IBMS BoneKEy. 2011 July;8(7):318-327
http://www.bonekey-ibms.org/cgi/content/full/ibmske;8/7/318
doi: 10.1138/20110520

PERSPECTIVES

Notch Signaling and Bone

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Abstract

The Notch receptors and their ligands, Jagged and Serrate, are transmembrane proteins that play a critical role in cell lineage specification. Notch signaling regulates skeletal development and homeostasis. Upon ligand-binding, activation of Notch canonical signaling requires cleavage of the receptor and release of the Notch intracellular domain (NICD), which translocates to the nucleus. There, it induces transcription after associating with CBF-1/RBP-Jκ, Suppressor of Hairless, Lag-2 (CSL) and Mastermind-like (MAML) proteins. Notch signaling suppresses differentiation of bone marrow mesenchymal cells and of cells committed to the osteoblastic or chondrocytic lineage. Notch induces expression of osteoprotegerin, an inhibitor of osteoclastogenesis, in osteoblastic cells. Misexpression of Hairy Enhancer of Split (Hes)1, a classic target of Notch signaling, does not phenocopy Notch misexpression in the skeleton, suggesting that other Notch target genes mediate Notch skeletal effects. In humans, aberrant Notch signaling is associated with diseases affecting skeletal development, such as Alagille syndrome, spondylocostal dysostosis and brachydactyly. *JAG1* polymorphisms are associated with bone mineral density, and *NOTCH2* mutations with Hajdu-Cheney syndrome. Overexpression of NOTCH1 is associated with osteosarcoma, and Notch is critical for breast cancer skeletal invasiveness. In summary, Notch suppresses the differentiation of skeletal cells and plays a role in the pathogenesis of developmental and postnatal skeletal disorders. *IBMS BoneKEy*. 2011 July;8(7):318-327.

Bone remodeling is the continuous process of skeletal tissue renewal that is carried out by the coordinated activity of osteoblasts, the bone matrix-producing cells, and osteoclasts, the bone matrix-resorbing cells (1). Osteoblasts derive from mesenchymal stem cells, whereas osteoclasts originate from multipotent hematopoietic cells (2). A network of intracellular signals regulates bone remodeling by maintaining a balance between osteoblast and osteoclast number and activity, and Notch signaling has emerged as a critical component of this network.

Notch and Its Ligands

Notch belongs to a family of evolutionary conserved receptors that regulate the lineage specification of mesenchymal and hematopoietic stem cells. There are four receptors, Notch1 to 4, and five classic Delta/Serrate/Lag-2 (DSL) ligands named Jagged (Jag)1 and 2, and Delta-like (Dll) 1, 3 and 4. Additional Notch ligands have been described, but it is not clear whether or not they activate canonical Notch signaling in mammalian cells. Notch and DSL ligands are single-pass transmembrane proteins that mediate interactions between neighboring cells. Following ligand-receptor interactions, Notch undergoes a series of cleavages, which result in the release of the Notch intracellular domain (NICD). Central to the cleavage of Notch is the γ-secretase complex, which contains the proteases *Presenilin1* and *Presenilin2*. Under basal conditions, the DNA-binding protein Epstein-Barr virus latency C promoter binding factor 1 (CBF1), Suppressor of Hairless and Lag1 (CSL), also known as RBP-Jκ in mice, is bound to DNA and interacts with co-

repressors of transcription (3). Following its cleavage, NICD is released and translocated to the nucleus, where it forms a ternary complex with CSL/RBP-Jκ and Mastermind-like (MAML), displacing transcriptional co-repressors and recruiting co-activators of transcription (3). This results in the transcription of Hairy Enhancer of Split (Hes) 1, 5, 6 and 7 and HES-related with YRPW motif (Hey)1, 2 and HeyL (4). Non-canonical Notch signaling results in NICD interactions with cytosolic proteins of the Deltex family and gene regulation in a CSL/RBP-Jκ-independent manner. In skeletal cells, the canonical Notch signaling pathway operates and mediates the known effects of Notch (5). However, undiscovered Notch actions dependent on the non-canonical pathway are possible.

Notch1 and Notch2 have overlapping functions and are required for embryonic survival, since the inactivation of *Notch1* and *Notch2* cause embryonic or perinatal mortality (6;7). Notch3 has a slightly different structural organization than Notch1 and Notch2, and its expression is restricted to osteoclasts and selected cell populations, such as thymocytes and neurons. Notch3 inactivation in mice is not lethal, although its constitutive induction recapitulates the cerebral autosomal-dominant arteriopathy with subcortical infarcts (CADASIL) syndrome in humans (8;9). Notch4 is involved in vascular morphogenesis in the embryo and is dispensable for development, since its functions overlap with those of Notch1 (10). Global inactivation of all classic Notch ligands, except for DII3, causes embryonic lethality, indicating that DSL ligands do not have compensatory functions (11). Mice with *Dll3* null mutations are viable and are characterized by vertebral and rib deformities secondary to developmental defects in the patterning of somites (11).

Hes and Hey are transcription factors regulated by Notch. There are seven Hes family members, Hes1 through 7, and except for Hes2 and Hes3, they are all targets of Notch canonical signaling (4). Hes1, 3 and 5 inhibit the differentiation of precursor cells during development or during the cell renewal process in adult tissues (12;13;14). Hes7 is necessary for proper somite segmentation, and Hes6 is expressed during neural development, where it suppresses Hes1 activity (4). Three Hey proteins, Hey1, Hey2 and HeyL, have been identified and are required for normal vascular and cardiac development and function. The inactivation of *Hey1* and *Hey2* mimics the loss of Notch signaling, suggesting that Hey1 and Hey2 mediate the effects of Notch (15).

Notch and Endochondral Bone Formation

During embryonic life, hyaline cartilage arises from the condensation and chondrogenic differentiation of precursor mesenchymal cells, and forms a template for bone formation by endochondral ossification. In this process, chondrocytes in hyaline cartilage undergo hypertrophy, deposit a mineralized matrix and become apoptotic; subsequently, blood vessels invade the calcified cartilage scaffold and allow skeletal cell precursors to colonize the hyaline cartilage template and replace it with bone (16).

Notch1 transcripts are detected during chondrocyte proliferation *in vitro*, and activation of Notch signaling inhibits chondrogenesis in murine chondrocytic ATDC5 cells and limb micromass cultures (17). Hes1 and Hey1 suppress *Col2a1* transcription, and Hes1 downregulation in mesenchymal progenitor cells increases the expression of chondrocyte gene markers and the secretion of a cartilage matrix (18;19). These observations indicate that Hes1 and Hey1 play a role in the inhibitory effects of Notch on chondrogenesis.

The *Prx1* enhancer, which is active in the limb bud during development from day 10.5 of embryonic life (20), was used to direct Cre recombinase and delete *Presenilin* to prevent Notch activation, or to delete *Notch1* and *Notch2* in conditional null models. Inhibition of Notch signaling by conditional inactivation of *Presenilin1* in the context of *Presenilin2* global inactivation, or by dual conditional deletion of *Notch1* and *Notch2*, caused an accumulation of hypertrophic

chondrocytes, resulting in dramatic malformations of the growth plate and the skeleton (21). The conditional deletion of *Notch2* caused a similar phenotype, suggesting that *Notch2* is the main regulator of endochondral bone formation. Confirming these results, overexpression of NICD in the limb bud impaired endochondral ossification, and this effect was lost by the concurrent deletion of *Csl/Rbpjκ*, indicating that it was secondary to the activation of canonical Notch signaling (19). Transgenic NICD overexpression governed by the *Col2a1* promoter impairs chondrocyte proliferation, confirming an inhibitory role of Notch in endochondral bone formation (22). However, these experiments did not determine which Notch target gene mediates inhibitory effects of Notch on chondrogenesis. Inactivation of *Hes1* and *Hes5* in cartilage does not affect growth plate organization, suggesting that neither *Hes1* nor *Hes5* mediate the effects of Notch in cartilage (23).

Notch and Osteoblast Differentiation

Osteoblasts derive from mesenchymal precursors found in the bone microenvironment during postnatal life (2). The role of Notch in osteoblastogenesis has been examined extensively *in vivo* and *in vitro*, and Notch has been reported to either suppress or induce osteoblastic differentiation *in vitro*, depending on the cell line studied and method used to induce Notch (24).

Studies in mice have established that Notch arrests the initial and terminal phases of osteoblast differentiation. Conditionally-activated NICD in the limb bud induces proliferation and suppresses the differentiation of adult mesenchymal precursor cells. These effects are opposed by the concurrent inactivation of *Csl/Rbpjκ*, indicating that canonical Notch signaling is responsible for the effects of Notch on mesenchymal cell differentiation (19). Accordingly, bone marrow stromal cells from conditional *Notch1;Notch2* or *Csl/Rbpjκ* null mice are depleted from mesenchymal progenitors, demonstrating that canonical Notch signaling maintains a pool of mesenchymal cells in an undifferentiated state (19;21).

Transgenic mice in which NICD is expressed under the control of the 2.3 kilobase (kb) *Col1a1* promoter exhibit an osteofibrotic phenotype associated with an increase in the number of dysfunctional osteoblasts depositing woven bone (25). Conditional deletion of *Csl/Rbpjκ* in the context of conditional NICD induction in osteoblasts reverses the phenotype, indicating that canonical Notch signaling mediates these effects (26). In contrast, expression of NICD controlled by the 3.6 kb *Col1a1* promoter causes osteopenia, secondary to a decrease in osteoblast number (27). The discrepancies between the two phenotypes observed appeared to be secondary to the different timing of activation of the 2.3 kb and 3.6 kb fragments of the *Col1a1* promoter (28). Expression of NICD under the control of the 2.3 kb *Col1a1* promoter is restricted to mature osteoblasts, and permits the early proliferation of cells that fail to mature as functional osteoblasts (25). Accordingly, NICD expression under the control of the 3.6 kb *Col1a1* promoter, which is expressed during the early stages of osteoblastogenesis, does not allow proliferation of undifferentiated precursors, leading to a reduced number of osteoblasts and osteopenia (27). Conditional deletion of *Notch1;Notch2*, or *Csl/Rbpjκ* where Cre recombinase is under the control of the 2.3 kb *Col1a1* promoter does not cause a skeletal phenotype, confirming that canonical Notch signaling suppresses osteoblastogenesis and not the function of mature cells (21;26).

The effects of Notch on cell proliferation can be explained by induction of cyclin D and cyclin E expression, whereas the inhibitory effects on osteoblast differentiation may involve diverse intracellular signals (25). NICD and Hey1 interact with Runt-related transcription factor (Runx)-2, a marker of osteoblast maturation (21). Central to the effects of Notch on osteoblast differentiation is the inhibition of canonical Wnt/β-catenin signaling, which is a critical regulator of osteoblastogenesis (27). Notch inhibits Wnt signaling by enhancing the pool of glycogen

synthase 3β, which phosphorylates β-catenin, favoring its degradation by ubiquitination. Additional mechanisms may operate, such as the induction of nemo-like kinase, which phosphorylates and degrades T cell factor, a nuclear protein that in conjunction with β-catenin induces Wnt target gene expression. Notch also interacts with the Nuclear Factor of Activated T-Cells (NFAT) signaling pathway. The role of NFAT signaling in osteoblastogenesis is controversial, but recently we have shown that NFATc1, like Notch, inhibits osteoblastogenesis (29;30). Canonical Notch signaling inhibits NFAT transactivation by suppressing NFATc1 transcription and by direct interactions between CSL and NFATc1 that lead to a decrease in the binding of NFATc1 to DNA consensus sequences. It is of interest that NFATc1 can interfere with canonical Notch signaling, and the inhibitory effects of Notch and NFATc1 on their reciprocal transactivation might represent a local feedback mechanism to modulate osteoblastogenesis (30).

Transgenic overexpression of Hey1, not selective to the skeleton, causes a mild impairment of osteoblastic function, which is consistent with the effects of Notch signaling in the skeleton (31). Accordingly, mice heterozygous for a *Hey1* null allele in a *Heyl* null background exhibit increased bone mass (32). However, data from models using global misexpression are difficult to interpret since the skeletal phenotype observed could be secondary to systemic non-specific effects. Hes1 interacts with Runx2 to induce osteocalcin and osteopontin promoter activity, indicating distinct functions of Hes1 in the skeleton (33;34;35;36). In accordance with these *in vitro* observations, neither the overexpression of Hes1 in the bone environment nor its conditional inactivation replicate all the characteristics of the skeletal phenotype of mice misexpressing Notch. Transgenic female mice overexpressing Hes1 under the control of the 3.6 kb *Col1a1* promoter are osteopenic due to reduced osteoblast number and increased osteoclast number and bone resorption (37;38). The conditional deletion of *Hes1* in the male limb bud, in a *Hes3;Hes5* null genetic background, causes a developmental osteopenic phenotype, whereas conditional *Hes1* deletion in osteoblastic cells of male mice causes osteopenia due to enhanced osteoclastogenesis.

Notch and Osteoclastogenesis

Osteoclasts are multinucleated cells that form through the aggregation of bone marrow mononuclear cell precursors. Osteoclastogenesis is regulated by the receptor activator of nuclear factor κ-B ligand (RANKL)-osteoprotegerin axis, where RANKL induces osteoclast formation and its activity is opposed by osteoprotegerin, a soluble RANKL decoy receptor. The ratio of RANKL and osteoprotegerin is controlled by bone marrow stromal cells and osteoblasts, and it is critical to the regulation of osteoclastic activity (39).

Inactivation of *Notch1*, *Notch2* and *Notch3* in murine myeloid cells enhances osteoclast precursor proliferation and differentiation *in vitro* (37). Accordingly, exposure of osteoclast precursors to immobilized Dll1, infection with viral vectors expressing NICD, or co-culture of mononuclear precursors with bone marrow stromal cells expressing Jag1, suppress osteoclastogenesis (37;40). Conditional deletion of *Presenilin1* and *Presenilin2* in osteoblasts suppresses osteoprotegerin levels, inducing osteoclastogenesis and causing osteopenia (25). Accordingly, inactivation of *Notch1* in osteoblasts enhances osteoclast differentiation and resorptive activity by suppressing the expression of osteoprotegerin. These observations indicate that Notch inhibits osteoclastogenesis (37). However, Notch2 acts in conjunction with nuclear factor κ-B, possibly by regulating the *Nfatc1* promoter during the terminal phases of osteoclast differentiation, suggesting that under specific conditions Notch2 can induce osteoclastogenesis (41).

As indicated, Hes1 appears to play a distinct function from Notch signaling in osteoclastogenesis. Transgenic male mice

overexpressing Hes1 under the control of the 3.6 kb *Col1a1* promoter are osteopenic due to increased osteoclast number and eroded surface. Conversely, conditional inactivation of *Hes1* where the Cre recombinase is expressed under the control of the *Osteocalcin* promoter, in the context of the global ablation of *Hes3* and *Hes5*, reduces osteoclast number and increases trabecular bone volume in male mice. These observations, coupled to *in vitro* experiments, demonstrate a stimulatory effect of Hes1 on osteoclastogenesis (38).

Notch and Developmental Disorders

Inherited or *de novo* mutations in components of the Notch signaling pathway can lead to developmental skeletal defects (42). Both autosomal dominant and recessive mutations of *DLL3*, leading to the expression of a truncated protein or to amino acid substitutions cause spondylocostal dysostosis, which is characterized by trunk dwarfism secondary to rib anomalies and vertebral segmentation defects (42;43). The pudgy mutation, a natural occurring allele of murine *Dll3*, causes a similar phenotype (11). Mesoderm posterior 2 (MESP2) expression is induced by Notch signaling, and mutations in *MESP2* cause spondylocostal dysostosis type 2, which is manifested by marked segmentation abnormalities of the thoracic vertebrae (42). The interaction between Notch and its ligands is regulated by glycosylation of the extracellular domains of Notch. Fringe proteins mediate Notch glycosylation promoting Notch-Dll1 interactions and inhibiting the binding of Notch to Jag1 (44). *Lunatic Fringe* (*Lfng*) deletions in mice cause rib cage and vertebral abnormalities. Similarly, mutations of *LFNG* in humans are associated with hemivertebrae and rib anomalies typical of spondylocostal dysostosis type 3 (45;46).

Recessive brachydactyly is characterized by developmental abnormalities of hands and feet, micrognatia and short stature. This disease is associated with marked upregulation of JAG1 and Notch signaling caused by loss-of-function mutations of chondroitin sulfate synthase (*CHSY*)*1*, which encodes a transmembrane protein containing a Fringe domain (47).

Alagille syndrome is associated with *JAG1* alleles containing splicing site mutations, missense base substitutions, gene deletions, or premature termination codons leading to truncated proteins; rarely, mutations of *NOTCH2* are associated with Alagille syndrome. This disease is characterized by defective bile duct formation leading to liver failure, vascular abnormalities, and cardiac developmental defects leading to pulmonary artery defects and cardiac valve stenosis. Individuals with Alagille syndrome frequently present with defects of the anterior chamber of the eye and skeletal abnormalities consisting of aberrant vertebral segmentation, which results in butterfly vertebrae or hemivertebrae, and absence of sacrum (43). *Jag1* inactivation in mice is lethal, and *Jag1* heterozygous mice with a *Notch2* hypomorphic allele display growth defects resembling those found in patients with Alagille syndrome (43;48;49).

Notch and Osteoporosis

Individuals with Alagille syndrome can develop osteoporosis possibly related to liver failure and malnutrition (50). In addition, a genome-wide association study in a Chinese population and follow-up replication studies in populations of European and Chinese descent have demonstrated an association between *JAG1* polymorphisms and bone mineral density (51). Recently, mutations in *NOTCH2* leading to a premature truncation of the gene and absence of the PEST domain were identified in Hajdu-Cheney syndrome, which is characterized by focal areas of osteolysis and generalized osteoporosis (52;53). The mutations do not affect the ability of the NOTCH2 intracellular domain to induce the formation of an active transcriptional complex, and they reduce the capacity to activate the process of nonsense mediated decay, stabilizing the *NOTCH2* transcript. Furthermore, the absence of the PEST domain leads to NOTCH2 stabilization, leading to increased protein levels. These observations confirm *in vivo* and *in vitro*

studies on *Notch* misexpression indicating that Notch in excess causes bone loss. They also highlight the Notch pathway as a possible target in the treatment of bone disorders.

Notch and Malignancies

Activation of Notch1 is associated with T cell acute lymphoblastic leukemia and lymphoma, and dysregulated Notch signaling is involved in multiple myeloma (54). In these conditions, Notch may act by inducing cell proliferation or by inhibiting apoptosis. Notch signaling is associated with the invasive potential of osteosarcoma cells, and increased expression of JAG1, NOTCH1 and its target genes are found in human osteosarcoma (55;56). Therefore, Notch signaling may play a critical role in the pathogenesis and metastatic potential of osteosarcoma. A major clinical problem encountered in carcinoma of the breast is bone metastases, and Notch plays a role in skeletal cell-breast carcinoma cell interactions and in breast cancer skeletal invasiveness (57;58).

Conclusion

Canonical Notch signaling inhibits mesenchymal stem cell lineage specification, chondrogenesis, osteoblastogenesis and osteoclastogenesis, and as a consequence, it inhibits bone remodeling. Misexpression of Hes1, a classic target of canonical Notch signaling, does not phenocopy completely Notch, suggesting that other Notch target genes, such as members of the Hey family, mediate the effects of Notch in the skeleton. Alterations in Notch signaling lead to developmental skeletal disorders, and Notch may play a critical role in the development of osteosarcoma and the osteolytic potential of breast carcinoma.

Acknowledgments

This work was supported by grant DK045227 (EC) from the National Institute of Diabetes, Digestive and Kidney Diseases and by a Research Fellowship from the Arthritis Foundation (SZ). The authors thank Ms. Mary Yurczak for valuable secretarial assistance.

Conflict of Interest: None reported.

Peer Review: This article has been peer-reviewed.

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