Notch signaling in acute lymphoblastic leukemia: any role for stromal microenvironment?

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Running title: Notch, ALL and stromal microenvironment

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Abstract
Notch signaling pathway regulates many different events of embryonic and adult development: among them, Notch plays an essential role in the onset of hematopoietic stem cells and influences multiple maturation steps of developing lymphoid and myeloid cells. Deregulation of Notch signaling determines several human disorders, including cancer. In the last decade it became evident that Notch signaling plays pivotal roles in the onset and development of T- and B-cell acute lymphoblastic leukemia by regulating the intracellular molecular pathways involved in leukemia cell survival and proliferation. On the other hand, bone marrow stromal cells are equally necessary for leukemia cell survival by preventing blast cell apoptosis and favoring their reciprocal interactions and cross-talk with bone marrow microenvironment. Quite surprisingly, the link between Notch signaling pathway and bone marrow stromal cells in acute lymphoblastic leukemia has been pointed out only recently. In fact, bone marrow stromal cells express Notch receptors and ligands, through which they can interact with and influence normal and leukemia T and B cell survival. Here, the data concerning the development of T and B cell acute lymphoblastic leukemia has been critically reviewed in light of the most recent findings on Notch signaling in stromal microenvironment.
Introduction

In 1917, Thomas Hunt Morgan and colleagues described a strain of *Drosophila* with notches at the end of their wings \(^1\). This curious trait was attributed to a partial loss of function of what would be identified later as the Notch gene, encoding a type I transmembrane receptor, which was cloned in the mid-1980s \(^2\). In *Drosophila* only a single Notch protein and two ligands (Delta and Serrate) are present, while mammals, including mice and humans, possess four Notch proteins, Notch1-4, and five ligands, named Delta-like (DLL)1, 3 and 4 \(^3\), Jagged 1 and Jagged 2, which are both similar in structure to Serrate (Figures 1a and 1b) \(^4\).

Notch receptors consist of one extracellular amino-terminal subunit and one intracellular carboxy-terminal subunit that are non-covalently linked by the heterodimerization domain (HD) \(^5\) (Figure 1c). Ligand binding induces γ-secretase, which is the important target (also under a therapeutic point of view) that mediates the cleavage and translocation of Notch intracellular domain into the nucleus, where it interacts with the DNA-binding protein RBP-J to induce the expression of downstream target genes, such as *Hes*1 and *Deltex*1 \(^4\). Jagged1/2 and DLL1, commonly named as Delta/Serrate/LAG-2 (DSL) proteins, are ligands for all Notch receptors \(^4\); Delta 4 can bind and activate Notch1 and 4 receptors \(^4\), whereas Delta-3 can bind and activate Notch 1 or similar Notch receptors \(^4\).

Ligand binding to the extracellular amino-terminal end of the transmembrane subunit presumably induces a conformational change within the Notch receptors leading to the exposure of the S2 cleavage site for proteolysis. After the shedding of the extracellular domain, a second cleavage within the transmembrane domain (at S3 cleavage site) is mediated by the γ-secretase activity of a multiprotein complex. This liberates the intracellular domain of Notch receptors (NICD), which subsequently migrates to the nucleus and heterodimerizes with the DNA binding transcription factor CSL/RBP-Jκ to regulate gene expression. This signaling cascade is evolutionarily conserved and regulates many cellular processes, including proliferation, differentiation and survival. Once bound to CSL, NICD recruits other coactivators, including mastermind proteins (MAML1-3), which in turn recruit the MED8-mediator transcription activation complex to induce transcriptional expression of downstream target genes \(^3\), \(^4\), \(^5\) (Figure 2). Members of the Hairy enhancer of split (Hes) or Hairy related (Hey or Hrt) genes have been identified as Notch target genes in many tissues, while other targets are more tissue-restricted \(^4\), \(^5\). Studies via genome-wide expression and chromatin immunoprecipitation (ChIP) arrays point to the existence of a large number of genes that can be directly regulated by Notch \(^6\). The challenge will be now to distinguish the drivers from the passengers among the large number of target genes. Moreover, there is emerging data suggesting
that Notch, Wnt, and Hedgehog pathways can crosstalk to or cooperate with other signaling pathways (including NF-κB, hypoxia or TGF-β), thus broadening the spectrum of target genes that are influenced by the Notch system \(^7,9\).

Notch signaling is a highly pleiotropic pathway, whose deregulation is on the basis of different diseases, including cancer, where it may act as both oncogene and suppressor, depending on the tumour context \(^10\). The crucial role of Notch signalling in haematological malignancies, such as T-acute lymphoblastic leukemia (T-ALL) and, more recently, B-acute lymphoblastic leukemia (B-ALL) may be understood by considering its involvement in normal T and B lymphopoiesis occurring inside the stromal niches of bone marrow and thymus.

**Notch signaling, lymphocyte development and stromal microenvironment**

Notch signaling regulates multiple aspects of lymphoid development and functions. During T cell development, Notch signaling is required for the commitment of the earliest bone marrow progenitors and during other developmental stages occurring in the thymus. In particular, Notch signaling is directly involved in the regulation of T cell precursor development, with Notch1 acting as a key receptor responsible for both the T cell lineage commitment and the inhibition of other differentiation pathways \(^3\). In fact, the expression of Notch 1-IC in lymphoid progenitors blocks B cell differentiation and leads to the generation of immature CD4\(^+\)CD8\(^+\) T cells \(^11\). However, it is still unclear whether this B versus T cell decision occurs in the common lymphoid progenitors (CLP) or in the recently described early T cell progenitors (ETP) \(^12\). In the process of B versus T cell differentiation some coactivators, such as MAMLs, are important for Notch-dependent CSL transcriptional activation \(^13\). Dominant negative mutants of MAML are capable of inhibiting Notch signaling since DNMAML1 contains only the N-terminal ICN-binding basic domain that allows them to bind to ICN, but therefore lacks the activation domain. DNMAML1 antagonizes Notch1 signaling by inhibiting recruitment of transcription coactivators \(^14–16\). The expression of DNMAML1 leads to remarkable inhibition of early T-cell differentiation and the appearance of intra-thymic B cells, consistently with Notch1 inhibition \(^17\). Other factors are involved in this process of lymphoid progenitor commitment, such as Fbw7 that is the F-box component of the SCF-E3 ubiquitin ligase complex (SCFFBW7). Fbw7 targets Notch1 for ubiquitination and degradation \(^9\). \(^18–19\). The PEST domain in the C terminus of the Notch1 receptor is essential for phosphorylation-mediated and proteasome-dependent degradation of Notch1; CycC:CDK8 phosphorylates the ICN1 within the TAD and PEST domains, and CycC:CDK8 expression strongly enhances ICN1 hyperphosphorylation and PEST-dependent degradation by the Fbw7 in vivo \(^19\), thus
suggesting that Notch1 protein stability could be a critical regulator of intracellular signaling thresholds leading to T versus B cell differentiation.

Notch is also involved in the thymic commitment of αβ versus γδ T cell receptor (TCR)-expressing T cell lineages. Notch 1 activation favors the αβ choice, as shown by the evidence that low Notch 1 levels result in the increase of γδ cells. However, the role of Notch signaling during αβ versus γδ T lineage decision remains controversial.

As previously mentioned, Notch signaling seems to be the main inhibitor of the early stages of B cell development. By contrast, several late maturation stages of B cells, such as the development of marginal zone B lymphocytes, are characterized by the expression of Notch target genes, thus suggesting the important role of Notch signaling also in B cells.

The Notch pathway may profoundly affect T and B cell development through the interaction with bone marrow and thymic microenvironmental cells, which express some of the Notch molecules and release factors that favor the effects mediated by Notch engagement. For instance, DLL4 ligand is expressed by thymic epithelial cells and is essential for T cell commitment. In the αβ T cell development, the transition through the β-selection checkpoint depends on both Notch signaling and ligation of stroma-derived CXCL12 to CXCR4 expressed by T cells, thus activating PI3K signaling.

Similarly, B cells develop in direct contact with bone marrow stromal cells, which behave as thymic epithelial cells or nurse cells do with T cells in terms of cell support and selection. Some important information has been recently obtained on the basis of the concept that bone marrow stroma derives from adult multipotent non-hematopoietic stem cell precursors named mesenchymal stromal cells (MSCs). These cells are important components of the bone marrow hematopoietic niche and can support the maintenance and engraftment of hematopoietic stem cells. MSCs are capable of self-renewal and differentiation into various mesodermal cell lineages (fibroblasts, osteoblasts, adipocytes, and chondrocytes), display a homogeneous mesenchymal immunophenotype, including CD105, CD44, CD73, CD90, CD146 marker expression, and acquire, once activated, a strong and broad immune modulatory effect that is shared also by the fibroblastic progeny. Although initially described in the bone marrow, where they are the precursors of bone marrow stromal cells and bone cells, MSCs reside in virtually every organ containing connective tissue, including lymphoid organs, such as thymus and spleen. Recent data show that human bone marrow MSCs express at basal conditions both Notch receptors, such as Notch1, 2, 3 and 4 and Notch ligands, such as Jagged1, DLL3 and 4. The expression of Notch molecules by MSCs is dynamic, as it may vary following co-culture with other cells. Consequently, bone marrow MSCs are sensitive to the effect of specific Notch inhibitors, such as γ-
secretase inhibitor XII (GSI XII) \(^{29}\). GSI XII does not induce apoptosis or morphological changes of human bone marrow MSC at up to 20.0 \(\mu\)M concentration for 3 days; by contrast, MSCs become apoptotic, displaying active Caspase3 and clear morphological changes, at 40.0 \(\mu\)M concentration, without affecting significantly mesenchymal immunophenotype \(^{29}\). The effect of GSI XII on MSCs is specific, as the control (DMSO, the vehicle for GSI XII) has no effect \(^{29}\).

Different functions of MSCs are influenced by Notch signaling: for instance, it affects MSC differentiation into osteoblasts \(^{30}\), thus suggesting that it could favor the maintenance of a pool of bone marrow mesenchymal progenitors by suppressing osteoblastic differentiation \(^{31}\). However, MSC-derived osteoblasts regulate the hematopoietic stem cell niche through the Jagged1/Notch1 signaling \(^{32}\), and Notch signaling is one of the pathways involved in osteogenic differentiation of MSCs induced by soluble factors derived from endothelial cells \(^{33}\). This feature is stem cell-specific: in fact, Notch signaling acts differently in other stem cell populations, such as epidermal stem cells, as it promotes differentiation instead of having suppressive effects; in particular, Notch signaling initiates a terminal differentiation programme in human and adult mouse keratinocyte precursors \(^{34,35}\); conversely, in others tissues, such as intestine and brain, Notch signaling inhibits cell differentiation \(^{34,35}\).

In addition, one of the redundant mechanisms mediating MSC immune regulatory effect depends on the interaction between Jagged1 expressed by MSCs and Notch1 expressed by T cells \(^{33}\); this suppression of T-cell activation is prevented by neutralizing Jagged1 and \(\gamma\)-secretase activity, thus implying a role of impaired Notch receptor signaling in T cells \(^{36}\). Ghisi M et al \(^{37}\) showed that miR-150 targets Notch3, a member of the Notch receptor family that plays important roles both in T cell differentiation and leukemogenesis and forced expression of miR-150 reduces Notch3 levels in T cell lines and has adverse effects on their proliferation and survival. By contrast, there are a few data so far on the role of Notch signaling pathway in the interactions between B cell precursors and MSC: it is evident that the full characterization of this phenomenon may offer many explanations about how the onset and maintenance of B lymphopoietic stromal niche occur either in normal conditions, or following hematopoietic stem cell \(^{33,36,37}\), or in presence of leukemia B cell clones developing in the bone marrow \(^{29}\).

**Notch signaling, T-ALL and stromal microenvironment**

*Notch deregulation in T-ALL*

T-ALL accounts for approximately one third of all cases of acute lymphoblastic leukemia and develops from the neoplastic transformation of the bone marrow-derived T cell precursors residing
Deregulation of Notch signaling is involved in the pathogenesis of those cases of T-ALL with the t(7;9)(q34;q34.3) translocation involving Notch genes. This translocation results in the expression of the N-terminal truncated, dominant active and ligand-independent Notch1 receptor, which is named TAN1, i.e. translocation-associated Notch homolog. The formal proof that TAN1 is indeed causative for disease development was obtained by murine BM reconstitution experiments: mice transplanted with bone marrow progenitors expressing TAN1 developed T cell neoplasms within 2 weeks after transplantation. Other truncated Notch isoforms, including Notch2 and Notch3, were equally capable of inducing T cell leukemias when expressed by bone marrow progenitors or immature thymocytes. Notch1 mutations are located at specific hotspots and affect critical negative regulatory elements of the protein. Retroviral or transgenic overexpression of NICD in hematopoietic or T cell progenitors is widely used to induce T-ALL in mice. However, sporadic mutations in murine and human T-ALL were found only in Notch1, thus suggesting that only Notch1 has a pivotal role in T-ALL pathogenesis. In addition, spontaneous Notch1-activating mutations occur at a high frequency in mouse models of T-ALL, such as TAL1/SCL, OLIG2 and LMO1/2 transgenic mice.

The molecular mechanisms by which aberrant Notch1 signaling contributes to T-cell transformation are not fully understood yet. Mutated Notch1 probably cooperates with other oncogenic transcription factors, such as c-Myc, E2A-PBX and Ikaros, but the aberrant Notch1 signaling itself is not sufficient for leukemic transformation. Observations in animal models suggest that even non-mutational Notch1 activation contributes to leukemogenesis, probably through the activation of c-Myc that is a direct downstream target of Notch1. Actually, Notch1 pathway activation represents a common feature of T-ALL if compared to acute myeloid leukemia and B-ALL: more than 50% of human T-ALL cases have gain-of-function mutations that involve the extracellular heterodimerization domain and/or the C-terminal PEST domain of Notch1. Thus, the role of Notch1 in the etiology and molecular tumorigenesis of T-ALL has been significantly highlighted, suggesting the possibility to identify novel targeted therapies that may interfere with Notch signaling and therefore with T-ALL development.

**Regulation of other signaling pathways by Notch**

**a. c-Myc Activation.** c-Myc proto-oncogene is a basic helix-loop-helix leucine zipper (b/HLH/LZ) protein involved in cell growth and differentiation. c-Myc has been identified as a critical direct downstream target gene of Notch1 in leukemogenesis. Inhibitors of c-Myc prevent Notch1 from...
rescuing T-ALL cells treated with γ-secretase inhibitor (GSI), and overexpression of c-Myc is sufficient to rescue most human T-ALL cell lines from GSI-induced growth arrest. The recruitment of Notch1 to the CSL binding sites in the c-Myc promoter was confirmed by other Authors. c-Myc is upregulated in non-malignant as well as malignant ICN1-overexpressing cells at both mRNA and protein levels. Furthermore, the deletion of c-Myc at the CD4⁺CD8⁺ stage of T cell development prevents tumor formation induced by Notch1. Finally, dysregulation of c-Myc expression is displayed by both ICN1-induced murine T-ALL and most human T-ALL with Notch1 gene mutations. These results suggest a synergistic effect of c-Myc and Notch1 in oncogenesis.

b. Cell Cycle Progression. Aberrant Notch signaling is directly linked to the regulation of cell cycle proteins. Protein amounts of the cell cycle-dependent kinase inhibitor p27Kip1 increased upon Notch inhibition. Notch signaling induces the transcriptional expression of the F-box protein Skp2, which is part of the E3-ligase complex that degrades p27Kip1 and p21Cip1, leading to enhanced G1-S transition. In mouse models, p27Kip1 deficiency itself was shown to contribute to T-ALL development. Another cell cycle protein involved in Notch-induced T-ALL is cyclin D3. Mice lacking cyclin D3 show greatly reduced susceptibility to Notch-induced leukemogenesis, suggesting that cyclin D3 might be an essential cell cycle protein through which Notch mediates its oncogenic effects.

c. ARF-Mdm2-p53 Pathway. As shown by a tetracycline-inducible mouse model for Notch-induced T cell leukemia, Notch can suppress p53 through repression of the ARF-mdm2-p53 surveillance network. Attenuation of Notch signaling led to increased p53 expression and to tumor regression by inducing apoptosis. Thus, Notch-mediated suppression of p53 appears to be another important event for T-ALL development.

d. PTEN/PI3K/Akt-mTOR Pathway. Microarrays profiling of the phosphorylation changes in a large number of signaling proteins in 13 T-ALL cell lines treated with GSI showed that the phosphorylation of multiple signaling proteins in the mTOR pathway is suppressed by inhibition of Notch signaling in a Notch-dependent manner; in fact, this phenomenon could be rescued by expression of ICN1 and mimicked by dominant negative MAML1, MAML1. This suggests that Notch signals positively regulate activity of the mTOR pathway in T-ALL. More importantly, the effect of GSI on the mTOR pathway can also be rescued by c-Myc, which is a direct transcriptional target of Notch. Inhibition of mTOR by rapamycin together with GSI treatment synergistically inhibits T-ALL cell growth. Notch inhibits p53 through the PI3K-Akt/protein kinase B-(PKB-) mammalian target of rapamycin (mTOR) pathway, and the inhibition of this pathway reverts the
PTEN, a tumor suppressor that negatively regulates PI3-kinase-Akt signaling pathway, is significantly downregulated in GSI-resistant T-ALL cell lines. By contrast, other groups showed in primary human and mouse T-ALL that cell growth remains dependent on Notch signaling and there is no correlation between PTEN status and resistance to Notch inhibition.

e. Other Pathways. The prognostic impact of aberrant Notch 1 was demonstrated in recent clinical studies, where the mutations resulted associated with good prognosis both in children and in adults. Despite the importance of Notch1 in leukemogenesis and T cell development, only a few Notch target genes have been identified in developing T cells. CSL-MAML-dependent Notch1 signaling controls T lineage-specific IL-7Rα gene expression both in early human thymopoiesis and in T-ALL cells. Notch signaling promotes proliferation and inhibits apoptosis of T-ALL cells. One of the first described target genes for Notch-induced transcriptional activation was the gene encoding the transcriptional repressor Hes1 that is essential for T cell development as well as Notch 1. In addition, deregulated Notch3 signaling may be important in T-ALL pathogenesis, although activating mutations of this gene have not been described; similarly, miR-150 is one of the most down-regulated miRs in ALL, thus suggesting a role for miR-150/NOTCH3 deregulation in this disease. Very low levels of miR-150 are detectable in T-ALL cell lines: forced miR-150 expression leads to functional effects ranging from the inhibition of cell proliferation to the induction of cell apoptosis, depending on the cell line. It is not clear whether these effects depend on the suppression of Notch3 or other targets of miR-150 in the hematopoietic process and, in particular, in T-cell differentiation and leukemogenesis. Very recently, two novel miRNAs (miR-451 and miR-709) have been identified in NICD1-overexpressing CD4+CD8+ T cells through miRNA expression profiling. Both miRNAs are transcriptional targets of the bHLH E2A tumor suppressor, which itself is degraded upon NICD1 induction in murine T-ALL cells. Increased Notch activity facilitates the degradation of E2A that may lead to the transcriptional downregulation of miR-451 and miR-709. Further studies of these and other miR-target interactions during normal T-cell differentiation will probably elucidate the key miRs involved in the regulation of this process and contribute to the identification of novel therapeutics for T-ALL and other T-cell malignancies.

Finally, even hypoxia may induce Notch activation and this mechanism is involved in the maintainance of hematopoietic stem cells in undifferentiated state, by increasing the number of multipotent clones; Notch and hypoxia have a role also in cell functions in normal and previously irradiated human skin. Taken together, these findings strongly suggest that the Notch signaling pathway can be under control of both oncogene activation and environmental conditions in...
physiological and pathological conditions, including ALL, although how these factors regulate Notch is still not fully understood.

**Role of stromal cells in T-ALL**

It is likely that Notch-mediated intracellular mechanisms are influenced, if not even determined, by the interactions of T-ALL blasts with stromal microenvironment. Coculture of primary human T-ALL cells, displaying either mutated or naive Notch receptor, with a mouse stromal cell line expressing ligand DLL1 permitted in a reproducible manner the maintenance of T-ALL-initiating cells and long-term growth of blast cells 76. However, quite surprisingly, very little information is available in literature about the role of Notch signaling in the cross-talk between T-ALL cells and bone marrow stroma. Actually, it has been clearly shown that the migration of T-ALL cells into human bone marrow stroma is necessary for the disease development 77, and that bone marrow stroma-supported cultures of T-ALL cells may predict treatment outcome in children 78 and quantify the efficacy of anti-leukemic drugs 79. It is matter of debate whether all malignancies are maintained by a small population of immature cancer stem cells or the majority of malignant cells normally possess stem cell-like properties 76. Studies employing NOD/SCID xenotransplant confirmed that TEL-AML1 ALL likely arises from a lymphoid progenitor rather than a malignant multipotent hematopoietic cell 80. Other studies with the same experimental model of xenotransplantation showed that CD34⁺CD10⁻ and CD34⁺CD19⁻ primitive ALL cells are enriched of leukemia-initiating cells 81; the nature of these cells remains to be defined, but it is likely that it depends on the cytogenetic abnormalities involved. The escape from dormancy of human T-ALL cells, as well as colorectal cancer cells, is associated with DLL4 expression in the tumor stromal microenvironment and increased Notch3 signaling in tumor cells 82. The link between Notch signaling pathway, which is pivotal for T-ALL onset and development, and bone marrow stromal cells, which are necessary for preventing blast cell apoptosis and favoring their reciprocal interactions and cross-talk with bone marrow microenvironment, could probably rely on the growth factors that stromal cells produce and release in presence of normal and neoplastic lymphoid cells. For instance, T-ALL cells in contact with bone marrow stromal cells are rescued from apoptosis through an IL-7-dependent mechanism 24, 83, which is in agreement with the control of the T lineage-specific IL-7Rα gene expression by CSL-MAML-dependent Notch1 signaling previously described 73. Notch-mediated regulation of cell-cell contact could be also involved, as shown in other diseases such as breast cancer 84: in fact, enhanced T-ALL cell survival on bone marrow stroma requires the involvement of adhesion molecules, such as LFA-1 and ICAM-1 85, which can be modulated by Notch activation 84. On this basis, an important challenge for the near future will
be to show formally the synergistic link between Notch pathway and bone marrow stromal cells in driving T-ALL development, to pave the way to combined therapeutic strategies aimed at interfering with the Notch signaling both in T-ALL cells and in supporting stromal cells.

**Notch signaling, B-ALL and stromal microenvironment**

B-ALL is the most common form of childhood and adult ALL and is characterized by the clonal expansion in the bone marrow of neoplastic B cell precursors at different developmental stages, which can be distinguished from the normal counterpart (B cell precursors) on the basis of the presence of aberrant antigen expression. The role of Notch signalling in B-ALL has been described more recently than in T-ALL. B-ALL cells express three of the four Notch receptors (Notch1–4) and five ligands (Delta-like1, Delta-like3, Delta-like4, Jagged1 and Jagged2). Microarray analysis of 207 children with high-risk B-ALL showed that Notch pathway expression is a common feature of these neoplasms. However, these studies also revealed the presence of additional transcriptional targets in GSI-I-dependent cell death, including genes in the unfolded protein response, nuclear factor-κB and p53 pathways. Z-LLNle-CHO blocks both γ-secretase and proteosome activity, inducing more robust cell death in B-ALL cells than either proteosome-selective or γ-secretase-selective inhibitors alone both in vitro and in vivo. In fact, in a nonobese diabetes/severe combined immunodeficiency (NOD/SCID) B-ALL xenograft model, Z-LLNle-CHO delayed or prevented engraftment of B-ALL cells in 50% of the animals, suggesting that this compound is worthy of further testing. These results have not been achieved in T-ALL in vivo so far, due to the limited ability of GSI to induce apoptosis in mouse and human T-ALL cells; in addition, severe gastrointestinal toxicity related to the inhibition of Notch signaling in the gut has been observed only in T-ALL in vivo models, but not in B-ALL models. However, alternative strategies, based on blocking antibodies against specific Notch receptors and/or ligands are currently being developed by several groups and pharmaceutical companies. The design of the new inhibitors is based on the X-ray structure of the CSL-NICD-DNMAML complex. Some groups designed and tested small α-helical peptides (hydrcarbon-stapled α-helical peptides) that block the intracellular protein-protein interaction. Treatment of leukemic cells with these peptides resulted in the suppression of the Notch-activated transcriptome. Most importantly, the peptide inhibited the proliferation of leukemic cells in vitro, as well as in a Notch1-driven in vivo T-ALL mouse model, without causing any gut toxicity. Whether these α-helical peptides are specific inhibitors of the Notch1 transcription complex remains to be...
investigated. Nevertheless, this is a very encouraging study showing that transcription factor complexes can indeed be targeted.

In B-ALL, Notch signaling may be involved as the result of other pathogenetic mechanisms. For instance, FBXW7 mutation may occur in adult B-ALL, as well as in T-ALL: the FBXW7 gene encodes a subunit of an ubiquitin protein ligase that regulates levels of Notch, cyclin E and other proteins.

*In vitro*, B-ALL cells require the contact with viable bone marrow stromal cells for optimal growth and survival; in absence of stromal cells, cultured B-ALL cells undergo apoptosis in a few days in a very reproducible manner. ALL blasts grow and accumulate in close association with bone marrow MSCs and this event is essential for the long-term survival and expansion of leukemic lymphoblasts *in vitro*. Inhibition of PI3K, mTOR and MEK signaling pathways promotes rapid apoptosis in B-ALL in the presence of stromal cell support. Stromal cells may protect B-ALL cell lines from cytarabine- and etoposide-induced cell death via a VCAM-1 dependent mechanism. Activation of caspase-3 by cytarabine or etoposide treatment is reduced upon co-culture of B-lymphoblasts with stromal cell layers. On the other hand, the interaction between VLA-4 on leukemia blasts cells and fibronectin or VCAM-1 on stromal cells activates phosphatidylinositol 3-kinase (PI3K)/Akt/Bcl-2 signaling, an important pathway that determines B-ALL chemosensitivity and contributes to the persistence of minimal residual disease in B-ALL patients.

Notch signaling plays both oncogenic and tumor suppressor roles, depending on cell type. The induction of Notch signaling in B-ALL cell lines leads to growth arrest and apoptosis, in contrast with T-ALL cell lines, primary B-ALL cells and B-CLL cells, where Notch activation promotes leukemogenesis. However, there are a few data about the role of Notch signaling in primary B-ALL cell survival and interaction with bone marrow stromal cells. Very recently, an *ex vivo* study has clarified the role of Notch signaling in stroma-dependent survival of human primary B-ALL cells, by studying both the B-ALL cell apoptotic rate in presence of bone marrow MSCs obtained from normal donors and B-ALL patients, and the capability of different Notch molecules expressed by either B-ALL cells or BM-MSCs to rescue leukemia cells from apoptosis. B-ALL cells express all Notch receptors and ligands; on the other hand, bone marrow MSCs express at basal conditions Notch1, 3 and 4 and DLL3 and 4, but following co-culture other molecules are induced. Notch receptors and ligands are functional, as shown by the evidence that the complete blockade of the Notch system by using GSI XII promotes the specific apoptosis of B-ALL cells either cultured alone or in co-culture with MSCs, thus suggesting that the reciprocal cell interactions via Notch signaling play an important role in leukemia cell survival. In particular, Notch3 and Notch4, Jagged1, Jagged2 and DLL1 are mainly responsible for the anti-apoptotic
effect derived from B-ALL cell and MSC co-culture, as shown by specific blocking experiments. Interestingly, Notch signaling also promotes the resistance of B-ALL cells to hydrocortisone when in direct contact with MSCs, effect that can be prevented by either anti-Notch 3 plus anti-Notch 4 blocking antibodies or GSI XII (Figure 3). Drug resistance mediated by the Notch system seems to be a general phenomenon, as it has been described also in solid tumors. A significant protective effect of short-term or periodic treatment with inhibitors (like GSI-I) has been reported in vivo in animal models, supporting the concept that targeting multiple proteases might sensitize B-ALL cells to chemotherapeutic reagents with less severe toxicity to normal organs. Additional pre-clinical studies will clarify the potential therapeutic effects of GSI-I in B-ALL. In addition, further useful information with potential therapeutic implication will derive from the study of the molecular mechanisms responsible for Notch-induced survival of B-ALL blasts in absence and presence of stromal cells and from in vivo blocking experiments in animal models. However, the role of Notch signaling in B-ALL cells does not seem so different from that played in T-ALL cells, thus suggesting a general mechanism of leukemia cell survival for lymphoid tumors.

Conclusions

Over the last decade it has become clear that Notch signaling is involved in the regulation of crucial cell fate decisions and differentiation processes during the development of the normal and leukemic hemato-lymphopoiesis. Although the oncogenic properties of Notch signaling in T-ALL were discovered approximately twenty years ago, the specific interest in this research field increased only after the finding that gain-of-function Notch1 gene mutations are the most frequent genetic alterations in T-ALL. All these intracellular mechanisms are likely the result of the interactions of T-ALL blasts with stromal microenvironment, which has a pivotal role in preserving T-ALL blasts from apoptosis. However, quite surprisingly, very little is known about the role of Notch signaling in the interactions between T-ALL cells and bone marrow stroma, but this gap will probably filled in the near future. Similarly, the recent discovery of the role of Notch signaling also in B-ALL development will probably lead to the re-interpretation of many important findings of the literature concerning the role of stromal support in B-ALL, thus hopefully opening new therapeutical possibilities. The complexity of the interactions between Notch signaling and several other intracellular signaling pathways involved in cell survival, proliferation and apoptosis makes difficult to predict both the role of each molecular component during the differentiation stages of normal and neoplastic lymphoid cell development, and the final effect on leukemia cells of Notch-targeted therapy with specific inhibitors that are already available (e.g., γ-secretase inhibitors,
neutralizing antibodies against DLL4 or Notch1). However, Notch targeting may represent a unique possibility to combine a direct anti-cancer treatment with the interference with the stromal support of the leukemia cell survival both in T-ALL and in B-ALL patients, and eventually to improve the prognosis of these diseases.
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Authorship

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References


Figure legends

Figure 1. Structure of Notch proteins and their ligands
a) Notch receptors: Notch 1-4 are presented on the cell surface as heterodimers. b) Notch ligands: two transmembrane-bound ligands for Notch have been identified in Drosophila, named Delta (Dl) and Serrate (Ser). The vertebrates possess three Delta homologues, called Delta-like (DLL)-1, -3 and -4, and two Serrate homologues, Jagged 1 (JAG1) and Jagged 2 (JAG2). Serrate, Jagged 1 and Jagged 2 harbour a cysteine-rich domain (CR) following the EGF-like repeats. Jagged 1, Jagged 2 and DLL 1 bind to all the Notch receptors; DLL 3 binds to Notch 1; DLL 4 binds to Notch 1 and Notch 4. c) Domain structure of the heterodimer Notch receptors: the ectodomain of Notch receptors contains EGF-like repeats and a cysteine-rich Notch/Lin12 domain (LN); this is followed by a transmembrane domain, the RAM domain and six ankyrin repeats (ANK), two nuclear-localization signals (NLSs), followed by the transactivation domain (TAD) and a PEST sequence.

Figure 2. Notch intracellular pathway
Interaction of Notch receptors with their ligands, such as Delta-like or Jagged, leads to a cascade of proteolytic cleavages. The truncated receptor is then the substrate for a multiprotein complex formed by presenilin, nicastrin, Aph1 and Pen-2 with \( \gamma \)-secretase activity that cleaves Notch within its transmembrane domain, thus leading to the release of the intracellular domain (Notch-IC or NICD) \(^{100} \). The first cleavage (S\(_2\)) is mediated by ADAM-type metalloproteases, called TACE (TNF-\( \alpha \) converting enzyme) in vertebrates or Kuzbanian in Drosophila, followed by a further cleavage at S\(_3\) within the transmembrane domain mediated by \( \gamma \)-secretase activity of presenilins, which liberates the cytoplasmic domain-Notch intracellular domain. The free Notch-IC enters the nucleus and binds to the transcription factor CSL, which displaces co-repressors (CoR) and recruits co-activators (CoA), leading to transcriptional activation of downstream target genes.

Figure 3. Notch signaling in the interactions between B-ALL cells and mesenchymal stromal cells
Interaction of Notch receptors and ligands expressed by bone marrow mesenchymal stromal cells (MSC), obtained from normal individuals and patients, and their counterparts expressed by B-ALL cells leads to the survival increase of leukemia cells and enhanced resistance to chemotherapeutical agents. Receptors and ligands written in bold mean strong expression, written in italics mean absent or weak expression. Jagged 1, Jagged 2 and DLL 1 bind to all the Notch receptors; DLL 3 binds to...
Notch 1; DLL 4 binds to Notch 1 and Notch 4. *Notch molecules involved in the anti-apoptotic effects. For references, see text.
Figure 2

Cytoplasm

- Notch ligand
- Notch receptor
  - S2 cleavage (ADAM-mediated)
  - S3 cleavage (γ-secretase-mediated)
- Notch-IC
  - Translocation

Nucleus

- Co-A
- CSL
- Hes 1, 5, 7
- Herp 1, 2
- GTGGGAA
- Activation of transcription induced by Notch-IC

Inactive complex

GTGGGAA
Notch signaling in acute lymphoblastic leukemia: any role for stromal microenvironment?

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