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Role of human Bone Marrow-Mesenchymal Stromal Cells-mediated Notch signaling on acute myeloid leukemia cells survival and proliferation in vitro

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Summary

Abstract

Notch signaling pathway activation is known to contribute to the pathogenesis of a spectrum of human malignancies, including T cell leukemia. However, recent studies have implicated the Notch pathway as a tumor suppressor in myeloproliferative neoplasms and several solid tumors. However, its role in human Acute Myelogenous Leukemia (AML) remains unclear.

In the present study, we investigated the role of Notch pathway in the development, progression and relapse of human AML. The expression of Notch receptors (Notch1, Notch2, Notch3 and Notch4) and their ligands (DLL1, DLL3, DLL4, Jagged1 and Jagged2) have been analyzed in human AML cell lines HL-60, THP1, U937, K562 and primary AML samples by flow cytometry, western immunoblotting and RT-PCR approaches. In addition, the phenotype of Notch receptors has been evaluated in the same cell populations cocultured both with human MSCs from healthy donors (hBM-MSCs) and from AML patients (hBM-AML-MSCs). Furthermore, we analyzed AML cell survival and proliferation upon treatment with Notch inhibitor GSI-XII and chemotherapeutic drugs. We observed that human AML samples expressed Notch receptors and ligands at activated levels and also downstream Notch targets, suggesting that Notch pathway is functional at basal levels in human AML. In addition, MSCs protect AML cells from apoptosis even in the presence of chemotherapeutic drugs. This study shows new possible interactions between the bone marrow stromal microenvironment and leukemia cells.

Introduction

1.1 Notch signaling pathway

Notch is a highly conserved cell signaling pathway present in most multicellular organisms. Mammals possess four different Notch receptors, referred to as Notch1, Notch2, Notch3, and Notch4 (Fig. 1.1) (Niessen et al. 2011). The Notch pathway mediates juxtacrine cellular signaling wherein both the signal sending and receiving cells are affected through ligand-receptor crosstalk by which cell fate decisions in neuronal, cardiac, immune, and endocrine development are regulated. The Notch receptor is a single-pass transmembrane receptor protein. It is a hetero-oligomer composed of a large extracellular portion, which associates in a calcium-dependent, non-covalent interaction with a smaller piece of the Notch protein composed of a short extracellular region and a single transmembrane-pass; this extracellular portion in its entirety is called "Notch Extracellular Domain" (NECD). Besides, Notch is constituted from a small transmembrane (TM), and intracellular (NICD) domains. In mammalian signal-sending cells, members of the Delta-like protein (DLL1, DLL3, and DLL4) and the Jagged (Jagged1, Jagged2) families serve as ligands for Notch signaling receptors. Upon ligand binding (Fig.1.2), the NECD is cleaved away from the TM-NICD domain by TACE (TNF-α ADAM metalloprotease converting enzyme). The NECD remains bound to the ligand and this complex undergoes endocytosis/recycling within the signal-sending cell in a manner dependent on ubiquitination by Mib. In the signal-receiving cell, γ-secretase releases the NICD from the TM (S3 cleavage), which allows for nuclear translocation where it associates with the CSL (CBF1/Su (H)/Lag-1) transcription factor complex, resulting in subsequent activation of the canonical Notch target genes: Myc, p21, and the HES-family members (Hori et al. 2013).

In the hematopoietic system, Notch is essential for the emergence of definitive hematopoietic stem cells during fetal life (Bigas et al. 2008) and indispensable for the commitment of progenitors to the T cell lineage (Benveniste et al. 2014). Moreover, Notch1 appears to be the central oncogenic trigger in T cell acute lymphoblastic leukemia (T-ALL) in both humans and mice (Weng et al. 2004). Indeed, Notch1 is commonly mutated, leading to constitutive activation of the Notch pathway in the majority of T-ALL patients (Malyukova et al. 2007; Maser et al. 2007). In contrast to the T cell lineage where the role of Notch signaling is well defined, there is conflicting information on the role of Notch signaling in the function of adult stem cells and multipotential progenitors and in the myeloerythroid compartment (Maillard et al. 2008; Delaney et al. 2010; Dahlberg et al. 2011). Initial in vitro studies suggested that Notch signaling accelerates myeloid differentiation (Tan-Pertel et al. 2000; Schroeder et al. 2003). However, subsequent studies contested this conclusion. Most notably, it was shown that Notch can suppress myelopoiesis in vitro (de Pooter et al. 2006) and Mercher et al. (2008) reported that Notch signaling can induce megakaryocyte differentiation. Laurence Bugeon et al. (2011) studies showed that inhibition of Notch signaling in vivo leads to the reduction of myelo-monocyte development. Hence, defects in Notch signaling affect definitive hematopoiesis, altering myelopoiesis from the early stages of development. Lobry et al. (2013) have recently shown that Notch signaling can function as an antagonist of the granulocyte/monocyte progenitor (GMP) cell fate and that loss of Notch signaling biases commitment toward GMP differentiation, eventually resulting in chronic myelomonocytic leukemia (CMML) (Klinakis et al. 2011), a myelodysplastic/myeloproliferative disease. They observed inactivating mutations in the Notch pathway in a fraction of CMML patients, suggesting that this pathway is targeted by genetic alterations. These data are consistent with subsequent reports of inactivating Notch pathway mutations in head and neck cancer (Agrawal et al., 2011; Stransky et al., 2011). However, there are still few studies that support the theory

that Notch may function as a tumor suppressor. Some in vitro studies based on the myeloid cell line 32D have shown contradictory effects of Notch1 activation on myeloid differentiation (Milner et al. 1996; Schroeder et al. 2000), but the study of Kawamata et al. (2002) are the first to show that Notch1 activation alters the ability of progenitors to mature along the myeloid lineage in vivo. Notch1 receptor is a known modulator of lineage-specific events in hematopoiesis (Stier et al. 2002). In particular, in haematopoiesis, Notch1 acts defining the increase of lymphoid precursor cells, while it determines a decrease of myeloid linage development (Fig1.3).

1.2 Acute Myeloid Leukemia (AML)

AML is a clonal myeloproliferative disease characterized by an uncontrolled proliferation and differentiation block of myeloid committed blood cells in the bone marrow (BM). Acute myeloid leukemia is a relatively rare cancer. There are approximately 3.7 cases per 100,000 people; 70% of new cases are over 60 years old and the mortality rate depends on the age of patients (Deschle et al. 2006).

The male: female ratio for AML is 1:1. Outcomes for AML patients remain poor; despite the use of cytotoxic chemotherapy and stem cell transplantation, most patients die of relapsed, refractory disease (Fröhling et al., 2005). The French-American-British (FAB) classification divides AML into eight subtypes, from M0 to M7, on the basis of blast cell morphology and maturation degree (Bennet et al. 1976, Table 1.1). However, WHO classification takes into consideration other biological features of AML cells: in fact, cytogenetic and molecular studies have shown that AML is a heterogeneous disease with a variety of cytogenetic and molecular alterations that have biological and clinical relevance (Dash and Gilliland, 2001; Armstrong et al. 2003; Döhner et al. 2010). Among them, chromosomal abnormalities

leading to the generation of leukemogenic fusion oncoproteins, including mixed lineage leukemia (MLL) gene fusions that are associated with adverse outcome. In addition, somatic mutations in tumor suppressors have been shown to contribute to leukemogenesis and AML risk classification (Bacher et al. 2010). However, molecular mechanisms linking these mutations to transformation are incompletely understood, and the role of the most recently identified genes, including TET2, ASXL1, and IDH1/2 in AML pathogenesis has not been fully clarified (Figueroa et al. 2010). Recently, a clear role for nucleophosmin NPM1 has been shown in the pathogenesis, clinical evolution and prognosis of the AML cases with apparently normal karyotype (Falini B et al.).

Current treatments for AML patients include dose-intensive chemotherapy and allogeneic hematopoietic stem cell transplantation, which are associated with significant and age-related toxicity, including Graft Versus Host Disease (GvHD), and high relapse rates (Rowe JM et al, 2010).

1.3 Human bone marrow mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) were isolated for the first time in the '70s by Friedenstein and coll. from the bone marrow, accounting for approximately 0.01% of all nucleated cells (Friedenstein et al. 1974). MSCs are stromal progenitors growing in culture as adherent cells with fibroblastoid morphology (Haniffa et al. 2009) and endowed with multi-lineage differentiation potential towards mesodermal cell lineages and extensive immune-modulatory properties. MSCs are the precursors of the bone marrow stroma that constitutes the normal hematopoietic stem cell niche (Quante et al. 2011), thus supporting hematopoietic stem cell homing, quiescence, self-renewal and differentiation into mature blood cells through cell-cell contact and secretion of growth factors (Jones et al. 2008; Kolf

2007; da Silva et al. 2008). Three main criteria have been identified by the International Society of Cellular Therapy (ISCT) to define MSCs: (i) Adhesion to plastic, (ii) expression of specific immunophenotypic marker combinations (CD73, CD90 and CD105), and lack of expression of hematopoietic markers (CD14, CD34 and CD45) and class-II major histocompatibility complex (MHC) molecules; (iii) capability of differentiating into mesodermal lineages (adipocytes, osteoblasts and chondrocytes) (Fig.1.4). Although the bone marrow is the main source of MSCs, they can also be extracted from adipose tissue (Kuhbier et al. 2010), umbilical cord blood, placenta, and many other tissues. Under artificial *in vitro* conditions MSCs may differentiate into cell types of tissues of different embryonic origin, such as nervous and hepatic tissue, but the spontaneous differentiation is still dabated (Devine et al. 2003; Vallabhaneni et al. 2010).

Growth and differentiation of hematopoietic cells require direct contact with stromal cells deriving from MSC-precursors (Konopleva et al. 2002). The importance of the stromal microenvironment in the maintenance and differentiation of normal hematopoietic progenitors has been emphasized in long-term bone marrow cultures. However, the molecular mechanisms of the interaction between stromal and hematopoietic cells are not well defined. Stromal cells produce a variety of growth factors, but direct cell-to-cell contact is needed for cell growth and differentiation (Konopleva et al. 2002).

As leukemic cells originate from the hematopoietic normal counterparts and also reside within the bone marrow microenvironment, it is likely that stromal cells influence the proliferation and apoptosis of leukemic cells. This interaction plays a crucial role in the pathogenesis of AML by promoting tumor cell growth and survival as well as drug resistance, which is the major challenge in the treatment of AML. For instance, the interaction between VLA-4 (α4β1 integrin) on leukemic blasts and fibronectin on stromal cells activates phosphatidylinositol 3-kinase (PI3K)/Akt/Bcl-2 signaling, an important

determinant of AML chemosensitivity and the level of minimal residual disease of AML patients (Becker et al. 2009). Thus, activation of the signaling cascades downstream of integrin engagement may play a critical role in the well-documented chemoresistance of bone marrow AML cells. However, the role of stromal cells is not yet entirely clear, although some data suggest that they may be responsible for the high frequency of relapse of the disease (Mayani H, 1996).

In this work we assessed whether the interaction between stromal cells derived from bone marrow MSCs and AML cells promotes leukemic cell survival and resistance to chemotherapy through the expression of the Notch system.

Materials and Methods

2.1 Samples and Patients

Cell lines

The following human AML cell lines were employed: HL-60 (acute promyelocytic leukemia cell line), THP1 (acute monocytic leukemia cell line), U937 (myeloid histiocytic sarcoma line) and K562 (myeloid blast crisis of chronic myeloid leukemia, CML). All the cell lines were purchased from the American Type Culture Collection (table 2.1).

Positive controls for gene and protein expression of Notch receptors and ligands are shown in Table 2.2.

AML samples

Bone marrow samples (fresh aspirates or cryopreserved cells, n=10) were obtained, after informed consent, from AML patients with high blast counts at diagnosis, admitted to to the Hematology Sections of Verona and Treviso. Bone marrow cells were further fractioned by Ficoll-Paque (Miltenyi Biotec) and analyzed by flow cytometry analysis to confirm the diagnosis (evaluation of CD11b, CD13, CD14, CD16, CD33, CD34, CD38, CD45, CD64 expression).

Human BM-MSC isolation and characterization

Human MSCs were obtained from bone marrow aspirates of healthy donors (hBM-MSCs, n=8) and AML patients (hBM-AML-MSCs, n=5) after informed consent. Whole unprocessed BM cells were plated in tissue cultures flask (BD Biosciences) at $10x10^4$ cells/cm^2 using α-MEM (Sigma-Aldrich) supplemented 10% fetal bovine serum (FBS), 1%

L-Glutamine solution 200mM, and 1% Penicillin-Streptomycin and incubated at 37°C and 5% CO_2 atmosphere. After 72h of colture, non-adherent cells were removed by washing with sterile 1X PBS. When reaching 70-80% of confluence, adherent cells were washed with sterile 1X PBS, trypsinized (0.05% trypsin at 37°C for 5 minutes, Sigma), harvested and expanded in large flasks at a lower density (100 cells/cm^2). MSC identity was confirmed by immunophenotyping at the end of third cell passage, according to the expression of CD73, CD90 and CD105. In addition, the lack of haematopoietic and endothelial markers (CD31, CD34, and CD45) was assessed. Further characterization of MSCs was carried out by using monoclonal antibodies (mAbs) specified for HLA-class I and II. All antibodies were purchased from Pharmigen/Becton Dickinson and Immunostep (Fig 2.1).

2.2 Cell cultures and co-cultures

Cells were counted in Neubauer chamber using Acridine Orange/Ethidium Bromide (Acr / EtBr) solution at 1:10 ratio. AML cells from patients were used immediately for flow cytometry analysis and/or co-cultures. AML cell lines and MSCs were expanded for about 24 and 72 hours, respectively. Human BM-MSCs were cultured at 4000 cells/cm^2 concentration in culture flasks (Becton Dickinson, Milano, Italy) with α-MEM (Sigma-Aldrich) supplemented with 10% FBS, 1% L-Glutamine solution 200 mM, and 1% Penicillin-Streptomycin. AML cell line cultures were performed by seeding 10 X 10^6 cells in T75 Cell Culture Flasks with RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycine. Both cell types were incubated at 37°C and 5% CO_2 atmosphere. Co-culture experiments were performed on a confluent monolayer of MSCs with RPMI (Sigma Aldrich) supplemented with 10% FBS, 1% L-Glutamine solution 200 mM, and 1% Penicillin-Streptomycin, in 24-well plates for 24, 48 and 72 hours.

Co-culture in presence of drugs

Gamma-Secretase Inhibitor XII (GSI-XII, Notch inhibitor) and some drugs normally used for AML treatment, i.e. Etoposide, Cytarabine (Ara-C) and Idarubicine chloridrate, were added in co-colture experiments at 1μM, 5μM, 10μM or 20 μM final concentrations for 72 hours. To determine EC50 dose, we performed the colorimetric MTT metabolic activity assay. AML cells collected during log growth were seeded in 96 wells plates (10^4 cells per wells) at 24, 48, and 72 hours, with or without different drugs. Cell viability was then assessed by adding 10 μL of methyl thiazolyl tetrazolium (MTT, Sigma-Aldrich) into each well and keeping in incubator for 3 hours. Metabolically active, viable cells converted MTT into a colored formazan, which was solubilized with a volume of acidic isopropanol equal to the volume of cell suspension. Optical density of formazan reflected cell viability. The product was then measured at 570nm in a spectrophotometric microplate reader (PerkinElmer VICTORX4). The viability was expressed as the percentage of optical density of treated cells compared to optical density of untreated cells. The MTT method was done in triplicate (Fig 2.2)

2.3 Flow Cytometry analysis

AML cells were identified as CD45+, CD34+, CD38- cells by flow cytometry (FACSCanto II, Becton Dickinson, Rutherford, NJ, USA) using the BD DIVA software. Cells were initially selected using a morphological gate based on forward scatter (FSC measured cellular size) and side scatter (SSC, measured cellular granularity) parameters. The subsequent evaluation was performed on CD45+ versus SSC and then CD34+ versus CD38- to identify myeloid blasts. A threshold was fixed on FSC to exclude cellular debris. The analysis of CD34 and CD38 expression was performed both in AML cell lines and in purified AML cells from patients, with acquisition of 30,000 morphologically gated events

per tube. The percentage of CD45+, CD34+, CD38- was used to evaluate the expression of Notch receptors and ligands compared to isotype-specific antibodies (Fig. 2.3).

Notch receptor immunophenotype

Immunophenotypic analysis was performed on fresh bone marrow aspirates or frozen mononuclear cells (MNCs) from AML patients as well as on AML cell lines. Expression of Notch receptors and ligands was analyzed by flow cytometry using Phycoerythrin (PE) conjugated antibodies against Notch receptors and ligands, as shown in Table 2.3. At least $3x10^5$ cells were incubated with the specific antibody or appropriate isotype control for 15 minutes in the dark at room temperature. Unbound antibodies were removed by washing the cells with 1X PBS. Cells were resuspended in 200-300uL of 1X PBS. Phenotype was assessed by FACS Canto II and data were analyzed by FlowJo software. Immunophenotyping was carried out on AML cell lines (5 times) and on 10 AML samples. Statistical analysis was performed with GraphPad Prism 6 software.

Apoptosis quantification

AML cells were seeded alone or in co-culture with MSCs at 10:1 ratio for 72h and then collected and washed twice with 1X PBS to remove FBS. After resuspension in 1X Binding Buffer, cells were stained with CD45 (Becton Dickinson, Milano, Italy) and Annexin V (Miltenyi Biotec), and incubated in the dark at room temperature. After 15 minutes of incubation and washing with 1 ml of 1X Binding Buffer, cells were resuspended in 200µL of 1X Binding Buffer with 10ul of Propidium Iodide and analyzed by flow cytometry. Cell viability was assessed in the selected CD45+ population using FLowJo software. The non-apoptotic cells percentage at the end of the experiment was calculated using a live cell population at time 0 as control (analyzed at the beginning of the experiment with fresh cells) (Fig 2.4).

Proliferation assay

Thawed AML cells were resuspended in appropriate culture medium, counted and washed. Cell pellets were resuspended at $1x10^6$ cells/mL concentration in 1X PBS with 0.1%BSA (Bovine Serum Albumin, Sigma Aldrich, Italy). Carbossifluorescein-di-acetate-succimidyl-ester (CFSE solution Gibco, Life Technologies, Milano, Italy) was added to cell suspension at 2µL/ 10^6cell concentration and incubated for 10 minutes at 37°C in the dark. Cells were washed 3 times with cold complete medium. Then, CFSE-labelling was confirmed by flow cytometry. After 24h, 48h, 72 hours of culture in complete medium cells were collected and stained with CD45PerCP and TO-PRO 3 (Invitrogen-Life Technologies) and acquired by flow cytometer. Proliferation was assessed in CD45+/TO-PRO 3- cells using FLowJo software, and the GeoMean of cycling cells was calculated with the following formula:

$$\text{CFSE Proliferation cells} = \frac{\text{GeomMen cells in culture - GeomMen cells in co-culture}}{\text{GeomMen cells in co-culture}} \text{ x100}$$

Cell Cycle assay

At 72 hours of culture and coculture, cells were collected and washed in 1X PBS. Cells were then resuspended in 1X PBS for 5 minutes, washed, resuspended in 70% ethanol in PBS, and incubated either 1 hour at -20°C or overnight at 4°C. Cells were then washed once and resuspended in 1X PBS. Ribonuclease A (Sigma Aldrich; Milan Italy) was added to 100 µg/mL final concentration and the samples were incubated at 37 °C for 1 hour in the dark. Propidium iodide (Sigma Aldrich; Milan Italy) was added to final concentrations of 40 µg/mL right before the acquisition on the flow cytometer. The percentage of mitotic cells was calculated by subtracting G0 and apoptotic cells (Cells with <2N DNA content) from total cells. Cell cycle assays were performed in triplicate.

2.5 RNA isolation and RT-PCR

Cell pellets were obtained by centrifugation at 30 minutes, 2h, 6h, 24h, 48h and 72h of culture with and without MSCs. TRIzol ® Reagent (Invitrogen™ - Life Technologies) was added in a ratio of 1mL per $5x10^6$ cells.

Phase separation

For each sample, 0.2 ml of chloroform per ml of TRIzol ® Reagent (Invitrogen™ - Life Technologies) was added. After incubation for 2-3 minutes, at room temperature, samples were centrifuged. The upper aqueous phase was removed and stored in a new tube. Then, 0.5 ml of 100% isopropanol were added to the aqueous phase per each mL of TRIzol used for homogenization. Samples were then incubated at room temperature for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C.

RNA wash and resuspension

After removing the supernatant from the tube, the RNA pellet was washed with 1 ml of 75% ethanol, air dried, resuspended in RNase-free H_2O and incubated in a water bath at 55°C for 10 minutes, then stored at -80°C until further use.

cDNA synthesis and qualitative PCR

For gene expression analysis, cDNAs were reverse-transcribed from total RNA (2ug) using SuperScript II Reverse Transciptase (Invitrogen™ - Life Technologies) according to manufacturer's instructions. The PCR reactions were performed with REDTaq ReadyMix PCR reaction mix with $MgCl_2$ (Sigma Aldrich) on a thermal cycler Verity (Applied Biosystem) with an initial denaturation at 95°C for 2 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The primers used, are listed in Table 2.4.

Electrophoresis Agarose gel

The PCR amplified products were observed by means of electrophoresis gel; 10 µL of PCR product was loaded onto a 2% agarose gel in TBE containing 1 µg/ml ethidium bromide. The Quickload 100 base pairs DNA ladder (New England Biolabs) was run on each gel at 110 V (7.3V/cm) for 45 minutes and visualized under UV light.

Gene expression by qPCR

RT-qPCR was performed to evaluate the quantitative expression of the *HES1* gene in AML cell lines. Complementary DNA was synthesized from 2 µg of RNA in a volume of 25 µL using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). cDNA was analyzed for the expression of target genes (HES-1 and GAPDH) by the SYBR Green double-stranded DNA binding dye assay, using a Platinum® Quantitative PCR SuperMix-UDG kit (Invitrogen™) and tested in a DNA Engine Opticon 2 Continuous Fluorescence Detector (MJ Research). UDG and dUTP in the SuperMix prevent the reamplification of carryover PCR products between reactions. dUTP ensures that any amplified DNA contains uracil, while UDG removes uracil residues from single- or double-stranded DNA, preventing dU-containing DNA from serving as template in future PCRs. A UDG incubation step before PCR cycling for 2 minutes at 50°C destroys any contaminating dU-containing product from previous reactions. UDG is then inactivated by the high temperatures during normal PCR cycling, thereby allowing the amplification of genuine target sequences. Reactions were denatured for 2 minutes at 95°C and then subjected to 50 two-step amplification cycles with denaturation at 95°Cfor 15 seconds followed by annealing/extension at 60°C for 1 minute. Primers used were:

FH3_GAPDH: 5'-CTC TGA TTT GGT CGT ATT GG-3'
RH3_GAPDH: 5'-GTA AAC CAT GTA GTT GAG GTC-3'

FH1_HES1: 5'-GCC TAT TAT GGA GAA AAG ACG-3'
RH1_HES1: 5'-CTA TCT TTC TTC AGA GCA TCC-3'

All primers were purchased from Sigma. Data were obtained with Optical monitor version 2.02.24 (MJ Research) software and were analyzed with GraphPad Prism (version 5).

2.6 Cells lysis and Western immunoblotting

For immunoblotting analysis, 1.8 x 10^6 cells were used. At >80% confluence cells were collected and lysed with an appropriate amount of ripa lysis buffer (25 nM Tris pH 7.6, 150mM NaCl, 1% NP40, 1% Na-deoxycholate, 0.1% SDS) supplemented by complete Protease Inhibitor (Sigma Aldrich) and 1 mM Na3SO4. Then cell lysates were centrifugated at 15.000 rpm at 4°C for 10 min. Supernatants were collected and tested with bicinchoninic acid (BCA) protein assay kits (Thermo Scientific). The BCA protein assay is a colorimetric, fast and sensitive microplate test, in which different sample concentrations can be compared with increasing amounts of Bovin Serum Albumin (BSA) in a standard curve. Deoxycholic acid is a strong detergent, capable of destroing all membranes.

Electrophoresis and western blotting (WB)

To assess protein expression and modulation, SDS polyacrylamide gel electrophoresis (PAGE) was performed. With this technique it is possible to identify proteins by sample size. Cells lysates were supplemented by 1x Laemmli Buffer solution (5x solution: 0.3M TRIS pH 6.8, 25% β-mercaptoethanol v/v by Sigma Aldrich; Milan Italy, 11.5% SDS w/v by Biochemical, 50% glycerol w/v 0.02% blue bromophenol to active denaturation and S-s reduction), then loaded into gel for electrophoresis. Gels were prepared as follows: 0.5mL of 40% solution of acrylamide: bisacrylamide 29:1(BDH), 1.25mL of solution M (0.5mL TRIS pH6.8 0.4% SDS w/v), 0.92 mL of 50% glycerol w/v, 2.35mL of ddH2O, 33.5µL of 10% APS w/v, 8.5 µL of TEMED (Jannsen). Of each sample, 20µg of total lysate was

loaded in the wells and gels were run in a running buffer consisting of 25mM TRIS, 192mM glycine for electrophoresis 0.1% SDS w/v. A pre-stained protein marker (Protein Sharpmass V prestained 11-250 kDa. Euroclone) was loaded to monitor the migration process and allow, by comparison, an approximate molecular weight estimation of the separated proteins. Electrophoresis was carried out at a constant voltage of 60V during the focusing phase, then the voltage was doubled during the running phase. After the run, samples were blotted on a 0.45µm nitrocellulose filter to perform further analysis. To this purpose, gel was unassembled and soaked for a couple of minutes in cold Transfer Buffer (25mM TRIS, 192mM glycine, 10% methanol v/v purchased from Sigma-Aldrich). In the meantime, the transfer "sandwich" was prepared by assembling the transfer cassette, a 3mm Whatman paper wetted with transfer buffer, the nitrocellulose, the gel and again the wetted paper. The transfer was achieved inserting the transfer cassette with the membrane oriented to the positive pole in the Transfer cell (Euroclone) in transfer Buffer and applying 500mA constant current for 2h at 4°C. After transfer, the nitrocellulose filter was stained with Ponceau Red solution (0.2% Ponceau S w/v 3% TCA w/v). Nitrocellulose membrane was saturated for 1h at room temperature (RT) in TBST (20mM TRIS, 150mM NaCl, pH8) supplemented with 0.1% Tween 20 v/v and 2% or 5% BSA (Sigma Aldrich). The incubation with primary antibody was always done at 4°C, under gentle shaking. The primary antibodies used to reveal specific proteins are reported in Table 2.5. After the incubation with the primary antibody, the blot was washed 5 times at RT for 5 minutes with TBST and then with the secondary antibody (anti-mouse, anti-rabbit both Sigma Aldrich) and anti-rabbit (GE Healthcare) labeled with Horse Radish Peroxidase (HRP) all used at working dilution of 1:10,000 in TBST 2% BSA and incubated at RT for 1h. At the end of the incubation time, the membrane was washed 5 times with TBST and then analyzed by incubation in chemiluminescent substrate. The labeled blots were then exposed to x-ray

film to visualize antibody binding. To ensure equal loading of protein samples, the blots were stripped of their primary antibody and re-probed for GAPDH.

2.8 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad software Inc. U.S.A.). Data were expressed as mean +/- standard error means (SEM). Statistical comparison among groups were performed by either ANOVA one-way test, when only one independent variable was considered, or Wilcoxon matched pairs test, when values in each row presented paired observation, or Mann-Whitney test, when data did not have Gaussian distributions. P value<0.05 was considered statistically significant.

Results

The expression of the Notch signaling molecules was assessed in human AML cell lines and MSCs. Specific antibodies against Notch receptors (Notch1, Notch2, Notch3, Notch4) and ligands (DLL1, DLL3, DLL4, Jagged1, Jagged2) were first used in HL-60, THP1, U937 and K562 (human AML cell lines). To validate flow cytometric data, parallel studies on the same cells were performed with Western blot and RT-PCR. Then, the expression of Notch receptors by bone marrow mononuclear cells (BM-MNCs) and MSCs cell populations from AML patients were analyzed. Finally, the expression of Notch receptors and ligands was analyzed in AML cell lines and AML primary cells cocultured with human MSCs from either healthy donors (hBM-MSCs) or AML patients (hBM-AML-MSCs). Furthermore, AML cell survival and proliferation was analyzed upon treatment with Notch inhibitor GSI-XII and chemotherapeutic drugs, in presence of absence of MSCs.

3.1 Notch molecule expression by AML and MSCs at resting conditions

Expression of Notch receptors and ligands

Using validated cDNA, we compared the mRNA expression of *NOTCH* genes 1–4 and their ligands (*DLL*1-4 and *JAGGED* 1 and 2) in AML cell lines, primary AML cells from patients, hBM-MSCs and hBM-AML-MSCs. To compare the data, we normalized the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Overall, *NOTCH1*,

NOTCH2 and *NOTCH3* were analyzed. Concerning MSCs we found expression of *NOTCH2* and *NOTCH3*, but no evidence of *NOTCH1 expression. NOTCH1* was detected at low levels in AML samples and *NOTCH2* was not expressed by K562 (Table 3.1 A). Expression levels of Notch ligand mRNA were similar in all the cell populations analyzed. *JAGGED1* and *JAGGED2* mRNA were expressed in all cell samples, while *DLL3* was expressed in hBM-AML-MSCs, we did not find *DLL1 and DLL4* mRNAs in any cell types analysed. Flow cytometry confirmed the data obtained at RNA level (Table 3.1 B), as Notch1, Notch2 and Notch3 molecules were expressed in all AML samples, with the highest expression of Notch1 in HL60 and U937 (median rMFI of 3.7 and 2.9 respectively) and Notch3 in THP1 cell line (median rMFI = 2.1). Interestingly, in primary AML blast cells (CD34+/CD38-) Notch receptor expression appeared slightly different from that observed in AML cell lines. Notch2 and Notch3 were more expressed (median rMFI 1.7 and 2.1 respectively), while Notch1 were expressed at different intensity according to the FAB subtypes: M1, M3, M4 and M5 cells expressed higher levels of Notch1 (from 1.5 to 2 folds) than M0 and M2 (Fig 3.1A).

Jagged-1 and Jagged2 and DLL3 resulted expressed at different levels both with RT-PCR and flow-cytometry: Jagged2 were expressed by all primary AML samples and cell lines, while Jagged1 was expressed by U937, MSCs both from healthy donors and AML samples and primary AML cells, particularly of M0 subtype, although not statistically significant (Fig 3.1 B). Notch1 and Notch2 expression was quite variable, but also Notch3 resulted more expressed in THP1 and primary AML samples. Notch1 was highly expressed in AML samples (rMFI=3.6), but also in some AML cell lines (HL60 and U937 with median rMFI= 2.7 and 3 respectively); Notch2 was particularly represented in AML samples (rMFI= 3) and HL60 (rMFI=5) and relatively expressed in other samples at different levels (Fig. 3.2 A). While Jagged 2 was expressed by all the samples analyzed, AML primary samples showed a high expression of Jagged1, which was not observed in

AML cell lines (Fig. 3.2 B). Some difference were observed also between hBM-MSCs and hBM-AML-MSCs. Notch1 receptor had a slightly higher expression in hBM-AML-MSCs than hBM-MSCs, while Notch2 and Notch3 were poorly expressed in AML-MSCs (Fig.3.3 A) and, similarly, Jagged1 and Jagged2 in hBM-AML-MSCs as compared to hBM-MSCs; DLL3 was expressed significantly only in the hBM-AML-MSCs (Fig 3.3 B).

Notch signaling is activated at basal level

To evaluate if Notch expression in AML is correlated with activation of Notch signalling, we analyzed the Notch intracellular domain NICD, the expression of Notch target gene HES1 and some genes involved in Notch signaling pathway, such as *ADAM17, ADAMDEC1, BCL2* and *NRARPn were analysed,* by using validated cDNA obtained from the same cell cultures used for previous studies. In AML cell lines, immunoblotting analysis showed the presence of NICD1 protein, consistent with Notch activation state, but HES1 was expressed at low levels, thus suggesting that Notch activation was present only at basal levels (Fig.3.4A). Hence, we characterized the genes involved in Notch signaling pathway by RT- PCR. Similarly to protein data, we observed the expression of HES1, ADAMDEC1 and BCL-2, and high levels of ADAM17. A very similar pattern of expression was found in AML samples. In addition, we did not observe the presence of NRARP RNA in all samples analyzed (Fig. 3.4 B). Human BM-MSCs from healthy donors and hBM-AML-MSCs showed lower expression of the genes involved in Notch activation, such as ADAM17 and ADAM DEC1 (Fig. 3.4C and D). The expression of BCL-2 depends on cellular differentiation and can be either pro- or anti-apoptotic (Oliver et al. 2011).

3.2 Expression of Notch receptors and ligands by AML and MSCs in co-culture conditions

We investigate the expression of Notch pathway by MSCs in co-cultures with AML cells. Slight differences were evident in co-culture with the different AML cell lines. However, in co-cultures with HL60, K562, and U937, Notch3 was expressed significantly (from rMFI value of 1.6 of control to 2.6, 2.7 and 1.7 respectively) (Fig 3.5A) by MSCs. Among Notch ligands, only Jagged2 expression resulted significant increased in K562 (Fig 3.5 B).

Among cell lines, the coculture determined a significant up-regulation of Notch1 and Notch2 only in HL60 (rMFI value of 6.37 vs 1.18). (Fig. 3.6 A), while Notch3 and Notch4 and Notch ligand expression did not change (Fig 3.6 B).

Interestingly, some differences were observed also in primary AML cells co-cultured with MSCs. Notch1 was expressed in all primary AML samples, with median rMFI values of 1.57 vs 0.99 ($P < 0.005$), while Notch2 and Notch3 ranged from 1.7 to 1.2 and from 1.9 to 1.4 median rMFI, respectively, even though the difference did not reach a statistical significance; Notch4 and all ligands remained unchanged (Fig. 3.7).

Co-culture activates Notch signaling in AML cell lines

To assess whether the co-culture-dependent increased expression of Notch 1 and 2 was directly proportional to the activation of Notch signaling, the quantitative expression of *HES1* mRNA was evaluated in AML cell lines at narrow intervals, both in coculture and culture alone condition. After 30 minutes, both HL60 and THP1 in coculture conditions showed a significant increase of levels of *HES1* mRNA, this increase was more evident in U937 both in coculture and culture alone conditions. Interestingly, K562 showed a significant decrease of *HES1*mRNA levels after co-culture with hBM-MSCs (Fig 3.8).

3.3 Effects of hBM-MSCs on AML cells viability and proliferation

Growth and differentiation in *vivo* of most types of hematopoietic cells require direct contact with stromal cells. The importance of the hematopoietic microenvironment in the maintenance and differentiation of normal hematopoietic progenitors has been emphasized in long-term bone marrow cultures.

Impact of hBM-MSCs on survival of AML cells

Our observations show that co-culture significantly promotes AML cell survival. Indeed, AnxV/PI assays in AML cell lines cocultured for 72h with MSCs in starvation condition demonstrated that the presence of the hBM-MSCs promoted the survival up to 40% in HL60, 20% in THP1 and 30% in U937, while K562 survival was not affected (Fig 3.9 A). The co-cultures performed with primary AML cells showed that the survival of leukemic cells was closely related to the presence of the MSC layer (Fig. 3.9 B). In particular, after 72 hours of co-culture with hBM-MSCs leukemic cell survival raised up to 20%.

Impact of hBM-MSCs on AML cell proliferation

AML cell lines showed a slight reduction in proliferation in presence of MSCs (Fig. 3.10), with lower percentage of cells in S and G2/M phases and higher percentage in G0/G1 phases in 3 out of 4 cell lines, although this effect was not statistically significant only in THP1 cell line (Fig. 3.11).

3.4 Effects of Notch pathway inhibition on AML cells and hBM-MSC functions

To assess the role of Notch signaling in AML cell proliferation and survival, increasing doses of γ-secretase inhibitor (GSI) XII were used on cells cultured alone or with hBM-MSCs (Fig 3.12).

Notch signalling is involved in human MSC-mediated promotion of chemo-resistance of AML cells

The effect of chemotherapeutic agents on AML survival was assessed by treating AML cell lines with increasing doses of Cytarabine, Etoposide, and Idarubicine chloridrate. As expected, the treatment resulted in a dose-dependent decrease of AML cells viability when cultured alone, but a consistent increase in the overall number of live cells when co-cultured with MSCs. We then assessed whether the blockade of Notch signaling could affect the apoptosis and proliferation of AML cell lines in culture alone or co-culture conditions in presence of drugs for 48 hours. To this aim, the EC50 dose of the drugs was added with increasing doses of GSI-XII. We noticed that except for THP1 cell lines, increasing concentrations of GSI-XII abrogated AML cell line chemoresistance induced by MSCs (Figures 3.13).

Discussion

The interactions between bone marrow stromal cells and myeloid hematopoietic precursors are essential for the regulation of survival, proliferation and differentiation of normal hematopoietic precursors and neoplastic cells (Konopleva M. et al. 2003; Fortney JE. et al. 2001). From a general point of view, the identification of signaling pathways involved in the stromal cell-dependent protection of neoplastic cells from apoptosis is crucial for the development of novel therapeutic targets. Many different signaling molecules are involved in the reciprocal interactions between bone marrow stroma and neoplastic cells. Among them, stromal Notch pathway activation represents a pivotal pathogenetic mechanism in T-cell acute lymphoblastic leukemia development (Chiaramonte R.et al. 2005; Kamdje N. et al. 2010) and may induce cell cycle arrest in a variety of neoplastic cells (Houde C. et al. 2004), including hematologic malignancies. For instance, culture of MSCs from multiple myeloma patients and normal donors may create a very efficient niche that supports the survival and proliferation of the myeloma cells (Corre J. et al.2007). Myeloma cells overexpress Jagged2 when in direct contact with stromal cells, and escape from apoptosis; Jagged-2 triggering induces the secretion of interleukin-6 (IL-6), VEGF, and insulin like growth factor-1 (IGF-1) in stromal cells (Houde C. et al. 2004).

Recently, our group showed that the treatment with combinations of anti-Notch molecules neutralizing antibodies resulted in the decrease in B-acute lymphoblatic leukemia (B-ALL) cell survival, either cultured alone or co-cultured in presence of stromal cells from normal donors and B-ALL patients. Our data suggested that the stromal cell-dependent anti-apoptotic effect on B-lineage ALL cells is mediated by Notch-3 and -4 or Jagged-1/-2 and

DLL-1 in a synergistic manner (Nwabo Kamdje et al. 2011). In addition, our group investigated the role of Notch signaling in the promotion of survival and chemoresistance of human chronic lymphocytic leukemia (CLL) cells in coculture with human BM-MSCs of both autologous and allogeneic origin. The presence of BM-MSCs rescued CLL cells from apoptosis both spontaneously and following induction with various drugs, including Fludarabine, Cyclophosphamide, Bendamustine, Prednisone and Hydrocortisone. The treatment with a combination of anti-Notch-1, Notch-2 and Notch-4 antibodies or GSI-XII reverted this protective effect by day 3, even in presence of the above mentioned drugs, thus suggesting that Notch blocking could be an additional tool to overcome drug resistance and improve the therapeutic strategies for CLL (Nwabo Kamdje et al. 2012).

We focused our research on the role of Notch pathway in the development, progression and relapse of human AML, because its role has not been elucidated so far. A recent paper showed that human AML samples express Notch receptors; however, Notch receptor activation and expression of downstream Notch targets are remarkably low, suggesting that Notch is present but not constitutively activated in human AML (Kannan et al. 2013). Induced activation through any of the Notch receptors (Notch1–4), or through the Notch target HES1, consistently leads to AML growth arrest and caspase-dependent apoptosis, which are associated with BCL2 loss and enhanced p53/p21 expression. Activated Notch1, Notch2, and HES1 all led to inhibited AML growth in vivo, and Notch inhibition via dnMAML enhanced proliferation in vivo, thus revealing the physiological inhibition of AML growth in vivo in response to Notch signaling (Kannan et al. 2013). Another recent paper showed that Notch signaling is silenced in human AML samples, as well as in AML-initiating cells in an animal model of the disease (Lobry et al 2013). In vivo activation of Notch signaling using genetic Notch gain of function models or in vitro using synthetic Notch ligand induced rapid cell cycle arrest, differentiation, and apoptosis of

AML-initiating cells; moreover, Notch inactivation cooperated in vivo with loss of the myeloid tumor suppressor Tet2 to induce AML-like disease (Lobry et al 2013).

In our work, the expression of Notch receptors and ligands were analyzed both in human AML cell lines (HL-60, THP1, U937, K562) and primary AML samples from patients, by applying different methodological approaches, i.e. flow cytometry, western immunoblotting and RT-PCR, and coculturing neoplastic cells with human MSCs from both healthy donors and AML patients. Finally, we assessed whether Notch signalling could affect the stroma-mediated support and chemoprotection towards AML cells.

We confirmed that AML cells express Notch receptors and ligands, representing as such an autocrine/paracrine system in AML. Accordingly, we found the presence of the active form of Notch1 (NICD1) as well as Hes1 in AML cell lines. These observations mean that the Notch pathway is activated in AML cells. Moreover, cell death occurring in GSI-XII - treated AML cells shows that Notch activation is necessary for AML survival.

The effect of GSI-XII implies a potent pro-survival role of Notch in AML. Consequently, as MSCs express Notch receptors and ligands, we hypothesized that stromal microenvironment can activate Notch signalling in AML cell lines and thus promote leukemic cell survival. We found that MSCs induced increasing expression of Notch1 and Notch2 in AML cell lines and this effect was correlated with a dramatic increase of Hes1 mRNA after 30 minutes. Previous studies showed that AML primary cells and cell lines are characterized by low activation of Notch (Kannan et al., 2013; Lobry et al.,2013). By contrast, according with our co-culture data, this phenomenon could not occur in the stromal microenvironment, as AML cells seem to be characterized by high levels of Notch receptors, associated with an important activation of this pathway, when in contact with stromal cells. Moreover, while Kannan et al. and Lobry et al. demonstrated that high levels of Hes1 were directly associated with AML cell death, in our study we did not observe cell death associated to the important level of Hes1 occurring in co-culture model. As GSI-XII

induced cell death in AML cultured alone, we inhibited Notch activation with GSI-XII in the co-culture model and then analyzed AML cell proliferation and survival. Our data show that GSI-XII was capable of abrogating AML chemoresistance induced by MSCs in a dose-dependent manner. This observation could indicate that stromal microenvironment activates Notch signalling in AML cells and this activation could be critical for the response to chemotherapy. With the aim to validate this new role of Notch in AML physiopathology, our group is currently implementing genetic inhibition of Notch by using dnMAML to confirm the results obtained with pharmacological inhibition. In addition, as we found that coculture induced Notch1 and Notch2 expression increase in AML cells, these two receptors could be responsible of the Notch pathway activation. Consequently, as we did previously in lymphoid malignancies (Nwabo et al., 2011 and 2012), we are currently analyzing the effects of specific anti-Notch blocking antibodies on the survival of AML cells in contact with MSCs in presence of chemotherapeutic agents. The validation of this hypothesis could pave the way for the development of therapeutic blocking antibodies targeting Notch signalling and possibly capable of interfering with stroma-induced AML chemoresistance.

Tables

Table 1.1 *The morphologic subtypes of AML also include rare types not included in the FAB system, such as acute basophilic leukemia, which was proposed as a ninth subtype, M8, in 1999 (Duchayne et al. 1999)*

Type	Name	Cytogenetic aberrations described in literature	Percentage of adult AML patients
M0	acute myeloblastic leukemia, minimally differentiated		5%
M1	acute myeloblastic leukemia, without maturation		15%
M2	acute myeloblastic leukemia, with granulocytic maturation	t(8;21)(q22;q22), t(6;9)	25%
M3	promyelocytic, or acute promyelocytic leukemia (APL)	t(15;17)	10%
M4	acute myelomonocytic leukemia	inv(16)(p13q22), del(16q)	20%
M4eo	myelomonocytic together with bone marrow eosinophilia	inv(16), t(16;16)	5%
M5	acute monoblastic leukemia (M5a) or acute monocytic leukemia (M5b)	del (11q), t(9;11), t(11;19)	10%
M6	acute erythroid leukemias, including erythroleukemia (M6a) and very rare pure erythroid leukemia (M6b)		5%
M7	acute megakaryoblastic leukemia	t(1;22)	5%

Table 2.1 *Genetic profile of human AML cell lines*

Name	FAB	description	Cytogenetic data
HL60	M2	acute promyelocytic leukemia	der(5)t(15;17), but RARa rearrangement
THP1	M5	acute monocytic leukemia cell line	T(9;11) MLL
U937	M5	myeloid histiocytic sarcoma line	T(10;11), t(1;5)
K562	CML	Chronic Myeloid Leukemia (CML) myeloid blast crisis	T(9;22) BCR-ABL1

Table 2.2 *Positive controls used in PCR and WB for the different Notch primers and antibodies*. Hek293, Embryonic Kidney was used to evaluate the expression of ADAM17, ADAMDEC, Notch receptors 1-4 genes, HES1, and Jagged1-2. SK-HEP1 was used for the Delta-like protein ligands and Jurkat to evaluate Hes1 and NICD protein in WB.

PCR primer	*positive control*	*WB*	*positive control*
ADAM17	*HEK293*	*Hes1*	*Jurkat, HEK 293*
ADAMDEC1	*HEK293*	*Nicd*	*Jurkat, HEK 293*
BCL-2	*Jurkat*	*Notch1*	*HEK293*
DLL1	*SK-HEP-1*	*Notch2*	*HEK293*
DLL3	*SK-HEP-1*	*Notch3*	*HEK293*
DLL4	*SK-HEP-1*	*Notch4*	*HEK293*
HES1	*HEK293*		
JAGGED1	*HEK293*		
JAGGED2	*HEK293, SK-HEP-1*		
NOTCH1	*HEK293*		
NOTCH2	*HEK293*		
NOTCH3	*HEK293*		
NOTCH4	*SKHEP1*		
NRARP	*SKHEP1*		
positive control	***Organism:***	***Tissue***	***disease***
HEK293	*Homo sapiens, human*	*Embryonic Kidney*	
Jurkat	*Homo sapiens, human*	*Peripheral Blood*	*Acute T Cell Leukemia*
SK-HEP-1	*Homo sapiens, human*	*Liver/Ascites*	*Adenocarcinoma*

Table 2.3*: Immunophenotyping for Notch receptors and Ligands (extracellular domain)*

Antibody	**Conjugate**	**Isotype**	**Manufacter**
mouse anti-human **Notch1**	PE	Mouse IgG1k	BioLegend San Diego, California - Biotechnology
mouse anti-human **Notch2**	PE	Mouse IgG2a	BioLegend - San Diego, California - Biotechnology
mouse anti-human **Notch3**	PE	Mouse IgG1k	BioLegend - San Diego, California - Biotechnology
mouse anti-human **Notch4**	PE	Mouse IgG1k	BioLegend - San Diego, California - Biotechnology
anti-human/mouse/rat **Jagged1**	FITC	Mouse IgG2b	R&D Systems
mouse anti-human **Jagged2**	PE	Mouse IgG1k	BioLegend - San Diego, California - Biotechnology
mouse anti-human Delta-like protein 1 (**DLL1**)	PE	Mouse IgG1k	BioLegend - San Diego, California - Biotechnology
polyclonal anti-human Delta-like protein 3 (**DLL3**)	PE	Goat IgG1k	R&D Systems
mouse anti-human Delta-like protein 4 (**DLL4**)	PE	Mouse IgG1k	BioLegend - San Diego, California - Biotechnology

Table 2.4: *Forward (FH) and reverse (RH) primers used in RT-PCR*

GENES	PRIMER	Sequence
ADAM17	FH	5'-CAGATTCGCATTCTCAAGTC-3'
	RH	5'-CTAGCAACATCTTCACATCC-3'
ADAMDEC1	FH	5'-CCGTCTTTACATCTAACCAG-3'
	RH	5'-CTAGCAACATCTTCACATCC-3'
BCL-2	FH	5'-CCGTCTTTACATCTAACCAG-3'
	RH	5'-CTAGCAACATCTTCACATCC-3'
DLL1	FH	5'-CCGTCTTTACATCTAACCAG-3'
	RH	5'-CTAGCAACATCTTCACATCC-3'
DLL3	FH	5'-CCGTCTTTACATCTAACCAG-3'
	RH	5'-CTAGCAACATCTTCACATCC-3'
DLL4	FH	5'-CCGTCTTTACATCTAACCAG-3'
	RH	5'-CTAGCAACATCTTCACATCC-3'
GAPDH	FH	5'-CCGTCTTTACATCTAACCAG-3'
	RH	5'-CTAGCAACATCTTCACATCC-3'
HES1	FH	5'-CCGTCTTTACATCTAACCAG-3'
	RH	5'-CTAGCAACATCTTCACATCC-3'
JAGGED1	FH	5'-CCGTCTTTACATCTAACCAG-3'
	RH	5'-CTAGCAACATCTTCACATCC-3'
JAGGED2	FH	5'-CCGTCTTTACATCTAACCAG-3'
	RH	5'-CTAGCAACATCTTCACATCC-3'
NOTCH1	FH	5'-CCGTCTTTACATCTAACCAG-3'
	RH	5'-CTAGCAACATCTTCACATCC-3'
NOTCH2	FH	5'-CCGTCTTTACATCTAACCAG-3'
	RH	5'-CTAGCAACATCTTCACATCC-3'
NOTCH3	FH	5'-CCGTCTTTACATCTAACCAG-3'
	RH	5'-CTAGCAACATCTTCACATCC-3'
NOTCH4	FH	5'-CCGTCTTTACATCTAACCAG-3'
	RH	5'-CTAGCAACATCTTCACATCC-3'
NRARP	FH	5'-CCGTCTTTACATCTAACCAG-3'
	RH	5'-AAAAGGTAACGAACCTTCAC-3'

Table 2.5 *Antibodies used for Western blotting*

Protein	**Manufacter**	**SPECIES**	**Dilution**	**MW**
Notch1	Abcam	Mouse	1/1000	230-290 kDa
Notch2	Santa Cruz	Rabbit	1/1000	265 kDa
Notch3	Abcam	Rabbit	1/1000	244 kDa
Notch4	Santa Cruz	Goat	1/1000	177-218 kDa
Hes1	Santa Cruz	Rabbit	1/500	35 kDa
Hes1	cell Signaling	Rabbit	1/1000	30 kDa
β-actina	Sigma	Mouse	1/1000	42 kDa
cleaved Notch1	cell Signaling	Rabbit	1/1000	110 kDa
cleaved Notch2	Sigma	Rabbit	1/500	100-130 kDa

Table 3.1 A) *mRNA expression of Notch receptors and ligands. Presence (+) or absence (-) of PCR product in RT-PCR experiments. B) Protein expression of Notch receptors and ligands in AML cells and MSCs. Values above (+) or below (-) 1.5 rMFI.*

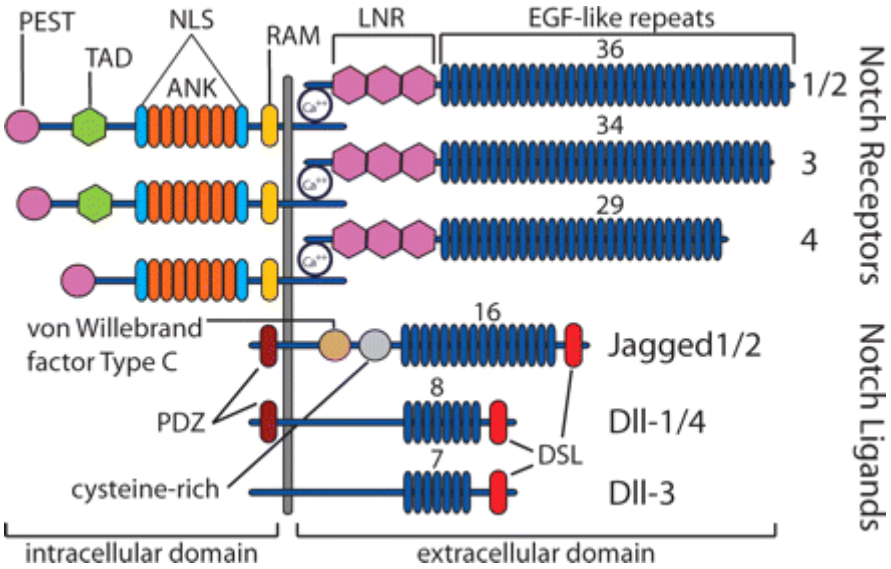
A

	NOTCH1	NOTCH2	NOTCH3	NOTCH4	JAGGED1	JAGGED2	DLL1	DLL3	DLL4
HL60	+	+	+	-	+	+	-	-	-
THP1	+	+	+	-	+	+	-	-	-
U937	+	+	+	-	+	+	-	-	-
AML Samples	±	+	+	-	+	+	-	-	-
K562	+	-	+	-	+	+	-	-	-
AML MSCs	+	+	+	-	+	+	-	+	-
MSCs	+	+	+	-	+	+	-	-	-

B

	Notch1	Notch2	Notch3	Notch4	JAGGED1	JAGGED2	DLL1	DLL3	DLL4
HL60	+	+	+	-	-	+	-	-	-
THP1	+	±	+	-	-	+	-	-	-
U937	+	+	+	-	+	+	-	-	-
AML Samples	+	+	+	-	±	+	-	+	-
K562	+	-	±	-	-	+	-	-	-
AML MSCs	+	+	+	-	+	+	-	+	-
MSCs	±	+	+	-	+	+	-	+	-

Figures



Fig, 1.1 Notch receptors and ligands. There are 4 Notch receptors (Notch1–Notch4) and 5 ligands [Jagged1/2, Delta-like (DLL)-1/3/4] in mammals. Notch receptors are expressed on the cell surface as heterodimers stabilized through calcium-dependent interactions. The extracellular domain contains 29–36 epidermal growth factor (EGF)-like repeats (human Notch receptors), 3 Lin-12/Notch (LNR) repeats, and a heterodimerization domain. The intracellular domain contains an RBP-Jκ-associated molecule (RAM) domain, 7 ankyrin (ANK) repeats, 2 nuclear localization signals (NLS), a transactivation (TAD) domain, and a PEST domain. Notch ligands are also expressed on the cell surface. The extracellular domains contain a Delta/Serrate/Lag2 (DSL) domain unique to Notch ligands and also contain multiple EGF repeats. Jagged1/2 also contains a cysteine-rich domain and a von Willebrand factor type C domain. The intracellular domains of Jagged1 and Dll1 have been shown to contain PDZ domains, which may interact with downstream signaling components to activate transcription. **Kyle Niessen et al. Cell Physiology, Published 1 July 2007 Vol. 293no. C1-C11DOI: 10.1152/ajpcell.00415.2006**

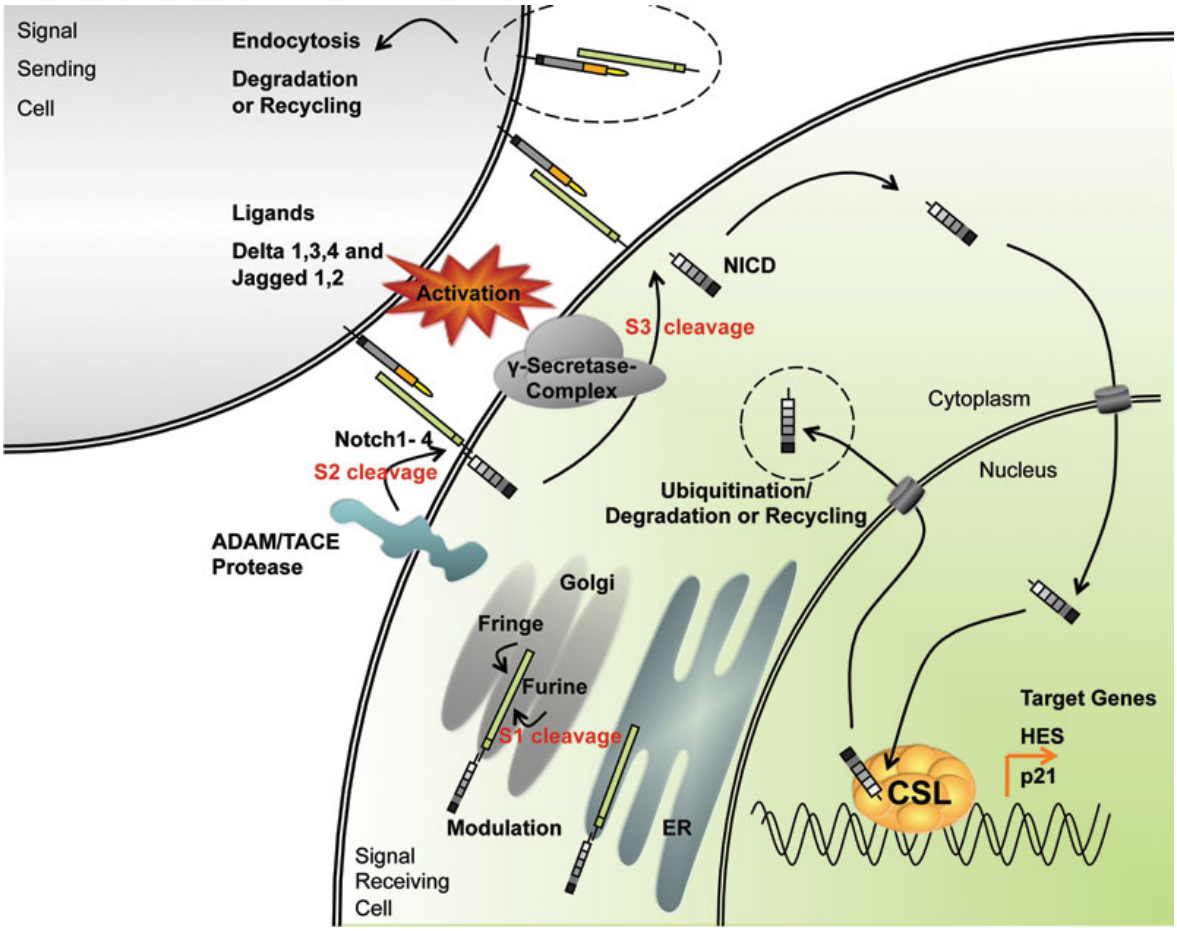


Fig. 1.2 *Notch signaling represents an evolutionarily highly conserved pathway in multicellular organisms that regulates cell-fate decisions through juxtacrine signaling among adjacent cells during development, in stem cells and in cancer cells. In mammals, four different Notch receptors (Notch 1-4), that are present in signal-receiving cells and that activated by binding to corresponding ligands in signal-sending cells, have been identified so far. They represent single-pass trans-membrane receptor proteins composed of functional extracellular (NECD), transmembrane (TM) and intracellular domains (NICD). In the signal-receiving cell, endoplasmatic reticulum (ER) and Golgi processing of Notch receptors causes cleavage, thereby producing a glycosylated, Ca2+-stabilized heterodimer composed of NECD no covalently attached to the TM-NICD inserted in the membrane (S1 cleavage). This processed receptor protein then translocates to the plasma membrane for binding to a corresponding ligand. In mammals, members of the Delta-like (DLL1, DLL3, DLL4) and the Jagged (JAG1, JAG2) families, which are in general present in the signal-sending cell, function as ligands that can activate corresponding Notch receptors. Following ligand binding, the NECD is cleaved (S2 cleavage) from the TM-NICD domain by the ADAM metalloprotease TACE (TNF-a converting enzyme). The NECD remains bound to the ligand. This protein complex is then processed by endocytosis and recycling/degradation within signal-sending cells. In the signal-receiving cell, a third cleavage event mediated by g-secretase releases the NICD from the TM (S3 cleavage), which then translocates to the nucleus and associates with the CSL family transcription factor complex, thereby causing activation of individual Notch target genes, including Myc, p21 and HES family members. In most cases, increased*

expression of ligands with subsequent Notch activation causes cellular differentiation (and cell growth arrest), thereby regulating the cluster size of cell populations. **(Reichrath et al. 2012)**

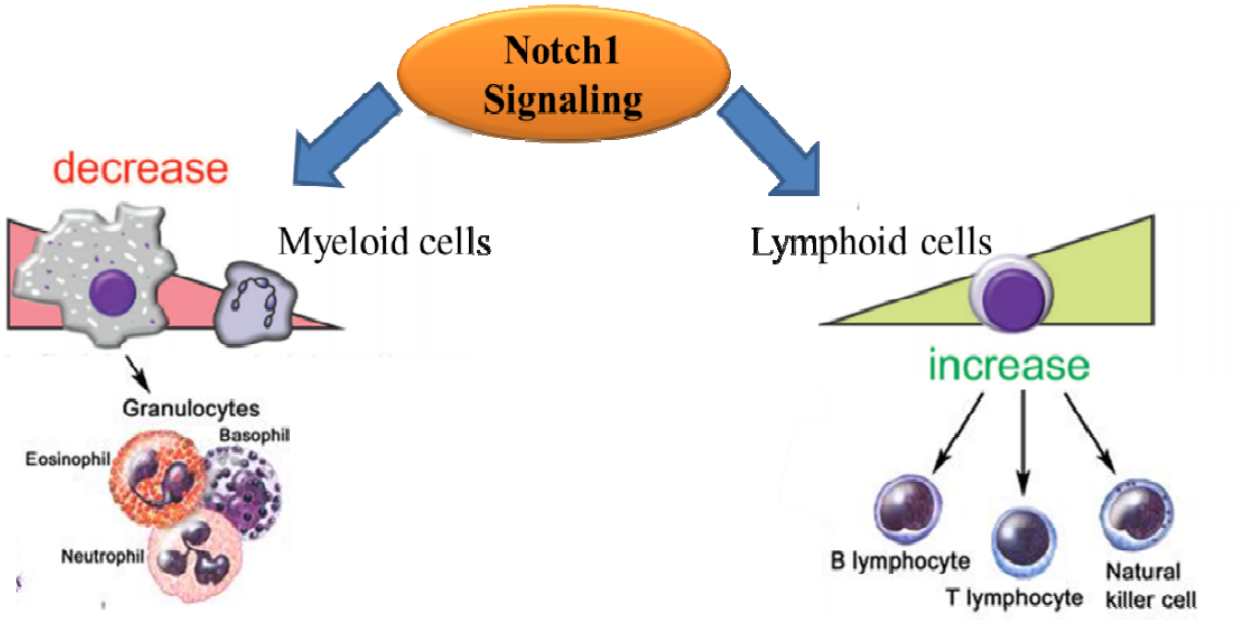


Fig.1.3 *Notch1 signaling influence generation of both myeloid and lymphoid cells (adapted by Stradoni from Stier et al. 2002)*

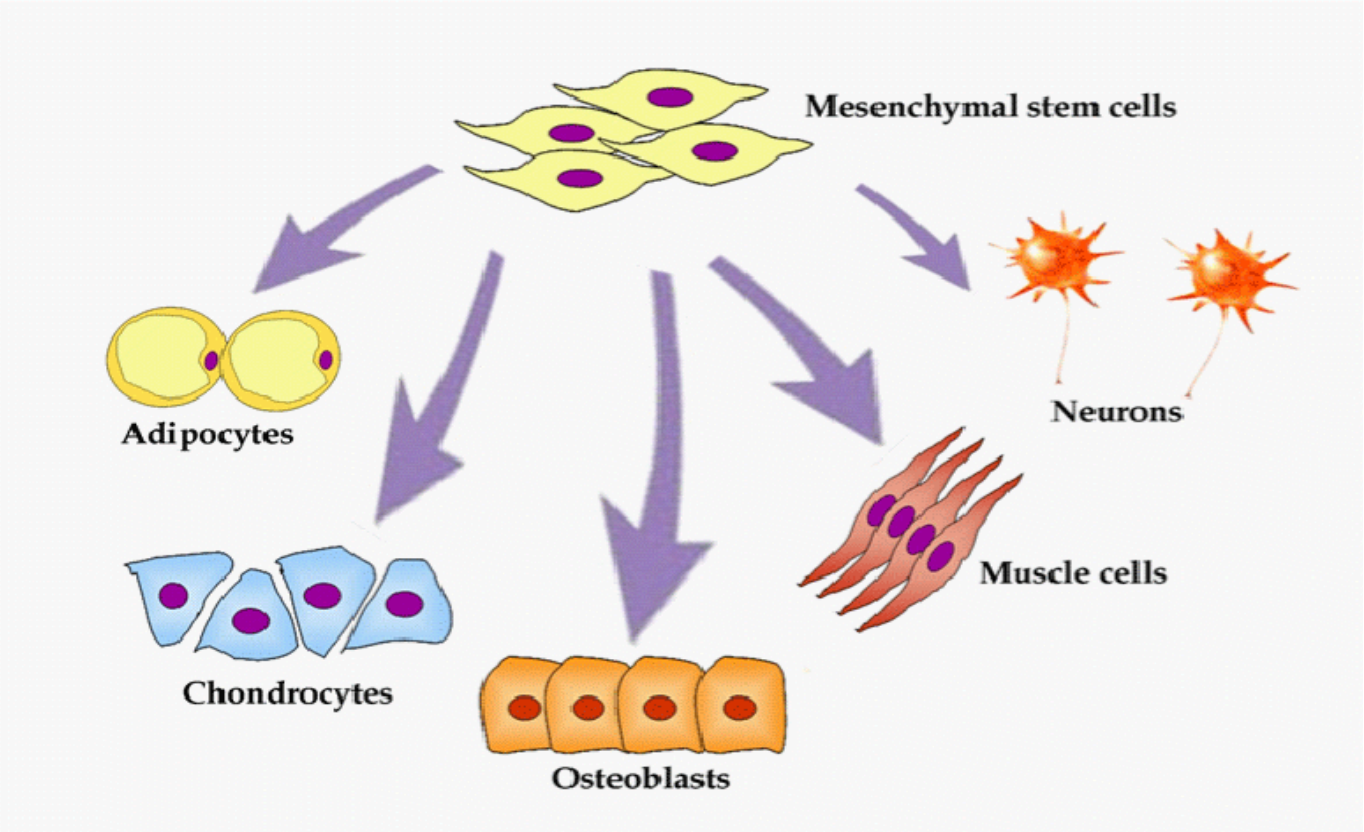


Fig.1.4 *Differentiation potential of mesenchymal stromal cells. MSCs can differentiate in a number of human tissues including osteogenic, chondrogenic, and adipogenic lineages. Recently, the presence of human MSC-like cells was shown in adult skeletal muscle (adapted by Stradoni from* Mergalli et al. 2011*)*

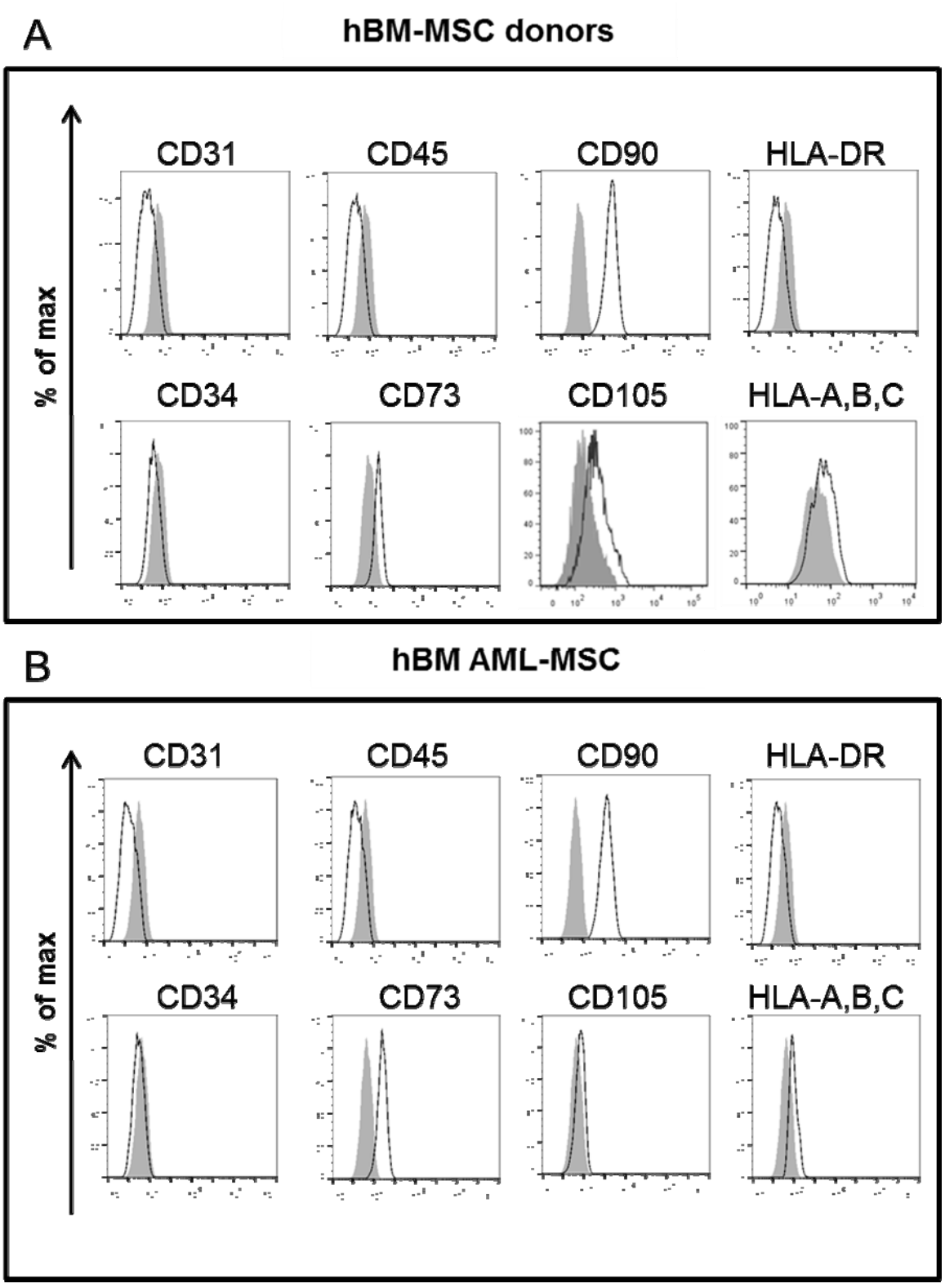


Fig.2.1. MSCs characterization according to ISCT phenotype. *Immunophenotype of hBM-MSC by flow cytometry. Isotype-matched mAb controls (grey)*

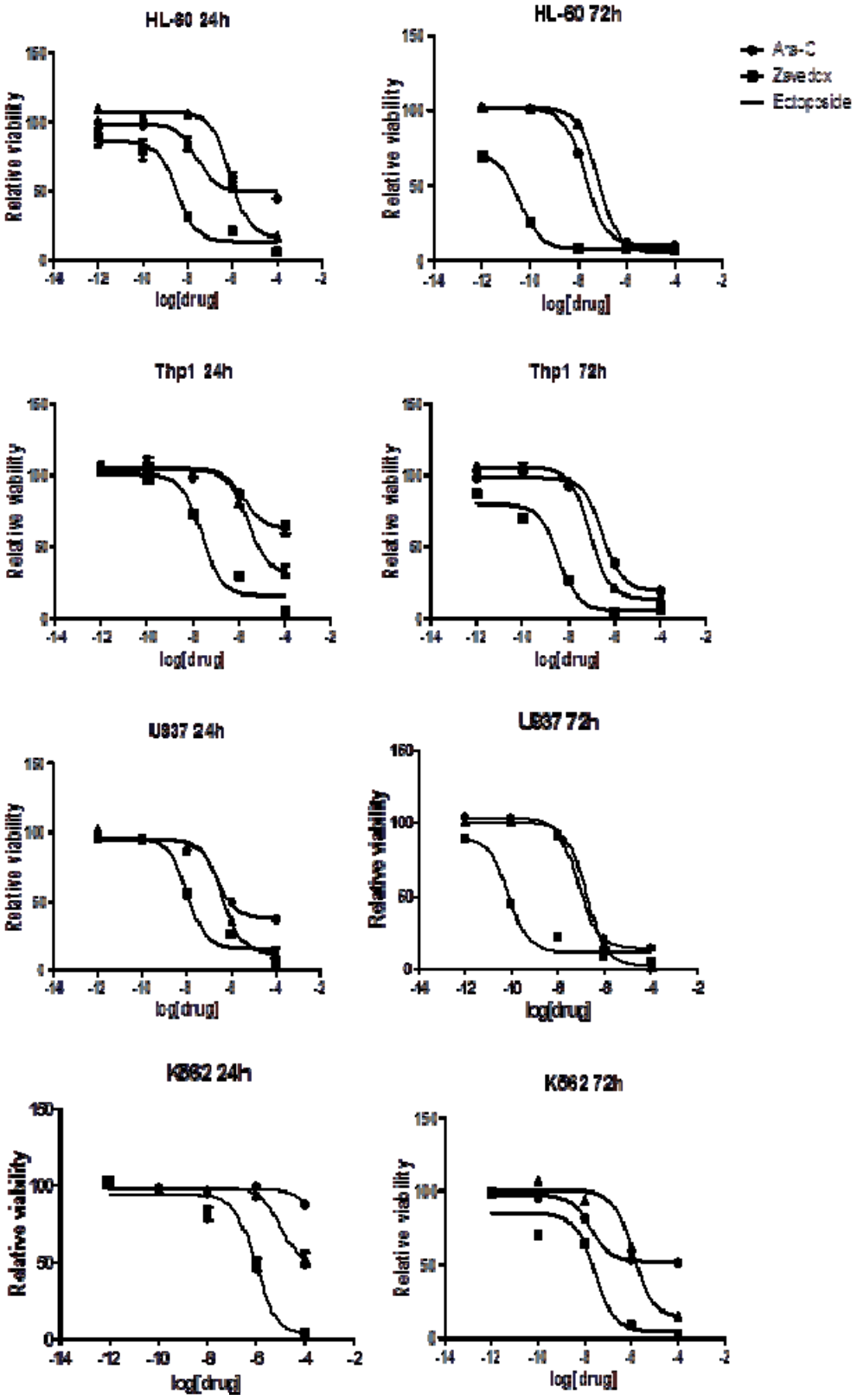


Fig 2.2 *Sensitivity of AML cell lines to drug treatment.*

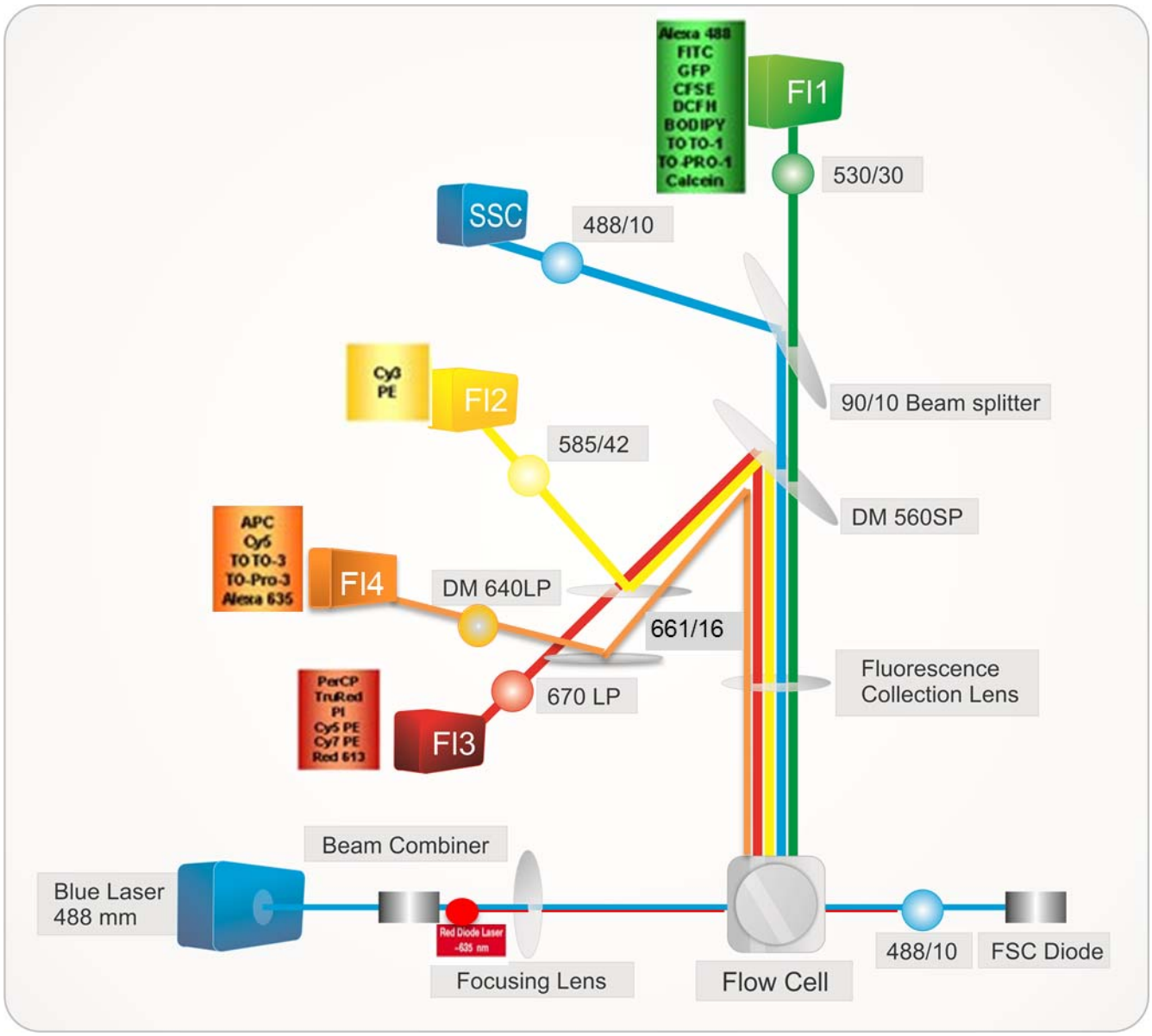


Fig. 2.3 *Schematic representation of a flow cytometer. The cell suspension pass through a needle using a system of pressurization. A laser intercept cells individually. The modifications that occur in this light beam due to cell characteristics are detected and measured by sensors (detectors) disposed adequately. Dispersed light is collected by an optical system which allows to identify cells by their size and complexity (FSC and SSC detectors). Fluorescence emitted by fluorochromes are also collected. To select these luminous signals emitted by fluorochromes, optical filters are used to block certain incident light wavelengths and let only the desired one pass. Each fluorescence emission is identified by different detectors (FL1, FL2, FL3, FL4), which convert luminous signals in electrical pulses and amplify this signal. (Baptista et al. 2011 adapted by Stradoni)*

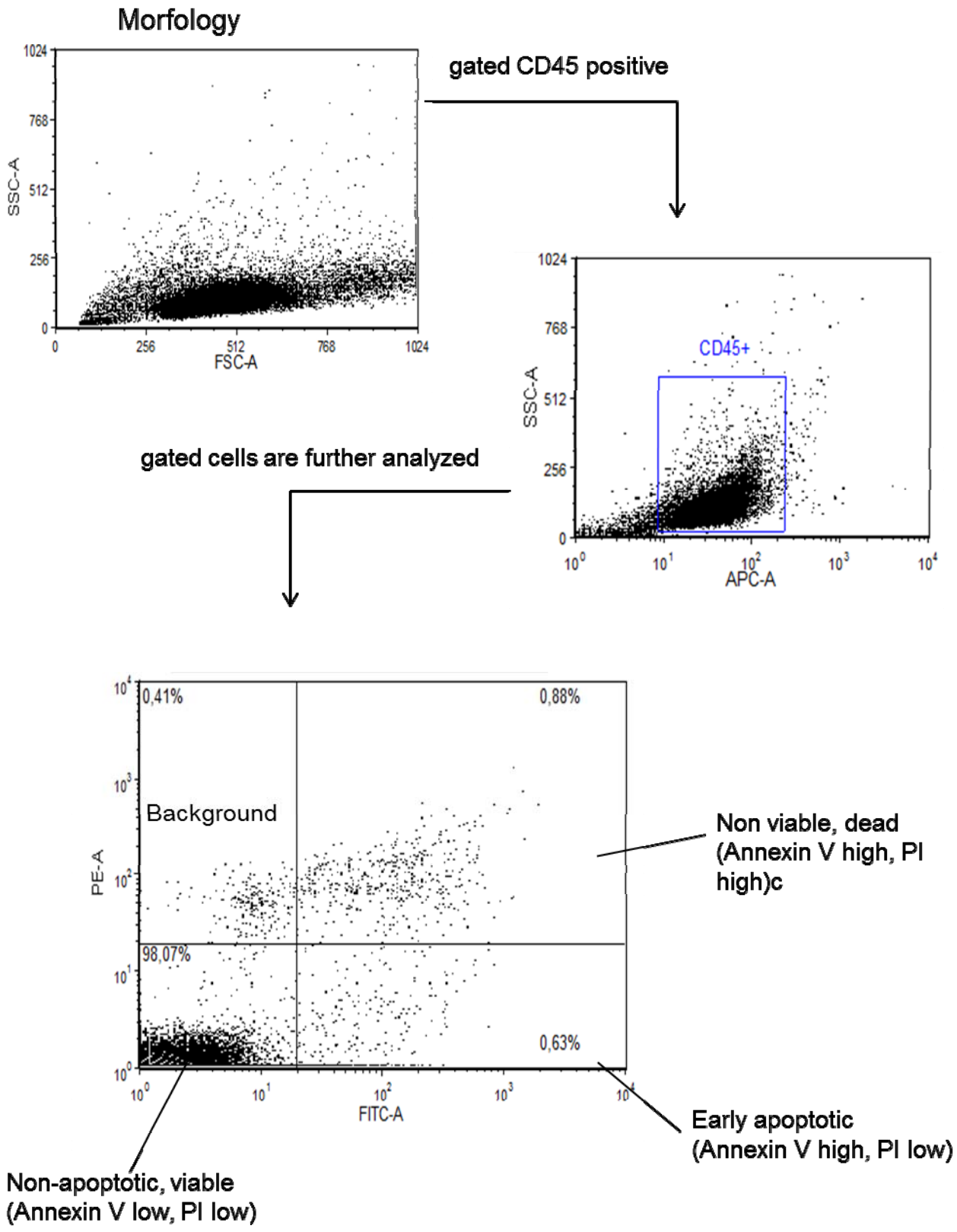


Fig 2.4 Analysis of apoptotic cells. *CD45+ population was gated and analyzed to define four cell subsets: 1) (AnxV low)+(PI low)=viable cells, 2) (AnxV high)+(PI low)=early apoptotic cells, 3) (AnxV high)+(PI mid)=late apoptotic cells, 4) (AnxV high)+(PI high)=non-viable cells, dead.*

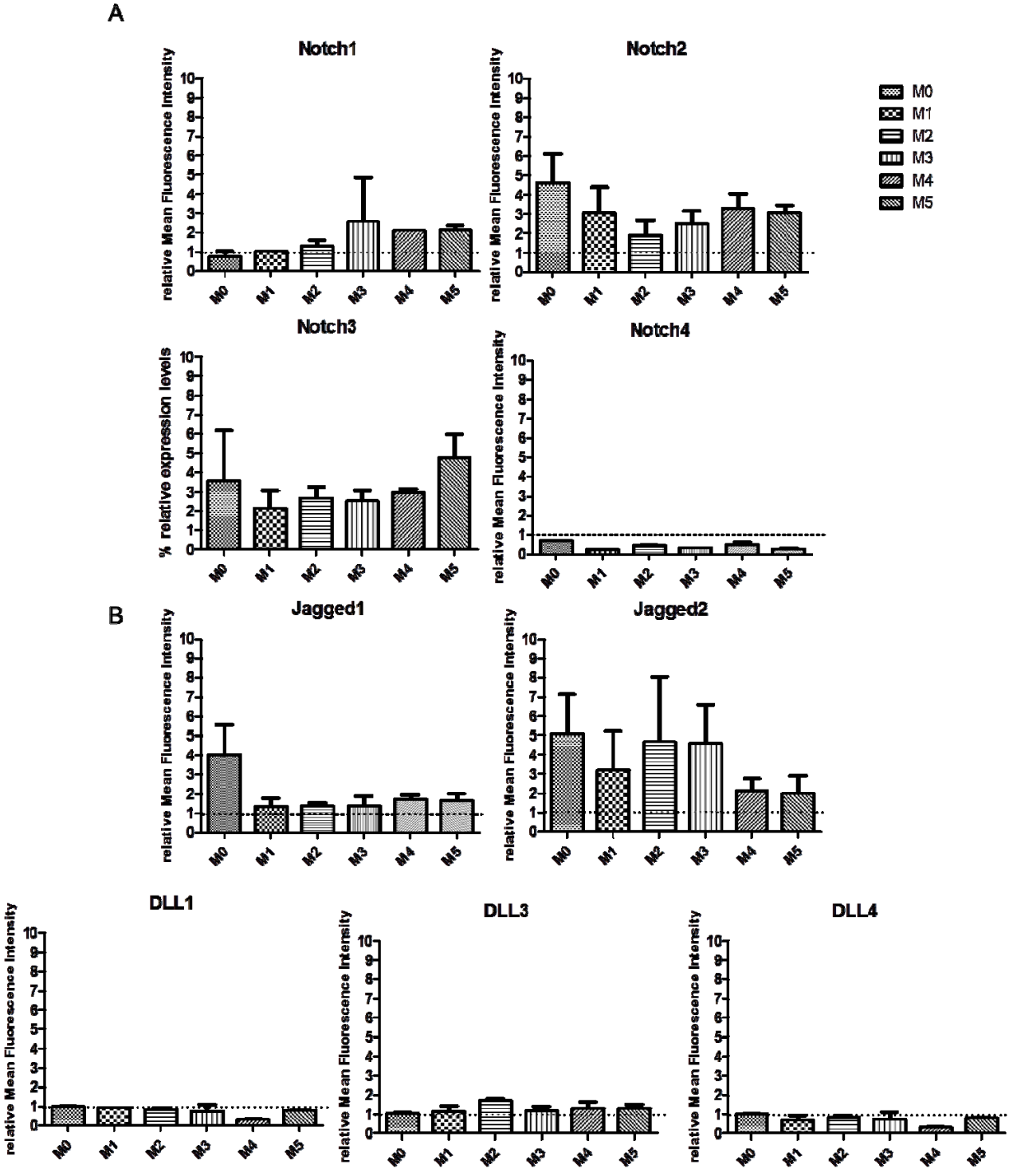


Fig. 3.1 Comparison of the expression of Notch receptors and ligands in different AML cell lines and primary AML cells. *FACS analysis for expression of Notch receptors (A) and Ligands (B) in a set of 3 AML samples according to FAB classification. Each value represents the median rMFI of three independent experiments.*

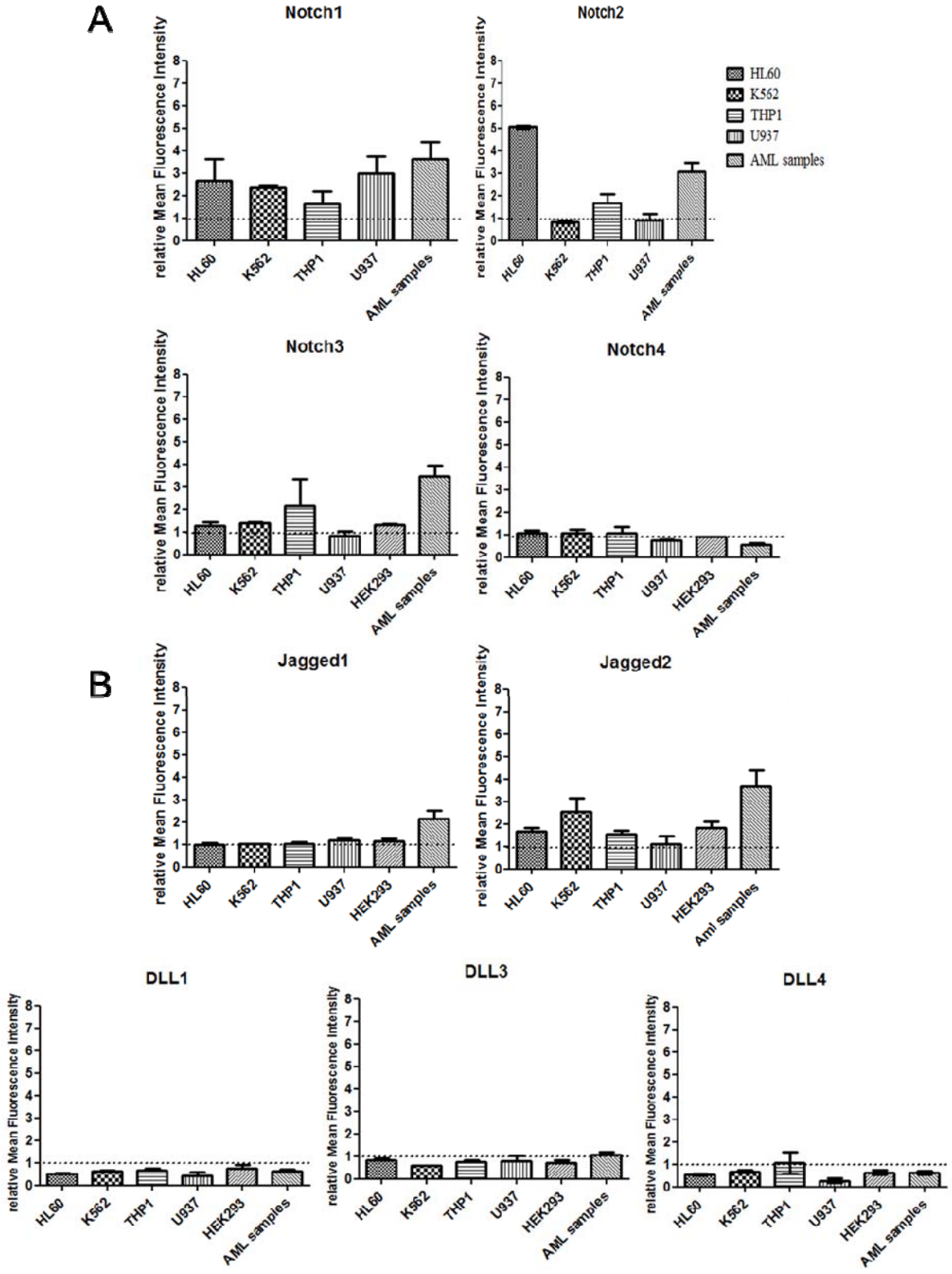


Fig. 3.2 Comparison of the expression of Notch receptors and ligands in AML cell lines and primary AML cells. *FACS analysis expression Notch receptors (A) and Ligands (B) were performed in a set of 4 AML cells lines and 10 primary AML samples. Each data represents the median rMFI of five independents experiments.*

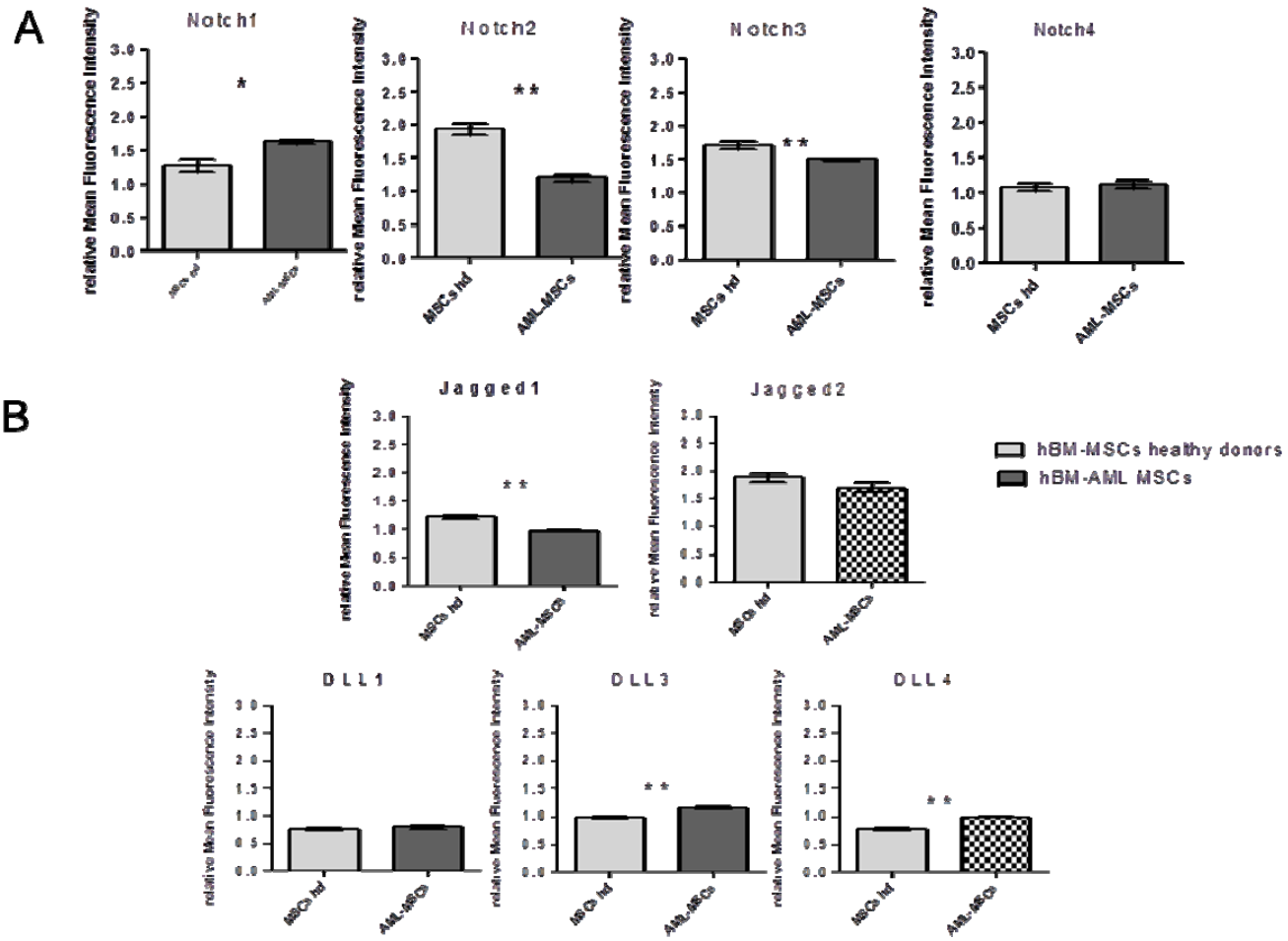


Fig. 3.3 Comparison of the expression of Notch receptors and ligands in hBM-MSCs from healthy donors and hBM-AML-MSCs. *FACS analysis for expression Notch receptors (A) and Ligands (B) were performed in a set of 8 hBM-MSC and 4 hBM-AMLMSC samples. Each data represents the median rMFI of five independents experiments. Statistical analysis was performed with Wilcoxon matched pairs test (** p<0.01).*

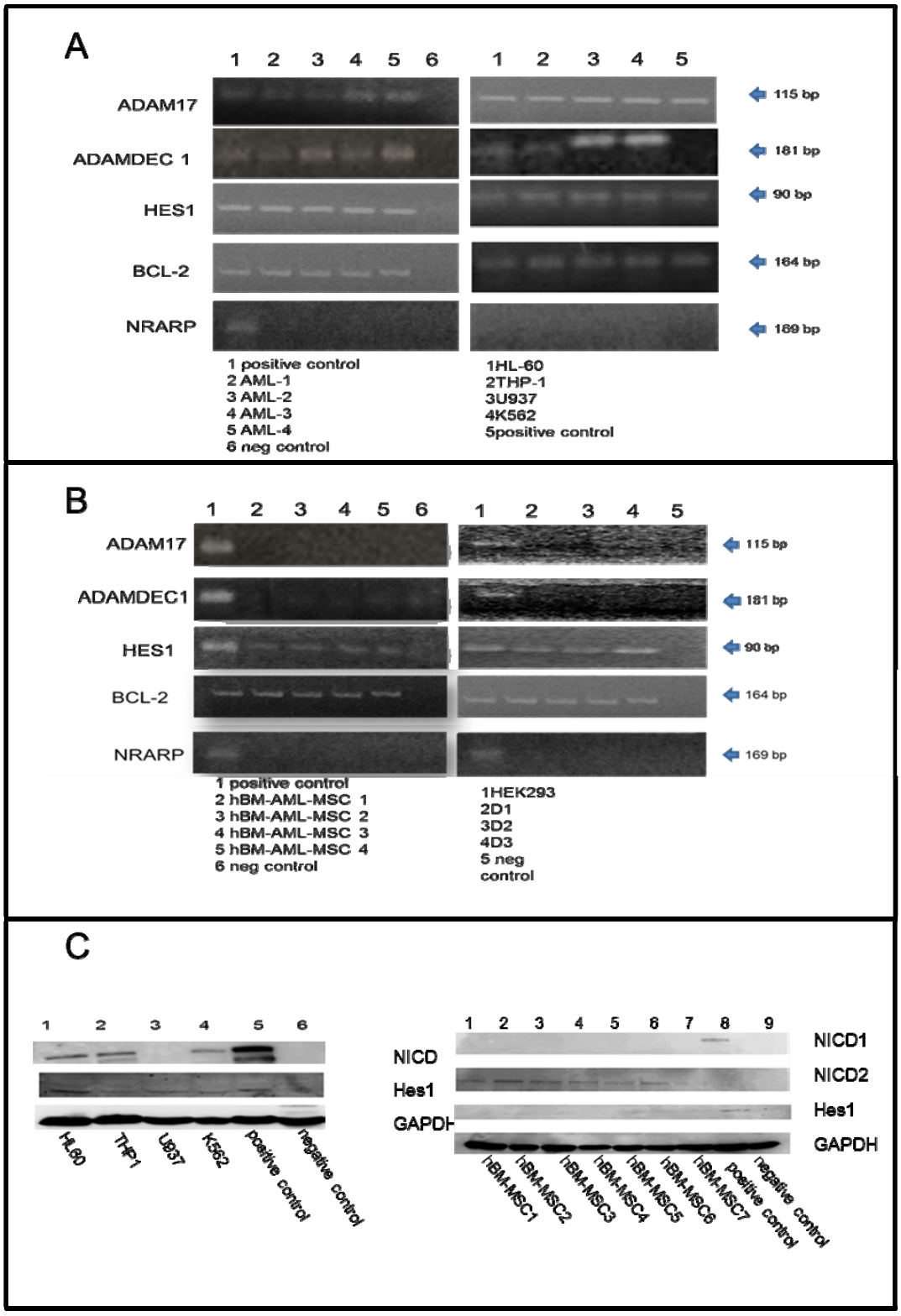


Fig. 3.4 Expression of Notch receptors targets. *(A) Immunoblot and mRNA expression by means of Qualitative PCR of Notch receptor target genes of AML cell lines probed for Notch receptor targets and B-actin. Positive controls HEK-293 (line 5) and Jurkat (line 6). (B) mRNA expression of Notch receptor target genes by Qualitative PCR in AML cells from patients. (C) Immunoblot and mRNA expression in hBM-MSCs for Notch Target genes of Notch receptors and B-actin. The analyses reported here were carried out in triplicate and with independent experiments. (D) hBM-AML-MSCs mRNA expression of Notch receptors target genes by qualitative PCR. The analyses reported here were carried out in triplicate and with independent experiments.*

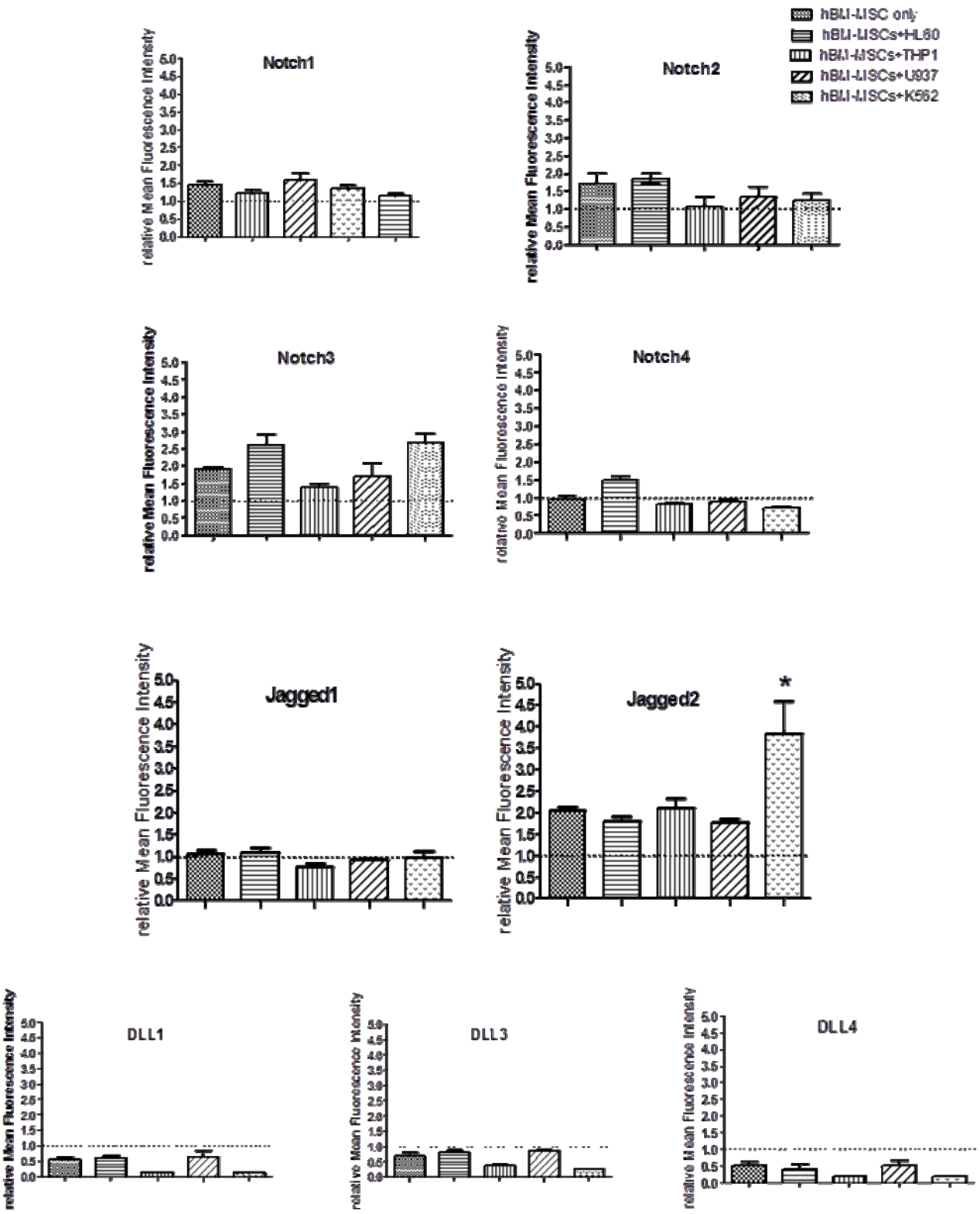


Fig.3.5 Expression of Notch receptors and ligands in hBM-MSCs cultured alone or in co-culture with AML cell lines. *Expression of Notch receptors and ligands in hBM-MSCs cultured alone or in co-culture with AML cell lines. Human mesenchymal cells cultured alone and cocultured with AML cell lines (1/10 ratio) were examined after 72h of culture by flow cytometry. Histograms reporting the median rMFI value of 5 independent experiments. * p<0.05, Wilcoxon matched pairs test. Notch receptors were not significant upregulated in all AML cell lines; but in co-cultures of hBM-MSCs with HL60 and K562 cell lines showed a slight increase in Notch3 expression. Five Notch ligands, Jagged1/2 and Dll1, 3 and 4, were examined. These ligands not showed increased except Jagged2 in co-cultures with k562.*

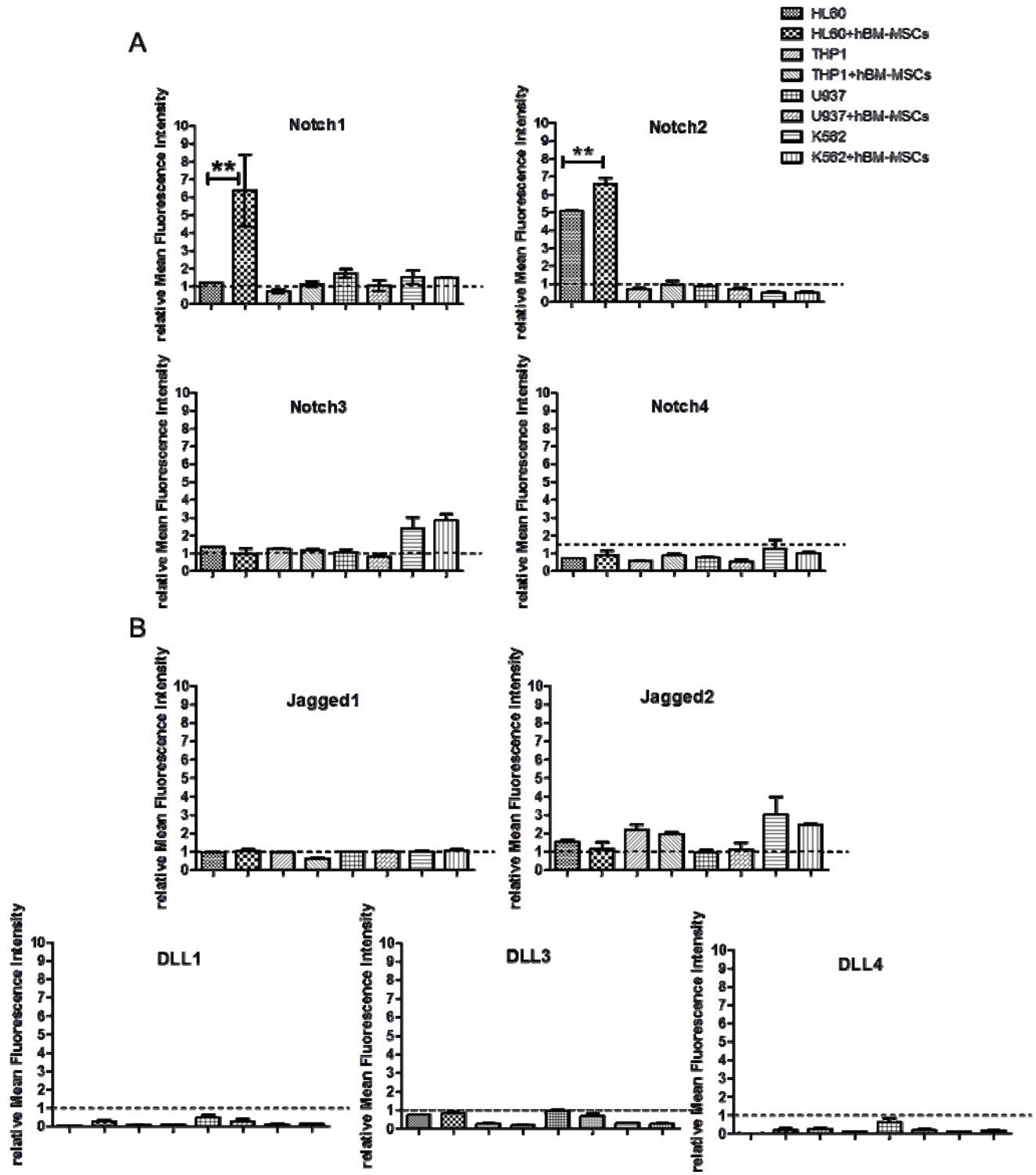


Fig.3.6 Co-cultures between AML cell lines and hBM-MSCs showed increased expression of Notch receptors and ligands. *AML cell lines cultured alone and cocultured with hBM-MSCs (10/1 ratio) were analysed by flow cytometry for Notch receptors (A) and ligands (B) after 72h of culture. Histograms reporting the median rMFI value of 5 independent experiments. **p<0.01, were considered statistically significant (ANOVA test).*

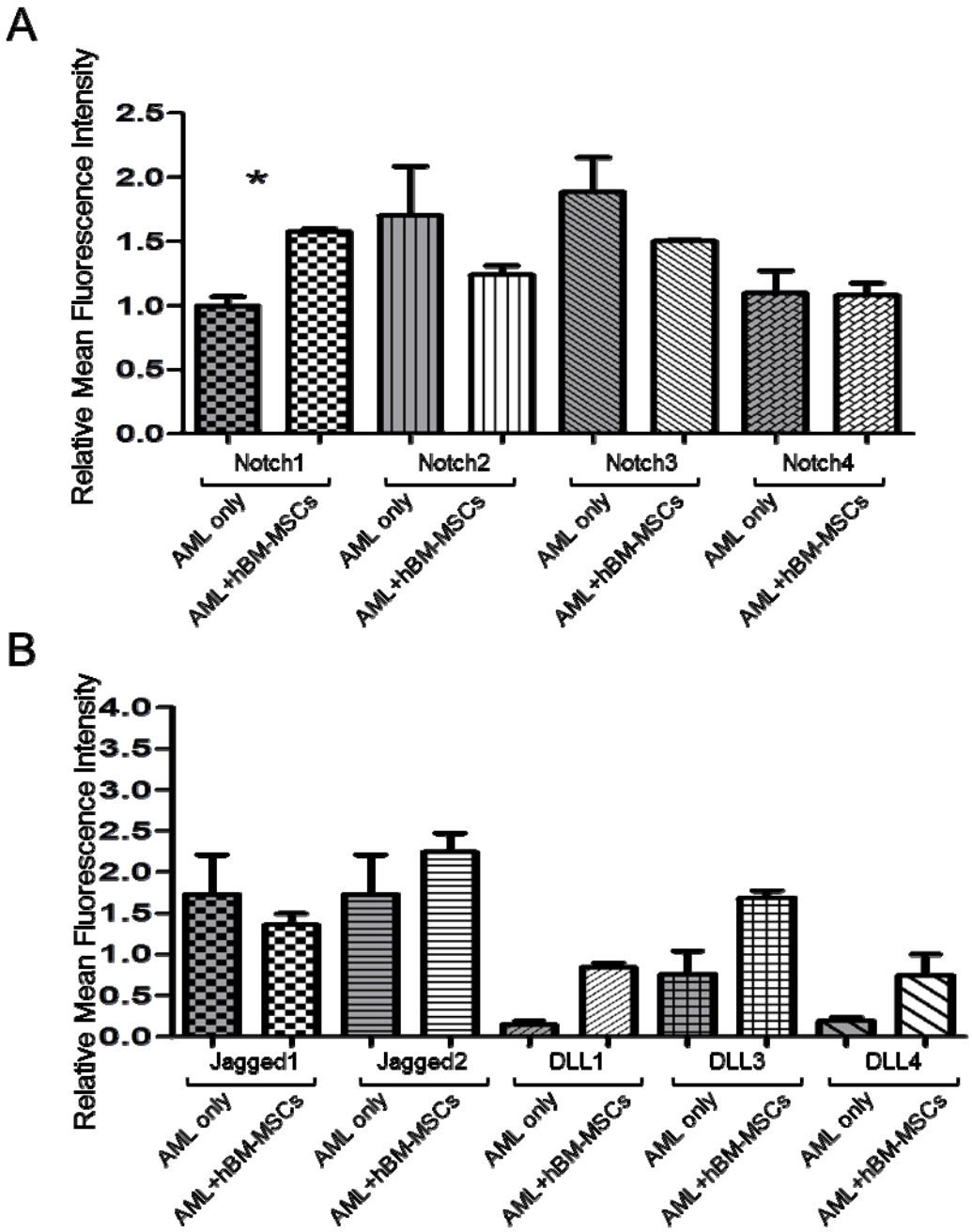


Fig. 3.7 Co-cultures between primary AML cells and hBM-MSCs showed increased expression of Notch receptors and ligands. *Primary AML cells cultured alone and co-cultured with hBM-MSCs (10/1 ratio) were analysed by flow cytometry for Notch receptors (A) and ligands (B) after 72h of culture. Histograms reporting the median rMFI value of 5 independent experiments. *p<0.01, were considered statistically significant (ANOVA test).*

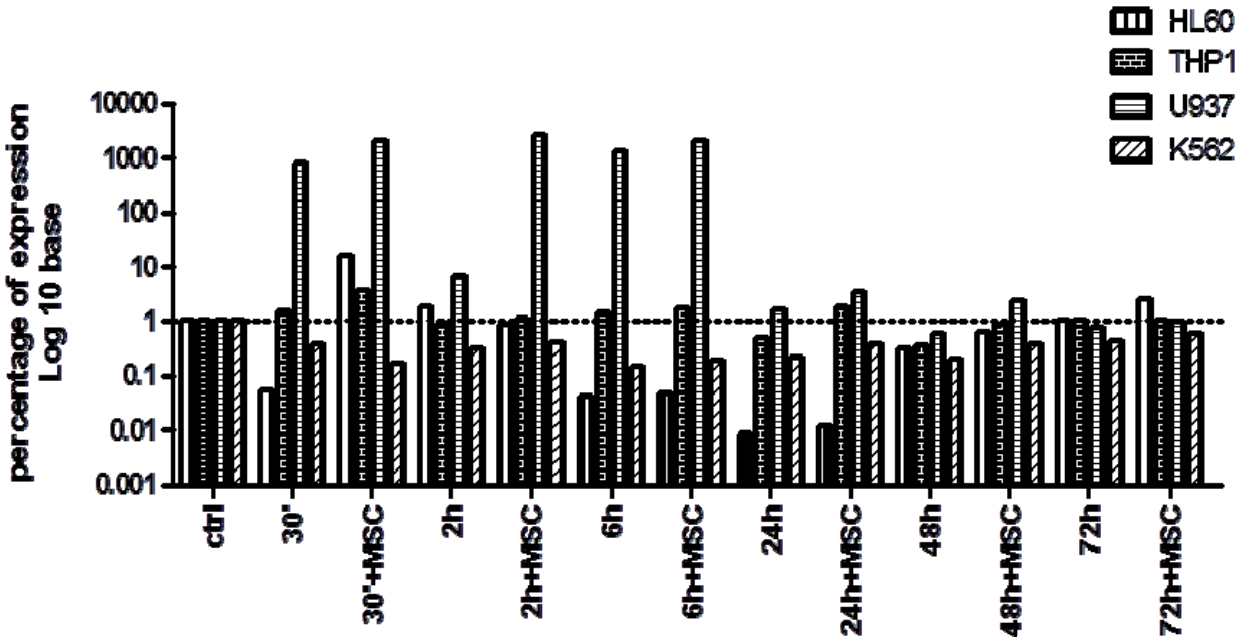


Fig. 3.8 Quantitative real-time PCR. *Total mRNA extracted from AML cell lines was amplified with HES1 primers by sybr green relative quantification assay.*

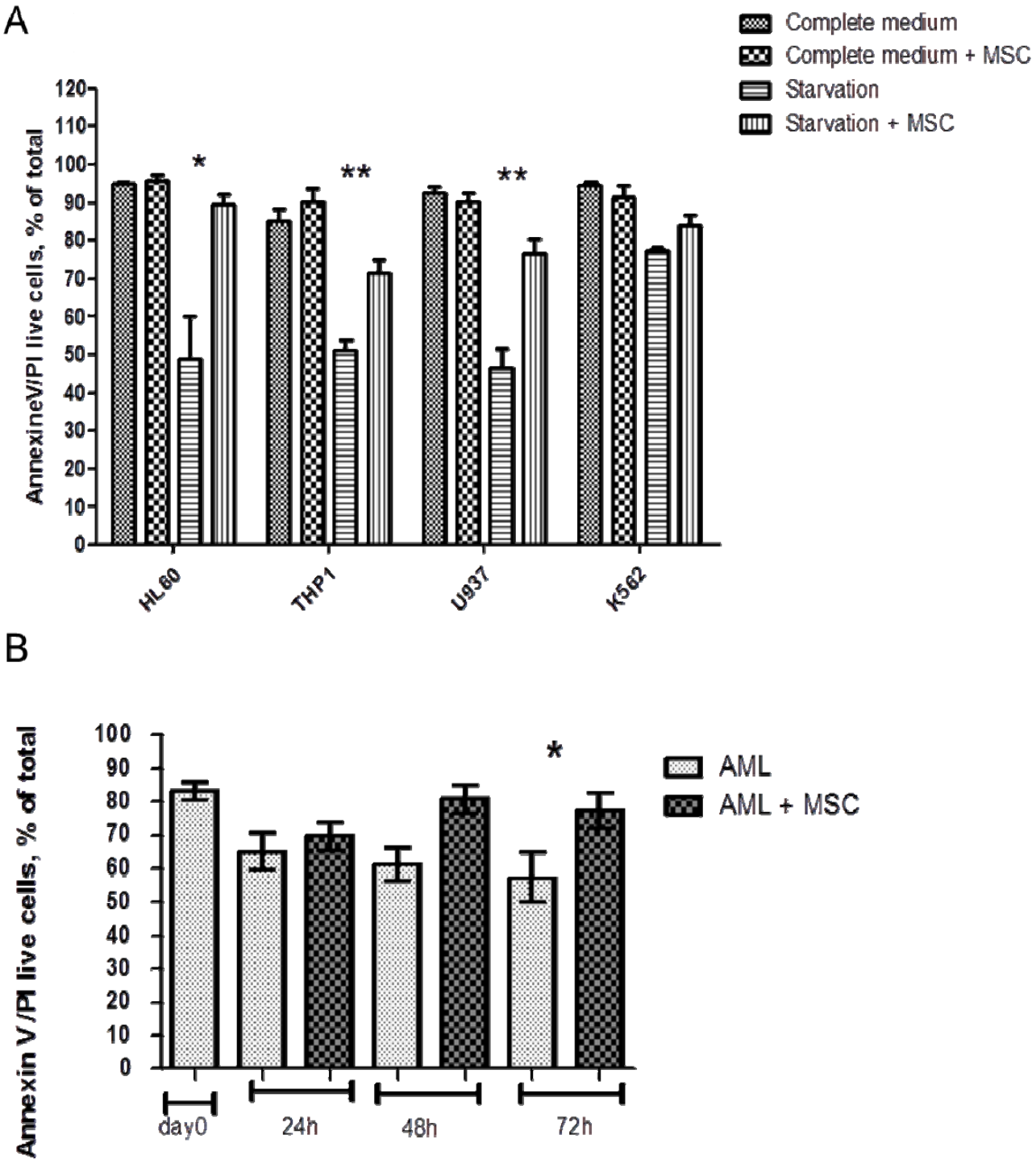


Fig. 3.9 hBM-MSCs affect AML cells viability when co-cultured in starvation condition. *Flow cytometric examination of cell viability with Annexin V/PI assay. Diagrams show viability of (A) AML cell lines after 72 h of co-culture and (B) AML patient samples at different time points of co-culture. Histograms show median of living cells percentage from 5 independent experiments. ANOVA test was used to compare the different groups; *p<0.05, **p<0.01 were considered statistically significant.*

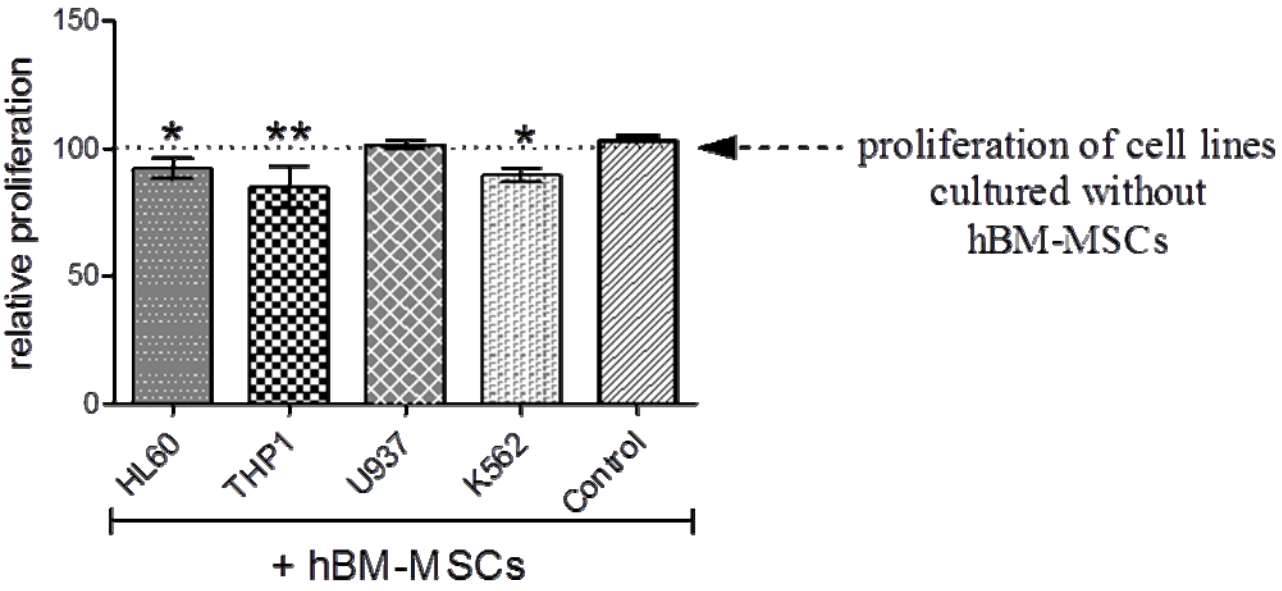


Fig. 3.10 Proliferation is inhibited under conditions of co-culture with HBM-MSCs. *AML cells were labeled with Carboxyfluorescein Succinimidyl Ester (CFSE) and cultured for 72h. Quantitation of AML cells proliferative capacity was performed by flow cytometry with added CD45 and TO-PRO-3 Iodide to discriminate dead cells. The data collected were normalized to isotype controls in each cell line (relative proliferation mean). Data were obtained from 5 independent experiments. ANOVA test was used to compare the different groups; *p<0.05 was considered statistically significant.*

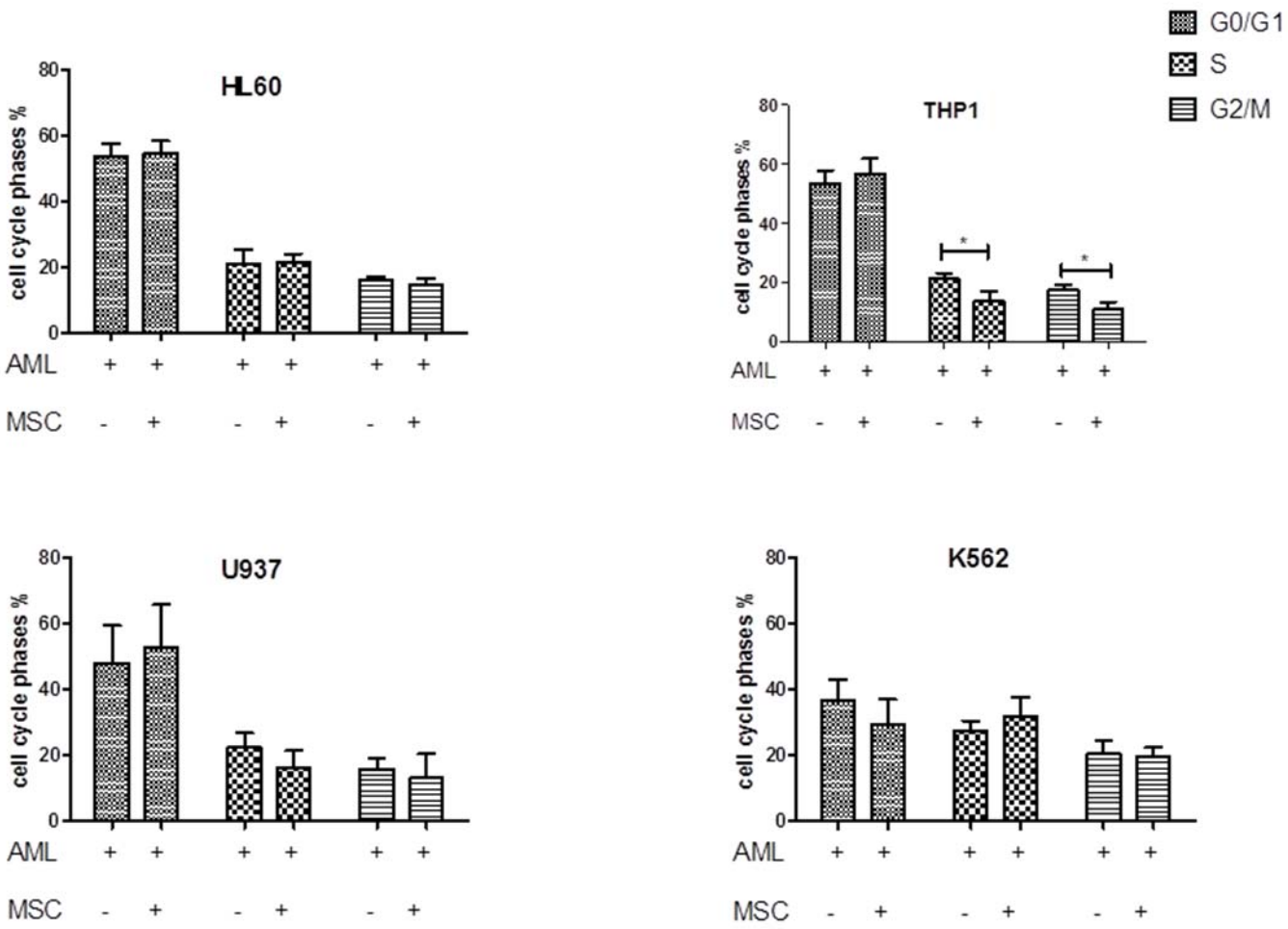


Fig. 3.11 The co-culture with stromal cells was corresponded with decrease of cellular division. *AML cells were stained with propidium iodide and subjected to cell cycle analysis by flow cytometry. Data were obtained from 5 independent experiments. ANOVA test was used to compare the different groups; *p<0.05 was considered statistically significant.*

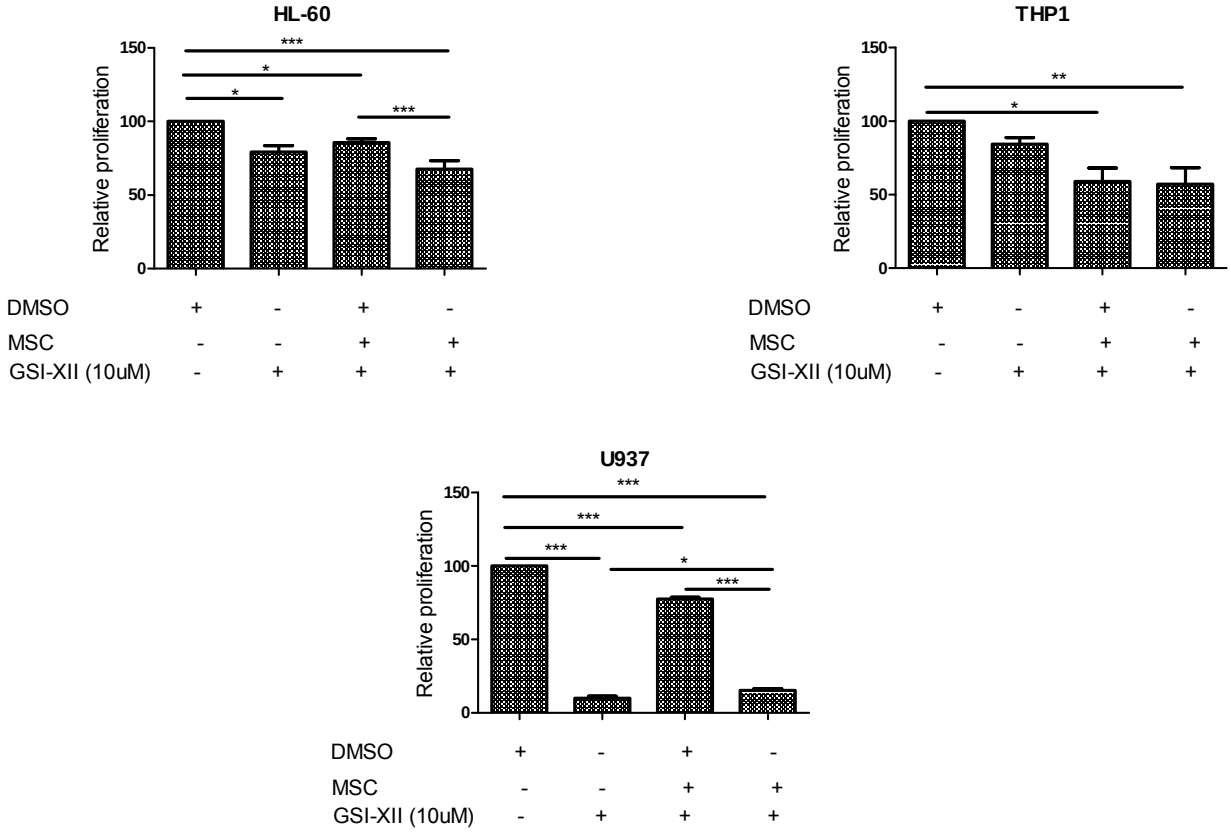


Fig. 3.12: Contribution of Notch to MSC-mediated proliferation of AML cell lines. *AnxV/PI (A) and CFSE (B) assays were performed by flow cytometry. Statistical analysis was carried out by using one-way ANOVA, Holm-Sidak test (*p<0.05).*

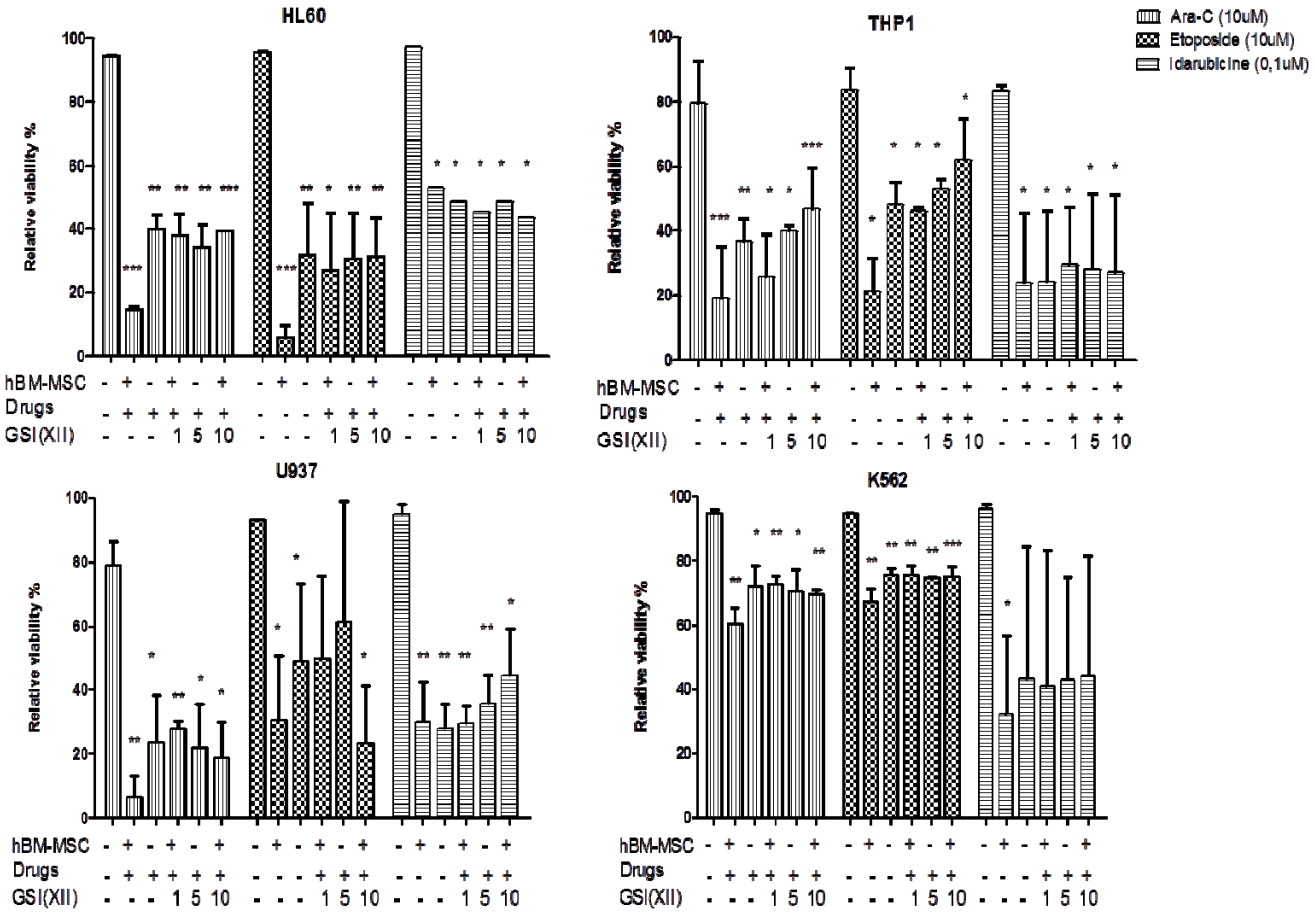


Fig. 3.13: Combined use of chemotherapeutic drugs and Notch inhibitor on AML cell lines. *Statistical analysis of AnxV/PI assay was carried out by using one-way ANOVA, Holm-Sidak test (**p<0.01).*

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