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Review

The role of notch signaling in bone development and disease

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ABSTRACT

During the last decade a considerable amount of data have been accumulated regarding the role of intracellular signaling pathways in the pathogenesis of human diseases. One of these, Notch signaling, well known for its significance in cellular development and tissue morphogenesis, has been increasingly recognized as a crucial participant in the pathogenetic mechanisms underlying certain skeletal disorders. A better understanding of the biology and regulation of this multifaceted pathway is considered an important step towards clarification of the pathogenesis of various skeletal diseases and the development of novel targets for therapeutic purposes.

Key words: Bone, Cartilage, Notch signaling, Osteoblasts, Osteoclasts

INTRODUCTION

Physiological development of complex organisms is based on cellular coordination in space and time. This transcellular communication regulates cell growth, proliferation, survival, fate, differentiation and morphogenesis. Intercellular signaling pathways mediated by receptors of the Notch family have been shown to be involved in all of these processes in a wide variety of developmental and physiological contexts in many organisms including humans.[1] Notch mediates lateral inhibition and formation of boundaries, both of which represent patterning processes of critical importance in the regulation of spacing of different cell types within tissues.[2,3] Therefore, Notch signaling has been increasingly implicated in various developmental disorders and endocrine diseases in humans.[4]

In this review we outline current knowledge regarding the participation of Notch signaling and its regulators in the development of cartilage and bone and its implication in the pathogenesis of certain skeletal diseases.

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1. NOTCH SIGNALING AND ITS COMPONENTS

Notch signaling, an evolutionarily conserved system which is essential for normal embryonic development, participates in the regulation of tissue homeostasis and maintenance of stem cells in adults. Upon activation by specific surface transmembrane proteins, Notch regulates a variety of cell types during specification, patterning and morphogenesis through effects on

differentiation, proliferation, survival and apoptosis. Its multiple functions can be categorized into two main modalities: "lateral inhibition" and "boundary formation".[5]

During "lateral inhibition", Notch signaling, having mainly a permissive role, contributes to binary cell fate choices in populations of developmentally equivalent cells by inhibiting one of the fates in some cells and allowing them to later adopt an alternative one. Lateral inhibition is a crucial patterning process that often results in the regular spacing of different cell types within a field. During the establishment of the developmental boundary, Notch signaling may instruct the adoption of a third cell fate at the border of neighboring populations of different cell types.[6,7]

A family of four Notch receptors (Notch 1, Notch 2, Notch 3, Notch 4) and five classic DSL (Delta/Serrate/Lag-2) ligands named JAG-1 and 2 (Jagged 1 and 2), DLL-1 (Delta-like 1), DLL-3 (Delta-like 3) and DLL-4 (Delta-like 4) are the main components of the Notch pathway. Both ligands and receptors are single-pass transmembrane proteins that mediate interactions between neighboring cells.

In mammals, Notch receptors display both redundant and unique functions. The extracellular domain (NECD) is involved in the ligand binding, while the intracellular domain (NICD) constitutes the active part of the molecule. Although there are broad variations between the Notch family members, several major structural features are highly conserved (Figure 1).

The NECD of all Notch proteins contains 29-36 tandem epidermal growth factor (EGF)-like repeats with embedded ligand binding sites. The EGF repeats are followed by a unique negative regulatory region (NRR), which is composed of three cysteine-rich Lin12-NOTCH repeats (LNR) and a heterodimerization domain. The NRR prevents receptor activation in the absence of ligands. The intracelluar domain consists of four distinct regions, the RAM (RBPj association module) domain, seven ankyrin repeats (ANK domain), the transcriptional activator domain (TAD) and a C-terminal proline, glutamic acid, serine, threonine-rich (PEST) domain that contains degradation signals and regulates the stability of NICD. Two nuclear localization sequences (NLS) are situated before and after the ankyrin repeats (Figure 1).

Notch receptors are synthesized as single polypeptides in the endoplasmic reticulum. After translation, the Notch protein is fucosylated on certain EGF repeats by the GDP fucose protein O-fucosyltransferase. Fucosylation appears to be essential for Notch signaling events that require regulation by Fringe glycosyltransferases.[9,10] This modification in the Notch ligand-binding domain can determine which ligands can bind to activate the receptor.[11]

In the Golgi apparatus, Notch receptors are cleaved by furin-like convertases into two domains, the extra-

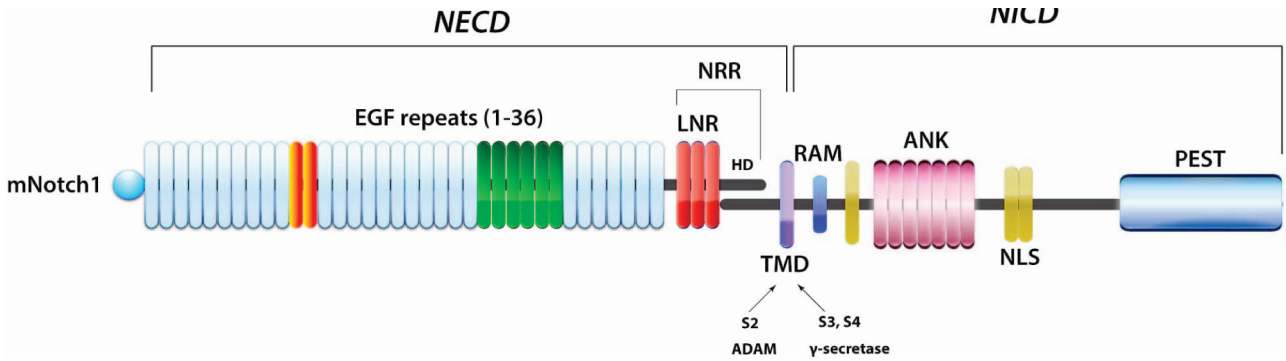

Figure 1. Structure of Notch receptors. Modified by Kopan et al.[8] The Notch receptor is a heterodimeric transmembrane protein composed of an extracellular domain and a transmembrane domain. The extracellular domain (NECD) is composed of EGF-like repeats and a nuclear regulatory region (NRR). NICD is composed of four domains (RAM, ANK, TAD and PEST) and two nuclear localization sequences. EGF: epidermal growth factor; LNR: Lin12-NOTCH; HD, heterodimerization domain; RAM: RBPj association module; ANK: ankyrin; TMD: transmembrane domain; NLS: nuclear localization sequences; PEST: proline, glutamic acid, serine, threonine-rich domain.

cellular and intracellular that form a NECD-NICD heterodimer held together by noncovalent bonds between the N- and C-terminal halves of the entire domain. This heterodimeric form is the one present in the cell membrane.

Notch ligands are also type I single-passage transmembrane proteins. The main class is characterized by three related structural motifs: an N-terminal DSL motif, specialized tandem EGF repeats and DELTA and OSM11-like proteins called the DOS domain. Both the DSL and DOS domains are involved in receptor binding. Proteins lacking DSL and DOS domains act as non-canonical ligands for Notch receptors (Table 1).[11,12]

2. NOTCH ACTIVATION AND TRANSCRIPTIONAL EFFECTS

Activation of Notch receptors is mediated by a sequence of proteolytic events.[12-15] Ligand binding leads to the cleavage of Notch by TACE (a TNF-a converting enzyme) of the ADAM (disintegrin and metalloprotease) family at site 2 (S2), which is located within the NRR of NECD. S2 cleavage is key regulatory step in Notch activation. The clipping of the extracellular domain creates a membrane-tethered intermediate called Notch extracellular truncation (NEXT). This intermediate is a substrate for γ-secretase, an intramembrane cleaving protease, which cleaves the truncate at sites 3 (S3) and 4 (S4). Gamma-secretase is composed of four membrane proteins: the catalytic component Presenilin 1 and Presenilin 2 and three limiting cofactors, Nicastrin, Pen2, and Aph1.[16] At this point the NICD is free to translocate into the nucleus.

Under basal conditions, the DNA-binding protein CSL (CBF1/Suppressor of Hairless/LAG-1), also known as Rbp-Jκ in mice, is bound to DNA and interacts with transcription co-repressors.

Table 1. Components and modifiers of the Notch pathway in mammals

Component function	Type	Effector
Receptor	Notch	Notch 1-4
Ligand	DLS/DOS	DLL-1, Jagged 1 and 2
	DSL only	DLL-3 and 4
	DOS co-ligands	DLK-1, DLK-2/EGFL9
	Non-canonical	DNER, MAGP-1 and 2, F3/Contactin 1, NB3/ Contactin 6
Nuclear effectors	CSL DNA-binding transcription factor	RBPjk/CBF-1
	Transcriptional co-activator	MAML1-3
	Trancriptional co-repressors	Mint/Sharp/SPEN, NcoR/SMRT, KyoT2
Receptor proteolysis	Furin convertase (site 1 cleavage)	PC5/6, Furin
	Metaloprotease (Site 2 clevage)	ADAM10/Kuzbanian, ADAM17/TACE
	γ-secretase (site 3/site 4 cleavage)	Presenilin 1 and 2, Nicastrin, APH-1a-c, PEN-2
Glycosyltranferase modifiers	O-fucosyl-transferase	POFUT-1
	O-glycosyl-transferase	
	β1,3-GlcNAc-tranferase	Lunatic, Manic and Radical Fringe
Endosomal sorting/ Membrane Trafficking Regulators	Ring Finger E3 Ubiquitin ligase (ligand endocytosis)	Mindbomb, Skeletrophin, Neutralized 1-2
	Ring Finger E3 Ubiquitin ligase (receptor endocytosis)	Deltex 1-4
	HECT Domain E3 Ubiquitin ligase (receptor endocytosis)	Nedd4, Itch/AIP4
	Negative regulator	Numb, Numb-like, ACBD3
	Neutralized inhibitors	
NICD Degradation	F-Box Ubiquitin ligase	Fbw-7/SEL-10
Canonical target bHLH repressor genes		HES/ESR/HEY

When NICD enters the nucleus, it is unable to bind DNA on its own but it interacts with CSL through its RAM domain.[11] The complex NICD-CSL recruits the mammalian co-activator MAML (Mastermind/Lag-3), which in turn displaces transcriptional co-repressors and recruits co-activators, such as the mediator transcription activation complex MED8 (Mediator of RNA polymerase II transcription subunit 8), thereby inducing up-regulation of downstream target genes (Figure 2). Transcription factors such as Hes (Hairy Enhancer of Split) 1, 5, 6 and 7 and Hey (Hes-related with YRPW motif) 1, 2 and Hey L are activated by canonical Notch signaling.

The nuclear environment that exists before the ar-

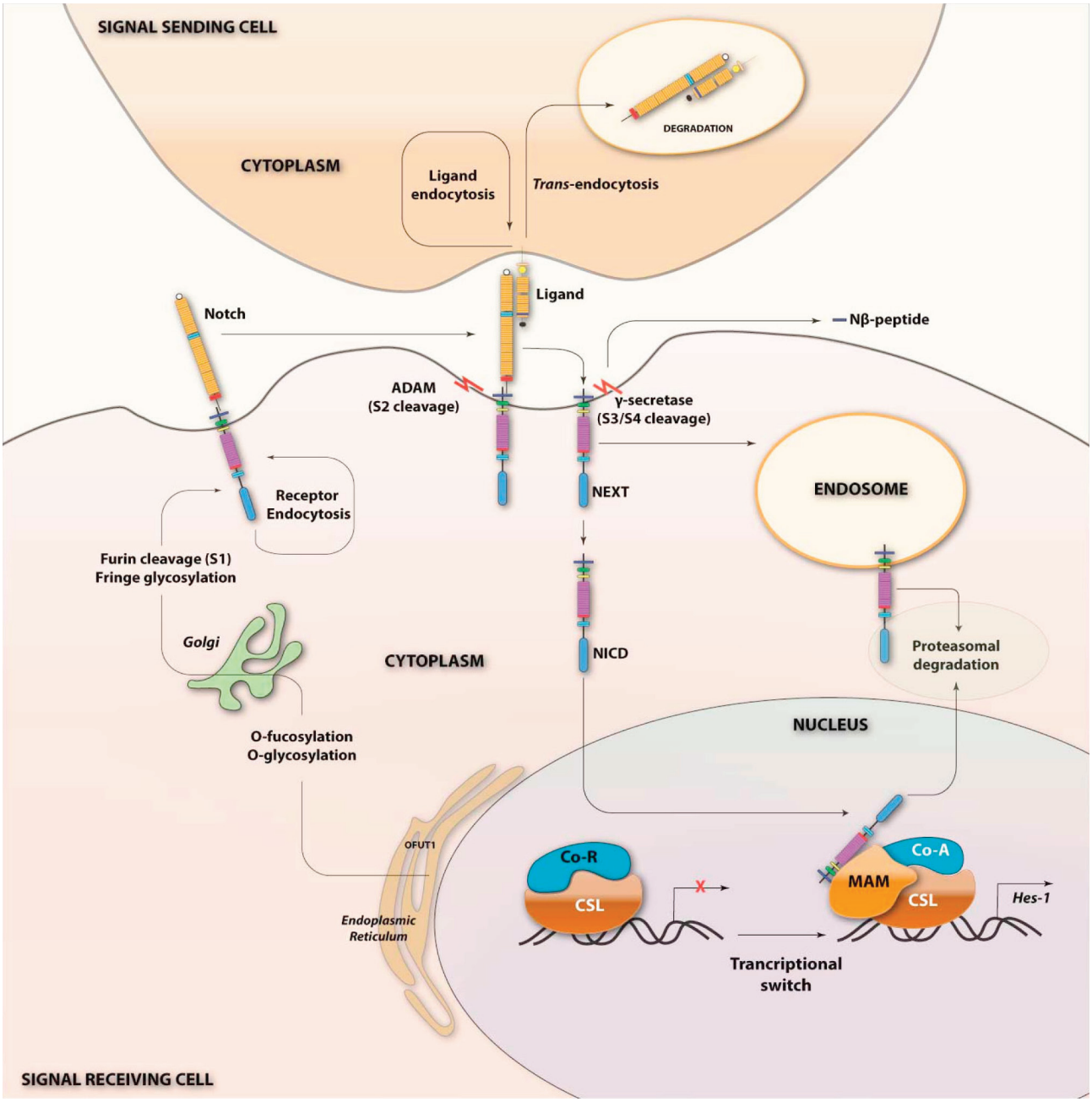

Figure 2. The Notch signaling pathway. NECD: NOTCH extracellular domain; NICD: NOTCH intracellular domain; NEXT: NOTCH extracellular truncated domain; CSL: CBF1/Suppressor of Hairless/LAG-1; MAML: Mastermind/Lag-3; Co-A: co-activators; Co-R: co-repressors; Nβ: short peptide released after cleavage at site 4.

rival of NICD will dictate which targets are available to CSL and thus can be activated by Notch (Figure 2). MAML is a potent, global and relatively specific inhibitor of Notch signaling. The tissue-specific target gene expression is controlled by the ability of different Notch paralogs to physically interact with diverse transcription factors bound with neighboring enhancers. Co-activators and co-repressors that are recruited during activation of Notch signaling are shared with other signaling pathways, and thus overexpression of NICD can affect the transcription of genes that are regulated by proteins outside the Notch pathway.

In humans, aberrant Notch signaling is associated with impaired development and disease. As recent knowledge uncovers the vulnerabilities in the intracellular pathways that lead to disease, we gain new insights into how we can restore the balance and achieve the desired biological outcome.

The Notch receptor is synthesized as a single transmembrane receptor that is glycosylated and undergoes proteolytic cleavage at site 1 (S1), yielding a bipartite heterodimeric receptor that is held together by noncovalent interaction and is expressed on the cell surface of a "signal- receiving cell". Activation begins when a ligand presented by the signal-sending cell interacts with the receptor. Conformational changes exposes site 2 (S2) in the Notch receptor for cleavage by ADAM metalloproteases, generating the membrane-anchored NEXT fragment, a substrate for the γ-secretase complex. Gamma-secretase then cleaves NEXT progressively from site 3 (S3) to site 4 (S4) to release the NICD and Nβ peptide. NICD then enters the nucleus where it associates with the CSL DNA-binding protein. The transcriptional co-activator MAML recognizes the NICD/CSL interface and this tri-protein complex recruits additional co-activators to activate transcription. In the absence of NICD, CSL may associate with ubiquitous co-repressor proteins and histone deacetylases to repress transcription of target genes (modified from Kopan et al).[8]

3. NOTCH SIGNALING IN BONE CELLS

During embryogenesis, the skeletal system, which is mainly comprised of the mesodermic tissues bone and cartilage, is formed by the coordinated action of chondrocytes and osteoblasts. In the adult skeleton, bone tissue is continuously regenerated by the coupled action of the bone-forming osteoblasts and the bone-resorbing osteoclasts.

Notch signaling has been extensively studied in the skeletal system and has emerged as an important regulator of skeletogenesis with important roles in chondrogenesis, osteoblastogenesis and osteoclastogenesis.

a) Notch signaling and chondrogenesis

Chondrogenesis is a process during which sequential aggregation, proliferation, differentiation and hypertrophy of chondrocytes provide the initial scaffold of the skeleton in vertebrates. The skeleton is divided into two parts: the *appendicular skeleton* that includes the pectoral girdle, the pelvic girdle and the upper and lower limbs, and the *axial skeleton* which consists of the skull, rib cage and vertebral column.[17] The vertebral column and rib cage develop from the mesenchymal sclerotome of the somites, whereas the appendicular skeleton develops from chondroosteoprogenitor (COP) cells in the limb buds. During embryonic development, mesenchymal progenitor (MPC) cells condense, differentiate, through a COP stage, into chondrocytes and form a cartilaginous scaffold, which subsequently is replaced by calcified bone through endochondral ossification.[18] The role of Notch signaling in chondrogenic commitment, proliferation, differentiation and maturation has just started to unfold and recent studies have shown that Notch acts on chondrogenesis through both CSL dependent and independent mechanisms (Figure 3).

In vivo,[19] it has been shown that regulation of Notch signaling is required for the appropriate balance of chondrogenic proliferation and differentiation at initial stages of somite compartmentalization and long bone development. During normal chondrogenic differentiation in endochondral bone formation, NICD is not expressed in the proliferative zone, but it is activated in prehypertrophic and hypertrophic cartilage. Increased NICD expression inhibits chondrocyte proliferation and prehypertrophic and hypertrophic chondrocyte differentiation, resulting in decreased bone formation. On the other hand, inhibition of Notch signaling in the chondrocyte lineage leads to increased proliferation and expansion of the hy-

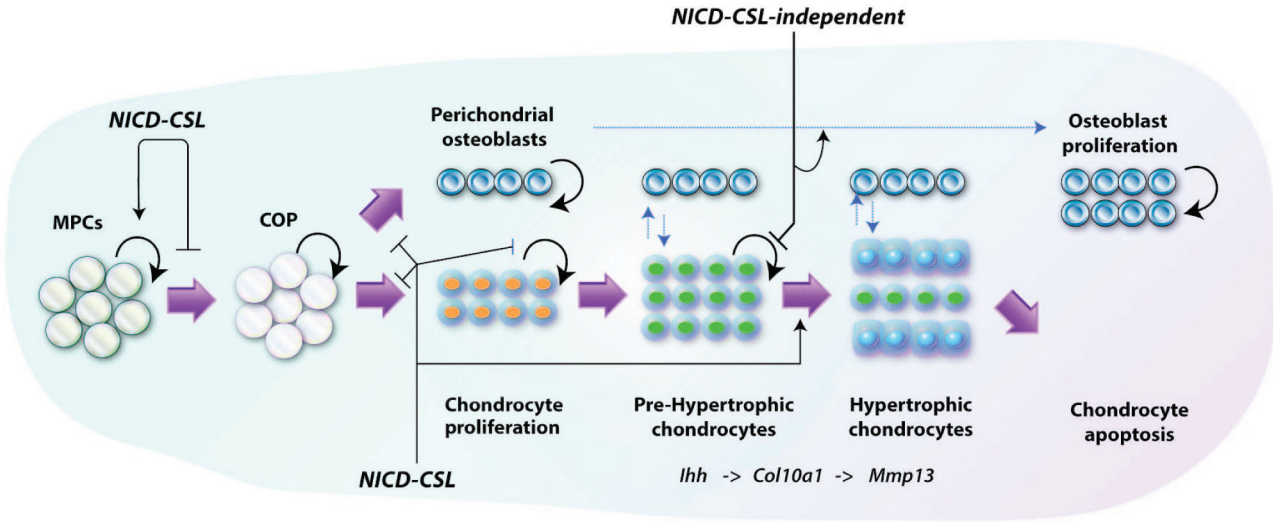

Figure 3. Notch signaling during cartilage cell proliferation and differentiation during development. Cartilage and bone development begin with a common precursor cell, the MPC. MPCs differentiate into COPs, which are lineage restricted and adopt the cell fate of either osteoblasts or chondrocytes. NICD: Notch intracellular domain; CSL: Epstein-Barr virus latency C promoter binding factor 1, suppressor of hairless and Lag-1; MPCs: mesoderm derived mesenchymal progenitor cells; COP: bipotential chondro-osteoprogenitor, Ihh: Indian hedgehog; Col1A1: Collagen 1A1; Mmp13: Matrix metalloproteinase 13.

pertrophic chondrocyte zone, which again results in decreased bone formation.[19] Conditional deletion of Notch 1 and 2 in the limb bud with the use of Prx1 (paired-related homeobox 1) enhancer, which is active from the 11th day of embryonic development and onwards,[20] leads to accumulation of hypertrophic chondrocytes and malformation of the growth plates and the skeleton.[21] Loss of Notch 2 expression alone results in a similar phenotype, this suggesting that Notch 2 is the predominant receptor in endochondral bone formation.[21]

At molecular level, Notch regulates the expression of sex determining region Y-box 9 (Sox9), which is considered a key transcriptional regulator of chondrocyte differentiation[22] and its target gene Crtl1 (cartilage link protein 1). Notch 1 has been found in proliferating chondrocytes in vitro.[23,24] Activation of Notch signaling and Notch-related transcription factors Hes1 and Hey1 suppresses chondrogenic differentiation by inhibiting the activity of collagen type 2 (Col2a1) promoter through binding in close proximity to the Sox9 enhancer,[19,24,25] while Hey7 suppresses Sox9 gene expression.[26] Moreover, down-regulation of Hey1 in mesenchymal progenitor cells increases the expression of chondrocyte gene markers.[19,24]

Recent data have also shown that cartilage-specific Notch signaling plays a significant role in the coordination of perichondrial osteoblast differentiation and bone formation, through CSL independent mechanisms, regulating the communication between chondrocytes and perichondrial osteoblasts and promoting chondrocyte proliferation and apoptosis. The latter effect is probably mediated by the Indian hedgehog (Ihh) signaling pathway.[27]

b. Notch and osteoblastogenesis

In osteoblasts, Notch signaling has been reported to either suppress or induce osteoblastic differentiation in vitro, depending on the cell line studied.

During osteoblastogenesis, precursors of osteoblasts, which like chondrocytes are derived from pluripotent mesenchymal cells, proliferate to expand, undergo maturation and, as mature cells, they mineralize.

In vivo studies have shown that activation of Notch signaling inhibits terminal differentiation of osteoblast progenitors, while it does not affect mature osteoblasts.[28,29] Using conditional activation of Notch signaling in cells of the osteoblastic lineage at various stages of differentiation and in osteocytes, it was found that Notch arrested differentiation of pre-osteoblasts, causing osteopenia, and when ex-

pressed in osteocytes suppressed bone resorption and increased bone volume.[30] Expression of NICD under the control of the 2.3-kb type I collagen promoter, which is a late marker of osteoblast differentiation showing expression in mature osteoblasts and osteocytes,[31,32] exhibited increased bone volume and growth retardation due to the deposition of woven bone by immature or dysfunctional osteoblasts.[29] In contrast, expression of NICD under the control of the 3.6-kb collagen type I promoter, which is active in early mesenchymal progenitors,[31,32] resulted in decreased bone volume that is secondary to a decrease in osteoblast number.[28] Differences in these two phenotypes can be explained by the arrest of osteoblastic cell differentiation at different stages of maturation.[31] On the other hand, Notch 1 overexpression under the control of the Prx1 promoter, which is a marker of early osteoblasts/mesenchymal cells, induced mesenchymal precursor cell proliferation and suppressed their differentiation, maintaining mesenchymal precursor cells in an undifferentiated state.[21]

In vitro it has been shown that Notch signaling suppresses osteoblastic differentiation through the inhibition of both early and late markers of differentiation such as collagen type 1, alkaline phosphatase, Runx-2 and osteocalcin.[28,33,34] By contrast, it has also been shown that activation of Notch signaling in MC3T3 cells, which represent early stages of osteoblast differentiation, stimulates osteoblast differentiation through the induction of calcific nodules, suggesting that the cell line studied and the cell culture condition used is important for exertion of the Notch-signaling effect on osteogenic gene induction.[35,36]

Activation or overexpression of Notch suppresses osteoblastic differentiation mainly through inhibition of Wnt/β-catenin signaling, which is also a critical regulator of osteoblastogenesis.[34,37] In contrast, transient induction of Notch signaling was found to enhance selected effects of bone morphogenetic proteins (BMPs) on osteoblastic cells.[35,36] These discrepancies could be due to differences between cell lines or culture conditions used to induce osteoblast differentiation. However, in the context of BMP stimulation, Notch appears to enhance the commitment of mesenchymal cells to the osteoblastic fate.[35,36]

At the molecular level, Notch enhances osteoblastic proliferation through the expression of the transcriptional factor Osterix and the cell-cycle related proteins Cyclins D and E and represses maturation by binding to Runt-related transcription factor 2 (Runx-2).[29] In addition, Notch inhibits the Wnt/β-catenin canonical signaling pathway, by phosphorylating β-catenin through activation of glycogen synthase 3 β (GSK3β) and thus promoting its ubiquitination by intracellular proteolytic systems.[28] In addition HES and HEY proteins appear to suppress osteoblastic differentiation, while HES1 interacts with Runx-2 to regulate osteocalcin and osteopontin promoter activity, suggesting certain additional functions (Figure 4).[33,38-40]

c. Notch and osteoclastogenesis

The bone-resorbing osteoclasts derive from hematopoietic cells of the macrophage-monocyte lineage and, coupled with the bone forming osteoblasts, maintain skeletal homeostasis. Key regulators of osteoclastogenesis are the osteoblast derived cytokines macrophage-colony- stimulating factor (M-CSF) and the receptor activator of nuclear factor kappa b ligand (RANKL).[41] RANKL activity is opposed by the soluble RANKL decoy receptor osteoprotegerin (OPG), which is also produced by osteoblasts.

In vitro and in vivo studies have shown that Notch signaling inhibits osteoclastogenesis both directly and indirectly through modulating the function of osteoblasts.

In vivo loss of function of Notch 1 and Notch 3 in osteoclasts increases the number of osteoclasts by stimulating cell proliferation.[42]

In in vitro studies, the constitutively active Notch 1 receptor reduces M-CSF expression and enhances RANKL/OPG expression thereby decreasing osteoclastogenesis in stromal cells.[43] Similarly, Jag-1 inhibits osteoclastogenesis in bone marrow macrophages.[42] When Notch 1 is inactivated in osteoblasts, RANKL expression is increased and OPG is decreased, leading to increased osteoclast formation.[42]

Although the majority of data indicate an inhibitory role of Notch signaling in osteoclastogenesis, two studies have questioned this notion. In one study, Notch 2 was reported to promote osteoclastogenesis through enhancement promoter activity and expression of nuclear factor of activated T-cells c1

(NFAT), demonstrating a new molecular cross talk.[44] In another study, transgenic male mice overexpressing Hes1 under the control of the 3.6 kb collagen type 1 (Col1a1) promoter became osteopenic due to increased osteoclast number and eroded surface.[45] Conversely, when Hes1 was inactivated in mature osteoblasts expressing osteocalcin, and Hes3 and Hes5 were globally ablated, trabecular bone volume was increased because of significant reductions in the number of osteoclasts (Figure 4).[45]

Collectively these data suggest that Notch signaling regulates multiple stages of osteoclastogenesis acting either as a stimulator or repressor of osteoclast formation and activity.

4. DEVELOPMENTAL DISORDERS AND BONE LOSS

Inherited or de novo mutations in components of the Notch signaling pathway can lead to developmental skeletal defects (Table 2).

Notch signaling is of critical importance for vertebrate evolution. It regulates the segmentation of paraxial mesoderm in the formation of somites, which are the precursors of the vertebrae, by boundary formation and is required for normal somite formation and vertebral column development in humans. Recessive mutations of Notch pathway genes involved in somitogenesis have been associated with four subtypes of spondylocostal dysostosis. Spondylocostal dysostosis comprises a heterogeneous group of axial skeletal disorders characterized by multiple segmentation defects of the vertebrae, malformation of the ribs with intercostal fusion and often reduction in the number of the ribs, with apparent physiologic appearance of the craniofacial skeleton and the limbs.[46]

Spondylocostal dysostosis type 1 is caused by mutation in the Notch ligand DLL-3 gene. Affected individuals present with multiple hemivertebrae, rib fusions and deletions with non-progressive kyphoscoliosis.[47] Mutations were reported to cause truncations

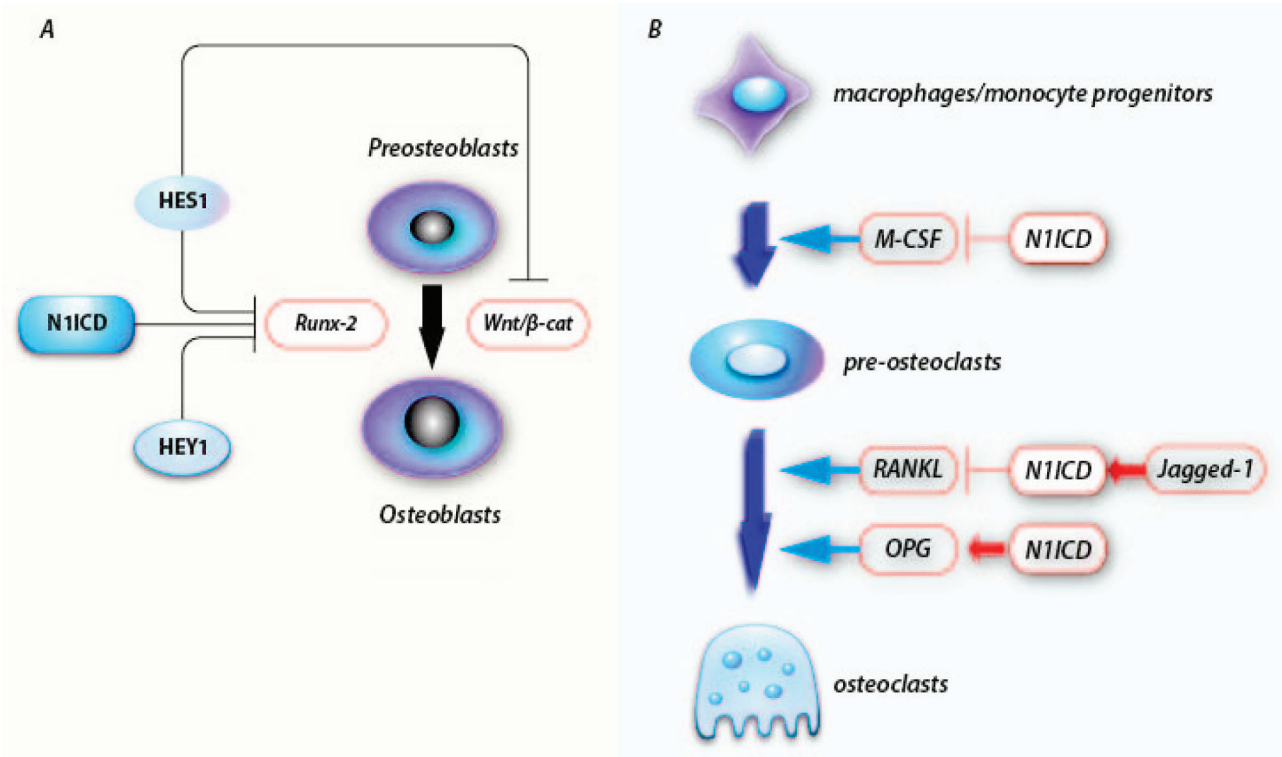

Figure 4. Notch signaling pathway regulation osteoblasts and osteoclasts. **A.** Regulation of osteoblast differentiation. N1ICD negatively regulates osteoblast differentiation through Hes1-mediated repression of Wnt/β-catenin signaling or through Hey1-mediated suppression of Run-2. **B.** Regulation of osteoclastogenesis. N1ICD inhibits MCSF and RANKL while activating OPG gene expression.

Table 2. Notch signaling and developmental diseases

Notch Component	Inheritance	Molecular Mechanism	Disease
Delta-like-3 (DLL-3) 19q13.2	autosomal recessive	Mutations on DLL-3 usually lead to expression of a truncated protein or to amino acid substitutions	Spondylocostal dysostosis type1 (trunk dwarfism secondary to rib anomalies and vertebral segmentation defects)
Mesoderm posterior 2 (MESP2) 15q26.1	autosomal recessive	Mutation in MESP2 produces a non-functional protein susceptible to nonsense-mediated RNA decay, up-regulating Notch signaling	Spondylocostal dysostosis type 2 (segmentation abnormalities of the thoracic vertebrae)
Lunatic Fringe (LFNG) 7p22	autosomal recessive	Missense mutations of LFNG up-regulates Notch signaling	Spondylocostal dysostosis type 3
Hairy Enhancer of Split 7 (HES7) 17p13.1	autosomal recessive	Missense mutations of HES7 up-regulates Notch signaling	Spondylocostal dysostosis type 4
Chondroitin sulfate synthase (CHSY)1*	autosomal recessive	Loss-of-function mutations lead to up-regulation of JAG-1	Recessive brachydactyly
JAG-1 (20p12.2), NOTCH 2 (1p12)	autosomal dominant	Heterozygous mutations	Alagille syndrome type 1 and type 2

*Encodes a transmembrane protein which contains a Fringe domain.

within conserved extracellular domains or affect the highly conserved glycine residue of the fifth EGF repeat of the gene, which reveals the important functional role of this domain.[48]

Spondylocostal dysostosis type 2 and the related disorder, spondylothoracic dysostosis, are caused by a loss-of-function mutation in the posterior 2 mesoderm (MESP2) gene, encoding a basic helix-loop-helix type transcription factor required for somite formation. Mesp2 is a target of Notch signaling and is directly involved in the somite-boundary formation and rostrocaudal patterning of each somite. Mesp2 knockout mice, like patients with spondylocostal dysostotosis, exhibit extensive malformations of the vertebrae and ribs.[49] In particular, the developing vertebral bodies in Mesp2-knockout mice are extensively fused showing rare insertions of intervertebral tissue, which are in turn longitudinally fused in the vertebral column. In addition, the differentiation of vertebral body chondrocytes was spatially disordered and delayed, demonstrating an increased cell proliferation rate that appears to associate with spatially impaired TGF-β and BMP signaling.[50]

Spondylocostal dysostosis type 3 is caused by a mutation of the Lunatic Fringe (LFNG) gene, which encodes a fucose-specific beta1,3-N-acetylglucosaminyltransferase.[51] LNFG modifies Notch receptors and alters Notch signaling activity.[52,53] The phenotypes of mouse embryos lacking DLL-3 and Lfng are virtually identical[54] and Lfng gene expression is severely disrupted in DLL-3 null embryos, showing that its expression is dependent on DLL-3 function.[55] A missense mutation causing spondylocostal dysostosis (type 4) was also identified in the DNA-binding domain of the HES7 protein which, apart from being a direct target of the Notch signaling pathway, participates in the negative feedback mechanism that attenuates Notch signaling.[56,57] The mutant HES7 is not able to repress gene expression by DNA binding or protein heterodimerization.

Alagille syndrome is an autosomal dominant disease characterized by cardiovascular defects, skeletal abnormalities, cholestatic liver disease and renal dysplastic anomalies (Table 3).[58]

It is associated with mutations of JAG-1, which leads to the expression of a truncated JAG-1 protein, although complete gene deletions and missense mutations are also described.[59] Rarely, mutations of Notch 2 have been found in patients with Alagille syndrome, either alone or together with mutations of JAG-1.[60] Dual heterozygous inactivations of Jag-1 and Notch 2 in mice are associated with most of the defects found in Alagille syndrome.[61] Moreover, selective inactivation of Jag-1 in cells of the cranial neural crest displays the abnormalities of the craniofacial skeleton of the syndrome,[62] confirming its association with impaired Notch signaling.

The most common skeletal finding is the "butterfly vertebrae" or sagittal cleft which is found in 33-87% of patients.[63] The affected vertebral bodies appear to be split into paired hemivertebrae, because of a failure of the fusion of the anterior arches of the vertebrae, and display a characteristic 'butterfly' appearance in radiographic images of vertebral spine.[63] Other skeletal abnormalities include narrowing of the interpedicular space in the lumbar spine, spina bifida occulta, fusion of the adjacent vertebrae, hemivertebrae, absence of the 12th rib, presence of a bony connection between the ribs and short fingers with broad thumbs.[64] Besides the craniofacial abnormalities, osteoporosis has been reported in patients with the disease but the exact mechanism is unknown, although liver failure and malnutrition may contribute.[65]

Genome-wide associations studies have also documented a relationship between JAG-1 polymorphisms and bone mineral density.[66]

Mutations in the Notch 2 receptor, which lead to premature termination of the protein product upstream of the PEST domain,[67] have been identified in the Hajdu-Cheney syndrome, which is characterized by focal areas of osteolysis and generalized osteoporosis (Table 4).[67]

The disease can be either sporadic, probably due to de novo mutations,[68,69] or inherited, transmitted with an autosomal dominant pattern. Since the PEST domain is responsible for ubiquitination and degradation of Notch in the proteasome, the mutations lead to increased expression of Notch 2 signaling. Although the skeletal abnormalities are severe, the mechanisms underlying the bone loss are largely unknown. Lesions in distal phalanges are osteolytic due to increased localized bone resorption, but the mechanisms responsible for the generalized osteoporosis remain elusive. The focal osteolysis is also accompanied by neovascularization, inflammation and fibrosis.[70] Iliac crest biopsies in these patients have shown decreased trabecular bone, normal or increased bone remodeling and normal or decreased bone formation.[71] Since Notch 2 induces osteoclastogenesis acting on osteoclast precursors, increased osteoclast-mediated bone resorption seem a plausible explanation for the observed lesions. Treatments with bisphosphonates and/or teriparatide did not show a clearly significant benefit.[72]

5. NOTCH SIGNALING AND BONE CANCER AND METASTASIS

As with Wnt and Hedgehog, the Notch signaling pathway regulates both development and tumorigenesis. In T-cell acute lymphoblastic leukemia, 50% of patients were shown to harbor activating mutations in Notch 1,[73] and it has also been implicated in the

Table 3. Characteristics of Alagille syndrome.

Craniofacial features	Skeletal features	Visceral manifestations
Craniosynostosis	Butterfly vertebrae	Bile duct atresia
Broad nasal bridge	Digit abnormalities	Cholestatic liver failure
Micrognathia -Pointed chin	Osteoporosis with fractures	Cardiovascular defects (Fallot tetralogy)
Prominent forehead	Short stature	Intracranial bleeding
Triangular facies		Renal failure
Deep set eyes		

Table 4. Characteristics of Hajdu-Cheney syndrome

Craniofacial features	Skeletal features	Systemic manifestations
Facial abnormalities	Acro-osteolysis	Cardiovascular defects
Micro- and retrognathism	Fibular deformities	Hearing loss
Periodontal disease -Tooth loss	Joint hyperlaxity	Neurological symptoms
Platysbasia	Osteoporosis with fractures	Polycystic kidneys
Open sutures	Wormian bones - Short stature	Delayed development

pathogenesis of other neoplasmatic diseases of the hematopoietic system, such as lymphoma and multiple myeloma.[74] In bone tissue, increased expression of JAG-1 and Notch 1 have been found in human osteosarcoma[75] and were associated with the invasive potential of the osteosarcoma cells.[76] In breast cancer, Notch 4 has been found to be hyperactive in breast cancer stem cells,[31] while Notch 3 and Jagged 1 appear to play a significant role in breast cancer stem cells skeletal invasiveness and osteolytic potential.[77,78] In addition, Notch signaling has been shown to regulate the epithelial-to-mesenchymal transition of tumor cells during cancer invasion. Several factors, such as transforming growth factor beta (TGF- β),[23,24] β-catenin and hypoxia, seem to participate in Notch-mediated regulations of bone metastasis. In particular, Jagged 1 released by tumor cells can activate the Notch pathway in pre-osteoclasts, increasing osteoclastogenesis and leading to severe osteolysis, while it also acts as a downstream mediator of TGF-β.[78] Delta-like 4 on the other hand, being up-regulated by VEGF, was shown to facilitate tumor-angiogenesis that is characterized by poor perfusion and increased hypoxia, resulting in blocking of tumor growth.[79]

Increasing understanding of the molecular mechanisms involved in Notch signaling mediated bone metastasis is opening up a new era in the quest for novel targets for anti-cancer therapy. Notch inhibitors, such as gamma secretase inhibitors that prevent the generation of the oncogenic (intracellular) domain of Notch molecules and suppress Notch activity, or monoclonal antibodies targeting Delta-like 4 or Jagged 1, hold promise for a potential therapeutic benefit for those tumors that harbor constitutively active Notch signaling. A Notch signaling inhibitor, MK0752, developed by Merck is currently being tested in a phase 1 clinical trial for patients with T-cell acute lymphocytic leukemia and advanced breast cancer.[80]

Future studies that will address puzzling issues, such as target specificity and possible side effects of the suppression of Notch activity, are a critical step forward for new target-based therapies for cancer.

6. CONCLUDING REMARKS

Despite the considerable expansion of Notch-related research over the past two decades, many issues concerning functionality and regulation of the pathway remain unanswered. The precise conditions of interaction between receptors and ligands and the exact core elements of the pathway are still not well defined, necessitating more intense genomic and proteomic approaches. Certain skeletal disorders in which the Notch signaling seems to play an important role in development, morphogenesis and morbidity figure among the most prominent of Notch-related diseases. A number of interventions are already underway and in the years to come Notch will undoubtedly be an important tool for understanding and treating many skeletal diseases.

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