HHS Public Access

Author manuscript

Bone. Author manuscript; available in PMC 2021 September 01.

Published in final edited form as:

Bone. 2020 September ; 138: 115474. doi:10.1016/j.bone.2020.115474.

Notch and the Regulation of Osteoclast Differentiation and Function

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Abstract

Notch 1 through 4 are transmembrane receptors that play a pivotal role in cell differentiation and function; this review addresses the role of Notch signaling in osteoclastogenesis and bone resorption. Notch receptors are activated following interactions with their ligands of the Jagged and Delta-like families. In the skeleton, Notch signaling controls osteoclast differentiation and bone-resorbing activity either directly acting on osteoclast precursors, or indirectly acting on cells of the osteoblast lineage and cells of the immune system. NOTCH1 inhibits osteoclastogenesis, whereas NOTCH2 enhances osteoclast differentiation and function by direct and indirect mechanisms. NOTCH3 induces the expression of RANKL in osteoblasts and osteocytes and as a result induces osteoclast differentiation. There is limited expression of NOTCH4 in skeletal cells. Selected congenital disorders and skeletal malignancies are associated with dysregulated Notch signaling and enhanced bone resorption. In conclusion, Notch signaling is a critical pathway that controls osteoblast and osteoclast differentiation and function and regulates skeletal homeostasis in health and disease.

Keywords

Notch; Jagged; tumor necrosis factor α; bone remodeling; inflammation; osteoclast; bone resorption

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Credit Author Statement

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Declaration of Competing Interest

All authors report no conflict of interest.

1. Introduction

Bone tissue continuously undergoes remodeling through the coordinated activity of cells of the osteoblast and the osteoclast lineages. A variety of molecules and signaling pathways control osteoclast differentiation and function either directly acting on osteoclast precursors or indirectly acting on osteoblasts, osteocytes and cells of the immune system that express osteoclastogenic factors under physiological conditions and in various disease states [1–5]. Notch signaling plays a critical role in cell fate determination and function and in the regulation of skeletal homeostasis [6–8]. Previous reviews on Notch signaling have described the function of Notch signaling in skeletal and non-skeletal cells, but have not addressed the specific function of each Notch receptor, an in particular the role of the receptors in osteoclastogenesis under physiological conditions and during inflammation. This is relevant since recent work has demonstrated distinct actions of each Notch receptor in bone remodeling and osteoclastogenesis. This review highlights recent insights into the role of Notch signaling in the regulation of osteoclastogenesis and bone resorption in physiological and selected pathological conditions affecting the skeleton.

2. Overview of Osteoclastogenesis

2.1. Regulation of Osteoclastogenesis

Osteoclasts are giant multinucleated cells that are responsible for bone resorption. Osteoclasts are derived from the differentiation and cell-cell fusion of myeloid progenitor cells that also have the potential to differentiate into monocytes, granulocytes and macrophages [1–4, 9, 10].

Macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) are indispensable cytokines for osteoclast differentiation and survival. M-CSF and RANKL are produced by osteoblasts, osteocytes, stromal cells, and by cells of the immune system [1–4, 11]. M-CSF is secreted under physiological conditions, and it is not secreted by macrophages. M-CSF triggers downstream signaling through its receptor, CSF-1 receptor (CSF-1R), and promotes the differentiation of myeloid progenitors into osteoclast precursors by upregulating RANK expression. M-CSF signaling also is essential for cytoskeleton re-arrangement and for the survival of osteoclasts. RANKL-RANK signaling plays a critical role in osteoclast differentiation and bone-resorbing activity. RANKL binds to RANK, and facilitates the activation of the IκB kinase complex (IKK), the phosphatidylinositol 3-kinase (PI3K)-AKT signaling pathway, and mitogen-activated kinases (MAPKs) including p38, ERK and c-Jun N-terminal kinase (JNK) [5, 12]. This leads to the activation of the transcription factors NF-κB, c-Fos and c-Jun, which are required for the induction of nuclear factor of activated T cells c1 (NFATc1) at an early stage of osteoclastogenesis. NFATc1 is a transcription factor that is self-induced in a process of auto-amplification during osteoclastogenesis [12–16]. NFATc1 promotes the expression of genes required for osteoclast differentiation and function, such as tartrate-resistant acid phosphatase (TRAP), dendritic cell-specific transmembrane protein (DC-STAMP), v-ATPase V0 subunit d2 (ATP6V0D2), osteoclast-associated receptor (OSCAR), αvβ3 integrin receptor, and cathepsin K [17–28].

There are transcriptional repressors that act as 'brakes' of RANKL signaling and are constitutively expressed in osteoclast precursors. For osteoclastogenesis to proceed, RANKL needs to overcome the activity of the transcriptional repressors, including that of inhibitor of differentiation and DNA binding (IDs), v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (MAFB), interferon regulatory factor 8 (IRF8) and B cell lymphoma 6 (BCL6) [29–32]. These transcriptional repressors modulate the expression or the transcriptional activity of NFATc1. The constitutive expression of transcriptional repressors is downregulated at an early stage of osteoclastogenesis following the induction of B-lymphocyte-induced maturation protein-1 (BLIMP-1) by the actions of RANKL. The activity of RANKL is opposed by osteoprotegerin (OPG), which acts as a decoy receptor of RANKL decreasing its interaction with RANK [33–35] (Figure 1).

2.2. Osteoclastogenesis and Inflammation

Osteoclast differentiation is excessively triggered under certain inflammatory conditions, such as infection, autoimmune disorders affecting the skeleton and fractures. Cells of the immune system, including macrophages, dendritic cells and T cells, as well as cells not forming part of the immune system, such as fibroblast-like synoviocytes secrete pro-inflammatory cytokines, including tumor necrosis factor α (TNFα), interleukin (IL)-1β, IL-6, and IL-17. These cytokines induce osteoclast differentiation by upregulating RANKL expression in osteoblasts and osteocytes, and through direct effects on osteoclast precursors that are either independent or dependent of RANKL signaling [3, 36, 37] (Figure 1).

TNFα is the main osteoclastogenic cytokine responsible for bone resorption during inflammation and in disease states characterized by an inflammatory process. TNFα induces the expression of IL-1β, IL-6 and TNFα itself. It can promote osteoclastogenesis independent of RANKL, although this has been an issue of controversy [38–41]. IL-1β alone does not induce osteoclast differentiation from osteoclast precursors although it has synergistic effects with RANKL and TNFα in the late stages of osteoclastogenesis and on bone-resorbing activity. As a result, IL-1β can cause bone destruction in a variety of diseases with an inflammatory component and affecting the skeleton [42–45]. IL-6 inhibits RANKL signaling in osteoclast precursors, however it increases osteoclastogenesis indirectly by upregulating RANKL production in osteoblasts [46, 47]. IL-17 enhances the sensitivity of osteoclast precursors to RANKL by augmenting RANK expression, and increases the expression of proinflammatory cytokines including TNFα, IL-1β and IL-6 [48–52].

3. Overview of Notch Signaling

3.1. Notch Receptors and Ligands

Notch receptors are single-pass type I transmembrane proteins that play a pivotal role in cell fate determination and function in a variety of cell lineages [6, 7]. In mammals, 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. Notch ligands also are transmembrane proteins and, when present in cells adjacent to those expressing Notch, their interactions with Notch result in signal activation. As a result, the Notch signaling pathway serves as a means of communication between neighboring cells.

Notch receptors have a complex structure; their extracellular domain consists of 29 to 36 epidermal growth factor (EGF) -like repeats and EGF repeats 11 and 12 interact with Notch ligands, although other EGF-like repeats modulate this interaction [53–55]. Notch receptors are cleaved (S1 cleavage) in the trans-Golgi network by a furin-like protease prior to their integration into the cell membrane as heterodimers [56] (Figure 2, top panel). At the junction of the extracellular and the transmembrane domain (TMD) rests the negative regulatory region (NRR), which consists of three Lin12-Notch repeats (LNR) that surround and protect the heterodimerization domain (HD) (Figure 2, lower panel). This is the site of cleavage (S2) required for Notch activation, and as a consequence plays a critical regulatory role in Notch signaling [57–60]. The intracellular domain of Notch (NICD) consists of a recombination signal-binding protein for Ig of κ region (RBPJκ)-association module (RAM) domain, nuclear localization sequences (NLS), and seven ankyrin (ANK) repeats; these domains are required to regulate transcription [61]. The C-terminus of Notch contains a proline (P)-, glutamic acid (E)-, serine (S)- and threonine (T)-rich (PEST) domain, which is the target of E3 ubiquitin ligases necessary for the proteasomal degradation of Notch [7, 62] (Figure 2).

The structure and downstream signal transduction of Notch receptors are highly conserved; however, each Notch receptor has a distinct expression pattern and function, as demonstrated in studies of Notch gene inactivation *in vivo*. Whereas *Notch1* null mice die during development due to widespread cellular death and hypomorphic *Notch2* alleles cause perinatal death due to vascular and renal defects, *Notch3* and *Notch4* null mice develop normally and mutant adults are viable and fertile although *Notch3* null mice have modest vascular alterations [63–67]. In the skeleton, NOTCH1, NOTCH2 and NOTCH3 and low levels of NOTCH4 are detected, and the most prevalent Notch ligand in skeletal cells is JAG1 [8, 68]. NOTCH 1 and NOTCH2 are expressed by osteoblasts, osteocytes and osteoclasts, whereas NOTCH3 is expressed by osteoblasts and osteocytes, and not by osteoclasts.

3.2. Activation and De-activation of Notch Signaling

Interactions of Notch with a Notch ligand present in an adjacent cell result in the endocytosis of the ligand and a pulling or hinge-like effect that unravels the NRR leaving the HD exposed to be cleaved by disintegrin and metalloprotease domain-containing proteins (ADAMs) (S2 cleavage) and the subsequent cleavage at the transmembrane domain by the γ-secretase complex (S3 and S4 cleavage) [69–71]. The “adjacent” cell expressing the Notch ligand required to activate Notch can be a skeletal cell or a cell in the bone marrow environment, and Notch activation by JAG1 in osteoblasts can regulate the hematopoietic stem cell niche [72]. Following endocytosis, the Notch ligand is recycled back to the cell surface, a process that involves ubiquitination by two E3 ubiquitin ligases [73–76]. Interactions of Notch with its ligands depend on a number of factors, including whether the ligand and Notch are in the same cell (cis) or in different adjacent cells (trans); cis interactions result in an inhibitory effect whereas trans interactions result in activation of Notch signaling. What determines or modulates the initial interaction of Notch with its ligand is not clear although does not seem to involve signals determining osteoclastogenesis, such as RANKL. Post-translational modifications of the Notch extracellular domain result in

differential regulation of its interactions with its ligands. For instance, the glycosyltransferase EOGT transfers N-acetylglucosamine linked to Ser or Ther (O-GlcNAc) to select EGF repeats of Notch enhancing the binding of DLL1 and DLL4 but not the binding of JAG1 to Notch [77]. Fringe glycosyltransferases also modulate differential interactions of Notch receptors with its ligands, and lunatic and manic fringe enhance the binding of DLL1 to Notch and decrease the binding of JAG1 whereas radical fringe enhances the Notch response to both ligands [78–81]. Whether a ligand activates Notch signaling is dependent on its expression in a given cell system. In skeletal cells, JAG1 is expressed in osteoblasts, osteocytes and osteoclasts, and with the exception of DLL3 and DLL4, which are detected in osteocytes other Notch ligands are not detected in skeletal cells [82]. JAG1 is presumed to be the most critical Notch ligand in bone and its inactivation in progenitor cells phenocopies the loss of Notch function in osteoblasts [83].

The proteolytic cleavage of Notch leads to the release of the NICD. The NICD translocates to the nucleus, where it forms a complex with RBPJκ and mastermind-like (MAML) to regulate the transcription of target genes [71, 84, 85]. RBPJκ is also termed C promoter-binding factor 1 (CBF1), Suppressor of hairless, Lin-12 and Glp-1 (Lag-1) or CSL. It is important to note that RBPJκ, and not the NICD, binds to DNA and that under basal conditions RBPJκ associates with co-repressors to inhibit transcription. The translocation of the NICD to the nucleus, leads to the displacement of transcriptional inhibitors and the recruitment of activators of transcription so that the NICD, RBPJκ, MAML complex induces gene transcription [86]. This is considered the canonical Notch signaling pathway whereas Notch actions that are independent of RBPJκ are considered non-canonical. Targets of canonical Notch signaling include members of the Hairy and enhancer of split (HES) and HES with an YRPW motif (HEY) families of transcription factors [87–90]. The activation of Notch signaling is terminated by the actions of cyclin-dependent kinases (CDK) that phosphorylate the PEST domain of the NICD, resulting in the disassembly of the NICD, RBPJκ, MAML complex, the ubiquitination of the NICD by E3 ubiquitin ligases and the degradation of the NICD [91]. FBW7 is an F-box protein that serves as a substrate receptor of the E3 ligase complex and binds to the PEST domain of the NICD to trigger E3 ubiquitin ligase-dependent proteasome degradation [92, 93]. Figure 3 illustrates the cascade of events that lead to the activation of canonical Notch signaling and induction of its target genes.

Although most of the activation of Notch depends on its interactions with ligands, a degree of basal activation has been reported under certain conditions, particularly for NOTCH3. Dysregulated activation of Notch signaling occurs in some malignancies and acute lymphoblastic leukemia and is associated with somatic mutations of the NRR that lead to the constitutive activation of Notch [6, 7, 94].

4. Notch Signaling and Osteoclast Differentiation and Function

4.1. Role of Notch Signaling in Osteoclastogenesis under Physiological Conditions

The role of Notch signaling in osteoclast differentiation has been studied by using *in vitro* cell culture systems and genetically modified mouse models. Results from this work have demonstrated unique actions of each Notch receptor on osteoclast differentiation and function (Figure 4). Although Notch gene misexpression can have developmental

consequences, studies to determine the function of Notch in the skeleton have utilized conditional genetic mouse models that for the most part have an impact on the adult skeleton. NOTCH1 inhibits osteoclast differentiation by acting directly on osteoclast precursors and indirectly through its actions on osteoblasts. The genetic deletion of *Notch1* in osteoclast precursors enhances osteoclastogenesis, whereas the overexpression of the NOTCH1 NICD (N1ICD) suppresses *Nfatc1* transcription and osteoclast differentiation [95, 96]. Signal activation of NOTCH1 in the osteoblast lineage suppresses osteoblast differentiation and increases the level of OPG resulting in a pronounced inhibition of osteoclastogenesis [95, 97, 98]. Indeed, the overexpression of N1ICD in osteoblasts and in osteocytes causes an osteopetrotic phenotype [99, 100]. In osteocytes this is the result of an induction of OPG as well as a suppression of the Wnt antagonists dickkopf1 and sclerostin. As a consequence, Wnt signaling is enhanced resulting in an increase in cortical bone formation and a decrease in osteoclast number and bone resorption through the direct and indirect inhibitory effects of Wnt on osteoclastogenesis [101–104]. The effect of NOTCH1 in osteocytes is dependent on canonical activation of Notch signaling since the induction of OPG and Wnt antagonists and the osteopetrotic phenotype are reversed following the inactivation of *Rbpjκ* [105]. It is important to note that under basal conditions RBPJκ is dispensable for the function of osteoblasts, osteocytes and osteoclasts since the inactivation of *Rbpjκ* in these cell lineages *in vivo* does not result in a skeletal phenotype [96, 100, 105]. This would suggest that the genes transcriptionally inhibited by RBPJκ do not play a role in skeletal physiology and that RBPJκ is mostly relevant following the activation of Notch signaling. Although the inactivation of *Rbpjκ* in the myeloid lineage does not cause a skeletal phenotype, it amplifies the effects of TNFα and to a lesser extent RANKL on *Nfatc1* transcription, osteoclastogenesis and bone resorption so that the activity of TNFα is comparable to that of RANKL [96, 106]. This has been interpreted to indicate that RBPJκ is an inhibitor of osteoclastogenesis. The mechanism by which RBPJκ suppresses *Nfatc1* induction is by attenuating c-Fos activation and suppressing BLIMP-1; as a result, the transcriptional repressor IRF8 is not downregulated and osteoclastogenesis does not progress.

In contrast to the inhibitory effects of NOTCH1, NOTCH2 induces osteoclast differentiation by direct and indirect mechanisms. By acting on cells of the osteoblast lineage, NOTCH2 induces the expression of RANKL, and as a result enhances osteoclastogenesis. Importantly NOTCH2 is expressed in the myeloid lineage, where it promotes osteoclastogenesis directly. The effect requires the activation of the Notch receptor, since it is prevented by γ-secretase inhibitors and by antibodies directed to the NRR of NOTCH2 [107, 108]. Interactions of the NOTCH2 NICD (N2ICD) with the p65 subunit of NF-κB in osteoclast precursors lead to the transcription of *Nfatc1* [109]. The inactivation of *Notch2* in *Lyz2* (LysM) expressing myeloid cells does not result in a skeletal phenotype [110]. However, the genetic deletion of *Fbw7* in mature *Ctsk* expressing osteoclasts results in the stabilization of NOTCH2 and a gain-of-NOTCH2 function and causes enhanced osteoclastogenesis and osteopenia [93]. An analogous phenotype is observed in mutant mouse models expressing a truncated NOTCH2 protein that lacks the PEST domain resulting in the stabilization of NOTCH2 and a generalized NOTCH2 gain-of-function [111]. The lack of a phenotype in mice where *Notch2* was deleted in the myeloid lineage might be related to inefficient Cre-mediated

recombination or to low levels of *Notch2* gene expression in undifferentiated cells [112]. This is possible since the levels of NOTCH2 increase substantially as cells of the myeloid lineage differentiate into mature osteoclasts in the presence of RANKL. Although some effects of NOTCH2 are due to direct interactions of the N2ICD with NF-κB on the *Nfatc1* promoter, some are dependent on the induction of Notch target genes. Osteoclast precursors express *Hes1* and low levels of *Hes3* and *Hes5* but do not express *Hey1*, *Hey2* or *HeyL* transcripts [8]. Therefore, *Hey* genes cannot mediate the actions of Notch signaling in cells of the osteoclast lineage. The levels of HES1 increase during osteoclast differentiation in parallel to those of NOTCH2 [109, 111, 113]. Importantly, osteoclastogenesis is attenuated under conditions of *Hes1* inactivation and the downregulation of *Hes1* reverses the enhanced osteoclastogenesis caused by NOTCH2 demonstrating that HES1 is responsible for the effects of NOTCH2 on osteoclast differentiation [113].

NOTCH3 is not expressed by osteoclast precursors, but its activation in osteoblasts and osteocytes increases RANKL resulting in an induction of osteoclast differentiation by this indirect mechanism [114]. NOTCH4 is mostly detected in vascular cells and its levels in osteoclast precursors and in the osteoblast lineage are low and there is limited information on its role, if any, in skeletal physiology.

4.2. Role of Notch Signaling in Osteoclastogenesis under Inflammatory Conditions

Notch receptors and ligands are induced by pro-inflammatory cytokines in a variety of cell lineages, and Notch signaling plays a role in osteoclast differentiation during inflammation acting by direct and indirect mechanisms [115–117] (Figure 4). Overexpression of the N1ICD in osteoclast precursors suppresses TNFα-induced osteoclast formation and osteolysis *in vivo*, and the inactivation of *Rbpjκ* in myeloid cells enhances the osteoclastogenetic potential of TNFα [96]. This suggests that NOTCH1 canonical signaling has a direct inhibitory effect on TNFα-induced osteoclast differentiation. However, there is evidence indicating an indirect and stimulatory effect of NOTCH1 signaling on osteoclastogenesis and bone erosion in the context of certain inflammatory conditions, such as those occurring in joints affected by rheumatoid arthritis (RA). NOTCH1 is overexpressed and activated in fibroblast-like synoviocytes, Th17 cells and M1 macrophages, cells that play a pivotal role in the pathogenesis of RA [115, 116]. NOTCH1 signaling accelerates the production of the pro-inflammatory cytokines TNFα, IL-6 and IL-17 in synoviocytes and inhibition of Notch signaling using either a γ-secretase inhibitor or the transgenic delivery of NOTCH1-antisense constructs ameliorates arthritis severity and bone erosion in experimental models of RA [115–118]. Th17 cells secrete IL-17 in RA and periodontitis and NOTCH1 induces the transcription of *Il17* in Th17 cells [119]. M1 macrophages produce pro-inflammatory cytokines, including TNFα, IL-6 and IL-1β so that M1 macrophages play a role in the pathogenesis of acute and chronic inflammatory conditions and the released cytokines induce osteolysis. In contrast, M2 macrophages synthesize anti-inflammatory cytokines, such as IL-4 and IL-10 and these are suppressed in RA [115, 120]. An imbalance between M1 and M2 macrophages is considered important in the pathogenesis of RA. The Notch inhibitor thapsigargin reduces TNFα-induced M1 macrophage polarization and attenuates inflammation and joint bone loss [121]. In the same context, microRNA-146a, which downregulates *Notch1* transcripts, promotes M2 and decreases M1 macrophage

polarization and ameliorates arthritis severity and bone erosion in a model of collagen-induced arthritis [122, 123].

NOTCH2 also plays a role in the bone loss associated with inflammation. Cells of the myeloid lineage expressing a truncated and stable NOTCH2 exhibit an enhanced osteoclastogenic response in the presence of TNFα [124]. TNFα induces *Jag1* and *Notch2* transcripts and NOTCH2 induces *Hes1* as cells become differentiated into osteoclasts under the influence of TNFα. In this specific context, the effects of TNFα are independent of NF-κB and reversed following the inactivation of *Hes1*. In addition, a HES1-dependent increase in *Il1b* mRNA levels by TNFα is observed in osteoclasts harboring a NOTCH2 gain-of-function. Anti- JAG1 and anti-NOTCH2-NRR antibodies prevent TNFα-induced osteoclastogenesis *in vitro*, and osteolysis *in vivo*, confirming the NOTCH2 activation-dependency of the effects observed. In line with its actions in the myeloid lineage, TNFα enhances the expression of *Notch2* and *Hes1* in fibroblast-like synoviocytes from RA patients, and NOTCH2 contributes to the production of IL-6 by these cells [125].

Notch3 transcript levels are upregulated during the activation and differentiation of collagen II-specific Th1-Th17 expansion. The proliferation of Th17 cells is attenuated by a specific neutralizing antibody targeting NOTCH3, suggesting that NOTCH3 could have an indirect role in osteoclastogenesis by modulating the Th17 cell population [126].

5. Notch and Disorders of the Skeleton Associated with Altered Bone Resorption

5.1 Notch and Congenital Disorders of the Skeleton Associated with Altered Bone Resorption

Although there is a variety of congenital disorders of the skeleton that are associated with mutations in genes encoding various components of the Notch signaling pathway, most of them do not manifest alterations in osteoclastogenesis or bone resorption [7, 8]. Hajdu Cheney Syndrome (HCS) and Lateral Meningocele Syndrome (LMS) are possibly the only congenital disorders associated with mutations in Notch genes where bone resorption is known to be affected. HCS is a rare, inherited disease associated with nonsense mutations or deletions in exon 34 of *NOTCH2* upstream of the PEST domain leading to the formation of a truncated and stable NOTCH2 protein and a NOTCH2 gain-of-function [127–130]. HCS is characterized by osteoporosis with fractures, acroosteolysis of the hands and feet, craniofacial developmental defects, spinal deformities and short stature [131–138]. Acroosteolysis is frequently present and accompanied by inflammation and lysis of the phalanges, which leads to short and broad digits. Platybasia and basilar invagination can result in severe neurological complications, including hydrocephalus and central respiratory arrest causing sudden death. Occasionally, polycystic kidneys, cardiac septal defects and valve abnormalities are present [138–140]. Iliac crest biopsies reveal the presence of cortical bone osteopenia, woven bone and increased osteoclast number and bone resorption [141]. We created a *Notch2* mutant mouse model harboring a truncating mutation in exon 34 upstream of the PEST domain reproducing a mutation found in HCS [111]. The *Notch2* mutant mouse model, termed *Notch2*$^{tm1.1Ecan}$, exhibits pronounced cancellous and cortical

bone osteopenia secondary to increased osteoclast number and bone resorption due to direct effects of NOTCH2 on cells of the myeloid lineage as well as the induction of RANKL by cells of the osteoblast lineage. The skeletal phenotype of *Notch2*$^{tm1.1Ecan}$ mice is congruent with the skeletal manifestations of HCS. Moreover, an alternate mouse model of HCS presents with increased number of osteoclasts and bone remodeling, confirming some of the phenotypic characteristics of *Notch2*$^{tm1.1Ecan}$ mice [142]. Although the mechanism of the inflammatory osteolysis of fingers and toes is poorly understood, *Notch2*$^{tm1.1Ecan}$ mice are sensitized to the osteolytic actions of TNFα *in vivo* and to the effect of TNFα on osteoclastogenesis [124].

Due to the limited number of subjects affected by HCS, there are no controlled trials on the management of the osteoporosis in this patient population. Bisphosphonates alone or in combination with teriparatide have been used, but evidence of benefit is scarce [140, 143, 144]. Because NOTCH2 induces RANKL and enhances osteoclastogenesis, a consideration is the use of the anti-RANKL antibody denosumab [145]. Although parathyroid hormone suppresses Notch signaling, the use of teriparatide in the treatment of HCS could pose risks since there is evidence of Notch activation in osteosarcoma in humans and prolonged activation of Notch in mice can cause osteosarcoma [68, 146, 147]. Moreover, since the mechanism responsible for the bone loss in HCS is increased bone resorption, the use of teriparatide would result in an increase in bone remodeling and possibly worsen the bone loss. NOTCH2 itself could become a future target in the treatment of HCS, and the skeletal phenotype of *Notch*$^{tm1.1Ecan}$ mouse mutants was reversed by anti-NOTCH2 NRR antibodies and ameliorated by the use of antisense oligonucleotides (ASOs) targeting *Notch2* [108, 148, 149].

LMS is a rare congenital disorder characterized by craniofacial developmental abnormalities, intellectual disability, hypotonia, decreased muscle mass, syringomyelia, meningoceles and cardiac valve abnormalities [150]. Skeletal manifestations include short stature and scoliosis, increased density of the base of the skull, and increased bone remodeling and bone loss [151]. LMS is associated with point mutations or short deletions in exon 33 of *NOTCH3*, upstream of the PEST domain, leading to a truncated and stable NOTCH3 protein and a gain-of-function [152]. Our laboratory created a mouse model of LMS, where a tandem STOP codon was introduced into the mouse genome in exon 33 upstream of the PEST domain [114]. The mutant mouse, termed *Notch3*$^{tm1.1Ecan}$, presents with osteopenia due to enhanced bone resorption secondary to increased osteoclast number due an induction of RANKL by cells of the osteoblast lineage including osteocytes. Because NOTCH3 is not detected in cells of the myeloid lineage, the effects on osteoclastogenesis are indirect and were documented in co-cultures of osteoblasts and bone marrow-derived macrophages. The enhanced osteoclastogenesis and osteopenic phenotype are reversed by the administration of anti-NOTCH3 NRR antibodies, selectively preventing the activation of NOTCH3; however, there is no information regarding possible therapeutic avenues in individuals with LMS [153].

5.2. Notch and Acquired Diseases of the Skeleton Associated with Altered Bone Resorption

A number of acquired skeletal disorders have been associated with dysregulated Notch signaling, including osteosarcoma, fractures, selected forms of arthritis and malignancies, particularly those presenting with bone metastases. The latter can cause lytic lesions of the skeleton or display enhanced bone resorption, which is the focus of this review. A more comprehensive review of acquired diseases of the skeleton associated with dysregulated Notch signaling was published recently [7, 8]. Although post-menopausal osteoporosis is associated with increased bone resorption, there is little evidence of dysregulated Notch signaling in osteoporosis. A SNP of the Notch ligand *JAG1* is associated with bone mineral density [154]. Notch could play a role in the bone loss of aging since dysregulated gene expression of components of the Notch pathway is found in aging bone [155]. Aberrant Notch activation has been well established in hematological malignancies, where translocations that activate NOTCH1 and NOTCH2 as well as mutations that result in a truncated NOTCH1 or NOTCH2 receptor lacking a PEST domain are associated with leukemia, lymphoma and skeletal malignancies [94, 156–160]. NOTCH3 is constitutively active in carcinoma of the breast independent of ligand binding and NOTCH3 promotes carcinoma of the breast tumor growth *in vitro* and *in vivo* [161]. NOTCH3 also is expressed by myeloma cells and promotes cell growth and osteocytic invasion of myeloma cells [162]. Notch signaling plays a role in oncogenic transformation, in the epithelial-mesenchymal transition (EMT), on tumor invasiveness and in tumor angiogenesis favoring the metastatic potential of tumor cells [163, 164]. Because of these reasons, Notch plays an important function in tumor development and skeletal metastases in carcinoma of the breast and of the prostate, often altering the interactions between bone cells and metastatic cells.

Human bone marrow-derived osteoblasts induce the expression of *NOTCH3* and its ligand *JAG1* in human carcinoma of the breast cell lines. Inoculation of carcinoma of the breast cells, or their direct injection into the bone marrow of athymic mice, induces the formation of osteolytic bone metastases, and downregulation of *NOTCH3* reduces their metastatic potential [165]. This indicates that NOTCH3 not only plays a role in the growth of breast cancer cells but also in their invasive potential to bone. The expression of *JAG1* in mammary tumor cells correlates with tumor load and with the ability of tumors to form metastases in bone [166]. Tumor cells expressing *JAG1* activate Notch signaling, which by inducing IL-6 in osteoblasts, enhances osteoclastogenesis and the formation of osteolytic bone metastases. The bone lysis results in a release from the bone matrix of transforming growth factor β, which upregulates *JAG1* causing further activation of Notch signaling. This positive feedback loop favors the metastatic potential of tumor cells. Downregulation of *JAG1* or the prevention of Notch activation decreases the osteolytic potential in experimental models of carcinoma of the breast.

Carcinoma of the prostate frequently metastasizes to bone, inducing osteoblastic woven bone formation and osteoclastic bone resorption [167]. *NOTCH1*, *NOTCH3* and *JAG1* are expressed by carcinoma of the prostate, and their levels are associated with high-grade tumors and their metastatic potential [168–172]. By inducing matrix metalloprotease 9 and of urokinase plasminogen activator receptor, NOTCH1 promotes prostate tumor

invasiveness, which is associated with the presence of molecular markers of the EMT [173–175]. The observations suggest that Notch plays a role in EMT and tumor aggressiveness and in the invasive potential of carcinoma of the prostate.

Somatic mutations of *NOTCH1* and dysregulated Notch signaling are often found in acute lymphoblastic T-cell leukemia and hypercalcemia. However, radiographic abnormalities of the skeleton including osteolytic lesions are uncommon and appear to be related to the secretion of parathyroid hormone related peptide by T-cells, and not to enhanced Notch signaling [176, 177]. *NOTCH2* mutations resulting in a gain-of-NOTCH2 function are found in B-cell lymphomas and splenic marginal zone lymphomas, but do not seem to be associated with skeletal manifestations [159, 160, 178–180]. Chronic lymphoblastic leukemia is associated with osteolytic lesions and hypercalcemia, and precursor B-cell acute lymphoblastic leukemia is associated with bone loss and fractures, but the mechanism of the bone lytic lesions does not appear to involve dysregulated Notch signaling [181–184].

6. Ways to Control Notch Signal Activation

A variety of approaches have been developed to control Notch signaling including the use of biochemical inhibitors of Notch activation, small permeable molecules that prevent the formation of an NICD/RBPJκ/MAML ternary complex, antibodies to Notch receptors or to their ligands, and the use of antisense oligonucleotides targeting Notch genes. γ-secretase inhibitors block the cleavage of Notch receptors by interfering with the γ-secretase complex, but lack specificity since they target many substrates unrelated to Notch signal activation [185–187]. Thapsigargin is an inhibitor of the sarco/endoplasmic reticulum Ca^{2+}-ATPase that precludes the proper folding of the Notch receptor leading to a decreased level of Notch receptors at the cell surface [188]. Synthetic small cell permeable molecules compete with the binding of MAML to the NICD/RBPJκ complex leading to suppression of Notch signaling [189]. These approaches result in a nonspecific inhibition of all Notch receptors. Since each Notch receptor has a distinct function, it is important to selectively target individual Notch receptors. To this end, antibodies directed against the NRR of NOTCH1, NOTCH2 and NOTCH3 have been developed and shown to have neutralizing activity specific to each receptor [148]. The targeting of the NRR prevents the cleavage and activation of Notch. In recent studies, the systemic administration of anti-NOTCH2 NRR and anti-NOTCH3 NRR antibodies were shown to reverse the osteopenic phenotype of HCS or LMS mutant mouse models [108, 153]. It is important to note that there may be safety concerns with the protracted suppression of a Notch receptor.

Potential future interventions to temper Notch signaling in skeletal disorders could include the administration of ASOs. These are short, synthetic, single-stranded oligodeoxynucleotides that bind target RNA by Watson-Crick base pairing resulting in RNA degradation by RNase H, but also in the inhibition of translation by blocking ribosomal subunits from attaching and/or running along the target transcript [190, 191]. The administration of ASOs has emerged as a novel therapeutic approach because ASOs can target and downregulate transcripts harboring specific mutations. The use of ASOs has been successful in the silencing of mutant genes in the central and peripheral nervous system, retina and liver, but there is limited information about their usefulness in gene silencing in

the skeleton [192–201]. We demonstrated that the subcutaneous administration of Notch2 ASOs downregulated wild type and mutant *Notch2* transcripts in bone extracts from *Notch2*$^{tm1.1Ecan}$ mice and ameliorated the osteopenic phenotype in this experimental model of HCS [149]. *In vitro* studies revealed that Notch2 ASOs inhibited the enhanced osteoclastogenesis observed in osteoclast precursors from *Notch2*$^{tm1.1Ecan}$ mice as well as the induction of RANKL expression in cells of the osteoblast lineage.

Conclusions

Notch signaling regulates osteoclast differentiation and function either by acting directly on osteoclast precursors or indirectly in cells of osteoblast lineage through the regulation of the RANKL-OPG axis. Each Notch receptor plays a distinct role in osteoclastogenesis and dysregulated Notch signaling is associated with selected congenital and acquired diseases. In conclusion, Notch signaling is a critical pathway that controls osteoblast and osteoclast differentiation and function and regulates skeletal homeostasis in health and disease.

Acknowledgments

This work was supported by grants from the National Institute of Arthritis, Musculoskeletal and Skin Diseases [Grants: AR063049, AR072987, AR076747] and a grant from the National Institute of Diabetes, Digestive and Kidney Diseases [Grant: DK045227].

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Highlights

- Notch receptors determine cell fate and function, and regulate osteoclastogenesis
- NOTCH1 inhibits osteoclastogenesis directly and by inducing osteoprotegerin in osteoblasts and osteocytes
- NOTCH2 stimulates osteoclastogenesis directly and by inducing RANKL in osteoblasts, and NOTCH3 induces RANKL in osteoblasts and osteocytes
- Dysregulation of Notch signaling is found in congenital and acquired diseases and can be associated with alterations in bone remodeling

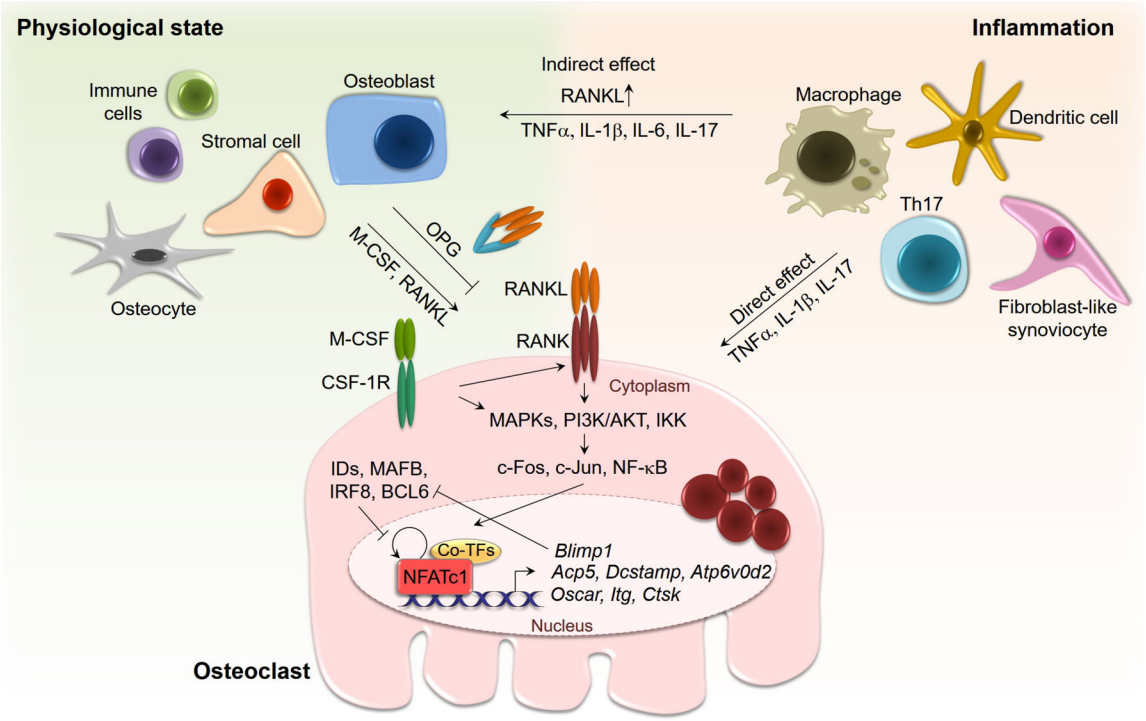

Figure 1.

Regulation of osteoclastogenesis. M-CSF and RANKL, synthesized by osteoblasts, osteocytes, stromal cells and cells of the immune system, trigger osteoclast differentiation via NFATc1 induction. NFATc1 promotes gene expression required for osteoclast differentiation and function including *Acp5* (encoding TRAP), *Dcstamp*, *Atp6v0d2*, *Oscar*, *Itg* (encoding integrin, αv and β3) and *Ctsk* (encoding cathepsin K). NFATc1 induces *Blimp1* which downregulates transcriptional repressors of NFATc1. During inflammation, macrophages, dendritic cells, Th17 cells and synoviocytes secrete the proinflammatory and osteoclastogenic cytokines TNFα, IL-1β, IL-6, and IL-17 and these stimulate osteoclastogenesis directly and by inducing RANKL in osteoblasts.

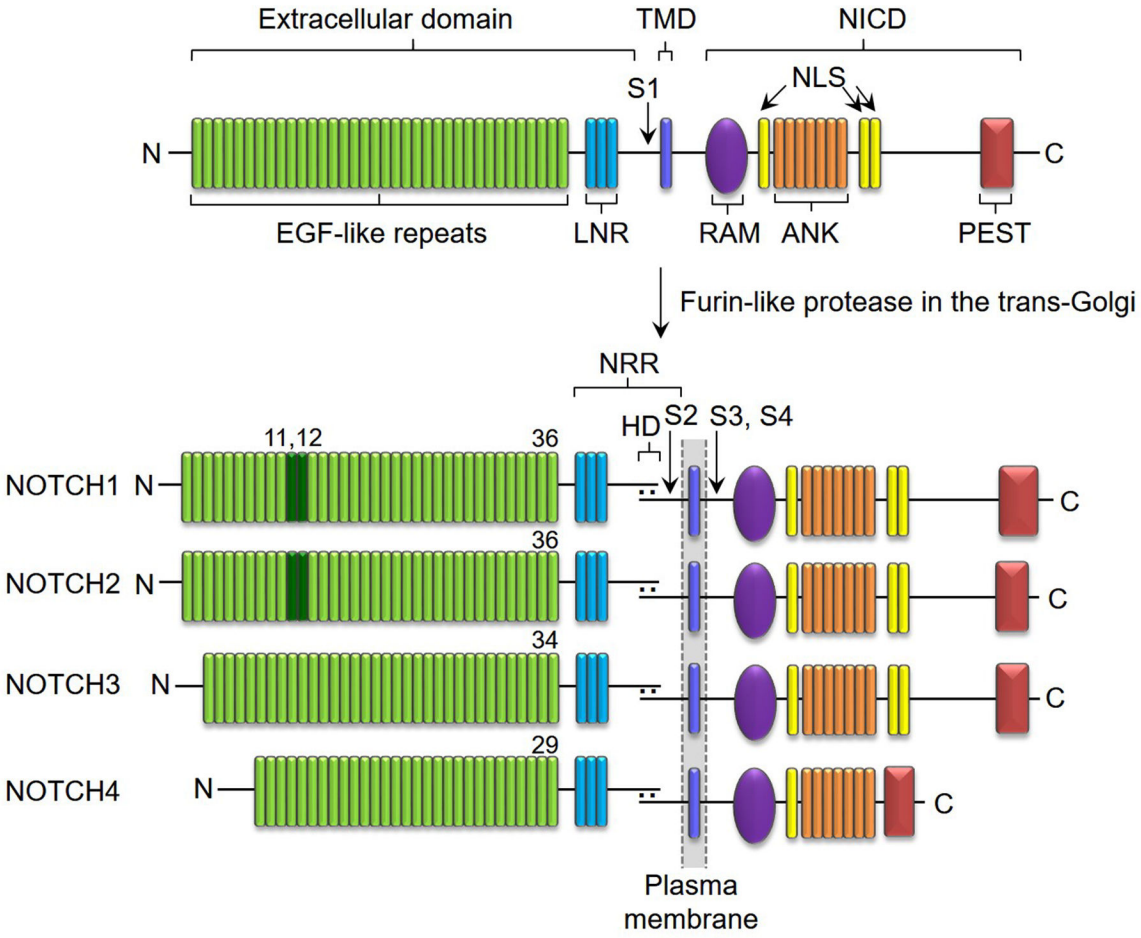

Figure 2.

Domain structure of Notch receptors. Notch consists of an extracellular domain, TMD and NICD. The extracellular domain contains EGF-like repeats and the NRR. The NICD comprises RAM, NLS, ANK and PEST domains. Notch receptors are cleaved by a furin-like protease (S1) in the trans-Golgi network to form a heterodimer at the cell membrane. The NRR consists of LNR repeats and the HD with a cleavage site for ADAMs (S2). Cleavage sites for the γ-secretase complex are in the TMD (S3, S4). NOTCH1 and NOTCH2 have 36 EGF-like repeats; dark green (EGF11, 12) indicate the binding site for ligands. NOTCH3 has 34 and NOTCH4 has 29 EGF-like repeats. NOTCH1 and NOTCH2 have similar NICD. Reproduced in part with permission from Zanotti and Canalis Endocrine Reviews 37:223–253, 2016.

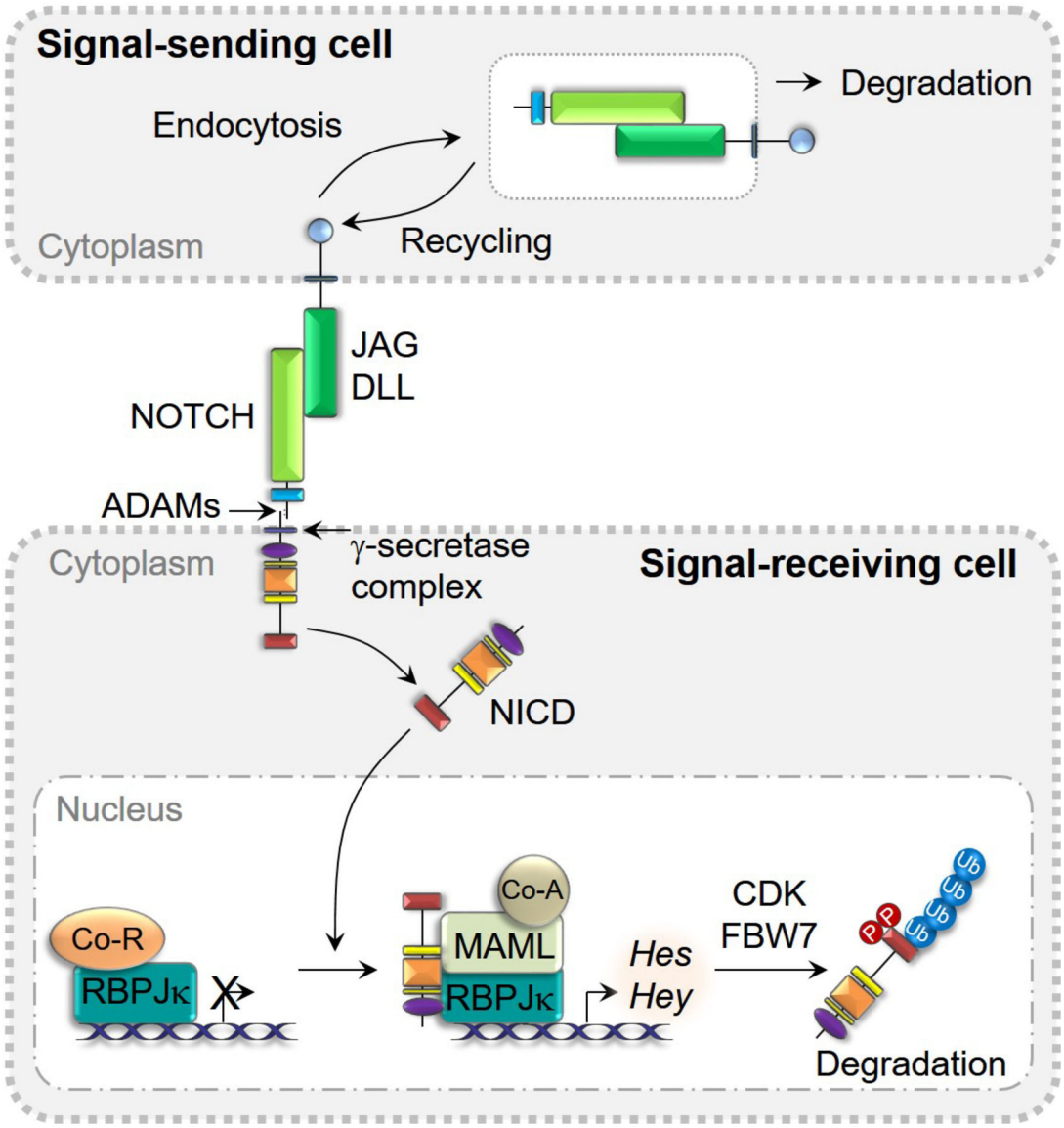

Figure 3.

Activation and de-activation of Notch signaling. Binding of JAG or DLL to NOTCH receptors results in the cleavage of NOTCH and the release of NICD to the cytoplasm and the endocytosis and degradation of JAG and DLL in signal-sending cells. NICD translocates into nucleus and binds to RBPJκ, MAML, and co-activators of transcription to induce *Hes* and *Hey*. The phosphorylation of NICD by CDK results in the disassembly of the complex and FBW7 promotes NICD degradation via E3 ubiquitin ligase in the proteasome.

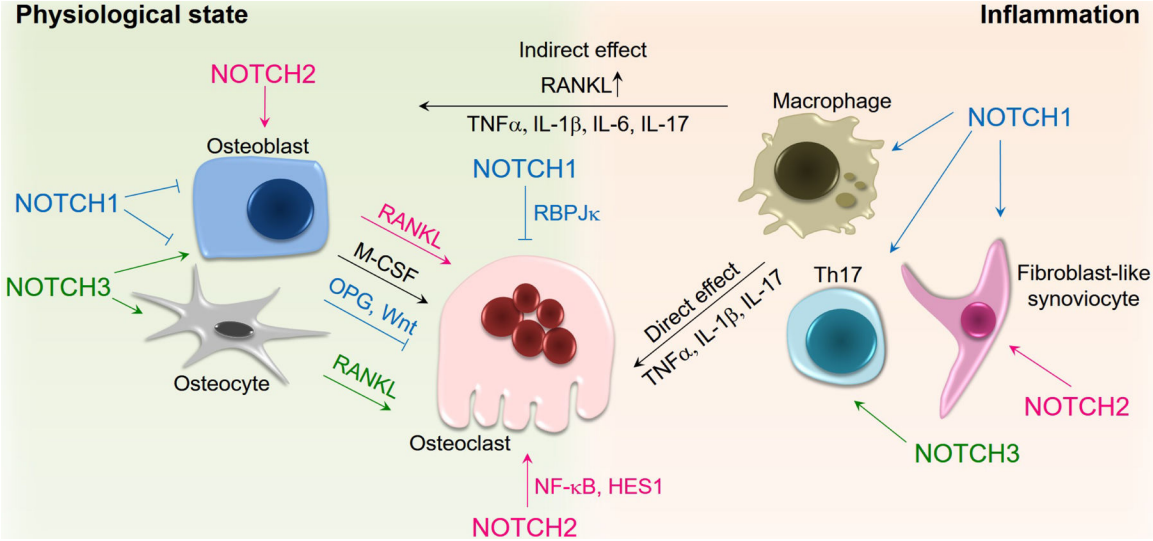

Figure 4.

Notch signaling and osteoclastogenesis. NOTCH1 inhibits osteoclastogenesis through the RBPJκ canonical pathway and by enhancing OPG levels and Wnt signaling in osteoblasts and osteocytes. NOTCH2 induces osteoclastogenesis through NF-κB and HES1-dependent mechanisms and by increasing RANKL in osteoblasts. NOTCH3 promotes osteoclastogenesis by enhancing RANKL levels in osteoblasts and osteocytes. NOTCH1, NOTCH2 and NOTCH3 act on the cells of the immune system during inflammation leading to the induction of pro-inflammatory and osteoclastogenic cytokines that can promote osteoclast differentiation and function either directly on osteoclasts or indirectly through the release of RANKL by osteoblasts.