# Fate of hematopoietic stem cells determined by Notch1 signaling (Review)

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**Abstract.** Regulation of the fate of hematopoietic stem cells (HSCs), including silencing, self-renewal or differentiation into blood line cells, is crucial to maintain the homeostasis of the human blood system and prevent leukemia. Notch1, a key receptor in the Notch signaling pathway, plays an important regulatory role in these properties of HSCs, particularly in the maintenance of the stemness of HSCs. In recent decades, the ubiquitination modification of Notch1 has been gradually revealed, and also demonstrated to affect the proliferation and differentiation of HSCs. Therefore, a detailed elucidation of Notch1 and its ubiquitination modification may help to improve understanding of the maintenance of HSC properties and the pathogenesis of leukemia. In addition, it may aid in identifying potential therapeutic targets for specific leukemias and provide potential prognostic indicators for HSC transplantation (HSCT). In the present review, the association between Notch1 and HSCs and the link between the ubiquitination modification of Notch1 and HSCs were described. In addition, the association between abnormal HSCs mediated by Notch1 or ubiquitinated Notch1and T-cell acute lymphoblastic leukemia (T-ALL) was also examined, which provides a promising direction for clinical application.

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*Abbreviations:* AA, aplastic anemia; AGM, aorta-gonadal-mesonephric; ALL, acute lymphoblastic leukemia; allo-HSCT, allogeneic hematopoietic stem cell transplantation; AML, acute myelogenous leukemia; auto-HSCT, autologous hematopoietic stem cell transplantation; bHLH, basic helix-loop-helix; CLL, chronic lymphoblastic leukemia; CML, chronic myelogenous leukemia; DLL, Delta-like; EE, early endosome; EGF, epidermal growth factor; EHT, transformation of hematopoietic endothelial cells into hematopoietic stem cells and progenitor cells; ESCRT, endosomal sorting complexes required for transport; ETP, T-cell precursor; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; Fbxw7, F-box and WD repeat domain containing 7; GVHD, graft-vs.-host disease; HD, heterodimerization; HSCs, hematopoietic stem cells; HSCT, hematopoietic stem cell transplantation; hSel-10, human Sel-10; ILVs, interluminal vesicles; MDS, myeloproliferative disorders; ME, maturing endosome; MM, multiple myeloma; MSCs, mesenchymal stem cells; MVBs, multivesicular bodies; NECD, Notch extracellular domains; NHL, Non-Hodgkin's lymphoma; NICD, Notch intracellular domains; N1ICD, Notch1 intracellular domain; OSCs, oligopotent stem cells; PSCs, pluripotent stem cells; P-SP, para-aortic splanchnopleure; TAD, transactivation domain; T-ALL, T-cell acute lymphoblastic leukemia; TSCs, totipotent stem cells; Ub, ubiquitin; UPS, ubiquitin-proteasome system; USCs, unipotent stem cells; YS, yolk sac

*Key words:* hematopoietic stem cell, Notch1, ubiquitination, stemness, T-cell acute lymphoblastic leukemia, ubiquitin ligase

## Contents



## 1. Introduction

Stem cells are undifferentiated cells that have the ability to proliferate (self-renewal) both *in vitro* and *in vivo* and differentiate into mature specialized cells (1). Stem cells can be divided into five groups: Totipotent stem cells (TSCs), pluripotent stem cells (PSCs), multipotent stem cells, oligopotent stem cells (OSCs) and unipotent stem cells (USCs) (2). TSCs have the highest differentiation potential, able to produce an entire living organism on their own, and most notably a zygote. PSCs can form cells in all the germ layers, with the exception of the cells that form structures outside the embryo, and embryonic stem cells are a prime example. Multipotent stem cells can produce certain lineages of cells, and the majority of adult stem cells are multipotent, including hematopoietic stem

cells (HSCs), mesenchymal stem cells (MSCs), and other adult progenitor cells (3). OSCs can differentiate into numerous cell types, such as bone marrow stem cells which may develop into white blood cells but not red blood cells. Eventually, USCs may form only one cell type, such as skin cells.

Among multipotent stem cells, HSCs are the most common multipotent stem cells with the ability to maintain homeostasis by self-renewal or differentiation into all blood cell lineages (4). The stemness of the HSCs combines the ability of the HSCs to perpetuate its lineage, to produce differentiated cells (such as lymphocytes and granulocytes) and to interact with the hematopoietic microenvironment to maintain a balance between quiescence, proliferation, and regeneration (5). In short, the stemness of HSCs helps maintain the homeostasis of the blood system by balancing the proliferation and differentiation of HSCs. When the stemness of HSCs is destroyed, abnormal production of blood cells occurs and further abnormal blood system tumors are produced, namely leukemia (6). Common subtypes of leukemia include acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myelogenous leukemia (CML) and chronic lymphoblastic leukemia (CLL). Therefore, a full understanding of the signaling pathways or regulatory factors that are capable of regulating the stemness of HSCs may provide further insight into HSCs and hope for a cure for leukemia.

In recent years, it has been gradually revealed that a series of signaling pathways, such as Wnt, Notch, the TGF-β family, Hedgehog and Hippo signaling, could affect the stemness of HSCs, and that dysregulations of these pathways alone or coordinated may lead to the development of leukemia (7,8). Among them, Notch signaling, an evolutionarily conserved signaling pathway, is essential for the establishment of the earliest embryonic HSCs and is closely associated with the emergence, development, and maintenance of HSCs in adulthood (9). In this signaling pathway, Notch1 receptor is most closely associated with the stemness of HSCs and plays a key role in T-cell development and transformation (10). Abnormally activated or mutated Notch1 receptors severely affect the balance of proliferation and differentiation of HSCs which triggers the continuous emergence of abnormal lymphocytes, thus leading to lymphocytic leukemia, particularly T-cell acute lymphoblastic leukemia (T-ALL). In addition to the Notch1 receptor itself, its post-translational modifications, such as glycosylation, phosphorylation, and ubiquitination, also affect the activation of the Notch1 pathway, thereby affecting the stemness of HSCs (11). Among these post-transcriptional modifications, particular attention has been paid to the ubiquitination modification of Notch1, since it affects the degradation of Notch1 receptor (12), the activation of Notch1 signaling (11), and the process of endocytosis that Notch1 receptor undergoes (13). Therefore, this suggests that the key enzymes responsible for the ubiquitination modification of Notch1 may also, directly or indirectly, affect the stemness of HSCs and the development of leukemia (14-16). In the present review, the structure of the Notch signaling pathway was firstly summarized in detail and the effects of the Notch1 receptor on HSC origin, proliferation, differentiation and associated T-ALL, were described. Subsequently, the ubiquitination modification of Notch1 receptor and its effects on HSCs were elucidated. Finally, the clinical application of HSCs, as well as the potential therapeutic targets and prognostic indicators of Notch were reviewed.

## 2. Overview of the Notch pathway

Notch signaling is a major mediator in determining cell fate during development, and it regulates a variety of cell functions, including differentiation, proliferation, and homeostasis (17). Evidence suggests that the Notch signaling pathway has markedly opposite functions in tumor development, possibly acting as an oncogene or a tumor suppressor (18). In the process of hematopoiesis, Notch signaling controls the fate of hematopoietic progenitor stem cells by inhibiting certain differentiation steps and inducing self-renewal or lymphatic lineage differentiation (19).

Notch receptor, Notch ligand and DNA binding sequence CSL [CBF1/SU(H)/LAG-1] are the three main components of the canonical Notch signaling pathway (20). There are four transmembrane Notch receptors (Notch1, Notch2, Notch3, and Notch4) and five typical transmembrane ligands [Delta-like (DLL) 1, DLL 3, DLL 4, Jagged1, and Jagged 2] in mammals (21). The extracellular region of the Notch receptor (NECD) contains 29-36 epidermal growth factor (EGF)-like repeats, three LIN12/Notch repeats and a heterodimerization (HD) domain. Notch intracellular domains (NICD) include a RAM domain, seven cdc10/ankyrin repeats, two nuclear localization sequences, a transactivation domain (TAD) (Notch1 and Notch2), and a C-terminal PEST motif (22) (Fig. 1A). Notch ligand is a type I transmembrane protein that contains extracellular EGF-like repeats, a Delta, Serrate and LAG-2 domain and a Delta and OSM-11-like protein domain, which together are responsible for Notch receptor interactions (21).

The Notch signaling pathway is activated when a ligand on a cell membrane binds to the Notch receptor on an adjacent cell. The Notch receptor passes through three different proteolytic cleavages (Fig. 1A). First, a single polypeptide precursor protein is cleaved in the Golgi by a furin-like convertase to produce a mature Notch receptor (S1) (21). When the mature Notch receptor binds to the ligand, a second cleavage (S2) is performed by TACE or Kuz of the A disintegrin and metalloprotease metalloproteinase family to release extracellular fragments (20). The remaining transmembrane and intracellular domains are cleaved by γ-secretase for a third time (S3), releasing the soluble NICD and transferring to the nucleus (20). Then, NICD binds to the DNA-binding protein CSL/RBPJκ in the nucleus, activating genes which belong to the *basic helix-loop-helix* (*bHLH*) family (20). The general consensus is that CSL/RBPJκ persistently binds to the promoter of the targeted gene. In the absence of NICD, CSL/RBPJκ binds with co-inhibitors (SMRT, histone deacetylase, etc.) to inhibit gene transcription. Conversely, when NICD enters the nucleus, it recruits co-activators such as MAML to promote the transcription of target genes (23). In mammals, genes known as the *Hes* family (*Hes1,5,7*) and the *Hey* family (*Hey1,2,L*) are the major components of the *bHLH* family (24-26). *Hes1* is important in the development of the nervous system, sensory organs (eye, inner ear), pancreas, endocrine cells, and lymphocytes (24). *Hes7* is essential for somitogenesis (25). By contrast, the *Hey* family play a key role in the cardiovascular system (26). In addition to the *bHLH* family, several other genes have also

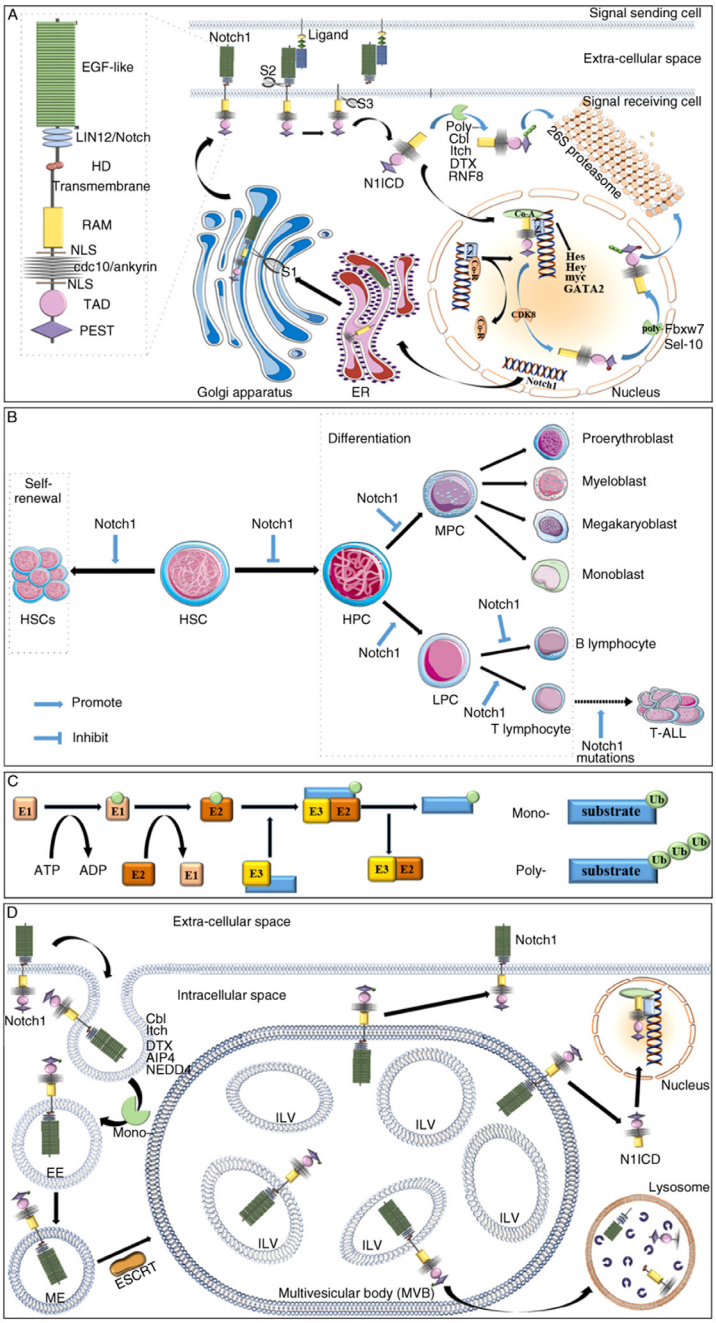


Figure 1. Notch1 and HSCs. (A) Notch1 receptor structure and Notch1 signaling. Notch1 receptors are composed of an intracellular domain (36 EGF-like repeats, three LIN12/Notch repeats and an HD domain), a transmembrane domain and an extracellular domain (RAM domain, seven cdc10/ankyrin repeats, two NLS, a TAD domain, and a PEST motif). The Notch1 receptor undergoes three proteolysis processes to become N1ICD. Above all, the precursor Notch1 receptor is cleaved by a furan-like convertase (S1) in the Golgi apparatus. When bound to the ligand, TACE or Kuz perform a second cleavage (S2). The remaining domain is cleaved by γ-secretase (S3) to release N1ICD. The N1ICD enters the nucleus and the target gene is detached from the Co-R. Then, N1ICD, together with SCL and Co-A, promotes the transcription of target genes. N1ICD in the nucleus and cytoplasm is degraded by 26S proteasome through a process of poly-ubiquitination modification. However, N1ICD in the nucleus is phosphorylated by CDK8 before the ubiquitination modification. (B) Effects of Notch1 signaling on self-renewal (proliferation) and differentiation of HSCs. In general, Notch1 promotes the proliferation of HSCs and inhibits their differentiation. However, when hematopoietic stem cells begin to differentiate, Notch1 promotes hematopoietic stem cells to differentiate into T lymphocyte lines rather than myeloid lines. In addition, Notch1 signaling drives T-cell development at the expense of the development of B cells. In the end, the most important carcinogenic pathway in T-ALL is the activation mutation of Notch1 signaling. (C) Processes and types of ubiquitination modification. The ubiquitin molecule is added to the substrate by the action of E1, E2 and E3 in turn. Ubiquitination modification mainly involves mono-ubiquitination modification and poly-ubiquitination modification. (D) The process of endocytosis. When no ligand binds, Notch1 undergoes endocytosis. Notch1 is mono-ubiquitinated before EE is formed. Then, EE will gradually mature into ME. Subsequently, the multiple MEs are fused into MVEs with the assistance of ESCRT. The position of Notch1 on the MEVs determines its fate. If Notch1 is present on the limiting membrane of MVBs, it may be recycled to the cell membrane for utilization. If Notch1 on the MVB-limiting membrane is cleaved and N1ICD is released, Notch1 signaling will be activated. However, the residual Notch1 in ILVs is transported to lysosomes for degradation. EGF-like, 36 epidermal growth factor (EGF)-like repeats; LIN12/Notch1, three LIN12/Notch repeats; HD, heterodimerization domain; transmembrane, transmembrane domain; RAM, RAM domain; NLS, nuclear localization sequences; cdc10/ankyrin, seven cdc10/ankyrin repeats; TAD, transactivation domain; PEST, PEST motif; ER, endoplasmic reticulum; S1, first proteolytic cleavage; S2, second proteolytic cleavage; S3, third proteolytic cleavage; N1ICD, Notch1 intracellular domains; Co-A, co-activators; Co-R, co-inhibitors; CSL, DNA-binding protein CSL/RBPJκ; HSC, hematopoietic stem cell; HPC, hematopoietic progenitor cell; MPC, myeloid progenitor cell; LPC, lymphoid progenitor cell; T-ALL, T-cell acute lymphoblastic leukemia; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; mono-, mono-ubiquitination; poly-, poly-ubiquitination; Ub, ubiquitin molecule; EE, early endosome; ME, maturing endosome; ESCRT, endosomal sorting complexes required for transport; ILV, interluminal vesicle.

been identified as Notch targets, including $p27^{Kip1}$ (27), *cyclin D1* (28), *myc* (29), and *Deltex1* (30).

The main substrates that undergo ubiquitination modification in this signaling pathway are Notch receptor, Notch ligand and γ-secretase (31). Among them, the ubiquitination modification of Notch receptor, particularly Notch1 receptor, may mostly affect the signaling strength of this pathway (31). The Notch1 receptor firstly undergoes either mono-ubiquitination or poly-ubiquitination, thereafter the Notch1 receptor is degraded or the Notch1 signaling is activated, thereby affecting the Notch1 signaling and the expression of downstream genes. The detailed process is subsequently described.

Since Notch1 receptor-mediated Notch1 signaling plays an irreplaceable role in the blood system, The Cancer Genome Atlas database (https://portal.gdc.cancer.gov/exploration?filters=%7B%22op%22%3A%22and%22%2C%22content%22%3A%5B%7B%22op%22%3A%22in%22%2C%22content%22%3A%7B%22field%22%3A%22cases.available_variation_ta%22%2C%22value%22%3A%5B%22ssm%22%2C%22cnv%22%5D%7D%7D%2C%7B%22op%22%3A%22in%22%2C%22content%22%3A%7B%22field%22%3A%22cases.project.project_id%22%2C%22value%22%3A%5B%22TARGET-ALL-P3%22%2C%22TCGA-DLBC%22%2C%22TCGA-LAML%22%5D%7D%7D%2C%7B%22op%22%3A%22in%22%2C%22content%22%3A%7B%22field%22%3A%22genes.gene_id%22%2C%22value%22%3A%5B%22ENSG00000148400%22%5D%7D%7D%5D%7D&searchTableTab=cases) and the International Cancer Genome Consortium database (https://dcc.icgc.org/genes/ENSG00000148400/mutations?donors=%7B%22from%22:1%7D&filters=%7B%22donor%22:%7B%22primarySite%22:%7B%22is%22:%5B%22Blood%22%5D%7D%7D%7D) were consulted, respectively, and data on the expression and mutations of *Notch1* were obtained by searching for *Notch1* mutations and selecting all hematology-related malignancies in the database, including various types of lymphomas and leukemias. The *Notch1* mutation rate was 32/241 (13.28%) in germinal B-cell derived lymphomas, 64/510 (12.55%) in CLL, 3/50 (6.00%) in T- and NK-cell lymphomas, 11/205 (5.37%) in AML and 1/136 (0.74%) in chronic myeloid disorders. Through further integrating these data into disease categories, it was identified that *Notch1* was mutated with a frequency of 9.72% in hematological malignancies. Furthermore, the mutation frequency of *Notch1* in lymphomas was 12.03 and 8.93% in leukemias.

## 3. Notch1 and HSCs

*Origin of HSCs*. HSCs are the cornerstone of the mammalian blood system (32). These stem cells self-renew to maintain a stable pool of HSCs, which are able to differentiate into myeloid, lymphatic and erythroid cells as required, thus maintaining blood cell homeostasis (32). The Notch signaling pathway, particularly the Notch1 receptor, plays a key role in maintaining undifferentiated HSCs and inducing self-renewal (9). Thus, Notch1 is biologically important in HSCs.

During human embryonic development, two distinct sites are involved in hematopoiesis: The extraembryonic yolk sac (YS) and the aorta-gonadal-mesonephric (AGM) region within the embryo (33). The hematopoiesis starts in the YS blood islands, travels to the fetal liver, and finally locates to the bone marrow during embryogenesis. Additionally, adult blood cells, including lymphocytes and hematopoietic progenitor cells (HPCs), are generated in the para-aortic splanchnopleure (P-SP) region, which then develops into AGM for long-term hematopoiesis before the HSCs reach the fetal liver (34). The first long-term regenerated HSCs are detected in the AGM region. By positively regulating Notch1 through the transcription factor SOX17, Saito *et al* (35) revealed that Notch1 intracellular domain (N1ICD) or its downstream target protein Hes1 transduced HSCs to maintain the ability of multipotent colony formation in AGM. By contrast, *Notch1* and *Hes1* gene knockout resulted in a decrease in the ability to form multipotent colonies. These results indicate that Notch1 and Hes1 are critical for maintaining the undifferentiated state of HSCs (35). Thus, Notch1 is critical to the production of HSCs during embryogenesis.

With regard to the origin of HSCs, it is generally considered that embryonic HSCs and progenitor cells are derived from the hematopoietic endothelium, and thus the transformation of hematopoietic endothelial cells into HSCs and progenitor cells (EHT) is required. Zhang *et al* (36) demonstrated that inhibition of Notch1 signaling can promote EHT by G protein-coupled receptor 183. In mouse embryos with *Notch1* deletion mutations, distinct hematopoietic endothelial cells were identified, but they did not develop into HSCs (37). Differences in the ligands that activate Notch1 may contribute to the paradoxical nature of the results. Through analysis of experimental data, Gama-Norton *et al* (38) revealed that 89% of endothelial cells co-expressed Jagged1 and DLL4 ligands, and only a few endothelial cells expressed Jagged1 ligands alone (3.8%) or DLL4 ligands alone (4.6%) or neither (2.5%). The balance of the DLL4-Notch1 and Jagged1-Notch1 signaling pathways may ensure the correct establishment of endothelial and hematopoietic cell fates in AGM. Furthermore, they suggested that the deletion of Jagged1 ligand leads to increased Notch activity in the aortic endothelium of AGM through the microarray analysis of AGM subpopulations, thereby improving the fate of endothelial cells at the expense of HSC formation. Conversely, when lacking the Jagged1-Notch1 signaling and experiencing high DLL4-Notch1 signaling, endothelial cells select the endothelial protocol, thus preventing the formation of HSCs. It was hypothesized that precursor hematopoietic cells responding to Jagged1 would attenuate the DLL4-Notch1 signaling, replacing it with an effective low Notch1 signaling, which is necessary and sufficient for activation of hematopoietic genes such as *GATA2* (38). In addition to *GATA2*, *Fox2* from the *Fox* gene family induced by N1ICD also plays a role in hematopoietic endothelium. Data from a study by Jang *et al* (39) established a pathway that binds Notch signaling to its downstream *Fox2* in hematopoietic endothelial cells, thereby promoting hematopoietic development. Collectively, these studies suggested that Notch1 has an indispensable role prior to HSC production (37).

*Proliferation and differentiation of HSCs*. Through downstream proteins or genes, particularly Hes1, the Notch signaling pathway mediated by Notch1 receptor promotes self-renewal of HSCs and inhibits their differentiation (Fig. 1B). Using *Rag-1*$^{-/-}$ mouse stem cells, Stier *et al* (40) documented Notch1-induced reduction of *in vivo* differentiation and an increased stem cell

population due to enhanced stem cell self-renewal. The research of Shao *et al* (41) revealed that endothelial Jagged1-Notch1 deficiency severely affects the development of fetal blood vessels and impedes the proliferation and differentiation of HSCs *in vitro* and *in vivo*. Additionally, it was specified that Notch1-Hes1 may act on hematopoietic precursor cells, which are produced following the fate of HSCs. Furthermore, Hes1 not only preserved the long-term recombination activity of HSCs *in vitro*, but also accumulated side population cells *in vivo* (42). These results suggested that Hes1 inhibits HSC differentiation. However, once HSCs enter the differentiation stage, Notch1 signaling promotes HSC differentiation, with a preference for the lymphatic rather than the myeloid line (40) (Fig. 1B). A previous study conducted by Henning *et al* (43) suggested that Notch1 signaling mediates this process via a p53-dependent pathway. Collectively, the main effect of Notch1 signaling on HSCs is to promote its proliferation and inhibit its differentiation.

*HSCs differentiate into T cells*. When HSCs first enter the thymus and become early T-cell precursor (ETP) cells, they receive high levels of Notch signaling regulation (44). On the one hand, excessive Notch1 signaling drives premature commitment of T cells, leading to loss of ETP cells and the fate of replacement cells (44). By contrast, complete loss of Notch1 signaling impairs ETP cell proliferation and leads to loss of ETP cells (44). Thus, maintaining a good balance of Notch signaling can maintain the stemness of HSCs.

In both mouse and human, Notch1 activation is the primary driver of inducing T-cell development in hematopoietic stem progenitor cells (Fig. 1B). The role of Notch1 in lymphogenesis has been well studied, and in particular the most prominent characteristic function of Notch1 signaling is maturation of T cells and lineage commitment in the mouse thymus (45). It has been demonstrated that the expression of *Notch1* transgene in HSCs leads to thymus-independent development of $CD4^+CD8^+$T cells (45). In addition, the study of Gerhardt *et al* (46) revealed that TAD in Notch1 drives T-cell development at the expense of common precursor development of B cells.

Notably, downstream target genes of Notch1, such as *Hes* and *myc*, are the driving force in the differentiation of HSCs into T cells. *Hes1* has been revealed to be expressed in both the thymus and thymus stroma, and its expression in the thymus was regulated by Notch signaling (47). More than 90% of *Hes1*$^{-/-}$ mice lacked thymus glands, suggesting that *Hes1* is critical for the *in vivo* proliferation of early T-cell precursors (48). In a recent study, De Decker *et al* (49) identified that Hes1 and Hes4 were upregulated in a Notch-dependent manner during early T-cell development and Hes1 acted as a differentiation inhibitor since it maintained quiescent stem cell characteristics in $CD34^+$ HPCs. However, Hes4 promoted the initiation of early T-cell development. Importantly, knockout of *Hes1* or *Hes4* significantly reduced human T-cell development. As for *myc*, in a well-established model of HSC T-lymphocyte differentiation *in vitro*, Haque *et al* (50) determined that Notch1 and 4 directly promoted *myc* expression. It was further demonstrated that overexpression of *myc* promoted T-cell differentiation, while dominant-negative *myc* delayed T-cell differentiation. These results confirmed that *myc* is an important mediator of Notch signaling in the differentiation of HSCs into T lymphocytes. The Notch1-mediated emergence of these two different effects on HSC differentiation into T cells may be attributed to the Notch ligand. OP9-cell co-culture experiments revealed that Jagged2 induced T-line differentiation and inhibited B cell and bone marrow development, as did DLL ligands (51). However, the results of Van de Walle *et al* (51) revealed a unique role of Jagged1 in preventing induction of differentiation of HSCs in T lines.

*T-ALL*. T-ALL is an aggressive hematologic tumor in which the malignant transformation of HSCs and HPCs lead to the development of T cells (52). Although T-ALL accounts for only 25% of ALL cases in adults and 15% in children, they have a higher risk of central nervous system recurrence in the presence of mutations activated by the Notch1 signaling pathway (53). Constitutive activation of Notch1 signaling is the most important oncogenic pathway in T-cell transformation, and >65% of T-ALL patients have *Notch1* activation mutations (52). In addition, Ma *et al* (53) concluded that Notch1 signaling promotes cell regeneration in human T-ALL. Most of the abnormal activation of Notch1 observed in T-ALL is due to mutations in its HD domain and/or PEST domain (54). Of the 15 T-ALL patients studied by Bhanushali *et al* (54), 6 (40%) patients had at least one *Notch1* mutation, with 2/15 (13%) occurring in the HD domain and 4/15 (27%) in the PEST domain. In addition, mutations are considered to occur in 4 out of 10 (40%) adult patients; in the pediatric cohort, two out of five (40%) had both mutations in the PEST domain (54). Mutations in the HD domain of Notch1 receptor render it more susceptible to protein cleavage and then release of N1ICD, while mutations in the PEST domain of Notch1 receptor inhibit proteasomal degradation of N1ICD by F-box and WD repeat domain containing 7 (Fbxw7), which is a ubiquitin ligase, thus prolonging its half-life in T-All cells. In addition, deletion or inactivation mutations of *Fbxw7* are frequently observed in T-ALL. In addition, Ding *et al* (55) revealed that fetal-derived T-cell precursor stem cells may play a role as leukemia initiation cells. This may be due to their discovery of overexpression of N1ICD in P-SP and YS cells. P-SP cells overexpressing N1ICD rapidly developed T-ALL, while YS cells exhibited no leukemia proliferation following N1ICD induction. To date, *Notch1* mutations have also been reported in CLL (56). Di Ianni *et al* (56) reported *Notch1* mutation in HSCs of CLL patients, and aberrant activation of Notch1 in HSCs of CLL patients without *Notch1* mutation.

## 4. Notch1 and ubiquitination

*Ubiquitination*. Ubiquitination is a common and important post-translational modification process that plays a key role in protein homeostasis (57). It is mainly achieved through labeling the ubiquitin (an 8.6 kDa regulatory protein) to the substrate, which is then degraded in the 26S proteasome to release the ubiquitin molecule (58). In addition, ubiquitination also includes certain non-proteolytic functions, such as receptor internalization (59), multiprotein complex assembly (60), inflammatory signaling (61), DNA damage repair (62), cell death (63), metabolism (64) and signaling activation (65,66).

Ubiquitination involves three different biochemical steps: activation, conjugation, and ligation, which are catalyzed by three types of ubiquitination enzymes: Ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3), respectively (67) (Fig. 1C). Initially, E1 causes the C-terminal adenylation of ubiquitin (Ub) to catalyze the activation of ubiquitin in an ATP-dependent manner (68). The mature ubiquitin is then transferred to cysteine at the active site of the E2 binding enzyme via trans-thiesterification (58). Finally, E3 and E2 jointly catalyze the formation of isopeptide bonds between Ub and the substrate protein (69). Once attached to the target protein, ubiquitin can be ubiquitinated on any of its lysine residues (K6, K11, K27, K29, K33, K48, K63) or on its N-terminal methionine (M1). The human proteome contains two E1s, ~50 E2s, and 600 E3s (70). Since largely determining substrate specificity, E3 plays a key role in the entire process of ubiquitination modification. E3s could be roughly divided into three families: the HECT family, the RING family, and the RBR family (64).

The E1-E2-E3 cascade is capable of producing several types of Ub modifications, resulting in the different fates of substrates (3). In general, two types of ubiquitination modification are prevalent in cells: Mono-ubiquitination and poly-ubiquitination (Fig. 1C). On the one hand, mono-ubiquitination is the addition of a single Ub molecule to the substrate. Poly-ubiquitination, by contrast, is the addition of Ub chains to one or more lysine residues of the substrate (3). In most cases, membrane-bound proteins are mono-ubiquitinated, which contributes to their endocytosis and lysosomal degradation (31,71). In addition, mono-ubiquitination is also involved in meiosis and chromatin remodeling. However, poly-ubiquitination plays a role in the ubiquitin-proteasome system (UPS), DNA repair, and immune signaling transduction (72).

*Ubiquitination modification of Notch1 receptor.* Recent studies have suggested that the ubiquitination modification of Notch1 receptor plays an irreplaceable role in the regulation of Notch signaling (73-81). The ubiquitinated Notch1 receptor has three distinct fates: Transferring to the 26S proteasome, promoting N1ICD-mediated signaling activation, and the endocytosis of Notch1 receptor. The fate of the Notch1 receptor that transfers to the 26S proteasome is degradation. However, the entry of Notch1 receptors into the process of endocytosis has three different outcomes: Cycling back to the cell membrane, becoming N1ICD and functioning in the nucleus or being degraded in lysosomes.

Above all, the most important function of ubiquitinated Notch1 is degradation in the 26S proteasome (Fig. 1A). The E3s that mediate this process are mainly Sel-10, Fbxw7 and RNF8. When N1ICD enters the nucleus, it forms complexes with MAML and CSL. Among them, MAML can recruit CDK8 to phosphorylate the PEST domain of N1ICD. Subsequently, Fbxw7, an E3, modifies the phosphorylated N1ICD for poly-ubiquitination and then enters into the 26S proteasome for degradation (12). Similarly, Wu *et al* (82) demonstrated that human Sel-10 (hSel-10) and Sel-10 bind N1ICD proteins in a region-specific manner and that the interaction between Sel-10 and N1ICD is phosphorylation-dependent. *In vitro* ubiquitination modification experiments also revealed that Sel-10 and hSel-10 mediated ubiquitination modification of N1ICD, which were subsequently degraded by the 26S proteasome in cells. As for RNF8, it acts as a negative regulator of Notch signaling through ubiquitination modification of N1ICD, leading to its degradation, thereby regulating Notch1 signaling and cell fate determination in lumen progenitor cells of the breast (83).

Another essential role of the ubiquitination modification of Notch1 is the activation of Notch signaling. Pettersson *et al* (11) revealed that MDM2 also regulates Notch signaling through direct interaction with N1ICD, leading to ubiquitination modification of N1ICD. However, this type of ubiquitination modification does not result in the degradation of N1ICD, but triggers the activation of the Notch signaling pathway. In addition, MDM2 also interacts with Notch regulator NUMB and induces its ubiquitination modification and degradation (11).

With the exception of the Notch1 proteasomal degradation and signaling activation, ubiquitination modification also regulates the endocytosis of Notch1 (Fig. 1D). In the absence of a ligand, Notch1 is continuously internalized and then degraded in lysosomes (84), circulating back to the plasma membrane (85,86) or activating Notch signaling. This mechanism is a way to maintain Notch1 function and ultimately regulate Notch signaling strength by targeting Notch1 levels on the cell surface. Notch1 begins the process by its internalization in the early endosome (EE) vesicles and then fuses with the EE. The EE then matures and merges into a maturing endosome (ME). Finally, multiple MEs fuse to form multivesicular bodies (MVBs). In this step, Notch1 has the three distinct aforementioned fates, specifically, it either returns to the membrane by circulating endosomes, remains in MVBs, or activates the Notch1 signaling. These different fates depend on the position of Notch1 in MVBs. If Notch1 is present on the limiting membrane of MVBs, it can be recycled, and when the part of Notch1 present on the MVB-limiting membrane is cleaved to release N1ICD, Notch1 signaling can be activated (31). However, Notch1 remaining in MVB interluminal vesicles (ILVs) can be further degraded by lysosomes. MVB formation is controlled by endosomal sorting complexes required for transport (ESCRT), a sequentially acting macromolecular protein complex that ultimately allows ILV formation (87,88). Mono-ubiquitination modification of Notch1 has been revealed to be necessary for effective recruitment to the endosomal membrane by the ESCRT machinery components and formation of ILV (89). If the ESCRT mechanical component is not functional, the mono-ubiquitinated Notch1 accumulates on the limiting membrane of MVBs, resulting in aberrant signaling activation. This suggests that mono-ubiquitination modification may be directly or indirectly involved in Notch endocytosis regulation and vesicular transport. The E3s that mediate this process include Su(dx)/Itch/AIP4, Cbl, NEDD4, and Deltex (DTX). The results of their effects depend on the cell contexts, as well as their abundance. In addition, Su(dx)/Itch/AIP4, DTX, and NEDD4 may also enable Notch1 to be labeled by poly-ubiquitination modification and then degraded into proteasome (73-81) (Table I).

*Effects on HSCs.* Regulation of N1ICD through ubiquitination modification is absolutely critical for proper Notch signaling, as maintaining Notch signaling over long periods of time can lead to severe diseases. For example, either a deletion of the *Notch1* gene, leading to a deletion of the PEST domain of

Table I. E3s of Notch1 receptor.

| E3 | Substrate | Species | E3-type | Ubiquitination | Effect | (Refs.) |
|---|---|---|---|---|---|---|
| Sel-10 | N1ICD | *Caenorhabditis elegans* | RING | poly- | Proteasome degradation | (73) |
| hSel-10 | N1ICD | Human | RING | poly- | Proteasome degradation | (82) |
| Fbxw7 | N1ICD | Mammal | RING | poly- | Proteasome degradation | (12) |
| RNF8 | N1ICD | Mammal | RING | poly- | Proteasome degradation | (83) |
| MDM2 | N1ICD | Mammal | RING | mono- | Signaling activation | (11) |
| Su(dx) | Notch1 | *Drosophila* | HECT | mono- | Endocytosis | (74,75) |
| | | | | poly- | Proteasome degradation | |
| Itch | Notch1 | Mammal | HECT | mono- | Endocytosis | (76,77) |
| | | | | poly- | Proteasome degradation | |
| AIP4 | Notch1 | Human | HECT | mono- | Endocytosis | (85) |
| | | | | poly- | Proteasome degradation | |
| NEDD4 | Notch1 | *Drosophila*, mammal | HECT | mono- | Endocytosis | (77,78) |
| | | | | poly- | Proteasome degradation | |
| DTX | Notch1 | *Melanogaster*, mammal | RING | mono- | Endocytosis (upgrade signaling) | (13,79,80) |
| | | | | poly- | Proteasome degradation | |
| Cbl | Notch1 | Vertebrate | RING | mono- | Lysosomal degradation | (16,81) |
| | | | | poly- | Proteasome degradation | |

N1ICD, Notch1 intracellular domains; E3, ubiquitin ligase; DTX, Deltex.

Notch, or a mutation in the *Fbxw7* gene, encoding an inactive or absent enzyme is associated with T-ALL (88). In the present study, three E3s were focused on, all of which affect the stemness of HSCs through Notch1 receptor.

Cell cycle quiescence maintains the stemness of HSCs by protecting them from differentiation or senescence (90). Fbxw7 can induce the degradation of positive regulators such as myc and Notch1 in the cell cycle. Iriuchishima *et al* (91) revealed that Fbxw7 maintained HSCs and inhibited leukemia by mediating ubiquitin-dependent degradation of myc and Notch1. Thompson *et al* (92) also demonstrated that the *Fbxw7*$^{-/-}$ severely affected the maintenance of HPCs in the bone marrow, and the cell autonomy defect of stem cell self-renewal led to the defect of HSC silencing and self-renewal, which was attributed to the loss of the function of *Fbxw7* deletion to ubiquitination modification and degradation of Notch1 or myc. Therefore, Fbxw7 serves as a key fail-safe device to prevent premature loss of HSCs and the development of T-ALL (93).

In addition, Cbl is a new negative regulator of HSC development and functional characteristics. Rathinam *et al* (94) determined that HSCs of *Cbl*$^{-/-}$ mice had increased pool capacity, increased proliferation, and increased long-term regeneration. Furthermore, Zhu *et al* (16) revealed that flavone promoted Cbl-induced ubiquitination modification and degradation of N1ICD, resulting in resistance to T-ALL.

Ultimately, HSCs in *Itch*$^{-/-}$ mice exhibited increased frequency, ability, and long-term regenerative activity. Rathinam *et al* (95) demonstrated that Itch-deficient HSCs exhibited accelerated proliferation rates and sustained progenitor cell properties due to increased accumulation of Notch1 activation, as well as increased Notch1 signaling by the transcription factor. Therefore, E3 ubiquitin ligase Itch negatively regulates the development and function of HSCs.

## 5. Clinical application

Multipotent stem cells, particularly HSCs and MSCs, are widely used in clinical practice due to their characteristics of self-renewal, multidirectional differentiation as well as numerous others. For example, MSCs have paracrine, anti-inflammatory, and immunomodulatory effects in addition to their role in tissue regeneration (96). MSC-derived chambers or substances (including exosomes, microvesicles, and microRNA) can serve as practical tools for diagnosing, following up, managing, and monitoring disease. In addition, Tehrani *et al* (97) suggested that MSCs could serve as a vehicle for gene-directed enzyme prodrug therapy, in which suicide genes are directed to tumor cells, attributing to their remarkable homing properties to the tumor sites. Mirzaei *et al* (98) considered that MSCs could carry 5-fluorouracil, suicide genes such as *pigment epithelium-derived factor*, *INF-α*, *INF-β* and *INF-γ* to melanoma sites to inhibit tumor growth. More specifically, interferon-γ-induced protein 10 kDa (IP-10) secreted by human adipose-derived MSCs may be involved in this process (99). In addition, it has been gradually determined that this method of gene therapy can also be applied to the treatment of osteoarthritis (100), cardiovascular disease (101) as well as other diseases, in recent years. However, MSCs also secrete certain growth factors, chemokines, and cytokines, which increase the burden of tumors, and this may be the most important unresolved issue with this treatment approach (102).

Table II. HSCT.

| Type | Recurrence rate at 100 days following HSCT | 5-Year survival rate | Main risk factor for late mortality | Indications for malignant tumors | Indications for other diseases | (Refs.) |
|---|---|---|---|---|---|---|
| Auto-HSCT | 57% | 88% | Relapse | MM, NHL, HL, AML, ALL, neuroblastoma, ovarian cancer, germ-cell tumors, etc. | Autoimmune disorders, amyloidosis, etc. | (106-109) |
| Allo-HSCT | 46% | 83% | Chronic GVHD | AML, ALL, CML NHL, HL, CLL, MM, MDS, myeloproliferative disorders, etc. | AA, PNH, Fanconi's anemia, sickle cell anemia, Wiskott-Aldrich syndrome, etc. | (107,108,110) |

HSCT, hematopoietic stem cell transplantation; MM, multiple myeloma; MDS, myeloproliferative disorders; NHL, non-Hodgkin's lymphoma; HL, Hodgkin's lymphoma; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; AA, aplastic anemia; PNH, paroxysmal nocturnal hemoglobinuria.

Since the Notch1 signaling pathway and its UPS mainly affect the stemness of HSCs, attention should be paid to the progression of HSCs in the treatment of leukemia, so as to provide a better direction for the treatment of leukemia. HSC-related therapies are gradually applied in the treatment of leukemia. Transplantation of HSCs from bone marrow, peripheral blood or cord blood is currently one of the most popular stem cell therapies in blood system diseases (103). HSC transplantation (HSCT) is a therapeutic method in which patients receive a massive high-dose of radiotherapy or chemotherapy (usually a lethal dose of radiotherapy or chemotherapy), occasionally combined with other immunosuppressive drugs to remove tumor cells and aberrant clonal cells in the body, and then reinfuse the HSCs collected from the patients themselves or other individuals to reconstruct normal hematopoietic and immune functions (104). HSCT is widely used in the treatment of hematological malignancies, such as acute leukemia, CML, lymphoma, multiple myeloma (MM), myelodysplastic syndrome (MDS), and certain hematological non-malignant tumors, such as severe aplastic anemia (AA) and thalassemia (105). The process may be autologous (using the patient's own cells), allogeneic (stem cells from a donor), or syngeneic (from identical twins) (2). For numerous types of leukemia, allogeneic HSCT (allo-HSCT) is a more suitable standard cell treatment option than autologous HSCT (auto-HSCT) (106-110) (Table II).

In view of the fact that HSCs are derived from the patients themselves during the process of auto-HSCT, there will be no graft rejection and graft-vs.-host disease (GVHD) and there are few transplantation complications. The low transplant-related mortality and favorable quality of life following transplantation are due to the no limitation of donor constraints. However, given the lack of graft antitumor effect and the possibility of residual tumor cells in the graft, the recurrence rate is high. Auto-HSCT has become a routine treatment option for patients with lymphoma (111), certain low-risk acute leukemias (112), highly invasive, relapsed/refractory non-Hodgkin's lymphoma (NHL) (113) and MM (114). For example, the clinical efficacy of auto-HSCT for AML has gradually improved. A group of European researchers retrospectively analyzed the survival outcomes of 809 patients with AML in their first complete response and identified that the 2-year leukemia-free survival rate and overall survival rate were 51 and 65%, respectively, and the non-recurrence mortality rate was only 3.7% (115). Taking it a step further, Passweg (116) revealed that the 3-year overall survival rate of AML was 34 (21-56)% following chemotherapy, but 75 (60-95)% following consolidation with auto-HSCT. In fact, a large number of studies have revealed that auto-HSCT is associated with lower recurrence rates and an acceptable non-recurrent mortality rate in AML patients compared with chemotherapy alone (115). In addition, in certain AML patients, auto-HSCT was comparable to allo-HSCT in overall survival (116).

The HSCs in allo-HSCT are derived from normal donors without tumor cell contamination. Considering the immune-antitumor effect of the graft, it has a low recurrence rate, a high long-term disease-free survival rate (also known as cure rate), a wide range of indications, and is even the only cure for certain diseases (114). However, due to the limited sources of donors, GVHD is prone to occur with numerous transplant complications, leading to high graft-related mortality. Therefore, patients need to be treated with immunosuppressants for a long period of time, and the quality of life of long-term survivors may be poor. Patients at moderate or high risk for acute leukemia (117), AML (118), MDS (119), severe AA (120), and thalassemia (121) are suitable for allo-HSCT (122). To date, allo-HSCT remains the only radical treatment for CML. Allo-HSCT is exhibiting better results in

the treatment of CML due to improved *HLA* gene matching techniques, the use of tyrosine kinase inhibitors, advances in postoperative immune status and fusion gene monitoring and improvements in postoperative complications, particularly GVHD (123). Similar to CML, allo-HSCT is effective in alleviating highly complex and severe AML. However, relapse is a major cause of treatment failure for AML patients undergoing allo-HSCT. Therefore, an effective and safe approach is required, in the future, to improve survival following remission of AML (117). In addition, a female patient with adult T-cell leukemia/lymphoma involving bone, skin, and skeletal muscle exhibited a favorable post-transplant course after receiving cyclophosphamide following allo-HSCT from her son in a clinical case report (124). There has been no progression of disease for more than two years, suggesting that this approach offers a well-tolerated and potentially curable treatment for this hard-to-treat disease (124). Given the high toxicity of this treatment, graft-anti-leukemia response and the high recurrence and mortality rate, novel post-transplantation maintenance regimens need to be studied.

In a series of studies, it was revealed that the *Notch1*/*Fbxw7* mutations in T-ALL patients may be useful biomarkers for predicting the prognosis of T-ALL. In a survey of 50 patients with T-ALL in southern India, there were 20 out of the 50 (40%) patients with *Notch1*/*Fbxw7* mutations among which the 13 out of the 20 (65%) T-ALL patients with *Notch1*/*Fbxw7* mutations exhibited favorable prednisone responses (P=0.01) and improved clinical outcomes compared with patients without *Notch1*/*Fbxw7* mutations (P=0.03) (125). In the survival analysis of the sample (n=50) studied by Valliyammai *et al* (126), it was determined that patients with *Notch1*/*Fbxw7* hotspot mutation had earlier response to treatment and improved survival. Additionally, it was suggested that *Notch1*/*Fbxw7* hotspot-mutated T-ALL cases responded better to the ALL BFM-95 protocol. Furthermore, pediatric T-ALL patients with either double *Notch1* mutations ($Notch1^{Double}Fbxw7^{WT}$) or mutations in both genes ($Notch1^{MUT}Fbxw7^{MUT}$), hereafter termed as $Notch1{\pm}Fbxw7^{Double}$, had an improved outcome (127). Jenkinson *et al* (127) screened 162 pediatric T-ALL patients treated in the MRC UKALL2003 trial for *Notch1*/*Fbxw7* gene mutations and associated genotypes in response to treatment and long-term outcomes. Of the 162 patients, 57 (35%) patients were both *Notch1* and *Fbxw7* wild-type, 62 (38%) patients had single *Notch1* mutations, 5 (3%) patients had single *Fbxw7* mutations, and 39 (24%) patients had $Notch1{\pm}Fbxw7^{Double}$. It was revealed that while 14 $Notch1{\pm}Fbxw7^{Double}$ patients were classified as high risk, only 2 patients progressed in disease and all survived. Collectively, these data suggested that detecting the *Fbxw7* mutations adds important prognostic value to the separate assessment of *Notch1* status, justifies individual treatment stratification of T-ALL (128), and allows the identification of the majority (72%) of *Notch1*/*Fbxw7*-mutated T-ALL patients with a relatively favorable prognosis, who cannot be treated with more classical, clinical, immunophenotypic or carcinogenic markers (128). Conversely, loss of *Fbxw7* in primary T-ALL has also been reported to provide a favorable prognosis for patients. Loss of *Fbxw7* reduces ubiquitination modification and degradation of glucocorticoid receptor α, thus enhancing glucocorticoid sensitivity. This increased sensitivity can enhance glucocorticoid response to treatment and provide a favorable prognosis for T-ALL (129).

In addition, several studies identified Cbl and Fbxw7 as new targets for anti-Notch1 therapy. Saito *et al* (130) revealed that flavonoids induced N1ICD degradation through the UPS by increasing Cbl in T-ALL. Flavonoid-induced resistance to T-ALL was also revealed, and Cbl was identified as a new N1ICD binding partner critical for regulating its stability and carcinogenic function. In the case of Fbxw7, oridonin has exhibited an anti-leukemia activity *in vitro* and *in vivo* by promoting Fbxw7-mediated ubiquitination modification and degradation of myc (131). These studies suggest that flavonoid and oridonin are potential drugs for T-ALL.

## 6. Conclusions and perspectives

Notch signaling, particularly Notch1 receptor, is the primary regulator of HSC stemness in embryos and adulthood, and its role in inducing leukemia (e.g., T-ALL) has been detailed in a variety of studies (52-55,132). In general, Notch1 can promote the proliferation of HSCs and inhibit its differentiation (133). However, due to the context dependence of the Notch signaling pathway and activation by different ligands, Notch1 receptor can also partially inhibit the proliferation of HSCs and promote their differentiation. Additionally, the lifetime and activity of the Notch1 receptor is largely determined by the UPS that regulates Notch1 receptor degradation, activation of Notch1 signaling, and Notch1 receptor endocytosis and its subsequent fate. Additionally, the signaling enhancement or mutations of the Notch1 pathway and the dysregulations or mutations of Notch1-related UPS have been demonstrated to be closely associated with the aberrancy of HSCs and the occurrence of leukemia (134). Therefore, further revealing the details of this pathway and the factors that regulate the UPS could help improve the treatment and prognosis of leukemia.

Since the present review is limited to the effects of Notch1 receptor and its ubiquitination modification on HSCs, other receptors and ligands of the Notch signaling pathway, or other regulatory modes of this pathway such as phosphorylation, require clarification. In addition, several studies have revealed that Notch signaling interacts closely with other signaling pathways, such as the Wnt, hippo, TGF-β family and Hedgehog that regulate stem cell properties, but these associations have not been well elucidated (135-139). Therefore, future research may also focus on the interactions or crosstalk between these pathways.

Currently, Notch1 and Fbxw7 are mainly used as prognostic indicators of T-ALL (140). However, studies on these two proteins and their regulators as treatments for leukemia are urgent. In particular, their application as therapeutic targets for leukemia or in combination with other chemotherapeutic agents require further study. In addition, it is necessary to examine whether they can be used as prognostic indicators for auto-HSCT or allo-HSCT.

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**Authors' contributions**

XJ, MY, YG, HZ, JW and JL made substantial contributions to conception and design. XJ, YG, HZ, JW and JL were involved in drafting the manuscript and revising it critically for important intellectual content. XJ, MY, YG, HZ, JW and JL agreed to be accountable for all aspects of the work. All authors read and approved the final manuscript for publication. Data authentication is not applicable.

**Ethics approval and consent to participate**

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**Competing interests**

The authors declare that they have no competing interests.

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