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Alteration of Notch signaling in skeletal development and disease

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Abstract

Notch signaling is an evolutionarily conserved mechanism for specifying and regulating organogenesis and tissue renewal. Human and mouse genetic studies have demonstrated mutations in many components of the Notch signaling pathway that cause skeletal patterning defects. More recently, the *in vivo* effects of Notch signaling on osteoblast specification, proliferation, and differentiation have been demonstrated, in addition to its regulation of osteoclast activity. However, while our understanding of canonical Notch signaling in skeletal biology is rapidly evolving, the role of non-canonical Notch signaling is still poorly understood. In a pathological context, aberration of Notch signaling is also associated with osteosarcoma. These studies raise the question of how Notch may interact with other signaling pathways like Wnt. Finally, manipulation of Notch signaling for bone-related diseases remains complex because of the temporal and context dependent nature of Notch signaling during mesenchymal stem cell and osteoblast differentiation.

Keywords

Notch signaling; skeletal defects; osteoblast differentiation; non-canonical signaling; osteosarcoma; osteoclasts; chondrocytes

Introduction

The mammalian skeleton is functionally required for support and protection for vital organs and tissues including the brain, spinal cord, heart, lung, and hematopoietic cells, as well as for mineral homeostasis. To perform these functions, three parts of skeleton, i.e., the skull, the axial skeleton and the appendicular skeleton, have evolved during development. The progenitor cells forming the majority of the skull undergo intramembranous ossification and are derived from neural crest, whereas the ones forming the axial and appendicular skeleton undergo endochondral ossification and are derived from paraxial and lateral plate mesoderm1. Later, those progenitor cells develop into mesenchymal stem cells, which can give rise to a variety of cell types including adipocytes, chondrocytes and osteoblasts, contributing to the basic cellular content of bone tissues. In contrast, osteoclasts, the most important cell type for bone resorption, originate from bone marrow hematopoietic stem cells. Bone remodeling is a process specified by a balance between bone deposition by osteoblasts and bone resorption by osteoclasts. An imbalance in bone remodeling

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contributes to several pathological conditions including osteoporosis, osteosclerosis, and osteopetrosis.

Over the past three decades, discoveries in human genetics have led to the identification of genetic signaling pathways important for skeletal health and development. Not surprisingly, the human skeletal dysplasias that were first solved genetically were found to be structural mutations in the most abundant components of bone and/or cartilage matrix, i.e., the collagens. For example, the first cause for a human prototypical chondrodysplasia was identified in the gene encoding type II collagen (*COL2A1*)2. Often the correlation of a novel gene mutation with a human phenotype might point to a previously unappreciated function of that gene and the corresponding signaling pathway during development and/or homeostasis. In the case of the spondylocostal dysostoses (SCDO), inherited disorders characterized by abnormal vertebra formation and patterning, mutations first in delta-like 3 (Drosophila) (*DLL3*), and subsequently, in mesoderm posterior 2 homolog (mouse) (*MESP2*), and Lunatic Fringe, a O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase (*LFNG*), linked Notch signaling to the pathogenesis of these diseases3. While this work first correlated with the known developmental function of this signaling pathway in somitogenesis, and hence vertebral segmentation, it brought to light potential other functions for example during bone homeostasis and formation.

Notch signaling is one of several evolutionarily conserved signaling pathways in the development of multi-cellular organisms. Its temporal-spatial expression effects can specify diverse cellular events, including proliferation, differentiation, apoptosis, stem cell maintenance, and binary cell-fate specification. Physiological functions of Notch have been studied in organogenesis and tissue renewal in many different systems such as muscle, nerve, kidney, skin, circulatory system including blood vessels and cells4. In mammals, there are four Notch receptors (Notch1-4), and eleven ligands (Jag1-2; Dll1,-3,-4; DLK1-2; MAGP1-2; DNER and NB3)5. Notch signaling requires cell-cell contacts through physical interaction between ligand and receptor, both of which are membrane-bound proteins. Notch activation also requires proteases that liberate the Notch intracellular domain (NICD), which traffics to the nucleus to form an active transcription complex with nuclear partner proteins such as RBPJ and MAML1,2,3 (Figure 1). The mode of its transduction relies on a “on” and “off” pulse signal without any amplification. Additionally NICD has a short lifetime. These characteristics enable the Notch signal to be delivered between cells in a high-fidelity manner. The amplitude and duration of Notch signals are tightly modulated by the availability of ligands, receptors and proteolytic enzymes in the membrane and the rate of NICD degradation in the nucleus. The detailed mechanism of Notch activation through the canonical pathway has been reviewed in recent papers5. In contrast, the non-canonical Notch signaling pathway is poorly understood in mammals. The majority of recent advances have focused on the role of canonical Notch signaling in osteoblasts and osteoclasts.

While clearly important for developmental patterning, a homeostatic function for Notch postnatally has also been appreciated. Aberrant Notch signaling plays an important role in pathogenesis of leukemia and several other types of cancer. Intensive studies have indentified novel activating mutations in Notch1 receptor, which are responsible for more than 50% of human T-cell acute lymphoblastic leukemia (T-ALL) samples6. Notably, Notch may function as an oncogene in certain tissues such as intestine, cervix and mammary gland, but as a tumor suppressor in others such as skin. Clinical treatment using small-molecule inhibitors of the Notch proteolytic enzymes proved to be promising in T-ALL. Therefore manipulation of Notch signaling has also emerged as a therapeutic approach in leukemia and other cancers.

Human and mouse skeletal phenotypes due to mutations in the Notch signaling pathway

Understanding of genotype-phenotype correlation in Notch signaling began in Drosophila in 1917 when a phenotype characterized by a serration at the ends of Drosophila wings was linked to a gene called *Notch*7. After 68 years this gene was identified and its human ortholog *NOTCH1* was also indentified for causing a malignant blood disease, T-ALL8,9. During the last two decades, the number of components in mammalian Notch signaling pathway has increased to more than fifty, with their functions classified into five groups. They include Notch receptors, ligands, effectors, bHLH targets, and enzymatic modifiers (Table 1). Human genetic studies showed that some of those components are associated with human developmental disorders or diseases in diverse tissues such as skeleton, heart, brain and CNS, liver, blood vessel, and blood.

There are four mammalian paralogs of Notch receptor that have been associated with four different types of human diseases: T-ALL and familial aortic valve disease (NOTCH1), Alagille syndrome (NOTCH2), CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) (NOTCH3), Schizophrenia (NOTCH4)6. Not surprisingly, locus heterogeneity in some of these phenotypes has been linked to other components of Notch signaling. For example, Alagille syndrome is caused by not only the NOTCH2 receptor, but also the Notch ligand JAG1. Spondylocostal dysostosis (SCDO) is caused by mutations in different Notch signaling components, including the ligand (DLL3), the enzymatic modifier (LFNG), the bHLH targets (MESP2 and HES7)3. Those findings suggest that the ability of different components to interact are not equal, but instead cell-type specific.

Aberrant Notch signaling is responsible for human skeletal defects in Spondylocostal dysostosis (SCDO) and Alagille syndrome. Homozygous mutations in the human DLL3 gene cause axial skeletal defects in autosomal recessive SCDO type 1 (SCDO1); this is characterized by abnormal vertebrate segmentation (AVS) throughout the vertebrate column and by irregularly aligned ribs with variable points of fusion and deletion. The abnormal hemivertebrae in these patient have smooth, rounded outlines – a radiological appearance, termed as “pebble beach” sign10. In vertebral development, Notch signaling is active in a narrow domain of cells at the anterior portions of the presomitic mesoderm. Once DLL3 is lost, this domain is expanded, resulting in the formation of irregular somites that lead to formation of abnormal vertebrae3. Like *DLL3*, mutations in *MESP2, LFNG*, and *HES7* genes cause SCDO2, 3 and 4, respectively, all of which have similar vertebral anomalies. Although these four genes that cause SCDO belong to three distinct functional groups in the Notch signaling pathway, all of them are expressed in the presomitic mesoderm at the same time. Several lines of evidence suggested a regulatory network forming between them and Notch receptors, in which the ligand DLL3, unlike DLL1, is an *in cis* inhibitor of Notch signaling and physiologically localized to the Golgi; LFNG modifies NOTCH1 receptor in Golgi and derepresses Notch signaling in presomitic mesoderm; the bHLH transcription factor MESP2 and HES7 are both direct targets of Notch receptors, and MESP2 activates transcription of *LFNG* and Ripply2 homolog (zebrafish), *RIPPLY2,* while HES7 represses expression of itself and LFNG3. Future work on elucidating the genetic interactions between these components will show whether this network is functionally relevant *in vivo*.

On the other hand, Alagille syndrome is an autosomal dominant disorder characterized by abnormalities in multiple organ systems including the liver, eyes, kidney and skeleton. The primary skeletal defects are variable and include butterfly vertebrae, or sometimes hemivertebrae, but much less severe than in SCDO. Mutations in *JAG1* gene were identified in more than 90% of patients, while around 6% of patients harbored mutations in the *NOTCH2* gene. Current data support that haploinsufficiency of JAG1 is a major cause of Alagille syndrome, but a dominant negative effect of soluble, mutant forms of JAG1 has not

been ruled out. In summary, six components in the Notch pathway have been associated with human skeletal disorders. It is worthwhile noting that other components in the Notch signaling pathway remain excellent candidate genes for contributing to a broad spectrum of human skeletal patterning defects.

Recent studies on skeletal phenotypes of mutant mouse models for Notch signaling have further broadened our view of the physiological functions of Notch in development and also provided insights into the pathogenesis of Notch-related human diseases. *Dll3* knockout mice or *pudgy* homozygous mice display pure axial skeletal defects that strongly resemble those seen in SCDO1 patients. Similar skeletal defects were later found in *Lfng, Mesp2* and *Hes7* knockout mice, leading to the recent identification of disease causing mutations in the orthologous genes in SCDO patients. Intriguingly, *Psen1* knockout newborn mice have skeletal defects, though mutations in human have not yet been associated with any skeletal phenotypes. *Jag1* and *Notch2* knockout mice are lethal by E11.5, prior to skeletal development. However, double heterozygous mice of Jag1 and Notch2 display multi-system defects that bear a resemblance to human Alagille syndrome, suggesting that Jag1 and Notch2 are linked through ligand and receptor interaction during skeletal development. Although defects in somitogenesis have been found in knockout mice of many genes including *Notch1, Dll1, Rbpj*, and *Psen1*, they are often lethal between E9.5 and E12 prior to skeletal formation. Hence, while the late effects on vertebral formation might be appreciated from early effects of somitogenesis and boundary formation, later effects on skeletal cell homeostasis have been relatively unstudied. The later function of Notch signaling has since emerged with the use of conditional and tissue-specific gain of function and loss of function mouse models.

Cell autonomous & non-cell autonomous Notch function in bone development and homeostasis

Recent *in vivo* studies identified crucial roles of Notch in osteoblastogenesis. In a loss-of-function study, Psen1 and Psen2 were removed by crossing with Prx-Cre mice (Prx1-Cre; $Psen1^{f/f}Psen2^{-/-}$, PPS mutants) to abolish functions of all the four Notch receptors in limb and calvarial mesenchymal and osteoblast-lineage cells11. The PPS adolescent mice had a high bone mass phenotype with decrease in mesenchymal stem cell (MSC) number. With aging, these mice developed severe osteopenia due to reduced active osteoblasts and increased bone resorption. Selective deletion of Notch1 and Notch2 receptors via Prx-Cre mice (Prx1-Cre; $Notch1^{-/f}Notch2^{f/f}$, PNN mutants) resembled the postnatal skeletal phenotypes of PPS mice, indicating that loss of Notch function was responsible for the bone phenotypes in both settings. In a complementary study, Notch gain-of-function mouse model was generated by expressing Notch1 ICD (NICD) under the control of 3.6 kb Col1a1 promoter driven in MSCs and preosteoblasts12. These transgenic mice died within 4 weeks and had low bone mass or severe osteopenia due to a decreased number of pre-osteoblasts and mature osteoblasts, suggesting that Notch inhibits osteoblast differentiation from bone marrow MSCs. Together, these studies show that during the early stages of osteogenesis Notch signaling in the mesenchymal stem cell (MSC) maintains its self-renewal potential and inhibits osteoblast cell fate commitment. Upon loss of Notch signaling, these cells adopt an osteoblastic fate (Figure 2).

An important pathological role of Notch in late stages of osteoblastogenesis was demonstrated by our recent study, in which activated Notch1 ICD (NICD) driven by 2.3 kb Col1a1 promoter was selectively expressed in committed osteoblasts13. These transgenic mice displayed a severe osteosclerotic phenotype with a dramatic increase in osteoblast number, proliferation and formation. Histological analyses of these mice indicated highly disorganized woven bone formation suggesting a maturation defect in committed osteoblastic precursors. Notably, the differential activation of the 2.3- and 3.6-kb fragments

of the type I collagen promoter contributes to the differences in their phenotypes and the arrest of osteoblastic cell differentiation at different stages. Thus, our study suggested that NICD over-expression later in osteoblastogenesis repressed the terminal differentiation of osteoblasts and promoted the proliferation of early osteoblastic cells. To study the physiological role of Notch in late osteoblastogenesis, we generated loss-of-function mouse models in committed osteoblasts by deleting *Psen1* and *Psen2* using Col1a12.3kb-Cre transgenic mice (Col1a1-Cre; $Psen1^{f/f}Psen2^{-/-}$, DKO mutants) or deletion of Notch1 and Notch2 receptors via Col1a12.3kb-Cre mice (Col1a1-Cre; $Notch1^{-/f}Notch2^{f/f}$, C1NN mutants)11,13. These mice were viable and did not show any obvious skeletal phenotypes at two months old. It is possible that lack of bone phenotypes may be due to incomplete deletion of targeted genes at least in C1NN mice. Thus, other osteoblast-specific Cre mice are worthy of further investigation for the developmental functions of Notch. In contrast, aged DKO mutant mice started to show a significant low bone mass phenotype due to increased osteoclastogenesis mediated by decreased Osteoprotegerin (OPG) levels. This indicated that Notch signaling may regulate formation of mature osteoclast in a non-cell autonomous manner through control of OPG regulation expression (Figure 2). However, *in vitro* studies have also shown that osteoclastogenesis can be regulated negatively by Notch signaling in a cell autonomous process14. Here, inhibition of Notch signaling in osteoclast precursors led to increased osteoclast differentiation. Hence, both cell autonomous and non cell autonomous processes may specify Notch dependent regulation of osteoclast differentiation.

Recent data suggest that osteoblast can directly regulate hematopoietic stem cells (HSCs) in a Notch dependent manner. For example, up-regulated expression of Jag1 in osteoblasts resulted from over-expression of PTH1R and this led to an increase of HSC number and activation of Notch1 receptor in these cells15. In this situation, the osteoblast is acting as a "sending" cell presumably to the HSC which acts as the Notch "receiving" cell. However genetic removal of canonical Notch signaling within the HSC population suggested that this "receiving" activity might be dispensable for HSC function *in vivo*16. This implies that osteoblasts may require other cellular components within the bone marrow microenvironment such as stromal cells, osteoclasts, and/or endothelial cells in order to regulate the HSCs. It is also possible that when interacting with these cells osteoblasts may act as both sender and receiver cells in term of Notch signaling. Furthermore, since osteoclastogenesis can be regulated by Notch in a non-cell autonomous manner it raises the possibility that HSCs can also be regulated by osteoblast in the same fashion. It will be of great interest in the future to study whether gain- and loss-of-function of Notch receptors and Presenilins in osteoblasts can alter HSC phenotypes.

Evidence of canonical vs. non-canonical signaling in Drosophila and mammals

In both Drosophila and mammals, canonical Notch signaling requires the DNA binding protein RBPJ/Su(H) as a nuclear effector for signal transduction. In this pathway, binding of transmembrane Notch receptors (one in Drosophila and four in mammals) to its ligands triggers ADAM10- and Presenilin-mediated proteolytic cleavages that then liberate the membrane-bound Notch intracellular domain (NICD), which eventually translocates into the nucleus. NICD forms a transcriptional complex with the nuclear factor RBPJ and co-activator MAML to regulate transcription of downstream genes, such as HES1 and HEY1, the two classic bHLH targets of Notch (Figure 1).

Emerging data support the notion that non-canonical signaling or RBPJ/Su(H)-independent Notch signaling also occurs in Drosophila and mammals. The first evidence came from the loss-of-function studies In Drosophila where loss of the Notch receptor caused stronger phenotypes in embryos than the one caused by loss of *Suppressor of Hairless* (*Su(H)* (the *RBPJ* ortholog in Drosophila). Gain-of-function of Notch in sense organ precursor (SOP)

cells prevented SOP differentiation and this phenotype could not be rescued by removal of *Su(H)* function17. Mechanistically, the mediator(s) of RPBJ/Su(H)-independent Notch signaling are not well defined although several proteins were identified to physically interact with Notch extracellular domain (NECD) or NICD 18. One of them is deltex, a cytoplasmic adaptor protein, which may mediate the RBPJ/Su(H)-independent signaling17.

Concurrently, in mammals, a handful of *in vitro* cell culture studies support that Notch non-canonical signaling exists in different cell types19-28. In the C2C12 myoblast cells, a RBPJ-independent signal could block both myogenesis and osteogenesis26. In CHO cells, Notch could activate integrin via the small GTPase R-Ras independent of RBPJ transcription27. In T cell, osteoclasts, and cervical cancer cell lines, NICD could interact with components in the NF-kB pathway in cytoplasm and nucleus and influence expression of NF-kB target genes20,21,23,24. In Jurkat cells, high level of NICD could interact with LEF1 and activated expression of non-canonical Wnt targets25. Together, these RBPJ-independent signals may mirror direct interactions of NICD with either cytoplasmic proteins or nuclear transcription factors, facilitating the cross talk between Notch and other signaling pathways (Figure 2).

Genetic evidence of RBPJ-independent signaling in mammals has also been reported recently. Here, Notch loss-of-function in the skin was generated from either deletion of all Notch receptors (Msx2-Cre; Psen1$^{flox/flox}$; Psen2$^{-/-}$; PSDCKO mice) or Notch1 and Notch2 (Msx2-Cre; Notch1$^{flox/flox}$; Notch2$^{flox/flox}$; N1N2CKO mice) or deletion Rbpj (Msx2-Cre; Rbpj $^{flox/flox}$; RBPJCKO). As a result, PSDCKO and N1N2CKO mice had much more severe phenotypes than that of RBPJCKO mice29. Finally, these reports suggest that similar discovery of non-canonical Notch signaling may be revealed in other organ systems in the future.

Osteosarcoma and Notch signaling

Osteosarcoma (OS), the most common form of bone tumor, is the second highest cause of cancer-related death after leukemia in children. OS has two peaks of incidence either in adolescents aged 15-19 years or in adults age 60 years and above30. The cure rates of OS patients are poor (as low as 20% survival depending on type), mostly due to unknown etiology and metastatic potential. The effective treatments are limited to a combination of limb-salvage surgery and chemotherapy. Most OS tumors localize at the distal femur, proximal tibia and proximal humerus. Histologically, OS can be divided into at least four subtypes including conventional osteoblastic OS (70%), chondroblastic OS (10%), fibroblastic OS (10%), and other OS types (10%)31. Osteoblastic OS tumor cells can make osteoid and are highly proliferative malignant osteoblast cells, which express early differentiation gene makers such as alkaline phosphotase (ALP), runt-related transcription factor 2 (RUNX2), osterix (OSX), but not the late marker, osteocalcin (OCN). Although the origin of OS tumor cells is unknown in human, recent studies showed that either murine mesenchymal stem cells (MSCs), or pre-osteoblasts, or immature osteoblasts could differentiate into OS tumor cells32-34 (Figure 2). It is still unknown whether mature osteoblasts can be de-differentiated into OS cells *in vivo*.

Because most OS occur sporadically, our understanding of the molecular basis of OS is still limited. However, a small percentage of OS patients are linked to familial cancer syndromes including hereditary retinoblastoma, Li- Fraumeni syndrome, Rothmund-Thomson syndrome, and Werner syndrome. Genes that are mutated in those syndromes including *RB, p53*, and *RECQ* helicase and some mutations have also been reported in sporadic OS. This suggests that these genes may be involved in initiation and/or progression of OS tumor. Recently, a mouse model for human OS has been established33. In this model, osteoblast-restricted deletion of p53 and pRb developed murine OS, which recapitulates the defining

features of human OS including cytogenetic complexity, gene expression signatures, histology, and metastatic behavior. This model provides a tool for understanding the molecular basis of osteosarcoma and for developing novel therapeutic strategies.

Dysregulation of several evolutionarily conserved signaling pathways in OS tumor cells has been repeatedly found in human OS tumor samples and cell lines. These signaling pathways include Wnt, Tgf/Bmp, Hedgehog(Hh), and Fgf31. For example, aberrant activation of Wnt signaling marked by elevated nuclear β-catenin has been detected in the majority of OS tumors and sporadic mutations of β-catenin have also been identified. Recently, our study and other groups both suggest that activation of Notch signaling contributes to the pathogenesis of human OS and its inhibition may provide a therapeutic treatment for OS or related mesenchymal tumors35,36. Notwithstanding those components in major signaling pathways, aberrant expression of many other genes has been associated with human OS and eventually can be applied for the diagnostic and prognostic markers 30. Notably, the current studies on OS were mostly carried out using human OS cell lines and/or OS tumors that are diagnosed at a late stage which have already accumulated complex molecular cytogenetic alterations. In the future, studying OS in genetically engineered mouse models will provide us clearer understanding of molecular pathogenesis of human osteosarcoma.

The interaction of Notch and Wnt signaling pathways

It is becoming clear that bone formation and homeostasis require at least six major universal signaling transduction pathways including Notch, Wnt, Tgf/Bmp, Hh, steroid hormone receptor (SHR), and receptor tyrosine kinase (RTK). However, it is less understood how Notch is interrelated to these other five signaling pathways in the context of bone formation in despite of data supporting this in Drosophila and mammals15,37-40. An interrelationship between Notch and Wnt in the context of osteoblastogenesis, is supported by our and others' studies that show both are essential regulators in the development and the maintenance of bone structure and function. In mammals, Wnt signaling pathway is composed of 19 Wnt ligands, 10 Frizzled receptors, 2 co-receptors (Lrp5 and Lrp6), extracellular antagonists such as Dkks (four genes), sFRPs (eight genes), and SOST, and more than 10 downstream effectors including cytoplasmic Dvl, GSK3-beta, AXIN, APC, beta-catenin and nuclear factor LEF1/TCF1. Since 2001, many genetic studies have found that loss-of -function mutations in the co-receptor genes of *Lrp5* and *Lrp6* cause early onset osteoporosis. In contrast to this, loss-of-function of the receptor antagonist *Sost* causes sclerosteosis, and gain-of-function mutations of Lrp5 cause high bone mass phenotypes41,42. Meanwhile, a variety of loss- and gain-of function mouse models have been generated to mimic Wnt-related diseases in human and they have been used for uncovering the molecular mechanisms of Wnt signaling43. Within the canonical Wnt signaling pathway, loss-of-function of the main components such as Lrp5, Lrp6, Wnt10b, Wnt7b, Wnt5a, Wnt3a, Dkk2, beta-catenin, Tcf1 decreased bone mass; this is consistent with the low bone mass phenotype found in the gain-of-function of antagonists such as DKK1 and SOST. Increased bone mass was found in loss-of-function of genes such as Dkk1, SOST, sFRP1, GSK3beta, Axin2, and in gain-of-function of genes such as Lrp5, Lrp6, Wnt10b, beta-catenin. More recently, non-canonical Wnt pathways were shown to play a role at different stages of bone formation. For example, Wnt5a induced expression of Runx2 in an osteoblastic cell lineage through Ca^{2+}-CaMKII–TAK1–TAB2–NLK signaling44. Wnt7b promoted bone formation through G protein-linked PKCdelta activation45. Together, those studies underscore the importance of Wnt signaling as a major regulator of bone formation.

Increasing evidence support that Wnt and Notch signaling interact with each other in both synergistic and antagonistic manners. This interaction exists throughout development and continues in a homeostatic context in many systems39. For instance, the synergistic interaction has been studied in the development of Drosophila wing, sea urchin germ layer,

and mammalian skin and somites. However interactions in bone have yet been reported. In the “Wnt on-Notch on” model, a positive feedback loop is formed as Wnt activates Notch ligand JAGs or DLLs, which in turn promote expression of Wnts. At early stages of osteoblastogenesis, both Notch and Wnt signaling are required to maintain the pool of MSCs and proliferative pre-osteoblasts. Inactivation of either beta-catenin or Notch1 and Notch2 genes in mice using Prx1-cre led to decreased numbers of MSCs and osteoblasts11,46. At later stages, inactivation of β-catenin genes using Col1a12.3 kb-Cre transgenic mouse caused osteopenia and increased osteoclastogenesis mediated by decreased ratio of OPG/RANKL47. This cell non-autonomous phenotype mimics that of Notch DKO and C1NN mice, as discussed earlier11,13. Additionally, gain of function of β-catenin or Wnt10b in mature osteoblasts resulted in high bone mass, which was seen in our NICD transgenic mice47,48. Together, in these contexts, Notch and Wnt may mutually regulate each other to synergize their functions in osteoblasts. On the other hand, their antagonistic interactions have been studied in the specification of precursors of nervous and muscle systems in Drosophila. In this “Notch on-Wnt off” model, phenotypes caused by gain-of- function mutations in Notch were partially rescued by gain-of-function Wnt signaling. One explanation for this is that physical interactions may occur between components of Notch and Wnt signaling. As an example, during osteoblastogenesis high Wnt and low Notch signaling are required for differentiation of MSCs to pre-osteoblast and to mature osteoblasts, suggesting that high Wnt signaling may repress expression of Notch. Moreover, the interaction between Wnt and Notch have been reported in many *in vitro* systems; however, significant *in vivo* interactions in the skeletal system will required detailed genetic analyses using both cell-type specific and temporally regulated loss of function and gain of function models.

Pharmacological targeting of Notch signaling

Finding novel targets for pharmacological intervention will benefit patients with osteoporosis and osteosclerosis/osteopetrosis. Since Notch signaling has a strong effect on bone homeostasis, it is tempting to modulate this signaling pathway by means of molecular agonists or antagonists. In theory, the components of Notch signaling could be targeted at many levels. These include the production of Notch receptors, ligand-receptor binding, proteolytic cleavage (S2 and S3 sites), endocytosis and NICD cytoplasmic transportation, interaction with nuclear partners, and degradation of NICD. In practice, many strategies have been experimentally employed to inhibit or activate Notch signaling in the last decade. A list of them include recombinant forms of soluble ligands and Notch ectodomain, monoclonal antibodies, RNA interference, dominant negative forms of RBPJ and MAML1, and enzymatic inhibitors49-52 (Table 2). Experimental and clinical approaches for modulating NOTCH signaling has been most successful when targeting the gamma-secretases, PSEN 1 and 2. A large amounts of data have been generated on small-molecular inhibitors of the gamma-secretase complex (GSIs), which were originally developed to treat Alzheimer disease and now for T-ALL, intestine tumors, stroke, and autoimmune encephalomyelitis6. Although GSIs present a promising therapeutic approach and as synthetic inhibitors have advanced into late-phase clinical trials, a major concern remains because of their off target effects including acting on other substrates such as E-Cadherin and ERBB449.

Notably, decrease of bone synthesis by the loss of gamma-secretase activity resembles the effects of loss of Notch function. In practice GSIs could be used to treat Notch gain-of-function diseases, and potential side-effects in bone should be assessed. In the case of osteosarcomas, this might be a direct effect and we have shown potential efficacy in a heterotopic graft model in nude mice35. However, in the case of low and high bone mass phenotypes, the temporal pattern of inhibition may ultimately affect the consequences of

treatment. On the one hand, transient use of GSIs might lead to increased bone formation by increasing the number of pre-osteoblasts by promoting differentiation of MSCs into the osteoblastic lineage. In this context, its use may be of benefit in bone healing. On the other hand, continuous usage of GSIs may cause bone loss since they can increase the activity of osteoclasts by inhibiting function of mature osteoblasts11. Thus, for the purpose of treating osteoporosis, whether enhancing and/or inhibiting Notch signaling is beneficial will depend on when and where the treatment occurs13.

In cell culture systems, artificial increase of Notch signaling has been effectively achieved by several approaches including expression of the active NICD fragment, use of immobilized ligand proteins (JAG1-Fc, DLL1-Fc), production of activating antibodies against Notch, and EDTA (relieving of Ca^{2+} inhibition)37 (Table 2). Though these approaches are still far from therapeutic application, they warrant further investigation.

Conclusion

This review has highlighted the recent findings implicating Notch signaling in multiple aspects of skeletal development and bone homeostasis. It has been plainly appreciated that mutations in Notch signaling components can directly cause human skeletal disease and Notch signaling is required for osteoblast differentiation and osteoclast activity. However, our understanding of the role of canonical vs. non-canonical Notch signaling in skeletal biology is at best in its infancy. How upstream regulators and downstream targets mediate Notch function in the skeleton is still unknown. Similarly, how large a role Notch activation plays in bone cancer remains to be tested though this might offer an important new avenue of treatment since osteosarcoma lacks specific treatments. How Notch signaling is integrated within other signaling pathways and especially Wnt signaling will be an important topic of future research given the similarity of bone phenotypes in both systems. Finally, the proportional contributions of canonical vs. non-canonical versions of these pathways must be established and this model incorporate the autocrine vs. paracrine paradigm model of signaling and the duration and time of the signaling. Only by elucidating these aspects, will mechanistic and translational studies into the potential for regulating Notch signaling in a therapeutic context be more fully and rationally informed.

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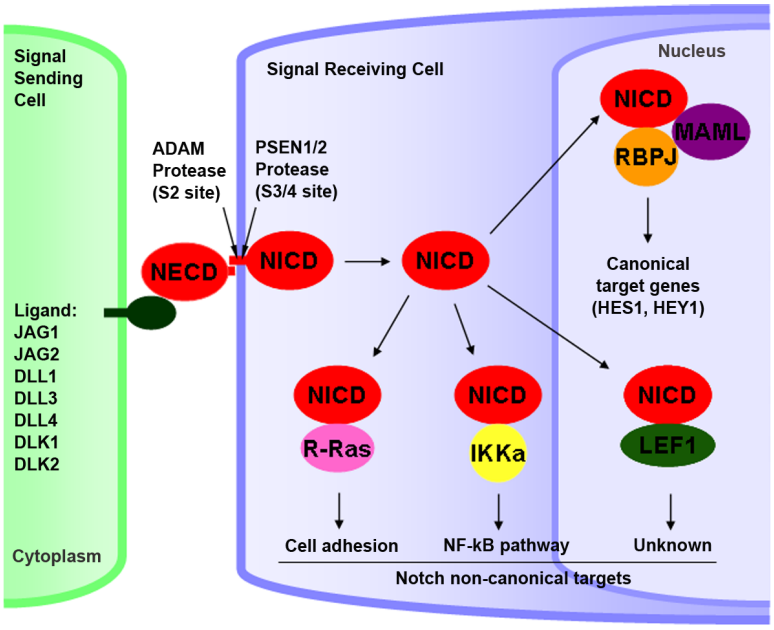

Figure 1. Canonical and non-canonical Notch signaling in mammals

In the canonical pathway, binding between transmembrane Notch receptors and its ligands triggers ADAM10- and Presenilin-mediated proteolytic cleavage. This liberates the membrane-bound Notch intracellular domain (NICD) to translocate into the nucleus, where NICD forms a transcriptional complex with nuclear factor RBPJ and the co-activator MAML to regulate transcription of downstream genes, such as *HES1* and *HEY1*, the two classic targets of Notch. The non-canonical pathway or RBPJ-independent Notch signaling occurs in Drosophila and mammals. Here, Notch can activate integrin via the membrane associated small GTPase R-Ras independent of binding RBPJ. In addition, NICD can interact with IKKα in the NF-kB pathway or LEF1 in the Wnt pathway.

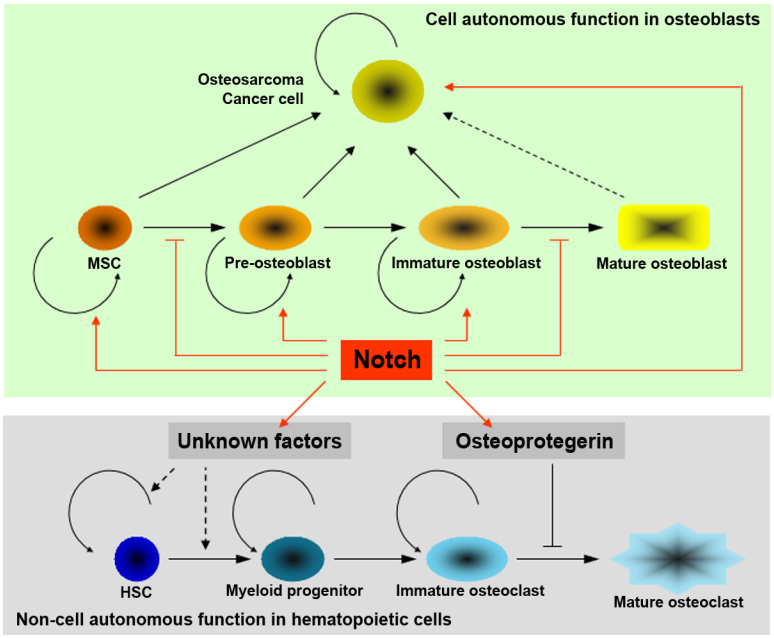

Figure 2. Cell autonomous and non-cell autonomous functions of Notch in osteoblasts

Notch regulates osteoblast differentiation through two different mechanisms: (1) to promote the self-renewal of MSC and the proliferation of pre-osteoblast and immature osteoblasts; (2) to inhibit the differentiation from MSC to pre-osteoblast or from immature to mature osteoblast. Osteosarcoma cells have a high level expression of Notch indicating pathological gain-of- function of Notch may contribute to tumorigenesis of bone cells. The de-differentiation of fibroblast into pluripotent progenitors opens the possibility that de-differentiation of mature osteoblasts may lead to a cancer stem cells or OS (dash line). Notch signaling in osteoblast not only regulates osteoblast function in cell autonomous fashion, but also influences other cell types which directly and indirectly interact with osteoblasts within bone marrow. Notch regulates expression of Osteoprotegerin (OPG), an important factor for inhibiting osteoclast differentiation. Similarly, osteoblastic cells may modulate activities of the hematopoietic stem cell (HSC) or HSC-derived cells through unknown factors in a Notch-dependent, non-cell autonomous manner.

Table 1

Human disease and mouse models

Protein Function	Human gene	Human disease	Mouse phenotypes
Receptor	NOTCH1	T-cell acute lymphoblastic leukemia (T-ALL), aortic valve disease	Lethal E10.5, somite defects
	NOTCH2	**Alagille syndrome (6%)**	Lethal E11.5
	NOTCH3	CADASIL[A]	Fertile, viable
	NOTCH4	?Schizophrenia[B]	Fertile, viable
	JAG1	**Alagille syndrome (90%)**	Lethal E10.5
Ligand	JAG2	Unknown	Perinatal lethality
	DLL1	Unknown	Lethal E12, somite defects
	DLL3	**Spondylocostal dysostosis type 1 (SCDO1)**	Viable, skeletal defects
	DLL4	Unknown	Lethal E9.5
Effector	RBPJ	Unknown	Lethal E10.5, somite defects
bHLH Target	**MESP2**	**Spondylocostal dysostosis type 2 (SCDO2)**	Postnatal lethality, skeletal defects
	HES7	**Spondylocostal dysostosis type 4 (SCDO4)**	Postnatal lethality or viable, skeletal defects
	HEY2	Tetralogy of Fallot	Postnatal lethality
	HEY1/2	Heart looping defects	Lethal E11.5
Enzymatic modifier	PSEN1	Alzheimer, Dilated cardiomyopathy	Perinatal, skeletal defect
	PSEN1/2	Alzheimer, Dilated cardiomyopathy	Lethal E9.5, somite defects
	LFNG	**Spondylocostal dysostosis type 3 (SCDO3)**	Perinatal or live, skeletal defects

PSEN1/2, PSEN1 and PSEN2 double knockout. HEY1/2, HEY1 and HEY2 double knockout. Bold letters, human

[A] CADSIL: cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy.

[B] remains controversial.

Table 2

Modulators of Notch signaling

Antagonists		Agonists	
Target	**Function**	**Target**	**Function**
GSIs	Gamma-secretase inhibitors (GSIs)	JAG1-Fc	Jagged1-Fc Fusion protein
sJAGI	Soluble Jagged1, binding to Notch	DLL1-Fc	DLL1-Fc Fusion protein
sNECD	Soluble Notch ectodomain	NICD	Notch intracellualr domain
mAb	Monoclonal antibodies to Notch	mAb	Monoclonal antibodies to Notch
RNAi	RNA interference for RBPJ, MAML	EDTA	Ca^{2+} depletion
sRNA	Small RNA or microRNA		
dnRBPj	Dominant negative form		
dnMAML	Dominant negative form		
DLL3	Interaction with Notch in Golgi	JAG1	Notch ligand
NUMB	Negative regulator	DLL1	Notch ligand
DTX1	E3 ubiquitin ligase	DLL4	Notch ligand
LFNG[A]	Glycosyltransferase	FURIN	Proteolytic enzyme (S1 site)
FBXW7	Ubiquitin ligase	ADAM	Proteolytic enzyme (S2 site)
CDK8	Kinase for Notch degradation	PSEN1, 2	Gamma-secretase (S3 site)
		RBPJ	Nuclear effector
		MAML	Nuclear effector

The area with light blue color: artificial recombinant protein or small molecules. The area with dark blue color: physiological protein components in the Notch pathway.

[A]LFNG could be an agonist in certain context.