REVIEW

MECHANISMS IN ENDOCRINOLOGY

Notch signaling in skeletal health and disease

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Abstract

Notch receptors are single-pass transmembrane proteins that determine cell fate. Upon Notch ligand interactions, proteolytic cleavages release the Notch intracellular domain, which translocates to the nucleus to regulate the transcription of target genes, including Hairy enhancer of split (*Hes*) and *Hes* related to YRPW motif (*Hey*). Notch is critical for skeletal development and activity of skeletal cells, and dysregulation of Notch signaling is associated with human diseases affecting the skeleton. Inherited or sporadic mutations in components of the Notch signaling pathway are associated with spondylocostal dysostosis, spondylothoracic dysostosis and recessive brachydactyly, diseases characterized by skeletal patterning defects. Inactivating mutations of the Notch ligand *JAG1* or of *NOTCH2* are associated with Alagille syndrome, and activating mutations in *NOTCH2* are associated with Hajdu–Cheney syndrome (HCS). Individuals affected by HCS exhibit osteolysis in distal phalanges and osteoporosis. NOTCH is activated in selected tumors, such as osteosarcoma, and in breast cancer cells that form osteolytic bone metastases. In conclusion, Notch regulates skeletal development and bone remodeling, and gain- or loss-of-function mutations of Notch signaling result in important skeletal diseases.

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Skeletal cells and bone remodeling

Skeletal tissue arises from distinct developmental processes. Cells of the cranial neural crest form the bones of the craniofacial skeleton, whereas the axial skeleton develops from somites (1, 2). Hyaline cartilage templates of appendicular bones originate from the proliferation and chondrogenic differentiation of mesenchymal cells residing in the limb bud. Chondrocytes in hyaline cartilage proliferate and acquire a hypertrophic phenotype, deposit a mineralized matrix, and ultimately become apoptotic. These events result in the formation of a calcified cartilage scaffold, which is vascularized and subsequently colonized by skeletal cell precursors that will replace cartilage with bone (3). Osteoblasts derive from mesenchymal cells that reside in the bone marrow and are the bone forming cells, which can differentiate further into lining cells or into osteocytes or can die by apoptosis (4, 5, 6). Osteocytes are terminally differentiated cells embedded in the mineralized matrix (7, 8). Osteoclasts, multinucleated cells that arise from the fusion of mononuclear precursors of the hematopoietic lineage, are the bone resorbing cells. Their formation requires receptor activator of nuclear factor κ B ligand (RANKL) (9) and macrophage colony stimulating factor, whereas

osteoprotegerin is a soluble inhibitor of RANKL. Therefore, the balance of RANKL and osteoprotegerin regulates osteoclastogenesis (9). Osteoblasts and osteoclasts play a critical role in the modeling of the growing skeleton, and the coupled function of these cells controls the remodeling of skeletal tissue throughout life (10).

The differentiation and function of cells of the osteoblastic and osteoclastic lineages are regulated by systemic and local signals, and the balance of their activities is essential to maintain bone remodeling. Notch has emerged recently as a local signal that plays a critical role in skeletal development, osteoblastic cell fate and function, and osteoclastogenesis (11). Consequently, it is not a surprise that gain- and loss-of-function mutations of various components of the Notch signaling pathway result in a variety of skeletal disorders. Furthermore, alterations in Notch signaling have been associated with selected malignancies and their skeletal metastatic potential (12).

Notch signaling in skeletal development and bone remodeling

Notch is a family of four (Notch 1–4) transmembrane receptors activated by Notch ligand interactions

(11, 13, 14, 15). Classical or canonical Notch ligands are the single-pass membrane proteins Jagged1 and 2 and delta-like (Dll) 1, 3, and 4. Following Notch ligand binding, the γ -secretase complex, containing the Presenilin1 and 2 proteases, cleaves the transmembrane domain of Notch, allowing the release and ultimate nuclear translocation of the Notch intracellular domain (NICD). In the nucleus, NICD interacts with the DNA-binding protein CSL (for Epstein-Barr virus latency C promoter-binding factor 1, suppressor of Hairless and Lag1), also known as Rbpjk. This leads to the assembly of the transcriptional complex formed by NICD, CSL/Rbpjk, and Mastermind-like (Mam1), and the subsequent displacement of transcriptional repressors and the induction of Notch target genes (Fig. 1) (15). Hairy enhancer of split (Hes) and Hes-related with YRPW motif (Hey) are classical targets of Notch signaling (14). The C-terminus of Notch contains a proline (P)-, glutamic acid (E)-, serine (S)-, and threonine (T)-rich (PEST) domain, which is necessary for the ubiquitinylation and subsequent degradation of the Notch protein in the proteasome, ensuring proper duration of the Notch signal (13, 15).

Notch signaling determines the segmentation of the axial skeleton during somitogenesis (16, 17), and its induction in the limb bud suppresses chondrogenesis, an effect that appears to be mediated by Hes1 (18, 19, 20, 21). The role played by Notch in mature chondrocytes is less clear (22, 23, 24, 25). Notch signaling regulates osteoblast and osteoclast differentiation and function and as a consequence controls bone remodeling. The effects of Notch in cells of the osteoblastic lineage are cell-context dependent and determined by the degree of differentiation of the cells targeted by Notch. Notch suppresses progression to a mature osteoblastic phenotype and osteoblast function, when expressed during the early stages of

the osteoblastic differentiation program, leading to suppressed bone formation and bone loss (19, 26, 27, 28, 29). Notch inhibits osteoclast formation and bone resorption by inducing osteoprotegerin expression in osteoblasts (26, 30). Accordingly, NICD overexpression in mature osteoblasts and osteocytes increases trabecular bone mass due to suppressed osteoclast formation and bone resorption (29). Most of the studies reported have examined the function of Notch1, so that less is known regarding the function of Notch2, 3, and 4 in the skeleton. There is evidence that Notch1 and 2 have distinct activities. For example, activation of Notch1 in osteoclast precursors prevents their differentiation toward mature osteoclasts, whereas activation of Notch2 enhances osteoclastogenesis (31, 32).

Developmental skeletal diseases associated with Notch signaling

Dysostoses of the axial skeleton

Spondylocostal dysostosis and spondylothoracic dysostosis are forms of trunk dwarfism caused by congenital abnormalities of the vertebrae and ribs secondary to defective somitogenesis (Table 1). Mutations of the various components of the Notch signaling pathway are associated with the diseases, and autosomal recessive inheritance is observed most frequently, although cases of autosomal dominant transmission have been reported (33, 34, 35). The phenotypes of delta-like 3 (*Dll3*) null mice recapitulate the manifestations of spondylocostal dysostosis, and mutations in *DLL3*, leading to the translation of a truncated or misfolded protein of this Notch ligand, are found in humans affected by the disease (36, 37, 38, 39). Mesoderm

Figure 1 Activation of Notch signaling. (A) Notch receptors and Jagged/delta ligands are expressed as single-pass transmembrane proteins. Epstein-Barr virus latency C promoter-binding factor 1, suppressor of hairless, Lag1 (CSL), also termed Rbpjk, is bound to DNA and inhibits gene expression by recruiting transcriptional repressors. (B) Receptor-ligand interactions lead to the cleavage of the Notch receptor mediated by Presenilin and release of the Notch intracellular domain (NICD) to the cytoplasm. (C) NICD translocates to the nucleus and forms a ternary complex with CSL and Mastermind-like (MAML), replacing transcriptional repressors with transcriptional activators and inducing expression of Notch target genes, such as Hairy enhancer of split (*Hes*) and Hes-related with YRPW motif (*Hey*).

Table 1 Skeletal diseases associated with *Notch* mutations.

Disease	Mutated gene	Major manifestations	Possibly impaired Notch function
Spondylocostal dysostoses	<i>DLL3, MESP2, HES7, LFNG</i>	Dwarfism Vertebral developmental defects	Regulation of the segmentation clock during somitogenesis
Spondylothoracic dysostoses	<i>MESP2</i>	Dwarfism Vertebral developmental defects	
Brachydactyly	<i>CHSY1</i>	Short digits Stunted growth	Patterning of the digits during development
Alagille syndrome ^a	<i>JAG1, NOTCH2</i>	Facial dysmorphism Vertebral abnormalities Bile duct atresia Cardiovascular defects	Craniofacial development; regulation of the segmentation clock during somitogenesis; vascular development
Hajdu–Cheney syndrome ^a	<i>NOTCH2</i>	Acro-osteolysis Osteoporosis Fibular deformities Polycystic kidneys	Unknown

DLL3, delta-like 3; MESP2, mesoderm posterior 2; HES7, Hairy and enhancer of split 7; LFNG, lunatic fringe; CHSY1, chondroitin sulfate synthase 1; JAG1, Jagged 1.

^aAdditional details are outlined in Tables 2 and 3.

posterior 2 (*Mesp2*) is a Notch target gene, which encodes a transcription factor critical for somitogenesis, and *Mesp2* null mice exhibit vertebral defects (17, 40). Accordingly, individuals that display abnormal segmentation of the thoracic vertebrae, typical of spondylocostal dysostosis, harbor homozygous nonsense mutations of *MESP2* (41). Similarly, spondylothoracic dysostosis, which is observed mostly in people of Puerto Rican descent, is associated with a mutant *MESP2* allele (42). *Hes7* regulates the transcription of lunatic fringe (*Lfng*), which, by regulating the glycosylation of Notch, changes the affinity of Notch receptors for its ligands (43). Inactivation of *Lfng* or *Hes7* in mice leads to abnormal development of the rib cage and vertebral column, and mutations in *LFNG* and *HES7* are associated with spondylocostal dysostosis in humans (44, 45, 46, 47, 48, 49).

Brachydactyly

Brachydactyly is characterized by shortening of the digits of the hands and feet (50). In mice, the global inactivation of the Notch ligand *Jag2* causes digit abnormalities and defects of the craniofacial skeleton that lead to perinatal death, indicating that perturbations of Notch signaling result in developmental defects that are reminiscent of brachydactyly (51, 52). A null allele of chondroitin sulfate synthase 1 (*CHSY1*) was discovered in members of a Jordanian family diagnosed with a syndromic form of brachydactyly that presented with stunted growth, micrognathia, and learning disabilities. *CHSY1* encodes a transmembrane protein containing a fringe domain, and cultured fibroblasts from affected individuals exhibited Notch activation secondary to upregulation of the ligand encoded by *JAG1*. Recombinant *CHSY1* inhibited Notch signaling *in vitro*, indicating that excessive activation of

Notch signaling is responsible for the disease (53). *Chsy1* null mice display abnormalities in digit patterning, although the phenotype appears to be secondary to dysregulation of Indian hedgehog and transforming growth factor β (TGF β) and not of Notch signaling (54).

Alagille syndrome

Alagille syndrome is an autosomal dominant disease that presents with cardiovascular defects, abnormalities of the craniofacial skeleton and vertebral column, cholestatic liver disease due to impaired formation of bile ducts, and renal anomalies, including dysplasia (Table 2) (55). In individuals affected by Alagille syndrome, vertebrae fail to fuse ventrally during development and assume a characteristic ‘butterfly’ appearance in radiographic images (56). Osteoporosis possibly secondary to liver failure and malnutrition has been reported in patients with the disease. Alagille syndrome is associated with mutations of *JAG1*, and these are mostly *de novo* mutations that lead to the translation of a truncated *JAG1* protein, although complete gene deletions and missense mutations are also observed (57, 58, 59). Rarely, mutations of

Table 2 Features of Alagille syndrome.

Craniofacial features	Skeletal features	Other features
Broad nasal bridge	Butterfly vertebrae	Bile duct atresia
Craniosynostosis	Digit abnormalities	Cholestatic liver failure
Deep set eyes	Osteoporosis	Cardiovascular
Micrognathia	secondary to	defects, including
Pointed chin	liver failure	Tetralogy of Fallot
Prominent forehead	Short stature	Intracranial bleeding
Triangular facies		Renal failure

Table 3 Features of Hajdu–Cheney syndrome.

Craniofacial features	Skeletal features	Other features
Facial dysmorphism	Acro-osteolysis	Cardiovascular defects
Microretrognathism	Fibular deformities	Developmental delay
Periodontal disease	Joint hyperlaxity	Hearing loss
Platysbasia	Osteoporosis	Neurological symptoms
Open sutures	with fractures	Polycystic kidneys
Tooth loss	Short stature	
Wormian bones		

NOTCH2 have been found to be associated with Alagille syndrome, either in isolation or in addition to mutations of *JAG1* (60, 61). Global *Jag1* null mice die during development, and the dual heterozygous inactivation of *Jag1* and *Notch2* in mice recapitulates most of the defects found in Alagille syndrome, confirming that the disease is secondary to mutations of these genes (62). In addition, inactivation of *Jag1* selectively in cells of the cranial neural crest phenocopies the abnormalities of the craniofacial skeleton that characterize Alagille syndrome, confirming its association with impaired Notch signaling (63).

Gain-of-function mutations of Notch signaling

Hajdu–Cheney syndrome

Hajdu–Cheney syndrome (HCS) is a devastating disease characterized by focal bone lysis of distal phalanges and by generalized osteoporosis (64, 65, 66, 67, 68). The disease was first described in 1948 in a 37-year-old accountant who died 12 years later, and the syndrome was reported by Cheney (1965) (Table 3) (64, 65). HCS is transmitted as an autosomal dominant disease, although many sporadic cases occur. Over 60 years after the original description, whole exome sequencing in individuals affected with HCS revealed the presence of point mutations in exon 34 of *NOTCH2* leading to the creation of a stop codon and the premature termination of the protein product upstream of the PEST domain (69, 70, 71). It is of interest that *NOTCH2* transcript levels were equivalent to those observed in controls, indicating a reduced capacity to activate the process of nonsense-mediated mRNA decay. As the PEST domain contains sequences necessary for the ubiquitinylation and degradation of Notch in the proteasome, the mutations lead to a stable protein and persistence of *NOTCH2* signaling as all sequences required for the formation of the Notch transcriptional complex are upstream of the PEST domain and are therefore preserved (Fig. 2).

Despite the pronounced skeletal abnormalities reported in HCS, little is known regarding the

mechanisms underlying the bone loss. Although the distal phalangeal osteolytic lesions would suggest increased localized bone resorption, there is no information on the mechanisms responsible for the generalized osteoporosis. The focal osteolysis is accompanied by neovascularization, inflammation, and fibrosis (72, 73, 74). Iliac crest biopsies have been reported in a small number of cases of HCS and revealed decreased trabecular bone, normal or increased bone remodeling, and normal or decreased bone formation (73, 75, 76, 77). Whether the osteoblast/osteocyte or the osteoclast is the cell responsible for the presumed change in bone turnover has not been established. In osteoclast precursors, Notch2 induces nuclear factor of T-cells 1 transcription and osteoclastogenesis (32). This effect is exclusively observed with Notch2 and not with Notch1, but whether this mechanism operates in HCS is not known.

Bisphosphonate therapy (alendronate and pamidronate) alone or in combination with anabolic therapy with teriparatide has been attempted for the treatment of the skeletal manifestations of patients with HCS, but there is no clear evidence that either therapy is beneficial (77, 78, 79). Serpentine fibula-polycystic kidney syndrome appears to be the same disease as HCS, and missense mutations in exon 34 of *NOTCH2*, upstream of sequences encoding for the PEST domain, were detected in patients affected by this disease (68, 80, 81). Although HCS affects a limited number of individuals, discovering a cluster of mutations in a single domain of *NOTCH2* in patients with HCS sheds light on potential mechanisms underpinning the development of osteoporosis. Multiple attempts to uncover genetic variants that contribute to the risk of osteoporosis have been

Figure 2 Structure of the NOTCH2 intracellular domain and mutations associated with Hajdu–Cheney syndrome. The intracellular domain of NOTCH2 (NICD) consists of a transcriptional domain formed by an Rbpjk association module (RAM) linked to ankyrin (ANK) repeats and a nuclear localization sequence (NLS). The C-terminus contains the proline (P)-, glutamic acid (E)-, serine (S)-, and threonine (T)-rich motif (PEST) domain, which is required for the ubiquitinylation and degradation of the NICD. Nonsense mutations in exon 34 associated with Hajdu–Cheney syndrome (HCS) and pointed by the arrow lead to the formation of a truncated protein consisting of all NOTCH2 sequences necessary for the formation of the transcriptional complex, but lacking the PEST domain needed for the ubiquitinylation and degradation of NOTCH2. As such, a stable and active NOTCH2 protein is synthesized.

relatively unsuccessful (82, 83). However, bone mineral density and risk of osteoporotic fractures were associated with a select *JAG1* allele in seven independent cohorts of women of Asian and European descent (84).

Notch and skeletal malignancies

Malignancies arising from cells of the hematopoietic lineage, such as T-cell leukemia, can exhibit activating mutations of *NOTCH1* and dysregulated Notch signaling, which may cause uncontrolled cell proliferation and inhibit apoptosis (85). Human osteosarcoma cells exhibit greater expression of the Notch ligand *JAG1*, of *NOTCH1*, and of the Notch target gene *HES1* than normal human osteoblasts, and the ability of osteosarcoma cells to metastasize correlates with increased Notch signaling (86, 87). Tumor burden in mice inoculated with human osteosarcoma cell lines was alleviated when cells expressing a dominant negative form of MAML, which inhibits Notch signaling, were used, or when γ -secretase inhibitors, to prevent Notch activation, were administered systemically (86). In addition, inhibition of γ -secretase prevented the growth of human osteosarcoma cells implanted subcutaneously in immunodeficient mice, confirming that activation of Notch signaling plays a critical role in the development of osteosarcoma (88).

Co-culture of human bone marrow stromal cells with cells from carcinoma of the breast induces NOTCH3 expression in the tumor cells, although the mechanisms mediating the effect are not known (89). Carcinoma of the breast cells induces osteolytic bone metastases in immunodeficient mice, and downregulation of NOTCH3 opposes this effect, suggesting a role for NOTCH3 in tumor invasion (89). Expression of *JAG1* in human breast cancer cells is associated with increased tumor burden and the ability of the tumor cells to metastasize to bone when implanted in immunodeficient mice. In this experimental model, *JAG1* expressing tumor cells stimulated osteoblasts to secrete interleukin 6, which in turn induced the maturation of osteoclast precursors and the formation of osteolytic metastases. This led to the degradation of the extracellular matrix surrounding the lytic lesions, and the release of TGF β , which promoted *JAG1* expression by tumor cells, creating a positive feedback loop favoring tumor invasion (90). In agreement with these findings, elevated expression of *JAG2* was associated with reduced metastasis-free survival in three cohorts of breast cancer patients, suggesting that activation of Notch signaling confers breast cancer cells the ability to form bone metastases (91).

Osteoblastic cells regulate the hematopoietic stem cell niche and influence stem cell function through the activation of Notch (92). Although defective Notch activation can lead to a myeloproliferative disease in the mouse, hematological malignancies have been

associated with the constitutive activation of *NOTCH1* and *NOTCH2* in humans (93). Activating mutations of *NOTCH1* leading to the expression of a truncated protein are present in over 50% of T-cell acute lymphoblastic leukemia (94, 95). Patients with acute lymphoblastic leukemia may present with osteopenia and vertebral fractures and the prevalence of fractures in newly diagnosed children is $\sim 16\%$ (96). Rarely, osteolytic lesions and hypercalcemia have been reported (97). Gain-of-function mutations of *NOTCH2* were found in a subset of patients with large B-cell lymphoma, but their association with specific skeletal disorders is not known (98).

Conclusions

Genetic mutations causing either gain- or loss-of-function of various components of the Notch signaling pathway are associated with diverse skeletal disorders and demonstrate that NOTCH is critical for human skeletal development and homeostasis. Findings in human diseases are consistent with results from numerous studies in mice and selected vertebrate model organisms, confirming that Notch signaling regulates skeletogenesis. Gain-of-function mutations of Notch lead to bone loss, and this is in agreement with the inhibitory effects of Notch on osteoblastogenesis, and with the stimulatory action of Notch2 on osteoclastogenesis. Various modalities to control Notch signaling have been reported, including the use of antibodies to the Notch extracellular domain or to its ligands, and the use of cell membrane permeable peptides that interfere with the formation of the Notch transcriptional complex, such as soluble MAML peptides (99, 100). However, reduced NOTCH signaling can result in the development of vascular tumors (101). There is strong evidence indicating that activation of NOTCH2 signaling causes HCS, and NOTCH2 could be a potential target for the treatment of this disease. However, for therapy to succeed, it needs to inhibit NOTCH2 activity specifically in affected tissues. Induction of NOTCH signaling is a critical event in the development and invasiveness of osteosarcoma and metastatic potential of carcinoma of the breast. NOTCH signaling could be targeted for the treatment of selected skeletal malignancies, but results from necessary clinical trials to establish the safety and efficacy of this approach are lacking. Targeting of NOTCH signaling in skeletal diseases associated with the loss of NOTCH activity would be even more problematic. Although strategies to activate Notch signaling were reported in a preclinical study, such approaches may result in serious complications due to the potential arrest of cell differentiation (102).

In conclusion, NOTCH signaling is required for skeletal development and bone homeostasis and gain- and loss-of-function mutations in genes that regulate

the NOTCH signaling pathway are uncommon but cause severe skeletal disorders.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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