Pleiotropic roles of Notch signaling in normal, malignant, and developmental hematopoiesis in the human

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Abstract

The Notch signaling pathway is evolutionarily conserved across species and plays an important role in regulating cell differentiation, proliferation, and survival. It has been implicated in several different hematopoietic processes including early hematopoietic development as well as adult hematological malignancies in humans. This review focuses on recent developments in understanding the role of Notch signaling in the human hematopoietic system with an emphasis on hematopoietic initiation from human pluripotent stem cells and regulation within the bone marrow. Based on recent insights, we summarize potential strategies for treatment of human hematological malignancies toward the concept of targeting Notch signaling for fate regulation.

Keywords hematopoiesis; human pluripotent stem cells; leukemia; Notch
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Introduction

The Notch gene was identified through mutational studies in *Drosophila* where a wing indentation phenotype corresponded to a gene locus identified to play an important role in embryogenesis [1]. Subsequently, a subset of acute T-cell leukemia in humans was shown to possess a gene located at a t(7;9)(q34;q34) breakpoint on chromosome 9 responsible for transcription of a human ortholog of *Drosophila* Notch and thus termed translocation-associated Notch-1 (*TAN-1*, later renamed *NOTCH1*) [2]. This observation initiated a series of studies into human Notch signaling and the search for mammalian homologs of *NOTCH1*.

Notch signaling is regulated by the direct interaction of evolutionarily conserved transmembrane receptors and their ligands. In mammals, there are four Notch receptors (*NOTCH1*, 2, 3, and 4) and five structurally related, single-pass membrane Notch ligands (*DLL1*, *DLL3*, *DLL4*, *JAG1*, and *JAG2*), which are all single-pass membrane

proteins [3]. Although *DLL3* has been shown to attenuate activation of Notch by other ligands, there is no evidence that *DLL3* physically binds Notch receptors [4], suggesting other elusive forms of cross talk. Within the hematopoietic system, Notch receptors and ligands are expressed in hematopoietic stem cells (HSCs) and stromal cells [5]. Notch signaling is activated when a NOTCH receptor binds its ligand on an adjacent cell. Upon binding, the receptor undergoes an intramolecular cleavage in the extracellular region (S2 cleavage), leading to a subsequent cleavage in the transmembrane domain (S3 cleavage) [6]. The final S3 cleavage releases the Notch receptor intracellular domain (NICD), which translocates to the nucleus and associates with transcription factors (for example, CBF1/RBP-J) and the known co-activator Mastermind-like (MAML) family [7], thereby displacing co-repressors that include c-repeat/dre binding factor 1 (CBF1) that associate with the DNA-binding protein LAG-1 (CSL) transcription factor. Different activating transcription factors are recruited to induce the expression of target genes such as *HES1* [8]. Downregulation of Notch transcriptional activation is mediated by F-box and WD repeat domain containing protein 7 (FBXW7) via ubiquitination that is followed by proteasomal degradation of NICD [9]. Alternatively, non-canonical CSL-independent pathways of Notch signaling have been suggested, but the importance of these pathways is presently unclear [10], may be cell context specific and requires further investigation in the appropriate systems.

Although the various modes of Notch signaling are well described, their regulation in a diverse set of cellular activities such as proliferation, differentiation, and survival/apoptosis in both physiological and pathological contexts [11,12] is not fully understood. Furthermore, the recently described important role of Notch in cell fate decisions, including stem cell regulation, has extended the potential impact of Notch regulation into regenerative medicine and clinically relevant hematopoietic regulation [13–15]. Future avenues and utility of Notch control extend to lineage specification of human pluripotent cells to mature cell types that could be used for disease modeling or cell replacement therapies in the human. As illustrated in Fig 1, this review attempts to capture these and other aspects of Notch involvement in normal and malignant hematopoiesis by detailing our current understanding the role of Notch in stem cell fate decisions.

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Figure 1. Roles of Notch signaling in human hematopoiesis and their relationships.

Notch signaling plays a role in both the self-renewal and differentiation of human HSCs through direct effects on HSCs and via indirect effects through regulation of the bone marrow niche. As such, Notch plays a multifaceted role in hematological malignancies by both regulating cancer stem cells along with regulation of the transformed bone marrow niche. Modulation of Notch signaling has been utilized for improving hematopoiesis from human pluripotent stem cells (both embryonic stem cells (ES) and induced pluripotent stem cells (iPS)). Furthermore, ES/iPS offers a unique human experimental system for better understanding the roles of Notch signaling in hematopoiesis as studies involving direct modulation of Notch are not feasible in the adults.

Complexity of Notch signaling in hematological diseases

Due to recent findings, the emerging role of Notch signaling in hematological diseases has become increasingly complex and multifaceted, ranging from initiation of human T-cell acute lymphoblastic

leukemia (T-ALL), to driving chronic lymphocytic leukemia (CLL), and to tumor suppressor roles in B-cell malignancies. An overview of the complex role(s) of Notch signaling in hematological diseases to date is given in Fig 2. Since the first identification of an activating *NOTCH1* mutation in human T-ALL [2], several other *NOTCH1*

Figure 2. Complex roles of Notch signaling in hematological diseases.

Notch has diverse functions in hematological diseases, which ranges from roles in cancer initiation, disease progression to even tumor suppression, which is being evaluated for clinical applications for treatment of hematological diseases.

mutations, all resulting in aberrant Notch activation and involvement in diverse oncogenic processes, have been characterized in T-ALL [16,17]. They likely lead to oncogenic *MYC* activation coupled with deletion of the tumor suppressor genes *P16/INK4A* and *P14/ARF*, which leads to malignant transformation of hematopoietic progenitors toward T-cell development. This suggests that Notch signaling is an initiator of T-ALL [18]. Recent studies have shown that normal T cells can undergo leukemogenic transformation by *KRAS* in conjunction with *NOTCH1* mutations [19]. Mutations in *NOTCH1* that drive leukemogenic transformation include mutations in exon 34 that encodes for the PEST domain in the C-terminal region [16] as well as type 1 deletions that remove exon 1 and a portion of the proximal *NOTCH1* promoter. Additionally, type 2 deletions, which have been identified to remove sequences between exon 1 and exons 26 to 28 of *NOTCH1*, have also been implicated in driving leukemogenic transformation of T cells [20,21]. Therefore, Notch1 activation plays an undisputed role as an initiator in T-ALL.

Activated Notch signaling has also been linked to CLL, albeit not causatively, in disease progression of a subset of patients. CLL is characterized by accumulation of $CD5^+$ B cells that fail to undergo apoptosis [22]. *NOTCH1* mutations are predictors of poor prognosis in CLL, and *NOTCH1* and 2, together with their ligands *JAG1* and *JAG2*, have been identified to be constitutively expressed in CLL B cells. As the Notch pathway is not constitutively active in normal B cells, these constitutive Notch signals are likely playing a role in preventing CLL B-cell apoptosis [23]. Thereby, *NOTCH1*-activating mutations (mainly a frameshift mutation at codon 2,515) that impair FBXW7-induced *NOTCH1* degradation have been identified at a frequency ranging from 8.3 to 12.2% in CLL patients, particularly in patients with a more clinically aggressive non-mutated IGV(H) subtype of CLL (20.4%), Richter syndrome (31.0%), and in chemorefractory CLL (20.8%) [24]. Interestingly, aberrant Notch signaling has also been identified to play a role in solid tumors and tumor angiogenesis [25]. Hereditary hemorrhagic telangiectasia (HHT), which is characterized by abnormal blood vessel formation,

is caused by inactivation of activin receptor-like kinase 1 (ALK1), an endothelial-specific member of the TGF- β /BMP receptor family. In this context, aberrant angiogenesis has been shown to depend on inactivation of Notch signaling leading to hemorrhage in different organs [26]. These observations point toward a role of Notch in vascularization of tissue and organs, as well as abnormal blood cell growth and differentiation. The interplay between these tissue types in the context of Notch control has not been determined.

In contrast to T-ALL and CLL, Notch acts as a tumor suppressor in B-cell leukemia. Expression of constitutively active *NOTCH1* has been shown to inhibit growth and induce apoptosis in both mature and therapy-resistant B-cell malignancies like Hodgkin, myeloma, and biphenotypic mixed-lineage leukemia-translocated B-ALL lines [27]. Furthermore, expression of oncogenic RAS in endothelial cells has been shown to promote hyperproliferative myelo-erythroid disorders by suppression of Notch signaling [28]. In a recent study, Notch was identified as a tumor suppressor in human chronic myelomonocytic leukemia (CMML). In this disease, deletion of the γ -secretase component, *NICASTRIN*, results in inactivation of Notch in HSCs leading to aberrant accumulation of granulocyte/monocyte progenitor (GMP) cells and extramedullary hematopoiesis [29]. The effects on myeloid lineages suggest a potential role of Notch in acute myeloid leukemia (AML). Unfortunately, the role of Notch signaling in AML is quite complex, with reports indicating a role of Notch in both AML initiation as well as suppression, by acting in different cell populations. Interestingly, Notch activity was identified in a recent report to induce cell cycle arrest and apoptosis on the majority of cytogenetically normal patient AML cells [14]. Importantly, deletion of the frequently mutated oncogene *TET2* in mice coupled with inactivation of Notch signaling in the GMP subset has been shown to induce AML-like disease, thereby pointing toward Notch as being an instigator in AML through its effects on the GMP cell fraction [14]. In summary, while Notch seems to play a pivotal role in hematological malignancies, the underlying mechanisms are still not completely understood.

Further detailed analysis of Notch receptor interactions in specialized tissue microenvironments are needed to clarify whether the effects of Notch on cell fate decisions are responsible for the observed duality in hematological malignancies and may help understand the diverse effects of Notch in the human hematopoietic system that lead to malignancies.

Notch bone marrow microenvironment

Notch signaling within the bone marrow (BM) microenvironment or niche where HSCs reside has also been shown to initiate and promote tumor progression [30–33]. In multiple myeloma (MM), characterized by the accumulation of cancerous plasma cells in the BM and composed of extracellular matrix, BM stromal cells (BMSCs) play a major role in the survival of cancerous plasma cells along with disease progression [30]. BMSCs include mesenchymal stem cells that express Notch receptors *NOTCH1*, 2, 3, and 4 and Notch ligands *JAG1*, *DLL3* and 4 at basal conditions and are, thereby, sensitive to specific inhibitors [33]. Cell–cell contact between MM cells, and between MM cells and bone marrow cells/niche cells appear to induce Notch signaling, which induces MM proliferation, suppresses apoptosis, and ultimately leads to drug

resistance. Accordingly, inhibition of Notch signaling in the BM niche prevents proliferation and resistance of MM cells to apoptosis [32]. MM-induced Notch signaling in BMSCs has been shown to induce secretion of interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and insulin-like growth factor-1 by the stromal cells, which exert anti-apoptotic and proliferative effects on MM cells [31]. BMSCs have also been shown to suppress apoptosis and promote migration as well as drug resistance of CLL cells via Notch signaling [34]. In addition to MM and CLL, Notch signaling in BMSCs also plays a role in disease initiation in T-ALL. In this case, migration of T-ALL cells to the BM niche has been shown to be necessary for disease development [33]. BMSCs enhance survival of T-ALLs via induction of adhesion molecules such as integrins and ICAM-1, which are amenable to modulation by Notch signaling [35]. Conversely, induction of Notch signaling in AML appears to suppress angiogenesis by suppressing VEGF-induced endothelial cell proliferation [36]. Moreover, Notch signaling in the BM niche may also play a direct role in AML initiation. A recent study identified an activating mutation of beta-catenin in osteoblasts, resulting in the overexpression of the Notch ligand JAG-1. This further induced Notch signaling in HSCs and progenitors, leading to the development of myelodysplasia that transforms into AML in mice [37]. This aberrant notch activation in osteoblasts was also found in approximately 38% of human patients with myelodysplastic syndromes or AML [37].

While these findings are in contrast to reports of a suppressive role of Notch signaling in AML induction [14], they highlight the complex role of Notch in these malignancies, which could have opposing cell autonomous role(s) within different cells of the hematopoietic microenvironment. This potential complexity is exemplified by the recent demonstration of Notch regulation of microRNAs, where miR-155, expressed in the BM niche and previously implicated in tumorigenesis, was shown to be repressed by Notch through RBPJ binding to the miR-155 promoter [38]. Although miR-155 upregulation was correlated to a patient subset with myeloproliferative disease, whether this exact regulatory mechanism and a role of the Notch-miR-155 axis have an impact on human disease remains to be determined.

Inhibiting Notch for leukemia therapy

Notch signaling can have both direct and indirect effects on cancer development, resulting in this pathway being a promising target for cancer therapy. The effects of Notch signaling likely stem from its extensive crosstalk with other critical tumor pathways such as PI3K/AKT, RAS, and several tyrosine kinase pathways that all have direct roles in tumor growth as well as indirect effects on angiogenesis [39,40]. It may be particularly powerful to target Notch in cancer stem cells (CSCs) that are thought to be resistant to standard treatments, such as chemotherapy and radiation, yet appear to be especially sensitive to inhibition of pathways involved in stem cell regulation such as Notch. The Notch pathway appears to be highly active in CSCs in medulloblastoma and glioblastoma, and it has been reported that inhibition of Notch signaling results in the depletion of CSCs in glioblastoma [41]. Similarly and in addition, inhibition of non-canonical Notch signaling has been shown to preferentially impair neural CSCs [42]. In mouse lymphoma and human

AML, hypoxia inducible factor- α (HIF-1 α) is known to maintain CSCs by repressing a negative feedback loop in the Notch pathway and as such, inhibition of the pathway has been shown to specifically eliminate CSCs [43]. Therefore, multiple strategies to inhibit Notch signaling in cancer have been explored, and the major Notch inhibitors that have proved useful in experimental and clinical medicine are outlined below.

Gamma-secretase inhibitors (GSIs)

Over 100 GSIs have been synthesized to date and can be divided into three subclasses: peptide isosteres, azepines, and sulfonamides. The development of GSIs for T-ALL has been inhibited by limited efficacy in inducing tumor cell apoptosis in addition to the development of gastrointestinal toxicity due to the accumulation of secretory goblet cells in the intestine [44]. Although GSIs have shown limited efficacy in inducing T-ALL apoptosis, they do have a profound effect on the homeostasis of T-ALL lymphoblasts and likely sensitize them for chemotherapy [45]. Accordingly, a combinatorial therapy involving GSIs with glucocorticoids has been reported to revert glucocorticoid resistance in glucocorticoid resistant T-ALL, thereby inducing tumor apoptosis while preventing gut toxicity in mice [46]. Furthermore, *in vitro* studies have also indicated that GSIs, by blocking Notch activation, can efficiently trigger apoptosis in CLL by inhibiting proteasome activity and enhancing endoplasmic reticulum stress [47]. Preclinical *in vitro* studies have also documented efficacy of GSIs in suppressing proliferation of tumor cells in MM and non-Hodgkin's lymphoma [48].

Alpha-secretase inhibitor (ASI)

Following Notch activation, the Notch extracellular domain is cleaved by metalloproteinases of the disintegrin and metalloproteinase (ADAM) family (α -secretases), indicating that ASI could be a potential inhibitor of Notch signaling. Although ASI-mediated Notch inhibition has been shown to suppress glioblastoma growth [49], its utility in hematological malignancies warrants further investigation [48].

Antibody inhibitors

Antibodies against Notch, its ligands Delta and Jagged, or other components of the Notch pathway have been investigated for Notch immunotherapy. Antibodies have been developed to specifically target the extracellular negative regulatory region of Notch, thereby preventing the conformational change that is required for ADAM protease cleavage [50]. Although antibodies are able to suppress oncogenic Notch signaling, the efficacy of Notch1 antibodies targeting the negative regulatory domain of Notch may be limited compared to GSIs, [51] and therefore, combinatorial approaches have been considered. Treatment of pediatric T-ALL tumors delays their engraftment in immunocompromised mice, and further treatment with dexamethasone in combination with an anti-NOTCH1 antibody increases T-ALL apoptosis, decreases cell growth, and induces a strong suppression of Notch target gene expression [52]. Moreover, even though antibodies against NOTCH2/3 have been shown to suppress solid tumor growth [53] and 293T cell growth [54], their utility in hematological malignancies remains unexplored [55]. In addition to Notch receptors, antibodies against Notch ligands have also been developed. Despite the efficacy of anti-DLL4 antibodies in suppressing solid tumors through anti-angiogenic

effects [56] in mice [57], their potency in human hematopoietic malignancies remains to be tested. At the moment, phase I clinical trials are underway only for non-hematological malignancies [58].

Other small molecule inhibitors

Synthetic, cell-permeable peptides have been developed to target the protein–protein interface of the Notch transactivation complex. For example, the SAHM1 peptide antagonizes Notch activation and exerts anti-proliferative effects on cultured T-ALL cells as well as in murine model of *NOTCH1*-driven T-ALL [59]. Since the Notch extracellular domain is cleaved by metalloproteinases of the ADAM family, inhibition of ADAM10 suppresses *NOTCH1* signaling in T-ALL lymphoblasts [60]. The proteasome inhibitor bortezomib has been shown to be cytotoxic in T-ALL by repressing *NOTCH1* and its downstream effectors along with downregulating the major transactivator Sp1. In addition, bortezomib induces dissociation of Sp1 from the Notch promoter, resulting in activation of NF- κ B signaling [61]. However, inhibition of proteasomal Notch-IC degradation by mutation of the *NOTCH1* PEST domain, or mutation of its E3 ligase FBXW7, has been identified to be an oncogenic event [62,63] and, with bortezomib being a proteasome inhibitor, may act as a double-edged sword for treatment of T-ALL. Sarco/endoplasmic reticulum calcium ATPase inhibition has been reported to impair the maturation and activity of mutated Notch1 receptors and to induce cell cycle arrest in *NOTCH1*-mutated human leukemia cells and is being investigated as a novel inhibitor of Notch signaling in cancer therapy [64].

Role of Notch in HSC self-renewal and differentiation

Self-renewal and differentiation are the two processes that maintain homeostasis of HSCs. Notch signaling is known to play a role in both processes, as soluble DLL1 ligand increases the number of putative CD34⁺CD38⁻ human HSCs [65] as well as of CD133⁺ primitive hematopoietic progenitors of cord blood cells *in vitro* [66]. In line with these *in vitro* observations, soluble DLL1 increases the regenerative capacity of human SCID repopulating cells (SRCs) detected by *in vivo* transplantation [65] with long-term survival capacity [66]. However, depending on protein concentration, immobilized DLL1 has also been shown to have opposite effects on putative HSCs: While low doses *in vitro* cause an observed increase in both the number of hematopoietic progenitors and human SRCs, high doses result in apoptosis of SRCs [67]. In addition, DLL4, another Notch ligand, has been reported to inhibit CD34⁺CD38⁻ cell proliferation and short-term SRCs while maintaining long-term SRCs [68]. These observations are concomitant with reports showing that active forms of NOTCH1 and 4 lead to an increase in long-term culture initiating cells (LTC-IC) *in vitro* as well as increase in engraftment levels of SRCs [69].

In addition to self-renewal, Notch signaling also affects stem cell differentiation. For example, DLL1 induces erythroid differentiation [70] and inhibits formation of myeloid progenitors and mature monocytic cells [71]. Similarly, DLL4 augments HSC proliferation in *ex vivo* cultures [65] and induces erythroid differentiation [72]. Moreover, the T-cell differentiation program is activated by DLL4 [73]. Further elucidation of the role of Notch signaling in mediating lymphoid differentiation program comes from genetic targeting of

RBP-J, which functions immediately downstream of Notch receptors and mediates transcriptional activation through their intracellular domains [74,75]. Genetic targeting of RBP-J in B cells has been shown to induce loss of marginal B cells with an increase in follicular B cells, pointing toward a role of Notch signaling in regulating B-cell fate [74]. Furthermore, loss of RBP-J during early stages of T-cell development enhances the number and accelerates migration of gamma-delta T-cell while arresting alpha-beta T-cell development, pointing to a role of Notch signaling in this process [75]. A complex of NICD with RBP-J recruits transcriptional co-activators of MAML family and overexpression of dominant negative MAML has confirmed a role of Notch signaling in regulating lymphoid differentiation [76,77]. However, RBP-J can also be controlled independently of Notch, for example by the transcription factor *PTF1A* [78], and further studies are needed to fully understand the role of Notch-dependent RBP-J signaling in regulating differentiation. Aside from lymphoid regulation, Notch signaling has also been implicated in regulating myelopoiesis. Membrane-bound JAG1 augments the number of myeloid colony frequencies in CD34⁺CD38⁺ cells [71]. When Notch signaling is constitutively activated through transducing the NICD into HSC, both a delay in hematopoiesis [79] and decrease in myeloid colony formation [69] are observed. Reduction in proliferation is due to a p21-mediated and *HES1*-independent upregulation of apoptosis [80]. These results demonstrate the important functions of Notch signaling in HSCs, but do not clarify how these pathways are activated or controlled extrinsically.

Notch and the bone marrow niche of HSCs

Although intrinsic roles for Notch signaling in HSC cell fate have been studied, the extrinsic role of ligand presentation from the HSC microenvironment is only recently coming to light. The adult hematopoietic niche is a specialized microenvironment in the BM that consists of supportive endosteal, vascular, endothelial, stromal, and adipose cells. Soluble factors, niche cell-HSPC interactions, and the physical structures of the BM facilitate the regulation of the hematopoietic hierarchy through modulation of HSPC self-renewal and differentiation. Osteoblastic niche cells are also capable of regulating major signaling pathways involved in self-renewal and differentiation through Notch signaling. Osteoblasts express the Notch ligand JAG1, which is markedly upregulated following osteoblast activation by parathyroid hormone, which, through activation of the parathyroid hormone 1 receptor, induces Notch signaling in HSCs and increases the stem cell pool [81]. However, conditional deletion of both *JAG1* and *NOTCH1* from BM cells does not affect HSCs. These opposing observations suggest that Notch signaling may affect HSCs only under circumstances of physiologic challenge or that other members of the Notch family play a compensatory role following deletion of a single Notch receptor or ligand. On the other hand, Notch signaling may also be suppressed in putative HSCs. The identification of the proto-oncogene *LRF* (Leukemia/lymphoma Related Factor, encoded by the *ZBTB7A* gene and also known as Pokemon) as a negative regulator of Notch signaling in BM progenitors indicates that Notch signaling must be repressed or under very stringent control in HSCs in order to prevent ectopic T-cell differentiation in the BM [82]. The mechanism of *LRF*-mediated

suppression of Notch signaling in HSC or progenitor cells is currently unknown.

Although Notch ligands are presently used to expand murine and human hematopoietic progenitors, there is limited evidence that Notch can be used to expand long-term HSCs [83,84]. We showed that the functional capacity of HSCs depends on regional localization within the BM, which, in itself, is possibly dependent on Notch signaling within the HSC niche [13]. In functional xenotransplantation assays, we found that human HSCs localizing to the trabecular bone area (TBA) have superior regenerative and self-renewal, along with preferential myeloid differentiation, capacity compared to HSCs localizing to the long bone area. Although this lineage discrepancy could potentially be explained by the observed upregulation of specific myeloid genes in HSCs homing to the TBA, such as *MEIS1* and *CEBPA* [85,86], our data indicates that the Notch pathway may modulate this myeloid deviation as most HSCs homing to the TBA were associated with JAG1⁺ osteoblasts, therefore corroborating previous studies that revealed the direct implication of Notch ligands in both HSC expansion and myelopoiesis *in vitro* [65,87,88]. Furthermore, genetic studies in mice have shown that defective Notch activation in the BM microenvironment leads to myeloproliferative disease when normal HSCs are transplanted into defective Notch recipient mice [89]. This is further supported by the identification of JAG-1-expressing osteoblasts being drivers of myeloid leukemia [37]. Interestingly, overexpression of NOTCH1 and 4 receptors in hematopoietic progenitors inhibits lymphoid differentiation, which in turn drives an increase in myeloid differentiation decisions by HSCs under the instruction of Notch ligands expressed by BM niche cells [69,79]. Nevertheless, further studies will be needed to identify the physiologically relevant combination of Notch receptors and ligands implicated in the regulation of myelopoiesis *in vivo*. These findings indicate the importance of maintaining tight control on both level and duration of Notch signaling in HSC fate decisions. A better understanding of these complex interactions should yield novel approaches for manipulating HSCs in culture without losing their self-renewal and regenerative properties.

Notch signaling in hematopoietic differentiation of hPSCs

Significant advances in cellular reprogramming technologies and hematopoietic differentiation from human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) have enabled successful production of multiple lineages of blood cells *in vitro* and opened novel opportunities to study hematopoietic development, model genetic blood diseases, and manufacture immunologically matched cells for transfusion and cancer immunotherapy. Hematological diseases usually require transplantation of hematological cells, which is limited by the donor pool. As such, hESCs/hiPSCs have been considered as a renewable source of hematopoietic cells and several protocols have been developed for the generation of different blood cells from hESCs/hiPSCs. However, generation of hematopoietic cells with robust and sustained multilineage engraftment has not yet been achieved from hESCs/hiPSCs. Therefore, a better understanding of the mechanisms regulating the formation of hematopoietic lineages from hESCs/hiPSCs is required.

HSCs arise from specialized endothelial cells (ECs) with hemogenic potential under specific culture conditions of mouse ESCs (reviewed in [90]). Notch activity initiates hematopoietic commitment toward differentiation into all three embryonic layers (mesoderm, ectoderm, and endoderm) with a transient requirement of *HES1* expression for further hematopoietic differentiation [91]. During the first wave of hESCs/hiPSC-derived hematopoiesis, Notch signaling needs to be silenced to achieve correct primitive erythroid progenitor development [92]. This observation is supported by genetic studies demonstrating that Notch-mediated regulation of early embryonic hematopoiesis vs. stem cells in adults is cell context specific [93]. Although *NOTCH1*^{-/-} mice show impaired hematopoiesis in the embryo, caused by defective hemogenic ECs, no impairment in *NOTCH2*^{-/-} mice was observed. This highlights the complexity in the role of Notch1 vs. 2 signaling in regulating initiation and emergence of the hematopoietic lineage in the developing embryo [94]. Furthermore, *NOTCH1* has been shown to be dispensable for primitive hematopoiesis and yolk sac embryonic hematopoiesis. Additionally, deletions of other Notch signaling components such as *JAG1*, *RBPI*, and Mind bomb-1 also result in defective hematopoiesis in knockout mice [95]. Taken together, these findings underscore the complexity of Notch signaling during early development and suggest that specific transitions of Notch signaling will be essential to appropriately control step-wise hematopoietic specification of hESCs/hiPSCs *in vitro* toward generation of putative HSCs. This also implies that the delicate coordination of other pathways such as Wnt and BMP that regulate self-hematopoietic differentiation of hPSCs [96] has to be considered.

To better understand the specific role of Notch signaling in human hematopoietic development, modulation of Notch signaling has been attempted *in vitro* in hESCs/hiPSCs cultures in a niche-dependent manner or via chemical/genetic manipulation. During hESCs/hiPSC hematopoiesis, Notch signaling could be activated in target cells by co-culturing hESCs/hiPSCs with Notch ligand-expressing bone marrow stromal cells (OP9, S17, MS-5) to mimic the *in vivo* HSC niche and as such this strategy has been employed to drive both lymphopoiesis as well as myelopoiesis from hESCs/hiPSCs. Chemically, chelating agents (e.g., EDTA) or soluble/immobilized ligands activate Notch signaling, whereas GSI treatment inhibits Notch signaling in hESCs/hiPSCs. Notch signaling can also be manipulated by regulating downstream effectors such as the *HES/HEY* family, which have been implicated in hESCs/hiPSCs hematopoiesis [92]. Manipulation of Notch signaling in an *in vitro* system for hESCs/hiPSCs hematopoiesis enables gain of function studies (overexpression of Notch target genes) along with loss of function studies (siRNA or lentiviral shRNA to inhibit Notch signaling), that is extremely difficult to perform using adult hematopoietic cells. Recent work from our group has provided further insight into the regulation of hematopoietic differentiation of hESCs/hiPSCs using the Notch pathway [97]. Using both pharmacological (Notch ligands/inhibitors) and genetic (lentiviral overexpression, siRNA, shRNA) modifiers of Notch signaling, we examined the role of Notch signaling in three different phases of hESCs/hiPSCs specification, emergence of bipotent hemogenic precursors, and finally hematopoietic cell maturation from progenitors derived from hemogenic precursors. Our findings indicate that Notch signaling through Notch1 is a molecular determinant for hematopoietic

induction from hESCs/hiPSCs, and its effects were mediated by *HES1* expression from human hemogenic precursors at the expense of endothelial commitment [97]. These observations are similar to early mouse hematopoietic development, suggesting that hESCs/hiPSCs may be an excellent surrogate system to study the mechanistic role of Notch in humans.

Although manipulation of Notch signaling has been strategically used for generating various blood cells derived from hESCs/hiPSCs, repopulation *in vivo* has had limited success, thus restraining the use of hESCs/hiPSCs-derived hematopoietic cells for cell replacement therapies. Recently, it has been reported that co-transplantation of hPSCs with Notch ligand expressing OP9 stromal cells into a mouse xenograft leads to hematopoietic differentiation *in vivo*, and resulting cells are capable of secondary reconstitution [98]. Although these are interesting observations that require broader use of additional hESCs/hiPSCs lines and reproduction from other groups, these initial observations suggest that even though putative HSCs may have been generated, the resulting cells do not share the same molecular profile, cell surface phenotype, or robust multilineage hematopoietic reconstituting capacity of SRCs obtained adult or cord blood sources. For example, experimental systems such as the SRC assay do not require such co-transplantation of other cell types and are able to home function appropriately in primary and secondary transplants based on their intrinsic regenerative capacity [99]. In the context of Notch signaling, although this study did not directly address the role of Notch signaling in the generation of repopulating hematopoietic cells, it was only upon injection of OP9 stromal cells together with hiPSCs that led to engraftment and detection of CD34⁺CD45⁺ cells. This suggests a potential role of Notch in the process, similar to T-lymphopoiesis shown in transplantation settings previously. hESC-derived CD34⁺ cells transplanted into human thymus/fetal liver grafts in SCID-hu mice have been shown to generate T cells, including CD4⁺CD8⁺ T-cell precursors [100]. Again, although a direct role of Notch signaling in the generation of T cells from hESCs has not been documented *in vivo*, *in vitro* generation of T cells from hESCs and iPSCs has been achieved using OP9 cells expressing the Notch ligands (*DLL1* or *DLL4*) [101–104]. Similarly, T-cell potential could also be detected when Notch signaling is activated in CD34⁺CD43⁻CD45⁻ cells expressing endothelial markers [101]. Collectively, these studies indicate that Notch signaling plays a role in the generation of cells with pan-myeloid and lymphoid potential from hESCs/hiPSCs. Overall, this suggests that Notch signaling plays an important, but yet not fully understood role in hematopoietic differentiation from hESCs/hiPSCs that requires niche regulation similar to that described in the adult hematopoietic system. These recent observations underscore the power of Notch signaling to regulate human hematopoiesis, independent of ontogenic stage, where embryonic *in vitro* systems and adult *in vivo* hematopoiesis will likely be bidirectional and complementary approaches to understand the true role of Notch signaling complexity in human hematopoiesis.

Conclusion and perspective

Our review delineates and describes the various roles of Notch in hematopoiesis that span adult hematopoietic regeneration,

Sidebar A: In need of answers

- How do stem cells balance the physical interactions, combinations, and affinity between Notch ligands and receptors toward a single cell fate decision?
- Is Notch involved in the autonomous regulation of human HSC self-renewal through asymmetric divisions at the single cell level or in non-autonomous regulation of a population of HSCs acting via cells comprising the bone marrow niche?
- Notch signaling can play a role in both initiation and suppression of AML by acting in a cell autonomous fashion. How can the Notch ligand and receptor interactions in any specialized tissue microenvironment account for this dual effect, and why are normal stem cells not impacted by these changes?
- How and why do Notch receptor and ligand interactions in specialized tissue microenvironments differ?
- What are the mechanisms that induce Notch activation-dependent accumulation of B cells in CLL while suppressing other B-cell leukemias?
- Which signaling stimuli govern or instruct the Notch pathway to initiate self-renewal vs differentiation pathways?
- What is the role of Notch-dependent RBP-J signaling in regulating lymphoid differentiation since there are Notch-independent pathways, which also regulate RBP-J?
- Does Notch signaling play a role in the generation of repopulating hematopoietic cells from pluripotent stem cells? Is temporal Notch signaling critical to generating repopulating hematopoietic cells from human PSCs *in vitro*?
- Can Notch activation or repression be harnessed as a therapeutic strategy for targeting hematological malignancies, and what will be the *in vivo* impact to normal HSCs that also depend on Notch for homeostatic regulation of hematopoiesis?

malignant hematopoiesis, and BM niche regulation in both normal and leukemic settings (Fig 1). Since the identification of *NOTCH1* activating mutations in T-ALL patients, the Notch signaling pathway has been extensively investigated in hematological malignancies. With the idea that ‘cancer stem cells’ are critical to leukemia initiation and emerging evidence for a role of Notch signaling in self-renewal and fate decisions of human HSCs, it is expected that manipulation of the Notch pathway will form the basis of therapeutic drugs to combat hematopoietic malignancies; it may also improve HSC transplantation via the BM niche that utilizes Notch signals. However, mouse and human studies of hematopoietic tissues have primarily relied on gain or loss of function approaches, where knock down of a single Notch receptor/ligand may result in compensation by other Notch members. In addition, temporal regulation to appropriately control Notch has not been examined, which limits our understanding of the physiological role(s) of Notch in regulating hematopoiesis. Due to these limitations, we propose that recent studies revealing a role for Notch signaling in hematopoietic lineages derived from hESCs/hiPSCs may provide a surrogate model to better understand the diverse role of Notch signaling and more precisely define the roles of its different receptors and ligands that ultimately control cell fate. A detailed understanding will be essential to improve current therapeutic drugs believed to impact Notch signaling in hematopoietic cells, but also to test and design new drugs with more precise control of Notch targets from signal transduction to transcriptional control of downstream gene targets.

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Author contributions

All authors participated in the writing and editing of this manuscript with CH additionally creating the illustrations and MB providing final edits and overview prior to submission.

Conflict of interest

The authors declare that they have no conflict of interest.

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