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Notch signaling in pathogenesis of diseases

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Abstract

The notch signaling pathway have critical role in regulating cellular processes during development as a linear mechanism. Therefore, Mutations in Notch signaling pathway members cause developmental phenotypes that affect various organ in human body. It has been established that Notch signaling regulates multiple steps of T and B cell development in both central and peripheral lymphoid organs. Relative to the well documented role of Notch signaling in lymphocyte development, less is known about its role in regulating myeloid lineage development and function, especially in the context of acute and chronic inflammation. In this review article, we will describe the evidence accumulated during the recent years to support a key regulatory role of the Notch pathway in innate immune and inflammatory responses and discuss the potential implications of such regulation for pathogenesis and therapy of inflammatory disorders.

Keywords: Notch signaling, Innate immunity, Cancer

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Introduction

The Notch pathway is one of the basic signaling pathways used repeatedly in development, and it is involved in both cell type specification and organogenesis. The role of the Notch signaling pathway in Drosophila development has been studied since a dominant notched wing phenotype was first reported in 1914 [1]. In 1996, *NOTCH3* mutations were found to cause CADASIL, a disorder characterized by stroke and dementia, with onset in the 3rd or 4th decade. CADASIL is caused by mutations in the extracellular domain of NOTCH3 [2], resulting from the gain or loss of cysteine residues in the epidermal growth factor-like repeats (EGFR-like). Pathological studies of tissue from CADASIL patients reveal accumulation of NOTCH3 protein in brain lesions.

Notch ligand structure

The DSL ligands of the Notch receptors have been also conserved throughout evolution [3]. *Drosophila* Notch has two DSL ligands, Delta and Serrate, whereas there are five

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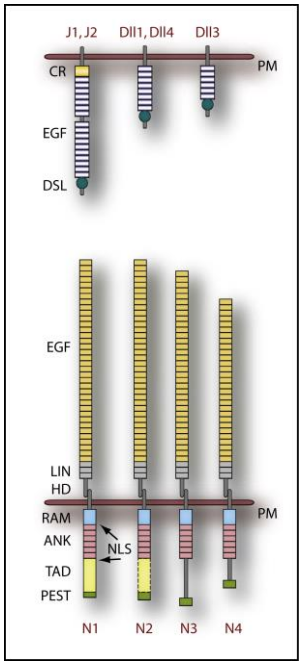
mammalian ligands, three of which belong to the Delta-like family (DLL1, DLL3 and DLL4) and two belong to the Jagged family of Serrate homologs, Jagged 1 and 2 [also known as JAG1 and JAG2, respectively.

DSL ligands are transmembrane proteins with an extracellular domain that contains a characteristic number of EGF-like repeats and a cysteine-rich N-terminal DSL domain. The DSL domain is a conserved motif that is found in all DSL ligands and is required for their interaction with Notch. Serrate, Jagged 1 and Jagged 2 contain an additional cysteine-rich domain. In contrast to the canonical DSL ligands, non-canonical ligands lack the DSL domain and comprise a group of structurally diverse proteins, which includes integral and glycosylphosphatidylinositol (GPI)-linked membrane proteins, and are presumed to modulate Notch receptor activity [4].



Notch ligands are known: Jagged1 (J1), Jagged2 (J2), Delta-like1 (Dll1), Delta-like3 (Dll3), and Delta-like4 (Dll4). A common structural feature of all ligands is an amino-terminal domain called DSL (Delta, Serrate, and Lag-2) involved in receptor binding followed by EGF-like repeats. A cysteine-rich domain (CR) is located downstream of the EGF-like repeats of J1 and J2 close to the plasma membrane (PM). Vertebrates have four Notch receptors (Notch1–Notch4; N1–N4). The extracellular domain of the receptors contains EGF-like repeats (36 in N1 and N2, 34 in N3, and 29 in N4) followed by three cysteine-rich LIN domains that prevent ligand-independent activation and the heterodimerization domain (HD). The cytoplasmic domain contains a RAM domain followed by six ankyrin repeats (ANK) that bind to the CSL transcription factor, two nuclear localization signals (NLS), a transactivation domain (TAD; present in N1 and N2), and a PEST sequence involved in regulating protein stability [5].

Notch Signaling in regulation inflammation and immunity

Recently, active Notch signaling has been observed under a variety of inflammatory conditions including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), primary biliary cirrhosis, also during bacterial and viral infections [6]. Many published manuscripts showed association of active Notch signaling with rheumatoid arthritis inform expression of Notch receptors and ligands were detected in the RA synovial tissues and aberrant activation of Notch1 was observed in primary synoviocyte cultures from RA patients [7]. Thus, there is compelling evidence suggesting that the Notch pathway is activated in RA and may modulate disease activities.

Notch signaling regulates numerous cellular processes during development and adult life [2]. Thus, it is not surprising that dysregulation in Notch signaling pathway has been implicated in

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several pathological processes including inflammatory diseases [8]. Despite the numerous reports that support a crucial role for Notch signaling in many inflammatory disorders, the mechanistic role of Notch signaling in these conditions requires more nvestigation. However, significant progress has been reported in the involvement of Notch signaling in immunity through regulation of immune cells development and function [9].

Inflammatory cytokines such as TNF and interlukin-1β (IL-1β) are abundantly present during the course of innate immune and inflammatory responses and are essential for host defense against a variety of pathogens. However, under conditions of uncontrolled inflammation and in autoimmune diseases, dysregulated production and/or action of inflammatory cytokines can be detrimental and pathogenic.

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Moreover, in osteoclast precursors, Notch-RBP-J signaling is activated by TNF and in turn inhibits osteoclastogenesis and attenuates TNF-mediated inflammatory bone resorption in a feedback manner [10]. Also, Notch activation is observed in a mouse pancreatic cancer model where TNF promotes expression of Notch target genes Hes1 and Hey1 [11]. TNF appears to function as an activator of Notch signaling in several cell types. IL-1β is another important pro-inflammatory cytokine. It is reported that IL-1β induces Notch target gene Hes1 [12].

Notch in cancer

Notch participates in many tumors development, the ability of Notch to potentially function as an oncoprotein or a tumor suppressor in certain contexts is unusual, but perhaps not surprising given its diverse roles during normal development. Notch and Lymphoid Neoplasms. In the late 1980s, Jeff Sklar's group identified a recurrent translocation t(7;9) associated with a small subset of T-ALLs [13].

Published researches showed that the breakpoints on chromosome 9 fell within the Notch1 locus and resulted in the juxtaposition of the T-cell receptor-β promoter/ enhancer region with the 3´ end of NOTCH1 on the derivative chromosome 9.1 As TCRβ is continuously expressed in T cells, the translocation caused dysregulated expression of a series of tumorspecific 5´-deleted NOTCH1 mRNA transcripts [14]. All known t(7;9) breakpoints fall within a single intron within the coding sequence EGF repeat 34 of Notch1. However, biochemical studies have shown that the t(7;9)-specific transcripts encode a predominantly nuclear ICN1-like molecule [14].

Insights into possible mechanisms relevant to the pathogenesis of Notch1-related T-ALL have come from studies looking at the role of Notch signaling in normal T cell development. Both Notch1 expression and activity appear to be triphasic during normal thymocyte development

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[15]. Levels are highest at the earliest stages of T cell development (CD4- CD8- (DN) cells), low in CD4+CD8+ DP cells, and intermediate in both the CD4+ and CD8+ single positive (SP) cells [16]. A variety of gain and loss of function studies have shown that Notch1 signaling through CSL is required for T cell commitment from a common lymphoid progenitor.

Role of Notch in lung cancer

Notch has also been linked to the pathogenesis of small-cell lung cancer (SCLC), Notch signaling is involved in both normal pulmonary development and tumorigenesis. During this process, Notch signaling controls pulmonary epithelial cell fate by activating HES, which in turn suppresses genes required for NE cell differentiation, such as ASH1. In experimental models of the developing lung, ASH1 is expressed in PNEC, while Notch1 and HES1 are strongly expressed in non-PNEC [17].

ASH1 forms a heterodimer with other ubiquitously expressed bHLH factors, and drives the expression of downstream genes needed for neuronal or NE differentiation Both HES1 and HES3 bind the hASH1 promoter and repress hASH1 transcription [18] providing one mechanism for downregulation of ASH1 by Notch. ICN1 may also induce hASH1 degradation through TAD-dependent polyubiquitination of the hASH1 protein. Mice lacking ASH1 have no detectable PNEC, while forced expression of ASH1 results in lung hyperplasia and metaplasia, though these cells displayed no detectable NE markers [19].

This suggests that while necessary, ASH1 is not sufficient for pulmonary NE differentiation. In contrast, enforced expression of both ASH1 and SV40 Large T Antigen results in aggressive lung adenocarcinomas with NE features, a phenotype previously found only in spontaneous murine tumors [20]. The targets of Large T antigen, p53 and Rb, are frequently inactivated in human lung cancers [21]. Human ASH1 is highly expressed in many SCLC line, but it is not detectable in non-SCLC (NSCLC) cell lines [22]. hASH1 transcripts are also highly over-expressed (1000-fold) in primary SCLC tumors, as compared to non-SCLC tumors and normal bronchial biopsies [4].

Notch Signaling in Pathogenesis of connective tissue diseases

connective tissue diseases are an autoimmune inflammatory tisue disorder, characterized by macrophages and lymphocytes infiltration, hyperplasia, and progressive tissue destruction [23]. Notch signaling has been shown to be implicated in various rheumatoid arthritis pathogenesis processes through mediate TNFα-induced RA synoviocytes proliferation and accelerate production of pro-inflammatory cytokines and immune responses including upregulation of anti-type II collagen (CII) antibodies [24]. Additionally, Notch signaling has been noted to mediate vascular endothelial growth factor (VEGF)/angiopoietin-2 (Ang-2) and hypoxia-induced angiogenesis and invasion in inflamed RA joint [24].

Moreover, Notch-3 and DLL-1 have been known to mediate CII-specific T-cells expansion and alter its response, which is usually elevated during the early phase of RA pathogenesis [23]. On the other hand, genetic and pharmacological inhibition of Notch signaling demonstrated

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relief in RA severity and had reduced pro-inflammatory cytokines levels in RA synoviocytes and collagen-induced arthritis (CIA) mice [24]. Interestingly, joints directed nanoparticles that bear either pharmacological or genetic Notch inhibitors successfully attenuate the severity of RA by reducing the progression of inflammation, and delay bone and cartilage damage in CIA mice [25].

These studies suggest that Notch signaling plays an essential role in RA pathogenesis. However, it remained unclear what specific cell type may be governed by Notch signaling in inflamed RA joints. Macrophages play a pivotal role in RA pathogenesis, evident by the numerous numbers and clear activation state of macrophages in synovial tissue, which are significantly correlated with disease severity [26]. Macrophages exhibit extensive pro-inflammatory, destructive, and remodeling properties, which significantly contribute to acute and chronic stages of RA pathogenesis [4].

Despite the ample evidence supporting the contribution of macrophages in RA pathogenesis, there is a lack of knowledge about the macrophage subsets in the RA synovial tissue. However, pro-inflammatory cytokines such as TNF-a and IL-1, which are consistently produced by M1 macrophages, are expressed abundantly in RA, whereas M2 characteristic cytokines such as IL-10 and IL-4 are relatively diminished in patients with RA [6]. Interestingly, M1 macrophages were predominately observed in high disease activity RA patients, whereas M2 macrophages are associated with low disease activity or clinical remission RA [8].

Most recently, the imbalance between M1 and M2 macrophages is considered one of the main causes of RA [27]. On the other hand, targeting unbalanced macrophage polarization may hold promise for treating RA by re-establishing homeostatic macrophages equilibrium. For example, the administration of human umbilical cord blood stem cells ameliorated the severity of CIA by promoting M2 macrophage polarization and suppresses the activation of M1 macrophages [8]. Besides, alginate nanoparticles loaded with IL-10 plasmid DNA and specifically designed to target macrophages have efficiently reduced the progression of inflammation and joints damage in experimental arthritis by re-polarizing macrophages from M1 to M2 phenotype [7]. Interestingly, many effective RA medications have been reported to manipulate M1/M2 polarization in favor of M2 macrophage polarization [21].

Consequently, cutting down M1 macrophage and promoting M2 macrophage polarization could offer a favorable treatment paradigm for RA. Given the implication of Notch signaling in the pathogenesis of RA and the crucial role of Notch signaling in the polarization of the macrophages, Notch signaling seems to play a causal role in M1/M2 imbalance in RA, which significantly implies in RA pathogenesis.

Overall, targeting Notch signaling in myeloid lineage may represent a potential novel therapeutic target for RA by controlling the balance of M1 and M2 macrophage polarization and re-establishing the homeostatic immune milieu. However, many clinical and pre-clinical studies are warranted to establish their therapeutic amenability in RA. Notch signaling seems to play a causal role in M1/M2 imbalance in RA, which significantly implies in RA pathogenesis. Using

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(TNF-α)- transgenic/(Hes-1)-GFP mice as RA model bearing Notch reporter transgene, Sun et al. identified M1 macrophages derived from bone marrow (BM) as the main cells with activated Notch signaling in the inflamed joint of (TNF-α)-transgenic mice. Additionally, they reported that RA synovial tissue promotes the activation of Notch signaling in BM-derived macrophages, leading to M1 polarization. While thapsigargin (Notch inhibitor) reduces TNF-α induced M1 macrophage polarization and attenuates inflammation and joint bone loss by switching M1 to M2 macrophages [9].

Cardiac and atherosclerosis diseases

In addition to the cardiac defects seen in association with ALGS, mutations in *JAG1* or *NOTCH1* have been found in individuals with non-syndromic right-sided cardiac disease that is similar in type to that seen in ALGS. *JAG1* mutations have been identified in patients with isolated TOF and in fewer cases, *NOTCH1* mutations have been associated with TOF as well [28]. *JAG1* sequence variants have been identified in 4% of patients presenting with pulmonic stenosis, peripheral pulmonic stenosis or pulmonary artery stenosis who did not meet the diagnostic criteria for Alagille syndrome [4].

We do not currently have a good explanation for why some patients with a *JAG1*mutation have the full range of clinical abnormalities associated with ALGS, while others have only cardiac disease. There was initial evidence that the *JAG1* missense mutation (G274D) identified in a large family segregating apparently isolated cardiac disease had a “leaky” phenotype, some of the protein appearing on the cell surface, with the ability to signal, but subsequent studies have shown that some cardiac only mutations appear to code for proteins with no activity leading to the hypothesis that there may be genetic modifiers [6].

NOTCH1 mutations are associated with structural abnormalities of the aortic valve, such as bicusupid aortic valve and have been found in individuals with more serious left ventricular outflow tract abnormalities such as aortic valve stenosis, coarctation of the aorta and hypoplastic left heart syndrome [2].

Functional work on the missense mutations associated with left ventricular outflow abnormalities have demonstrated reduced binding of the mutant receptor to the Notch ligands, as well as a reduction of the amount of receptor at the surface, with increased localization to the endoplasmic reticulum [11]. This reduced signaling is associated with defective epithelial-to- mesenchymal transition, which is necessary for proper formation of the aortic and pulmonary valves [15]. For a more in depth analysis of the role of Notch signaling in cardiac development there are several excellent reviews [19].

In the early stages of atherosclerosis circulating monocytes bind to ECs expressing adhesion proteins and migrate to the intima where they differentiate into macrophages. During the progression of atherosclerosis, monocytes attracted by inflammatory cytokines continue to infiltrate the growing plaque contributing to perpetuate the inflammation. Macrophages are classically divided into a high-inflammatory M1 subset and an anti-inflammatory (or less-

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inflammatory) M2 subset. M1 macrophages are classically defined as pro-inflammatory players secreting cytokines, such as IL-1, IL-6, IL-12, IL-15, IL-18, MIF, TNF-α able to trigger T cell-mediated responses. M2 macrophages hold anti-inflammatory activities able to resolve plaque inflammation and release different cytokines (IL-4, IL-10, and IL-13) from M1 [28]. TGF-β produced by M2 macrophages has a role in the biology of the vascular wall by influencing cell proliferation, differentiation, and production of extracellular matrix [20].

Studies on cultured monocytes found that Notch1 induces M1 macrophage differentiation and heightens inflammatory responses by increasing IL-6, MCP-1, and TNF-α production. Conversely, Notch1 inhibition drives in the direction of an increase of M2 differentiation promoting the secretion of anti-inflammatory cytokines IL-10 and IL-1RA [21].

In ApoE$^{-/-}$ mice, the treatment with Notch inhibitor DAPT reduced macrophages migratory activity and repressed ICAM-1 expression in macrophages that led to decreased macrophage infiltration in the atherosclerotic plaques [22]. Recently, a group of researchers demonstrated that Dll4 is the ligand involved in the Notch-dependent selection process promoting the differentiation of M1 macrophages and preventing the differentiation of M2 macrophages blocking the expression of M2 genes induced by IL-4. Noteworthy, Dll4 was also able to promote the induction of apoptosis selectively in M2 cells [27].

Consistent with a pro-inflammatory role of Notch signaling, Fukuda et al. have been shown in LDLr$^{-/-}$ mice that high-fat/high-cholesterol diet promotes expression of Dll4 in the atherosclerotic plaques and in fat tissue. Inhibition of the Notch signaling with anti-Dll4 antibody reduced atherosclerotic lesions, diminished plaque calcification while improving insulin resistance, and decreasing fat accumulation. These changes were associated with a reduction of macrophage accumulation and decreased MCP-1 levels. *In vitro* experiments revealed that Dll4-mediated Notch signaling increases MCP-1 expression by activating NF-κB. Noteworthy, also in this setting Dll4 induced macrophages M1 polarization [29].

Other reported that in a mice model of chronic kidney disease (CKD) accumulation of the uremic toxin 3-indoxylsulfate drives the expression of Dll4 in macrophages with consequent Notch signaling-induced pro-inflammatory responses. In this model an anti-Dll4 antibody was able to lessen both macrophage accumulation and atherosclerosis [3].

Notch signaling and hematopoietic stem cells

Hematopoiesis is the developmental process, whereby pluripotent hematopoietic stem cells (HSCs) give rise to committed progeny that undergo proliferation and differentiation in response to both positive and negative soluble and cell-bound factors and cytokines, resulting in the continuous production of mature blood cells of various lineages.

In the developing immune system, the Notch signaling pathway regulates interactions between HSCs, which express all four Notch receptors, and bone marrow stromal cells, which express various Notch ligands [6]. In this section, we will discuss recent progress in understanding the role of the Notch pathway in development and differentiation of myeloid cells. Although Notch

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signaling is thought to play a key role in myeloid cell differentiation from HSCs, there are discrepancies as to the mechanisms involved. One body of evidence demonstrates a role for Notch in the maintenance of progenitor cells and block of terminal differentiation of myeloid cells. In support of this hypothesis, retroviral transduction of the activated intracellular domain of Notch1 (NICD1) in 32D myeloid progenitor cells inhibited differentiation of mature granulocytes in response to granulocyte colony-stimulating factor (G-CSF), but not granulocyte macrophage colony stimulating factor (GM-CSF), without affecting proliferation of undifferentiated cells [9].

NICD2 inhibited differentiation of 32D cells in response to GM-CSF but not G-CSF [19]. These findings suggested that although both Notch1 and Notch2 inhibited myeloid differentiation, they may have distinct functions in HSCs depending on the specific differentiation signal involved. The Notch RAM domain, which contains the RBP-J binding region, was subsequently shown to be required for these Notch-mediated functions, implying that Notch signals through the canonical RBP-J-dependent pathway to inhibit terminal differentiation and enhance survival of 32D myeloblast cells. Over-expression of the downstream RBP-J target Hes1 resulted in a similar phenotype [17].

Notch signaling and osteoclastogenesis

Physiological bone development and remodeling represents a balance between bone formation by osteoblasts and bone resorption mediated by osteoclasts, which are multinucleated cells derived from the monocyte-macrophage lineage. Osteoclast differentiation is a multi-step process that culminates in expression of the osteoclast marker TRAP (tartrate-resistant acid phosphatase), multinucleation and bone-resorping activity.

Osteoclastogenesis depends on differentiation signals from stromal cells and synovial fibroblasts, and is physiologically triggered by RANKL (receptor activator of NF-κB ligand) in the presence of M-CSF and other co-stimulatory factors. Recruitment of these resorptive cytokines can be physiologically restricted by osteoprotegerin (OPG, also known as osteoclastogenesis inhibitory factor) [29]. RANKL stimulation of osteoclast precursors leads to the induction of cell signaling cascades resulting in activation of the master transcriptional regulator of osteoclastogenesis, NFATc1 (nuclear factor of activated T cells, cytoplasmic 1). Numerous inflammatory molecules, such as TNFα, IL-1β, IL-17, and TLR ligands, promote osteoclastogenesis in synergy with RANKL to induce pathological bone resorption in inflammatory settings.

As such, osteoclasts have been implicated in musculoskeletal tissue damage and the pathogenesis of diseases characterized by inflammatory osteolyis, including RA, psoriatic arthritis, and peridontitis. In these disease settings, abnormally enhanced osteoclast formation and activity causes bone loss that results in pain, deformity, osteopenia, osteoporosis and even fracture. The extent of bone destruction in inflammatory disease is determined by the balance between positive and negative regulators of osteoclastogenic factors [30].

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Notch signaling has been implicated in osteclastogenesis during normal bone homeostasis and inflammation. Notch receptors, ligands and target genes have been detected in osteoclast precursors and differentiated osteoclasts [29].

A role for Notch in promoting osteoclast differentiation has been described. Suppression of Notch signaling by GSI treatment or shRNA for Notch2 inhibited RANKL-induced osteoclast differentiation, whereas activation of Notch signaling by stimulation with Jagged1 or NICD2 over-expression increased NFATc1 promoter activity and romoted osteoclastogenesis [30]. DLL1 also decreased surface expression of the M-CSF receptor c-Fms on the bone marrow cells. Stromal cells over-expressing NICD1 reduced M-CSF production and enhanced RANKL and OPG production, resulting in the decreased capability of these cells to support osteoclastogeneis [5].

Subsequently, genetic approaches indicated that deletion of Notch1 or combined Notch1–3 enhanced osteoclastogenesis in response to M-CSF or RANKL, resulting in increased resorptive activity [12]. Osteoclast precursors with inactivated Notch1–3 exhibited increased expression of c-Fms. Overexpression of NICD1 or Jagged1 stimulation of wild type BMDMs blocked their differentiation into osteoclasts in response to M-CSF and RANKL [4].

Further studies have supported an inhibitory role for Notch in the context of TNFα-induced osteoclastogenesis in the inflammatory setting [22]. RBP-J was shown to strongly repress TNF-induced osteoclastogenesis, as myeloid specific deletion of RBP-J dramatically increased osteoclastogenesis and resulted in severe bone destruction in a TNF-induced inflammatory bone resorption model.

Additionally, knockdown of RBP-J expression in human osteoclast precursors by RNAi enhanced TNF-induced osteoclast differentiation. By activating RBP-J using forced expression of NICD1 in myeloid osteoclast precursors, TNF-induced inflammatory bone resorption was dramatically decreased. RBP-J was demonstrated to suppress induction of NFATc1 by attenuating cFos activation and inhibiting induction of Blimp1, thereby preventing the downregulation of transcriptional repressors such as IRF8 that block osteoclast differentiation [12]. Such inhibitory effects are possibly attributed to Notch-mediated crosstalk with other pathways such as immunoreceptor tyrosine-based activation motif-containing (ITAM-containing) receptors and adaptors [6] as well as TAK1 signaling [6]. Thus, the majority of studies have delineated a direct inhibitory role for Notch signaling in the physiological context of osteoclastogenesis and inflammatory bone resorption. In addition, Notch signaling may indirectly regulate osteoclast differentiation *in vivo* by regulating the differential expression of RANKL and OPG on osteoblast lineage cells [30].

Targeting developmental Notch pathways

Long-term therapeutic success in cancer is rarely achieved with monotherapy, and even targeting developmental pathways such as Notch will most likely require the development of combination regimens. Traditionally such regimens have been produced through a process of

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'clinical trial and error', often based on limited mechanistic information. Clinical experimentation will always be necessary, because no preclinical model completely recapitulates a patient. However, the more complete and accurate our mechanistic understanding of how the pathways we target cross talk with each other, the less guesswork will be involved in designing future therapeutic regimens.

The high evolutionary conservation of developmental pathways means that information from simpler model organisms is likely to be reliably predictive of human pathophysiology [31]. On the other hand, the context dependence of Notch signaling will require each specific cancer type to be studied independently, without preconceived notions. Our knowledge is still considerably incomplete, but evidence accumulated so far suggests that some combination regimens involving Notch inhibitors deserve further investigation. The examples that follow are not meant to be all-inclusive [32].

Inhibitors of the PI3-kinase–AKT–mTOR pathway may be useful in combination with Notch inhibitors, and there is evidence that this strategy may reverse resistance to GSIs in T-ALL that carry PTEN inactivating mutation [30]. Whether this strategy can be successful in other cancers characterized by loss of PTEN is still unclear.

The complex cross talk between Notch and NF-κB suggests that at least in some circumstances drugs that inhibit NF-κB activity directly or indirectly could be successfully combined with Notch inhibitors [16]. As intracellular Notch is degraded by the proteasome, and accumulates in cells treated with proteasome inhibitors, it is possible that these agents may benefit from the addition of a GSI. As DLL4 mAb appear to be effective independently of VEGF, they may be useful in combination with agents that block the VEGF pathway such as bevacizumab.

To design the best combination regimens including Notch inhibitors, indication-specific studies will have to be performed. These studies will need to include a whole range of approaches, from simple model organisms such as *Drosophila* or zebrafish to *in vitro* and *in vivo* studies in mammalian models to identify which genetic and epigenetic factors interact with Notch signaling.

These findings will require validation by studies of primary clinical specimens. Ultimately, the best use of these new therapeutic targets, as is the case for newest targeted agents, will be in the context of 'individualized medicine'. It will be necessary to identify groups of patients and/or subtypes of cancer who are most likely to benefit from Notch inhibitors. To that end, we will have to determine: (1) which cancers and specific subtypes are characterized by active Notch signaling; (2) what role do specific components of Notch signaling perform in these cancers (for example, Notch-2 versus Notch-1), and whether global or selective Notch modulation is most desirable and (3) what genes or pathways cross talk with Notch in specific cancers, indicating targets for combination regimens.

High-throughput system biology and bioinformatics will be important in this task. Simply determining expression levels of receptors and ligands in clinical specimens will not necessarily identify prospective responders. Because of extensive cross talk between Notch and other

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pathways, there is no simple correlation between expression and Notch activity. Thus, it will be important to develop accurate molecular tests that measure the level of pathway activity *in vivo*, possibly based on expression levels of multiple genes in the pathway. With the tools of today's cancer biology, these tasks are not as daunting as they would have been a few years ago. And the payoff for these efforts may be multiple new treatments for a whole range of human malignancies.

Conclusions

Notch signaling is essential in all cellular processes, and its dysregulation has been linked to many diseases. Nevertheless, Notch seems also to influence Th1 cell differentiation. Th1 and Th2 cell differentiation may depend on the ligand used to activate Notch.

The disease outcome of several patient-relevant experimental murine autoimmune models can be influenced by interfering with Notch signaling, suggesting that inhibitors or activators of Notch might be used to treat inflammatory and/or autoimmune diseases. In this context, it is important to note that many functions of Notch are conserved between mice and men. Nevertheless, the effects of interfering with Notch signaling in human systems remain to be systematically investigated.

The Notch pathway became an attractive therapeutic target and multiple tools (e.g., γ-secretase inhibitors, neutralizing antibodies against Dll4 or NOTCH1) that interfere with Notch signaling are currently being developed and tested in various murine cancer models or even in clinical trials. In the future, the same tools might also be exploited to treat immunological disorders.

Author Contributions

EM, SB wrote the paper. SB, TG, provided substantial revision. All authors reviewed and approved the final version of the manuscript.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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