Stimulation of osteoblastic cell differentiation by Notch. *J Bone Miner Res* 2002;17:231–9. [PubMed: 11811553]
12. Zamurovic N, Cappellen D, Rohner D, Susa M. Coordinated activation of notch, Wnt, and transforming growth factor-beta signaling pathways in bone morphogenic protein 2-induced osteogenesis. Notch target gene Hey1 inhibits mineralization and Runx2 transcriptional activity. *J Biol Chem* 2004;279:37704–15. [PubMed: 15178686]
13. Miele L, Miao H, Nickoloff BJ. NOTCH signaling as a novel cancer therapeutic target. *Curr Cancer Drug Targets* 2006;6:313–23. [PubMed: 16848722]
14. Miele L, Golde T, Osborne B. Notch signaling in cancer. *Curr Mol Med* 2006;6:905–18. [PubMed: 17168741]
15. Grabher C, von Boehmer H, Look AT. Notch 1 activation in the molecular pathogenesis of T-cell acute lymphoblastic leukaemia. *Nat Rev Cancer* 2006;6:347–59. [PubMed: 16612405]
16. Radtke F, Raj K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nat Rev Cancer* 2003;3:756–67. [PubMed: 14570040]
17. Katoh M, Katoh M. Notch signaling in gastrointestinal tract (review). *Int J Oncol* 2007;30:247–51. [PubMed: 17143535]
18. Leow CC, Polakis P, Gao WQ. A role for Hath1, a bHLH transcription factor, in colon adenocarcinoma. *Ann N Y Acad Sci* 2005;1059:174–83. [PubMed: 16382053]
19. Nakashima K, et al. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 2002;108:17–29. [PubMed: 11792318]

20. Galindo M, et al. The bone-specific expression of Runx2 oscillates during the cell cycle to support a G1-related antiproliferative function in osteoblasts. *J Biol Chem* 2005;280:20274–85. [PubMed: 15781466]
21. Pratap J, et al. Cell growth regulatory role of Runx2 during proliferative expansion of preosteoblasts. *Cancer Res* 2003;63:5357–62. [PubMed: 14500368]
22. Shen R, et al. Cyclin D1-cdk4 induce runx2 ubiquitination and degradation. *J Biol Chem* 2006;281:16347–53. [PubMed: 16613857]
23. Hill TP, Spater D, Taketo MM, Birchmeier W, Hartmann C. Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes. *Dev Cell* 2005;8:727–38. [PubMed: 15866163]
24. Day TF, Guo X, Garrett-Beal L, Yang Y. Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. *Dev Cell* 2005;8:739–50. [PubMed: 15866164]
25. Glass DA 2nd, et al. Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. *Dev Cell* 2005;8:751–64. [PubMed: 15866165]
26. Krishnan V, Bryant HU, Macdougald OA. Regulation of bone mass by Wnt signaling. *J Clin Invest* 2006;116:1202–9. [PubMed: 16670761]
27. Tu X, et al. Noncanonical Wnt signaling through G protein-linked PKCdelta activation promotes bone formation. *Dev Cell* 2007;12:113–27. [PubMed: 17199045]
28. Janssens K, ten Dijke P, Janssens S, Van Hul W. Transforming growth factor-beta1 to the bone. *Endocr Rev* 2005;26:743–74. [PubMed: 15901668]
29. Mezquita-Raya P, et al. The contribution of serum osteoprotegerin to bone mass and vertebral fractures in postmenopausal women. *Osteoporos Int* 2005;16:1368–74. [PubMed: 15711777]
30. Fahrleitner-Pammer A, et al. Osteoprotegerin serum levels in women: correlation with age, bone mass, bone turnover and fracture status. *Wien Klin Wochenschr* 2003;115:291–7. [PubMed: 12793029]
31. Arko B, Prezelj J, Kocijancic A, Komel R, Marc J. Association of the osteoprotegerin gene polymorphisms with bone mineral density in postmenopausal women. *Maturitas* 2005;51:270–9. [PubMed: 15978970]
32. Choi JY, et al. Genetic polymorphisms of OPG, RANK, and ESR1 and bone mineral density in Korean postmenopausal women. *Calcif Tissue Int* 2005;77:152–9. [PubMed: 16151677]
33. Hofbauer LC, et al. Estrogen stimulates gene expression and protein production of osteoprotegerin in human osteoblastic cells. *Endocrinology* 1999;140:4367–70. [PubMed: 10465311]
34. Bucay N, et al. osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev* 1998;12:1260–8. [PubMed: 9573043]
35. Wang LL. Biology of osteogenic sarcoma. *Cancer J* 2005;11:294–305. [PubMed: 16197719]
36. Wei CL, et al. A global map of p53 transcription-factor binding sites in the human genome. *Cell* 2006;124:207–19. [PubMed: 16413492]
37. Okuyama R, et al. p53 homologue, p51/p63, maintains the immaturity of keratinocyte stem cells by inhibiting Notch1 activity. *Oncogene* 2007;26:4478–88. [PubMed: 17237812]
38. Boyce BF, Xing L. Osteoclasts, no longer osteoblast slaves. *Nat Med* 2006;12:1356–8. [PubMed: 17151690]
39. Zhao C, et al. Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis. *Cell Metab* 2006;4:111–21. [PubMed: 16890539]
40. Zhou G, et al. Dominance of SOX9 function over RUNX2 during skeletogenesis. *Proc Natl Acad Sci U S A* 2006;103:19004–9. [PubMed: 17142326]
41. Ducy P, et al. A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. *Genes Dev* 1999;13:1025–36. [PubMed: 10215629]
42. Segall H, Yoo E, Sutton RE. Characterization and detection of artificial replication-competent lentivirus of altered host range. *Molecular Therapy* 2003;8:118–129. [PubMed: 12842435]
43. Dai C, McAninch RE, Sutton RE. Identification of synthetic endothelial cell-specific promoters by use of a high-throughput screen. *J Virol* 2004;78:6209–21. [PubMed: 15163714]

44. Sutton RE, Wu HT, Rigg R, Bohnlein E, Brown PO. Human immunodeficiency virus type 1 vectors efficiently transduce human hematopoietic stem cells. *J Virol* 1998;72:5781–5788. [PubMed: 9621037]
45. Xing L, et al. NF-kappaB p50 and p52 expression is not required for RANK-expressing osteoclast progenitor formation but is essential for RANK- and cytokine-mediated osteoclastogenesis. *J Bone Miner Res* 2002;17:1200–10. [PubMed: 12096833]

d cont.

Figure 1. Gain of Notch function in transgenic mice cause osteosclerosis

a, X-ray and skeletal preparations of 4 week-old transgenic mice show severe osteosclerosis in the skull, ribs, and long bones (white arrows). WT: Wild type mice; Tg: Transgenic mice. **b**, H&E staining of 4 week-old transgenic mice hind limbs show immature woven bone formation with little distinction between cortex and marrow (black arrow). B: Bone; BM: Bone marrow. Scale bar left panel, 500 μ m, and right panel, 50 μ m. **c**, Toluidine blue staining of lumbar sections of 11 week-old mice reveals increased number of osteoblasts (black arrow). Ob: Osteoblasts lining bone. Scale bar left panel, 500 μ m, and right panel, 50 μ m. **d**, Von Kossa staining of lumbar sections of 11 week-old mice and trabecular bone volume density (BV/TV) analyses of 4 week and 11 week old mice (n=4) demonstrate significantly increased mineralization. Scale bar, 500 μ m. Histomorphometric analyses of 4 week-old mice show increased osteoblast surface (Os.S/BS), and osteoid surface (OS/BS) (n=5). Goldner's staining of spinal trabecular bone also showed increased osteoid production in transgenic mice. Boxed area is enlarged in the right panel. Scale bar left panel, 500 μ m, and right panel, 50 μ m. **e**,

Osteoblastic cells from P6 calvaria showed increased proliferation by BrdU staining (n=5). Scale bar left panel, 200 μ m, and right panel, 20 μ m. **f**, Q-RT-PCR was performed by using RNA obtained from 4 week-old mouse calvaria (n=4) to elucidate the expression of osteoblast markers. Col1a1: type I collagen; Osx: Osterix; Alp: Alkaline phosphatase; Bsp: Bone sialoprotein; Oc: Osteocalcin. **g**, RNA obtained from 4 week-old mouse calvaria and forelimbs (n=4) was subjected to Q-RT-PCR to analyze the expression of bone remodeling markers. Rankl: Rank Ligand; Opg: Osteoprotegerin; Rank: RANK; Trap: Tartrate Resistant Acidic Phosphatase; M-Csf: Monocyte-Colony Stimulating Factor. * p<0.05 between WT and Tg.

Figure 2. Notch regulates key osteoblast transcription factors and cell cycle proteins

a, Relative luciferase activity in Cos7 cells transfected with Runx2 and Runx2-dependent osteocalcin enhancer (p6OSE2) luciferase reporter with increasing dosage of Notch1 ICD. Western analyses of cell lysates using respective antibodies on left is shown below. **b**, Transfection of Notch1 ICD inhibits endogenous Runx2 activity in ROS17/2.8 osteosarcoma cells as shown by relative luciferase activity of the Runx2-responsive osteocalcin enhancer (pX6OSE2). Western analyses of cell lysates using respective antibodies on left is shown below. **c**, GST pull down assay with amino-terminal-truncated (GST-NICD Δ ARA) and carboxy-terminal truncated (GST-NICD Δ TAD) Notch fusion proteins and *in vitro* transcribed/translated, 35 S-methionine labeled Runx2 (IVT-Runx2). Strongest binding is noted with the carboxy-terminal portion of Notch. **d**, Decreased Runx2 protein level was detected by western blot analyses and quantified with densitometry on P2 calvarial protein extracts in transgenic vs. wild type mice. **e**, Relative luciferase activity in C2C12 cells transfected with 1360 bp Osterix promoter luciferase reporter gene and Notch1 ICD. **f**, Q-RT-PCR of cell cycle markers on RNA obtained from 4 weeks old mouse calvaria (n=4). **g**, Western Blot analyses of P2 calvarial protein extracts to detect cell cycle markers and quantification by densitometry. * $p < 0.05$ between WT and Tg.

Figure 3. Loss of Notch signaling via Presenilin deletion causes osteoporosis

a, Von Kossa staining of 6 month-old lumbar vertebrae of *Ps1^{fl/f}; Ps2^{-/-}* (Control) vs. of *Ps1^{fl/f}; Ps2^{-/-}; Colla1-Cre* (DKO) mice demonstrated osteoporosis. Scale bar, 500 μ m. **b**, Micro-CT reconstruction of distal femur from 6 month-old DKO mice showing decreased trabecular bone. Scale bar, 1 mm. **c**, Histomorphometry of 3 month and 6 month-old DKO L3–L4 spine showing age-related penetrance of low bone mass phenotype. **d**, TRAP staining of 6 month-old lumbar vertebral sections of DKO and control mice indicated increased osteoclast staining (arrow). Scale bar, 200 μ m. **e**, Histomorphometry of 6 month-old DKO and control mice (n=5 each group) tibia revealed decreased bone volume/tissue volume (BV/TV), osteoclast number per bone volume (N.Oc/BV), osteoclast number per bone surface (N.Oc/BS), osteoclast surface per bone surface (Oc.S/BS), and eroded surface per bone surface (ES/BS) in DKO mice.

Figure 4. Loss of Notch signaling via Presenilin deletion increases osteoclastogenic pool
a, Co-cultures of P7 (n=2) spleen cells (WT or DKO) and osteoblasts (WT or DKO) stained and quantified for TRAP⁺ osteoclasts. **b**, Co-cultures of P7 (n=2) osteoblasts (DKO) transduced with Notch1 ICD lentivirus and WT spleen cells stained and quantified for TRAP⁺ osteoclasts. **c**, Bone marrow cells obtained from 3 months-old (n=3) DKO and WT mice were subjected to FACS to stain the CD11b⁺/Gr-1^{-/-} cells and c-Fms⁺ population specific for osteoclast precursors in both total gated and CD11b⁺/Gr-1^{-/-} gated populations. Representative histograms show total c-Fms⁺ cells, and c-Fms⁺ cells in CD11b⁺/Gr-1^{-/-} population. **d**, Q-RT-PCR for osteoblast markers in RNA obtained from P4 mouse DKO vs. control calvaria (n=5 each group).

Figure 5. Model for Notch's dimorphic effects in bone homeostasis

In established osteoblastic lineages, pathological gain of Notch function activates expansion of the immature osteoblastic pool by increasing transcription of *Osx*, *Cyclin D*, and *Cyclin E* and by repressing the function of *Runx2* by direct interaction and inhibition of its binding. Physiologically, it inhibits osteoclastogenesis by increasing *Opg* production over *Rankl* production.