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Notch in Skeletal Physiology and Disease

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Abstract

Notch (Notch 1 through 4) are transmembrane receptors that play a fundamental role in cell differentiation and function. Notch receptors are activated following interactions with their ligands in neighboring cells. There are five classic ligands termed Jagged (Jag)1 and Jag2, and Delta-like (Dll)1, Dll3 and Dll4. Recent work has established Notch as a signaling pathway that plays a critical role in the differentiation and function of cells of the osteoblast and osteoclast lineages and in skeletal development and bone remodeling. The effects of Notch are cell-context dependent, and the four Notch receptors carry out specific functions in the skeleton. Gain- and loss-of-function mutations of components of the Notch signaling pathway result in a variety of congenital disorders with significant craniofacial and skeletal manifestations. The Notch ligand Jag1 is a determinant of bone mineral density, and Notch plays a role in the early phases of fracture healing. Alterations in Notch signaling are associated with osteosarcoma and with the metastatic potential of carcinoma of the breast and of the prostate. Controlling Notch signaling could prove useful in diseases of Notch gain-of-function and in selected skeletal disorders. However, clinical data on agents that modify Notch signaling are not available. In conclusion, Notch signaling is a novel pathway that regulates skeletal homeostasis in health and disease.

Summary:

Notch receptors are activated following interactions with their ligands (Jagged and Delta-like) in adjacent cells. Notch and ligands are expressed by osteoblasts and osteoclasts and regulate their differentiation and function. As a consequence, Notch modulates skeletal remodeling and plays an important role in bone homeostasis in health and disease.

Keywords

Notch; Jagged; congenital disorders; osteoblast; osteoclast

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Conflict of Interest

None.

I. NOTCH PHYSIOLOGY

Notch Receptors

Notch are transmembrane receptors that play a fundamental role in cell differentiation and function. Work conducted over the past decade has established Notch as a signaling pathway critical for bone remodeling and a determinant of skeletal homeostasis [1–7]. There are four Notch receptors (Notch1 through 4) and five classic ligands termed Jagged (Jag)1 and Jag2, and Delta-like (Dll)1, Dll3 and Dll4 [5]. Notch ligands, like Notch, are transmembrane proteins and their interactions with Notch are required to activate Notch signaling. Since Notch and its ligands are transmembrane proteins, the signaling pathway serves as a means of communication between neighboring cells.

Notch receptors have a complex structure. Their extracellular domain is the site of interaction with Notch ligands, and at the junction of the extracellular and the transmembrane domain rests the negative regulatory region (NRR). This is the site of cleavage required for Notch activation, and as a consequence plays a critical regulatory role in Notch signaling [8]. The intracellular domain of Notch (NICD) consists of an RBPJκ-association module (RAM) domain, ankyrin repeats and nuclear localization sequences; these domains are required to regulate transcription. The C-terminus of Notch contains a proline (P)-, glutamic acid (E)-, serine (S)- and threonine (T)-rich (PEST) domain, which is targeted by ubiquitin ligases for the proteasomal degradation of Notch, and as such it defines the life span of Notch [5] (Fig. 1).

Interactions of Notch with its ligands lead to its proteolytic cleavage and release of the NICD, its translocation to the nucleus, where the NICD forms a complex with recombination signal-binding protein for Ig of κ region (RBPJκ) and mastermind-like (MAML) to regulate transcription [9, 10]. RBPJκ is also termed CBF1, Suppressor of hairless, Lag1 or CSL. RBPJκ, and not the NICD, binds to DNA and under basal conditions RBPJκ is a repressor of transcription. The translocation of the NICD to the nucleus, where it interacts with RBPJκ, leads to the displacement of transcriptional inhibitors and the recruitment of activators of transcription. As a result, the NICD, RBPJκ, MAML complex induces the transcription of target genes. Targets of this canonical signaling are members of the Hairy and enhancer of split (HES) and HES with a YRPW motif (HEY) families of transcription factors [11]. Cyclin-dependent kinases phosphorylate the PEST domain of the NICD, causing the disassembly of the NICD, RBPJκ, MAML complex followed by ubiquitination of the NICD by E3 ubiquitin ligases and the degradation of Notch [12]. A less well-characterized non-canonical pathway can operate under certain physiological conditions, and by definition it does not require RBPJκ. It is believed that in skeletal cells Notch operates through the canonical RBPJκ-dependent pathway.

It is important to recognize that there are structural and functional differences among the four Notch receptors and that their function is not redundant. Therefore, the independent study of the four Notch receptors in skeletal cells has been of utmost importance (Table 1). Functional differences among Notch receptors are related to structural distinctions in the NICD, to differential interactions of each NICD with RBPJκ, to the temporal and cellular expression of each receptor, to variations in the affinity of the extracellular domain of Notch

for its ligands and to NRR sequence differences conveying specificity to the activation of Notch [13, 14]. Notch1, 2 and 3 and low levels of Notch4 are detected in skeletal cells [1, 15]. Notch 1 and 2 are expressed by cells of the osteoblast and osteoclast lineage, whereas Notch3 is mostly expressed by osteoblasts and osteocytes, and not by cells of the osteoclast lineage.

Distinct cellular patterns of expression confer the Notch receptors a unique physiological role (Table 2). Moreover, the actions of Notch are cell-context dependent and cellular responses depend on the specific cell being studied and its stage of maturation at the time of Notch activation. For example, when Notch1 is activated in osteoblast precursors it inhibits osteoblast differentiation, whereas when activated in mature osteoblasts and osteocytes Notch1 inhibits bone resorption and causes an osteopetrotic phenotype [6, 7]. Depending on the cell environment, these differences in Notch activity have led to discrepant conclusions regarding the actual function of Notch in skeletal cells.

Notch, Bone Cells and Skeletal Remodeling

1. **Notch and Osteoblasts—**Initial studies performed *in vitro* demonstrated that Notch1 suppresses osteoblast cell differentiation and Wnt/β-catenin signaling [16]. Activation of Notch1 signaling in osteoblast precursors and mature osteoblasts causes profound osteopenia secondary to impaired bone formation [7].Transgenic overexpression of Notch1 in osteoblasts also causes osteopenia and the mechanism appears to be an inhibition of Wnt/β-catenin signaling [16]. In agreement with these findings, the deletion of *Notch1* and *Notch2* in osteoblast precursors causes an increase in osteoblast number, bone formation and cancellous bone volume [17]. It is of interest that mice harboring the activation of Notch1NICD in osteoblasts fail to form a compact cortical bone, which has the appearance of embryonic cortical bone suggesting that a compact cortex fails to develop in the presence of Notch [7]. Notch activation in the early phases of the osteoblast differentiation program prevents the maturation of cells capable to synthesize a mineralized matrix, whereas Notch induction in mature cells precludes further differentiation and causes an accumulation of dysfunctional osteoblasts [2, 4, 7]. These events are possibly mediated by suppression of RUNX2 transcription, and a decrease in Wnt signaling [2].

2. **Notch and Osteocytes—**Activation of canonical signaling by Notch1 in osteocytes induces osteoprotegerin and as a consequence causes an osteopetrotic phenotype. Both cancellous bone resorption and formation are reduced, so that bone remodeling is suppressed [6, 7, 18]. The phenotype is compartment-specific and cortical bone formation is increased, but cortical bone is porous, with the appearance of trabecular bone, possibly because it fails to reach maturity. The dual inactivation of *Notch1* and *Notch2* in osteocytes causes a modest increase in cancellous bone volume and a decrease in bone resorption [6].

Notch1 activation in osteocytes suppresses the Wnt antagonists sclerostin and dickkopf 1 (Dkk1); through this indirect mechanism Notch increases Wnt/β-catenin signaling [6, 18]. The induction of Wnt signaling seems paradoxical to the direct inhibition of Wnt signaling by Notch in osteoblasts. However, the mechanism in osteocytes is indirect; and since osteoblasts express minimal levels of *Sost*, an inhibition of *Sost* with a consequent increase

in Wnt signaling cannot occur in these cells. Therefore, the direct inhibitory effect of Notch on Wnt signaling prevails and is likely to be responsible for the impaired osteoblast maturation [16].

The interactions of Notch and Wnt in osteocytes are complex. Notch receptors are induced by Wnt signaling in osteocytes creating a positive-feedback loop where Wnt enhances Notch signaling and Notch, by downregulating *Sost*, increases the levels of Wnt activity [19]. Mechanical forces induce Notch signaling in osteocytes either directly or indirectly through the activation of Wnt [20].

3. Notch and Osteoclasts—Notch signaling regulates osteoclast differentiation and function, but the response is highly dependent on the Notch receptor being activated. Notch1 inhibits osteoclastogenesis by direct and indirect mechanisms, including the induction of osteoprotegerin [2, 4, 6, 7]. Notch1 has direct effects on osteoclast precursors and its activation suppresses osteoclastogenesis [1, 21]. The inactivation of *Rbpjκ* in myeloid cell cultures leads to enhanced osteoclastogenesis, indicating that Rbpjκ is an inhibitor of osteoclastogenesis and that effects of Notch1 in osteoclast differentiation are mediated by canonical mechanisms [22].

In contrast to the inhibitory effects of Notch1 on osteoclastogenesis, Notch2 induces osteoclastogenesis by direct and indirect mechanisms. Interactions of the Notch2NICD with nuclear factor (Nf)-kB in osteoclast precursors lead to the transcription of *Nfatc1*, a gene critical for osteoclastogenesis [3, 23]. Notch2 also enhances osteoclastogenesis by inducing receptor activator of NF-κB ligand (RANKL) in cells of the osteoblast lineage. A similar induction of RANKL is observed with the activation of Notch3. However, Notch3 is not expressed in the myeloid lineage and does not have direct effects on osteoclast precursors. The actions of Notch4 in either the osteoblast or osteoclast lineage are less certain and low levels of Notch4 are expressed in skeletal cells.

4. Notch Target Genes and Skeletal Cells—*Hes1*, *Hey1*, *Hey2* and *HeyL* are the Notch target genes expressed by skeletal cells (Table 2). Although HEYs are induced by Notch in skeletal cells, *in vivo* models of gene misexpression demonstrated modest effects of HEY1, HEY2 and HEYL on bone remodeling [24–26]. Studies conducted in our laboratory revealed that transgenics expressing *Hey2* under the control of a 3.6 kilobase *Col1a1* promoter develop osteopenia, but the inactivation of *Hey1*, *Hey2* or *HeyL* do not result in a significant or lasting skeletal phenotype [24–26]. *Hey1* and *HeyL* transgenics do not have an obvious skeletal phenotype. These findings indicate only a modest role of HEY proteins in bone physiology. In contrast to the modest role of *Hey* genes in skeletal homeostasis, initial studies from our laboratory on models of *Hes1* misexpression suggest a more significant role of HES1 in bone remodeling. *Hes1* inactivation in osteoblasts causes an increase in bone formation and suppressed bone resorption, and *Col1a1-Hes1* transgenics are osteopenic, have decreased osteoblast function and increased bone resorption [27]. These changes are compatible with the skeletal manifestations of Notch activation in bone, making *Hes1* a candidate gene responsible for the actions of Notch in the skeleton.

5. Hormonal Regulation of Notch

Parathyroid Hormone: Parathyroid hormone (PTH) induces the Notch ligand Jag1 in osteoblasts, and the effects of PTH on osteoblast differentiation are opposed by 03B3-secretase inhibitors, suggesting that Notch has the potential to mediate selected actions of PTH in osteoblasts [28]. However, γ-secretase inhibitors have nearly 100 substrates and therefore are not specific inhibitors of Notch signal activation [29]. In addition, PTH and Notch have opposite effects on bone formation and osteoblast activity. These apparently contradictory observations could be explained by additional interactions between PTH and Notch in osteoblastic cells.

PTH was examined further for its effects on Notch signaling in cells of the osteoblast lineage *in vitro* and *in vivo*. PTH was found to decrease *Hey1*, *Hey2* and *HeyL* mRNA levels in Notch-activated osteoblasts and suppress the expression of Notch target genes in osteocytes [15]. In agreement with these observations *in vitro*, administration of PTH *in vivo* suppressed *Hey1* and *HeyL* expression in bone extracts. Transactivation experiments with a Notch reporter construct and electrophoretic mobility shift assays in osteoblasts indicated that PTH acts by decreasing the capacity of Rbpjκ to bind to DNA and by interfering with the formation of an active NICD/Rbpjκ transcriptional complex.

Although Jag1 is induced by PTH, Notch signaling is downregulated by PTH in osteoblasts and osteocytes, suggesting that *Jag1* induction is not sufficient to overcome the inhibitory effect of PTH on Rbpjκ-mediated signaling. Alternatively, Jag1 induced by PTH may sequester and suppress Notch receptors [30]. The inhibitory effects of PTH on Notch signaling might contribute to the mechanisms of the anabolic effects of PTH in bone.

Cortisol: Cortisol causes a time-dependent increase in *Notch1* and *Notch2* mRNA levels in MC3T3 osteoblast-like cells, and has no effect on the expression of Notch ligands. Cortisol acts by increasing the rate of *Notch1* and *Notch2* transcription [31]. Although cortisol induces *Notch1* and *Notch2* expression, it does not enhance Notch activity. In primary cultures of osteoblasts, cortisol decreases the transcription of *Hey1*, *Hey2* and *HeyL* in Notch-activated cells. In addition, the administration of prednisolone *in vivo* decreases the expression of Notch target genes in bone. These results suggest that cortisol has the potential to oppose Notch activity.

II. NOTCH AND CONGENITAL DISORDERS OF THE SKELETON

Congenital Disorders Associated with a Gain-of-Notch Function

1. Hajdu Cheney Syndrome—Hajdu Cheney Syndrome (HCS) is a rare, inherited disease associated with *NOTCH2* mutations (Table 3). HCS is characterized by osteoporosis with fractures, acroosteolysis of the hands and feet, craniofacial developmental defects and short stature [32–34]. HCS is associated with nonsense mutations or deletions leading to the creation of a termination codon in exon 34 of *NOTCH2* upstream of the PEST domain [35–37]. Since the PEST domain is required for the degradation of NOTCH2, the mutations lead to the formation of a truncated and stable NOTCH2 protein and, as a consequence, to a NOTCH2 gain-of-function.

HCS has autosomal dominant inheritance, although sporadic cases occur. There is clinical variability and evolution of the clinical manifestations, and signs of the disease can be manifested as early as in the first two years of life [32]. Acroosteolysis is frequently present and accompanied by inflammation, and lysis of the phalanges which leads to short and broad digits. Spinal abnormalities include compression fractures, kyphosis and scoliosis. Long bone deformities can occur. Platybasia and basilar invagination can result in severe neurological complications, including hydrocephalus and central respiratory arrest causing sudden death. Polycystic kidneys are present in 10% of the cases and occasionally affected subjects present with cardiac septal defects and valve abnormalities [38]. Iliac crest biopsies reveal the presence of cortical bone osteopenia, woven bone and increased osteoclast number and bone resorption [39].

Our laboratory created a *Notch2* mutant mouse harboring a truncating mutation in exon 34 upstream of the PEST domain reproducing the mutation found in HCS and termed *Notch2*$^{tm1.1Ecan}$ [23]. *Notch2*$^{tm1.1Ecan}$ mutant mice exhibit cancellous and cortical bone osteopenia secondary to increased osteoclast number and bone resorption. *In vitro* studies demonstrated enhanced osteoclastogenesis and confirmed the stimulatory effect of Notch2 on osteoclast differentiation [3]. The mechanism involves a direct effect of Notch2 on osteoclast precursors as well as the induction of RANKL by cells of the osteoblast lineage. In an alternate murine model of HCS, an increased expression of interleukin 6 was reported in bone marrow cells, and this cytokine could contribute to the enhanced bone resorption observed [40]. *Notch2*$^{tm1.1Ecan}$ mutant mice are sensitized to the development of osteoarthritis, a mechanism that may also involve an enhanced expression of interleukin 6 by the chondrocyte of HCS mutant mice [41].

There are no controlled trials on the management of the osteoporosis in patients with HCS. Bisphosphonates alone or in combination with teriparatide have been used, but evidence of benefit is scarce [38, 42, 43]. Because Notch2 induces RANKL and enhances osteoclastogenesis, a consideration is the use of denosumab and the agent was used successfully in the treatment of a subject with osteoporosis and fractures [44]. Although PTH suppresses Notch signaling, the use of teriparatide in the treatment of HCS could pose risks [15]. There is evidence of Notch activation in osteosarcoma in humans and prolonged activation of Notch in mice can cause osteosarcoma, suggesting that Notch activation could be a risk factor for osteosarcoma [45, 46]. As such, teriparatide should be used with caution. Moreover, the mechanism responsible for the bone loss in HCS is increased bone resorption making teriparatide not an ideal choice in the management of HCS.

Notch2 itself could be a future target for the treatment of HCS, and the skeletal phenotype of *Notch2*$^{tm1.1Ecan}$ mouse mutants was reversed by anti-Notch2 antibodies that target the NRR of Notch2 and prevent the activation of Notch2 [47, 48]. There are no clinical trials on the use of this approach which carries the risk of a generalized downregulation of Notch2 with potential unwanted events.

It is of interest that somatic *NOTCH2* mutations causing loss of the PEST domain exhibit enhanced Notch2 activation and have been identified in B cell lymphoma, specifically in marginal zone B lymphoma of the spleen [49]. Notch2 is required for the development of the

marginal zone of the spleen, and mouse models of HCS exhibit a reallocation of marginal zone B cells at the expense of follicular cells but do not appear to develop marginal zone lymphomas [50, 51]. The reallocation of marginal zone B cells does not influence skeletal remodeling. There is no evidence of higher incidence of marginal zone B cell lymphomas in subjects with HCS.

2. Lateral Meningocele or Lehman Syndrome—Lateral Meningocele Syndrome (LMS) is a rare disorder characterized by meningoceles with relatneurological dysfunction [52]. Clinical manifestations of LMS include craniofacial developmental abnormalities, intellectual disability, hypotonia, decreased muscle mass, syringomyelia and cardiac valve abnormalities. Skeletal manifestations include short stature, scoliosis, pectus excavatum, wormian bones, increased density of the base of the skull, and increased bone remodeling and bone loss [53].

LMS is associated with point mutations or short deletions in exon 33 of *NOTCH3*, upstream of the PEST domain [54]. The mutations are analogous to those reported in exon 34 of *NOTCH2* in HCS and result in the translation of a truncated and stable product, devoid of the PEST domain. The stabilization of the Notch3 protein likely leads to a gain-of-function and enhancement of Notch signaling. Subjects with LMS share selected clinical features with HCS [55]. Although increased bone turnover and decreased bone mineral density (BMD) may occur in LMS, acroosteolysis, a hallmark feature of HCS, has not been reported. The inheritance in LMS is not clear and autosomal dominant inheritance has been suggested [53]. However, most cases reveal *de novo* heterozygous mutations of *NOTCH3*.

3. Brachydactyly—Brachydactyly is an autosomal recessive disorder characterized by bilateral pre-axial brachydactyly or shortening of the digits of the hands and feet [56]. Affected individuals display facial dysmorphism, dental anomalies, sensory hearing loss, and growth, motor and mental retardation. A null allele of chondroitin sulfate synthase (*CHSY*)*1* has been associated with brachydactyly and downregulation of *Chsy1* results in enhanced Notch signaling, suggesting that activation of Notch signaling is responsible for aspects of the clinical syndrome [57].

Congenital Disorders Associated with a Loss-of-Notch Function

1. Adams Oliver Syndrome—Adams Oliver Syndrome (AOS), a rare congenital disorder characterized by aplasia cutis congenita and terminal transverse limb defects, is often, although not always, associated with mutations of genes encoding components of the Notch signaling pathway. These include loss-of-function mutants in *NOTCH1*, *DLL4*, *CSL* and *EOGT*, encoding for EGF-domain-specific O-linked *N*-acetylglucosamine transferase (O-GlcNAc). The clinical presentation of AOS is variable. The terminal limb defects include oligodactyly, syndactyly, hypoplastic nails and transverse amputations [58]. Small vessel abnormalities and vascular thrombosis during development may be responsible for the terminal transverse limb defects, and these are consistent with the established role of Notch signaling in vascular development [59, 60]. Congenital cardiac defects, including ventricular septal defects, tetralogy of Fallot, and anomalies of arteries and cardiac valves are

uncommon and associated with peripheral vascular abnormalities, such as cutis marmorata telangiectatica congenita and retinal hypovascularization.

A variety of *NOTCH1* mutations across the length of the receptor are found in AOS. The majority of the gene mutations occur in the extracellular domain leading to structural changes and loss-of- function [59]. Loss-of-function mutations in CSL result in decreased binding of CSL to regulatory regions of Notch target genes [61]. Missense and nonsense mutations in the coding sequence of *DLL4* are predicted to cause loss-of-function [62]. Mutations in *EOGT* cause impaired O-GlcNAc activity and Notch1 glycosylation [63].

2. Alagille Syndrome—Alagille Syndrome is an autosomal dominant disease that presents with cardiovascular defects including tetralogy of Fallot, abnormalities of the craniofacial skeleton and vertebrae, cholestatic liver disease due to bile duct atresia and kidney anomalies causing renal failure [64]. Failure of the vertebrae to fuse ventrally during development causes a characteristic “butterfly” appearance in radiographic images [65]. Craniofacial developmental abnormalities cause craniosynostosis and characteristic facial features. Subjects have short stature, digit abnormalities and may present with osteoporosis, considered to be secondary to liver failure and malnutrition.

Alagille Syndrome is associated with loss-of-function mutations of the Notch ligand *JAG1* [66–68]. Rarely, mutations of *NOTCH2*, isolated or in conjunction with mutations of *JAG1*, have been reported in Alagille Syndrome [69, 70]. Individuals with *NOTCH2* null mutations frequently present with hypoplastic kidneys and renal insufficiency, since Notch2 is required for renal development [70].

Null mutations of *Jag1* or inactivation of *Notch2* in mice result in embryonic lethality [71, 72]. However, the combined heterozygous inactivation of *Jag1* and a hypomorphic *Notch2* allele in mice recapitulates features of Alagille Syndrome suggesting that the inactivation of these genes is responsible for the clinical manifestations

3. Spondylocostal and Spondylothoracic Dysostosis—Spondylocostal dysostosis and spondylothoracic dysostosis are characterized by vertebral segmentation defects and rib anomalies secondary to defective somitogenesis [73]. Dominant, recessive and sporadic loss-of-function mutations of genes encoding various components of the Notch signaling pathway are associated with these disorders. Mutations of *DLL3* are found in 20–25% of affected individuals, and inactivation of *Dll3* in mice recapitulates the manifestations of spondylocostal dysostosis [74, 75]. Spondylothoracic dysostosis is associated with a mutant mesoderm posterior bHLH transcription factor (*MESP*)*2* allele [76]. *MESP2* is a Notch target gene critical for somitogenesis, and *Mesp2* null mice exhibit vertebral developmental defects [77]. Hes7 regulates the transcription of lunatic fringe (Lfng) which regulates the glycosylation of Notch, and loss-of-function mutations of either *LFNG* or *HES7* also are associated with spondylocostal dysostosis [78, 79].

III. NOTCH AND ACQUIRED SKELETAL DISEASES

1. Osteoporosis

The actions of Notch signaling in skeletal physiology as well as the presence of severe bone loss in congenital disorders of Notch gain-of-function would suggest a possible role of Notch in osteoporosis. Genome-wide association studies revealed an association between *JAG1*, the gene encoding the Notch ligand Jag1 and BMD [80]. A SNP (rs2273061) of *JAG1* is associated with high BMD at the lumbar spine and femoral neck, and low risk of osteoporotic fractures. The findings suggest that *JAG1* is a candidate gene for BMD regulation and a potential factor in the pathogenesis of fractures.

2. Fractures

Fracture healing is a regenerative process that results in the formation of new bone after a fracture. The healing of a fracture occurs by either intramembranous bone formation, when the bone is mechanically stable, or by endochondral ossification, when the bone is unstable. The function of Notch in fracture healing is complex, and upregulation as well as downregulation of Notch signaling have been reported in experimental models of fracture healing [81]. Because of the known inhibitory effects of Notch on osteogenesis and chondrogenesis, downregulation of Notch signaling might be a requirement for the fracture healing process to occur. The administration of γ-secretase inhibitors, to prevent the activation of Notch signaling, accelerated fracture healing in an open mid-shaft tibial fracture mouse model [82]. Whereas these results suggest a negative role of Notch in fracture healing, it is important to note that γ-secretase inhibitors are not specific inhibitors of Notch activation. Interestingly, the downregulation of *Rbpjκ*, a main component of canonical Notch signaling, in chondrogenic-/osteogenic-expressing cells results in non-union fractures [83]. This suggests that a degree of Notch signaling is necessary for fracture repair.

Cell lineage tracing studies have demonstrated that osteoblast precursors move into the fracture site along with invading blood vessels, confirming the importance of vascularization for proper fracture healing [84]. Notch signaling plays a critical role in vascular development and physiology, and promotes endothelial cell proliferation and vessel growth in long bones [85]. The angiogenic effects of Notch may be necessary for the healing process of fractures to occur [86].

3. Osteosarcoma

Notch receptors and Notch target genes are upregulated in osteosarcoma [87, 88]. Gene expression analysis in tissue samples from subjects with osteosarcoma compared to unaffected human bone tissue or to normal osteoblasts has demonstrated upregulation of *NOTCH1*, *NOTCH2* and *JAG1* mRNA levels in osteosarcoma [45, 88]. Notch target genes, particularly *HEY1* and *HEY2*, are increased in osteosarcoma, demonstrating activation of Notch canonical signaling. The growth of osteosarcoma cell lines is suppressed by γ-secretase inhibitors and by lentiviruses delivering a dominant negative MAML, to prevent Notch-dependent activation or transcription, respectively. This suggests that Notch controls the growth of osteosarcoma cells [87].

The induction of the Notch1NICD in mature osteoblasts in mice causes the spontaneous development of osteosarcoma as mice age [46]. The development of osteosarcoma requires the activation of Notch canonical signaling since it is not observed in the context of *Rbpjκ* inactivation. The findings in humans and mouse models demonstrate that Notch canonical signaling plays a role in the initiation as well as in the invasive potential of osteosarcoma [89]. As a result, components of the Notch signaling pathway could become future therapeutic targets in osteosarcoma.

4. Skeletal Metastases

Skeletal metastases are complications of carcinoma of the breast and prostate, and Notch has been implicated in the interactions between osteoblasts and metastatic cells [90, 91]. Breast cancer cells expressing *JAG1* activate Notch signaling, which induces Interleukin 6 in osteoblasts and, as a consequence, enhances osteoclastogenesis and the formation of osteolytic bone metastases. This leads to the release of transforming growth factor (TGF)β from the bone matrix, which upregulates *JAG1*, causing further activation of Notch signaling and creating a positive feedback loop favoring the metastatic potential of the tumor. Downregulation of *JAG1* decreases the osteolytic potential in experimental models of carcinoma of the breast.

Carcinoma of the prostate frequently metastasizes to bone. *NOTCH1* and *JAG1* are expressed by carcinoma of the prostate, and their expression is associated with the metastatic potential and recurrence of the tumor [92]. Downregulation of *NOTCH1* by RNA interference in human prostate cancer cells decreases their invasive potential verifying a role of Notch in this malignancy.

IV. MODIFYING NOTCH SIGNALING

Notch signaling can be downregulated or tempered using a variety of approaches including the use of biochemical inhibitors of Notch activation, antibodies to Notch receptors or to their ligands, and the use of small permeable molecules that prevent the formation of an NICD/Rbpjk/Maml ternary complex [93]. γ-secretase inhibitors are used to prevent the cleavage and activation of Notch induced by presenilins [94]. Their limitation is a lack of specificity since nearly 100 substrates of the γ-secretase complex are known to exist [29]. Thapsigargin is an inhibitor of the sarco/endoplasmic reticulum Ca^{2+}– ATPase that precludes the maturation and folding of the Notch receptor and, as a consequence, prevents Notch activation [95]. Synthetic small cell permeable molecules that prevent the assembly of an active Notch transcriptional complex can be used for the inhibition of Notch signaling, but their long-term efficacy is unknown [96]. A limitation of these agents is that they interfere with the indiscriminate activation of all four Notch receptors.

To target specific Notch receptors, antibodies to the NRR of Notch1, Notch2 and Notch3 have been developed [48, 97]. The NRR contains the initial cleavage sites of Notch required for protein maturation and signal activation (Fig. 1) [98]. The epitope for the anti-Notch NRR bridges the domain so that the antibody locks the receptor in its quiescent state preventing Notch activation [48, 99]. The targeting of the NRR prevents the activation of

specific Notch isoforms, making the use of anti-Notch NRR antibodies ideal for the neutralization of individual Notch receptors.

Recently, anti-Notch2 NRR antibodies were tested for their effects on the skeletal phenotype of HCS mutant mouse models and shown to reverse the osteopenic phenotype of *Notch2HCS* mice [47]. However, information on the inhibition of Notch signaling in humans is quite limited, and widespread Notch neutralization is not without unwanted events; it may cause vascular tumors and gastrointestinal toxicity [100].

V. CONCLUSIONS

Notch is a determinant of cell differentiation and function in cells of the osteoblast and osteoclastlineages and plays a critical role in skeletal development and bone homeostasis. The effects of Notch are cell-context dependent, and the four Notch receptors carry out specific functions in the skeleton. Alterations in Notch signaling are associated with a variety of congenital disorders and there is evidence of altered Notch signaling in skeletal malignancies. In conclusion, Notch signaling is a novel pathway that regulates skeletal homeostasis in health and disease.

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Abbreviations

ANK	Ankyrin
BMD	bone mineral density
CHSY	chondroitin sulfate synthase
DII	Delta-like
Dkk1	dickkopf 1
EGF	epidermal growth factor
HES	Hairy and enhancer of split
EOGT	EGF-domain-specific O-linked *N*-acetylglucosamine transferase
HCS	Hajdu Cheney Syndrome
HEY	HES with a YRPW motif
HD	heterodimerization domain; n

Jag	Jagged
MAML	mastermind-like
LP	leader peptide
LMS	Lateral Meningocele Syndrome
LNR	Lin12-Notch repeats
Lnfg	lunatic fringe
NRR	negative regulatory region
NICD	Notch intracellular domain
Nf	nuclear factor
NLS	nuclear localization sequence
PTH	parathyroid hormone
PEST	proline (P)-, glutamic acid (E)-, serine (S)- and threonine (T)-rich
RANKL	receptor activator of NF-κB ligand
RBPJκ	recombination signal-binding protein for Ig of κ region
RAM	RBPJκ-association module
TGF	transforming growth factor
TMD	transmembrane domain

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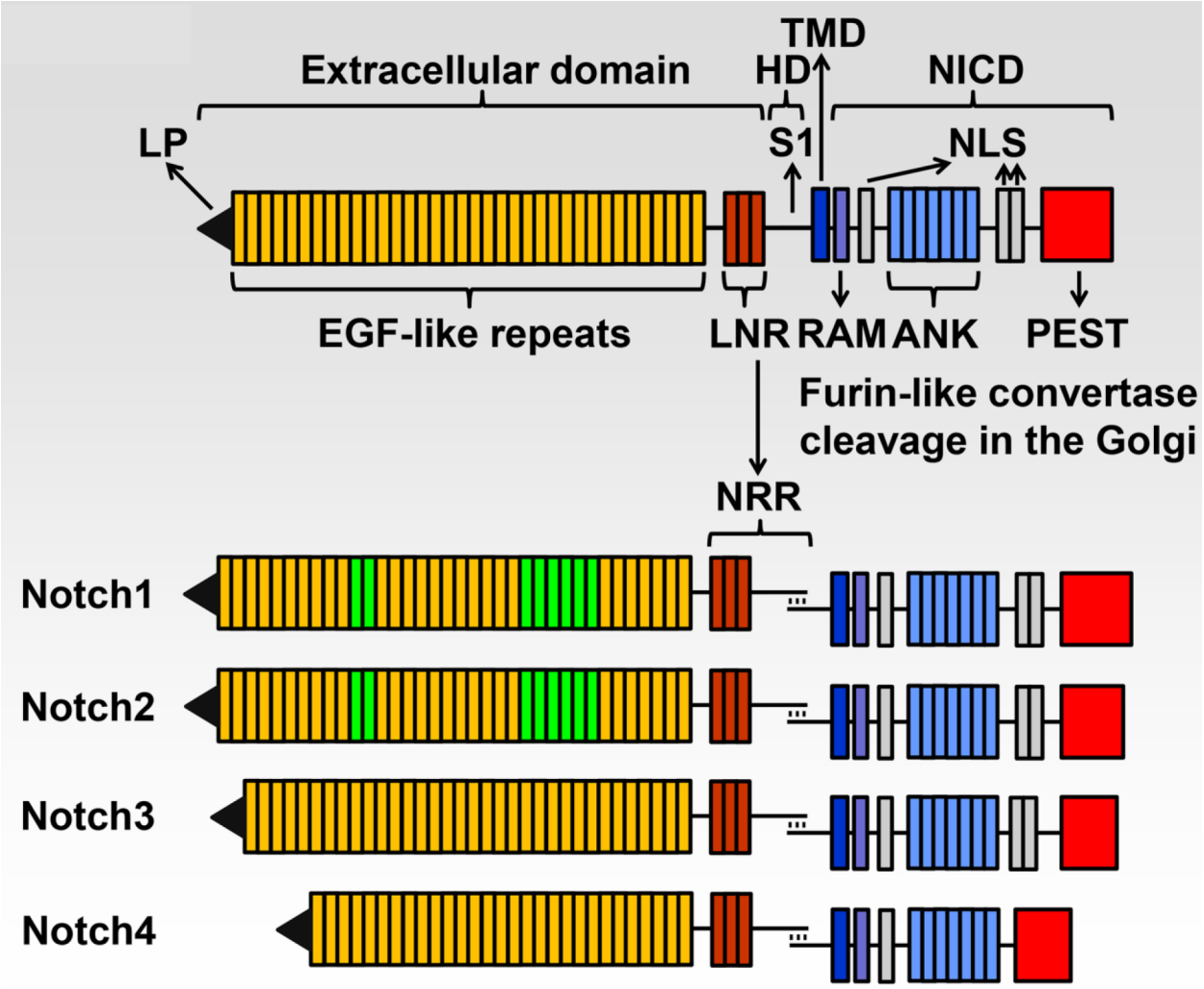

Fig.1.

Domains of the four Notch receptors. The upper panel shows the domain and motif organization of a generic human/murine Notch receptor before cleavage at the S1 site by furin-like convertases in the Golgi compartment. The extracellular domain contains a leader peptide (LP) and multiple epidermal growth factor (EGF)-like tandem repeats followed by Lin12-Notch repeats (LNR) and the heterodimerization domain (HD). The transmembrane domain (TMD) is located between the extracellular and intracellular domains. The Notch intracellular domain (NICD) contains an Rbpjκ-association module (RAM), a nuclear localization sequence (NLS), ankyrin (ANK) repeats and tandem NLS, which are followed by a proline (P)-, glutamic acid (E)-, serine (S)- and threonine (T)-rich (PEST) domain. The lower panel shows the domains and motifs of heterodimeric individual receptors, the negative regulatory region (NRR) is formed by the LNR and HD following cleavage at the S1 site. Notch1 and Notch2 have 36 EGF-like repeats; in green are those required for binding of Notch1 and Notch2 to cognate Delta/Serrate/Lag2 ligands. Notch1 and Notch2 have a similar NICD, and Notch3 has 34 EGF-like repeats and a shorter NICD than Notch1 and Notch2. Notch4 has 29 EGF-like repeats and an NICD that is shorter than that of other receptors and lacks the tandem NLS located between the ANK repeats and the PEST

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Table 1.

Functional differences among Notch receptors.

	Notch1	**Notch2**	**Notch3**	**Notch 4**
Extracellular Domain EGF Repeats	36	36	34	29
Extracellular Transmembrane Junction Distinct NRR	Yes	Yes	Yes	Yes
Intracellular Domain Similar NICD	Yes	Yes	No	No
Myeloid Lineage Expression	Yes	Yes	No	Yes
Osteoblast Lineage Expression	Yes	Yes	Osteocyte	Yes
Osteoblast Replication	No effect	No effect	Increased	Not studied
Osteoblast Differentiation	Inhibited	Inhibited	No effect	
Osteoclastogenesis	Inhibited	Enhanced Direct & Indirect Effect	Enhanced	
Indirect Effect				
Osteoprotegerin	Induced	No effect	Suppressed	
RANKL Induction	Modest	Robust	Robust	

EGF, epidermal growth factor; NRR, Notch regulatory region; NICD, notch intracellular domain; RANKL, receptor activator of NF-κB ligand

Table 2.

Expression of A. Notch receptors and their ligands, and B. Notch target genes in skeletal cells.

A.	Cells	Notch1	Notch2	Notch3	Notch4	Jag1	Jag2	Dll1	Dll3	Dll4
	Osteoblast	✓	✓	✓	✓	✓	ND	ND	ND	ND
	Osteocytes	✓	✓	✓	✓	✓	ND	ND	✓	✓
	Bone Marrow-derived Macrophages	✓	✓	✓	±	✓	ND	ND	ND	ND
	Osteoclast	✓	✓	ND	✓	✓	ND	ND	ND	ND

B.	Cells	*Hes1*	*Hes3*	*Hes5*	*Hey1*	*Hey2*	*HeyL*
	Osteoblasts	✓	ND	ND	✓	✓	✓
	Osteoclasts	✓	Low	Low	ND	ND	ND

✓= Detected by quantitative reverse transcription polymerase chain reaction

ND = Not detected

Table 3.

Genetic disorders associated with altered Notch signaling.

Disorder	Associated Gene Mutation	References
Hajdu Cheney Syndrome	*NOTCH2*	34, 35, 36, 37
Lateral Meningocele Syndrome	*NOTCH3*	52, 54
Brachydactyly	*CHSY1*	56, 57
Adams Oliver Syndrome	*NOTCH1, DLL4, CSL, EOGT*	59, 61, 62, 63
Alagille Syndrome	*JAG1, NOTCH2*	65, 66, 69, 70
Spondylocostal Dysostosis	*DLL3*	74
Spondylothoracic Dysostosis	*MESP2, LFNG, HES7*	76, 78, 79