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The role of NOTCH pathway in multiple myeloma progression: control of IL-6 expression in malignant plasma cells and bone marrow niche

PhD thesis:

Serena GALLETTI

R10586

Tutor: Professor Antonino NERI

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Summary

ABSTRACT

Multiple myeloma (MM) is an incurable hematological tumor characterized by the accumulation of malignant plasma cells (PCs) within the bone marrow (BM). MM cells create a very strictly interaction with the surrounding BM niche that directly supports tumor growth through adhesion molecules and soluble factors, the most important of which is interleukin-6 (IL-6). IL-6 is probably the most important factor for MM cells growth and survival *in vivo* and it mainly produced by non-neoplastic cells of the BM microenvironment, and at less extent by malignant plasma cells.

Recent studies suggest the involvement of NOTCH pathway in the pathogenesis of multiple myeloma. The NOTCH pathway is composed by four isoforms of receptors (NOTCH1-4) and two family of ligands, the Serrate-like ligands (JAGGED1 and 2) and the Delta-like ligands (DLL1/3/4). NOTCH pathway plays an important role in several cellular processes such as proliferation, survival, differentiation and stemness in various tissues and tumors. Regarding MM, the up-regulation of NOTCH signaling is due to the over-expression of both receptors (NOTCH1 and NOTCH2) and ligands (JAGGED1 and JAGGED2): these alterations promote tumor growth, pharmacological resistance, and bone disease. Furthermore, NOTCH pathway plays an important role in the pathological interaction between malignant PCs and the BM niche.

This study indicates that there is a correlation between levels of NOTCH signaling members (JAGGED1, NOTCH2, HES5 and HES6) and the malignant progression of the disease. Indeed, NOTCH pathway is involved in multiple myeloma progression since it is able to increase the release of IL-6 produced by BM stromal cells (BMSCs). Our *in vitro* studies show that NOTCH pathway induces *IL-6* gene expression in MM cells as well as in the surrounding BMSCs through the over-expression of JAGGED ligands. The activation of NOTCH pathway in the BMSCs renders these cells able to support tumor cells growth through the production of IL-6. These data are confirmed also by correlation analysis on gene expression profiles of MM patients and immunohistochemical studies. The ability of NOTCH pathway to positively regulate IL-6 in the non-neoplastic cells of BM microenvironment renders this pathway an important mediator of tumor-directed reprogramming of bone marrow niche. These results support the rationale for a NOTCH-direct therapeutic approach that can specifically target JAGGED ligands in order to reduce side effects associated with common NOTCH signaling inhibitor.

INTRODUCTION

1. Multiple Myeloma

Multiple Myeloma (MM) is a hematologic cancer characterized by malignant plasma cells (PCs) accumulation in the bone marrow (BM) [1]. It represents about 13% of all hematologic cancers and 1% of all neoplastic disease. The disease has an annual incidence of about 5.6 cases for 100000 individuals in western countries. The median age at diagnosis is about 70 years [2].

Malignant plasma cells are able to produce high amount of monoclonal immunoglobulins, mainly IgG or IgA, which represent important laboratory markers of the disease. MM is characterized by bone destruction, renal failure, anemia and hypercalcemia [3]. In MM the interaction between malignant PCs and the surrounding microenvironment plays a key role. In fact, MM cells and BM stromal cells (BMSCs) interact through soluble factors (such as cytokines and growth factors) and adhesion molecules: this interaction directly improves survival and growth of tumor cells and favors the osteolytic damage associated with MM [3-5].

Despite recent remarkable improvements in the treatment of the disease and the advances in investigational platforms, MM remains incurable with a median overall survival of 7-8 years [1].

1.1 Multiple Myeloma pathogenesis and progression

Malignant plasma cells are post-germinal center B-cells. Normally, B-cells in germinal center are characterized by three different Ig heavy chain light (IgH) gene rearrangements [6] (fig.1.1):

- VDJ loci recombination which occurred in the pre-B cell precursors in the bone marrow (necessary for the formation of a specific *B cell receptor* (BCR))
- constant region recombination (necessary for class switch);
- hypersomatic mutations of variable regions for antigen affinity.

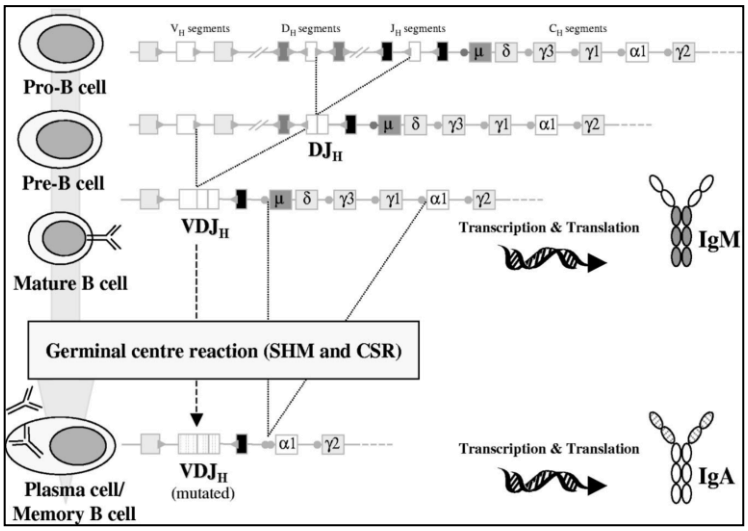

Figura 1.1 Scheme of IgH locus rearrangement during B-cells maturation.

The last two mechanisms occur in the germinative center of lymphoid follicles and are necessary for the selection of B-clones with high specificity for variable antigens. B-lymphocytes with functional superficial IgM (*naïve B-lymphocyte)* are able to migrate into secondary lymphoid organs, where they could:

- differentiate out of the germinal center to become short-lived PC with 3 day of life *(short-lived post-germinal center PC)*, expressing IgM without incurring somatic hypermutation;
- enter into the germinal center where somatic hypermutation and antigenic selection occur. Here, cells with poor antigenic affinity undergo apoptosis, while the remaining return to circle as memory B-cells or undergo switch-recombination to change immunoglobulin class. The latter are stimulated by the microenvironment in the BM to differentiate into long-lived PCs *(long-lived post-germinal center PC)*, which could survive for 30 days or several years [7] (Fig.1.2).

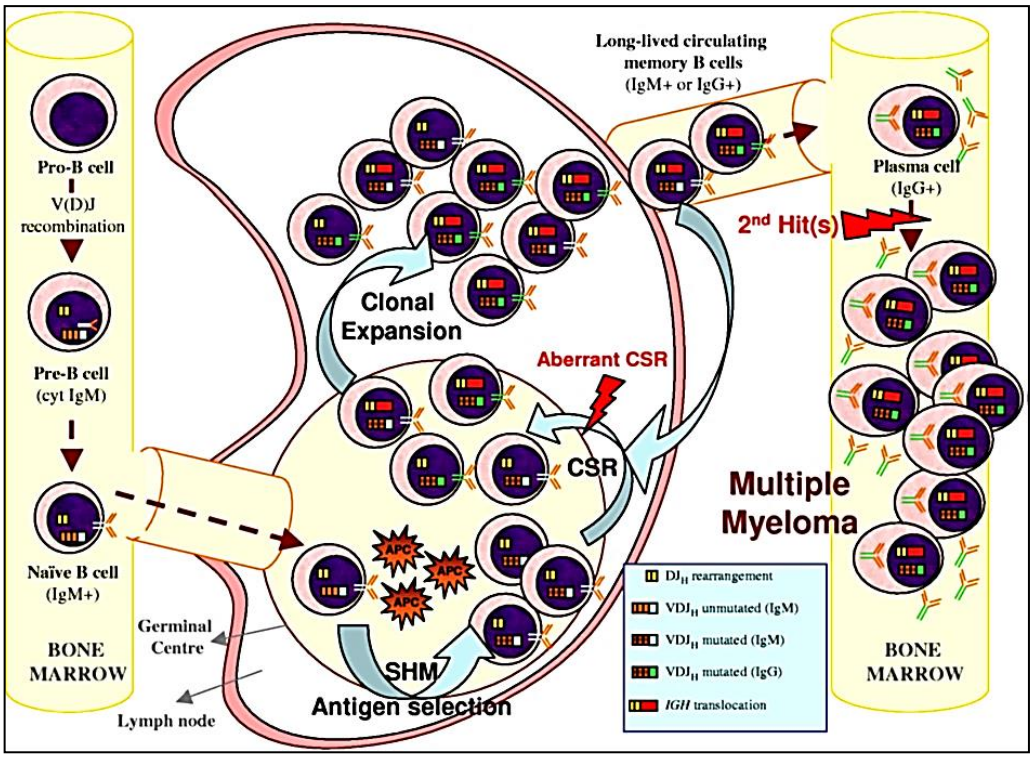

Figure 1.2 Multiple myeloma pathogenesis. *Naïve* B-cell in germinal center (GC) undergoes somatic hypermutation and antigen selection, followed by regions recombination for isotype switching. During this physiological process, double-strand breaks can be solved with altered junctions resulting in pathological translocation involving the 14q32 region of heavy chain of Ig genes. If this aberrant recombination prevails over the normal, there are different subpopulations of B-lymphocytes expressing IgM, IgG or IgA, which will have proliferative and survival advantages due to translocation. This could make them long-lived memory B cells without being fully malignant. When one of these subpopulations leaves the GC to become PC (expressing IgG or IgA) with homing in the bone marrow, it can occur secondary genetic modifications that will make it totally malignant.

Multiple myeloma is a highly biologically and clinically heterogeneous neoplasia. It is characterized by four distinguishable clinical phases that may not be discernible in each patient.

It may be preceded by a monoclonal gammopathy of undetermined significance (MGUS), an asymptomatic, indolent and premalignant phase characterized by a small clonal population (<10%) of PCs within the BM. MGUS may progress to MM at a rate of 1% per year. Intramedullary MM may present as an asymptomatic, smoldering, multiple myeloma (SMM), or associated with organ dysfunction including hypercalcemia, renal failure, anemia, and bone disease. SMM has an average risk of progression to MM of 10% per year [8, 9]. The final stage of MM is represented by plasma cell leukemia (PCL), defined as at least 20% of PCs or an absolute PCs count of more than 2×10^9/L in the peripheral blood. PCL is a rapidly progressive and fatal disease, which may occur as secondary (sPCL) in the context of a

preexisting refractory MM, or primary (pPCL) if presenting *de novo* in leukemic phase [10, 11] (fig.1.3).

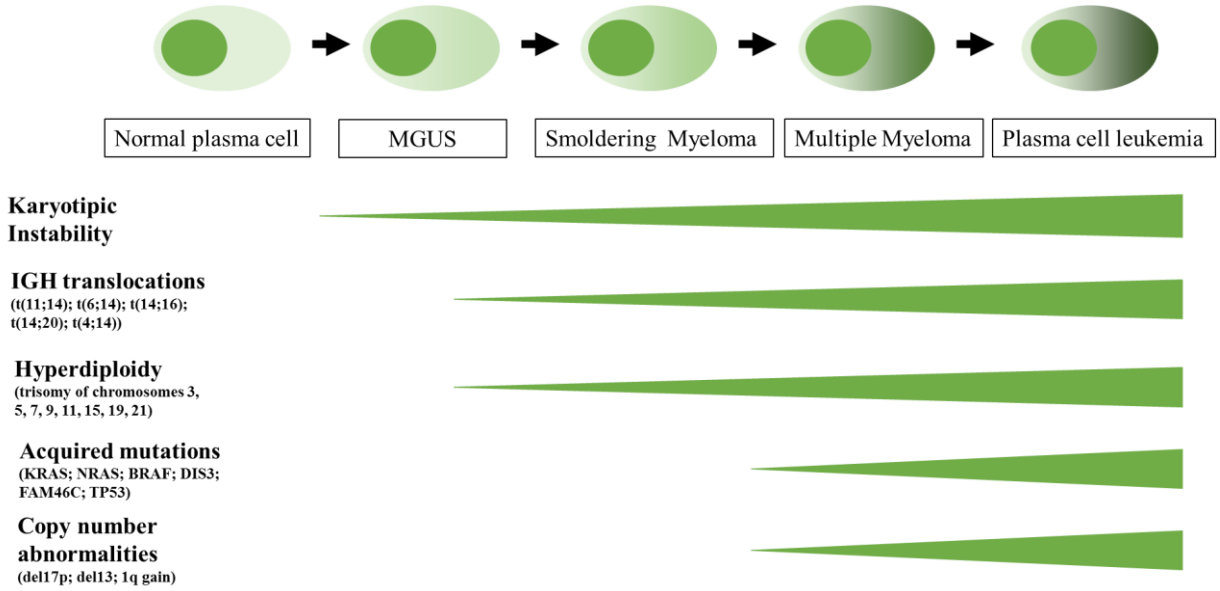

Figure 1.3. Schematic representation of MM progression and oncogenic events along the four clinical phases: MGUS, SMM, MM, PCL.

1.2 Genetic of Multiple Myeloma

Multiple myeloma is characterized by a profound genomic instability that involves both ploidy and structural rearrangements. The majority of MM patients are aneuploid and they could be divided into two different categories: hyperdiploid (HD) (about 50% of all patients) and non-hyperdiploid (comprising hypodiploid, pseudo-diploid or pseudo-tetraploid) [12]. HD patients are characterized by trisomies of odd chromosomes (including 3, 5, 7, 9, 11, 15, 19, and 21) and have a better prognosis [13]. The remaining non-HD tumors are characterized by the presence, in about 85% of cases, of translocation involving the 14q32 region of IgH genes locus and a prevalence of chr13 deletion and chr1 alterations. Translocations involving the 14q32 region play an important role in the pathogenesis of lymphoproliferative disorders. Chromosome translocations involving the IgH locus are the most important mechanism for oncogenic activation in mature B-cell neoplasia and in fact about 50% of MGUS and SMM, 55-70% of MM and 80% of PCL patients and virtually all human multiple myeloma cell lines (HMCLs) carried IgH translocations. Translocations involving the IgH locus cause the constitutive activation of important oncogenes such as CCND1 (11q13), CCND3 (6p21), MAF (16q23), MAFB (20q11), or FGFR3/ MMSET (4p16.3) [14, 15] (fig. 1.4).

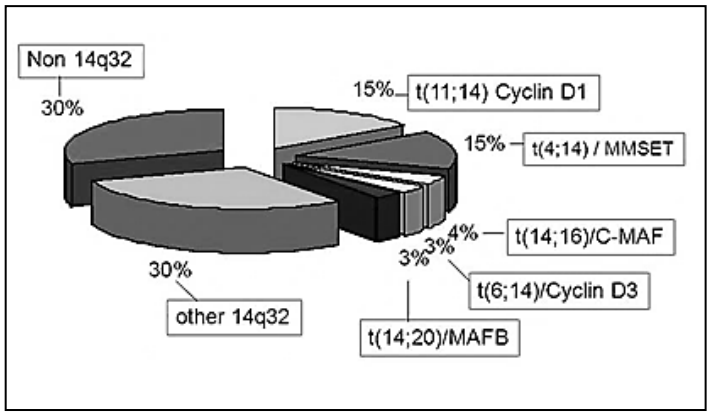

Figure1.4 Frequency and types of IgH translocation in MM.

The t(11;14), present in about 15% of patients, results in constitutive activation of *cyclin D1* (*CCND1*). Although biological consequences are still unclear, it has been suggested that cases bearing the translocation have lower proliferative capacity and a longer disease free survival [13]. Studies of gene expression profiling (GEP) have shown that the deregulation of at least one of the cyclin D1-3, which control cell cycle transition from the G1 to the S phase, is associated with nearly all MM patients. Based on this evidence, Bergsagel *et al.* [16] have proposed a molecular classification of the disease based on the presence of IgH translocations and expression of cyclins that was further investigated by our group [17] (Table 1.1).

Groups	Translocations	Cyclin	Risk
TC 1	t(11;14) t(11;16)	CCND1 CCND3	Low
TC 2	HS	CCND1	Low
TC 3		CCND2	Low/Moderate
TC 4	t(4;14)	CCND2	High
TC 5	t(14;16) t(14;20)	CCND2	High

Table 1.1. Molecular classification based on TC (translocation/cyclin) group. The prognosis gets worst from TC1 to TC5 classes. TC2 cases with 13q deletion have negative prognosis. TC3 class comprises heterogeneity cases, mostly overexpressing ciclyn D2 (CCND2). TC5 cases have the highest CCND2 levels and they have also a deregulation of MAF genes.

The t(4;14) occurs in about 15% of cases and causes the over expression of two genes: the *Fibroblast Growth Factor Receptor 3* (*FGFR3*), coding for a tyrosine kinase receptor, and the *MMSET* gene, encoding a histone methyltransferases. MMSET deregulation is considered the

most important molecular consequence of the translocation, which also results in the over expression of c-MYC. In fact, MMSET regulates MYC protein at post-transcriptional level by binding and subtracting a regulatory miRNA (miR-126) [18]. The translocation is associated with aggressive clinical forms and is generally considered as a poor prognostic factor.
The t(14;16) results in constitutive activation of the MAF gene and occurs in approximately 4% of MM patients; it is more frequent in PCLs. *MAF* and *MAF-B* genes are part of transcription factors family that positive regulates expression of *CCND2* and *integrin B7*, which are over-expressed in TC5 patients [19]. Patients with translocation involving MAF genes have poor prognosis [13].

TP53 is a tumor suppressor protein encoded by the *TP53* gene at chromosome 17p13. As known, it mediates the response to various stress signals (oxidative stress, ribonucleotide depletion, DNA damage and deregulated oncogene expression) [20]. Loss of TP53 function is frequent in MM due to deletions of *TP53* locus and/or mutations of *TP53* gene. The deletion of the 17p13 region, containing *TP53* gene, is predominantly monoallelic and occurs in about 10% of untreated MM and increases in PCL (from 35% to 75%) and it is higher in HMCLs (50%) [21]. Mutations of *TP53* are also rare events at disease presentation (0%-9.7%), but their frequency increases during progression (about 25-30% in PCL) and have the highest percentage in HMCLs (80%) [22-24]. It has been described a strong association between TP53 mutation and del(17p) [25] A recent study has identified *TP53* as the critical gene in del(17p13) in MM [26], which has a very negative prognostic feature [14].

The most common cytogenetic alteration in MM is represented by extra copy of the long arm of chromosome 1 (1q gain). 1q gain can be due to duplications, jumping translocations or can occur as isochromosomes [27]. Putative targets of this genetic alterations include genes involved in cell cycle progression (*CKS1B*) or in resistance to bortezomib (*PSMD4*). Patients with 1q gain are characterized by complex karyotype and very aggressive disease with poor prognosis.

Recent data based on whole exome/genome sequencing indicate a heterogeneous pattern of gene mutations in MM, frequently involving members of the ERK pathway (*NRAS*, *KRAS* or *BRAF*) and, at a lesser extent, other genes such as *DIS3* or *FAM46C* [15, 28-32].

1.3 Diagnosis and clinical features

MM diagnosis is based on the presence of at least 10% clonal PCs in the BM and of monoclonal protein in serum (>1g/100ml) or urine (>200mg for 24hours) [33]. There are some recommended laboratory tests for the diagnosis. They include complete blood count, protein electrophoresis with immunofixation on serum and urine and chemical analysis and quantification of the monoclonal proteins. There are some tests made on BM sample, such as cytogenetic analysis or fluorescent in situ hybridization (FISH) on BM biopsies. There are also additional tests including conventional radiography of the spine, skull, chest, pelvis, humeri and femora in order to identify myeloma-related bone lesions [34]. From a clinical point of view, symptomatic MM patients have organ dysfunction including hypercalcemia, renal failure, anemia, and bone disease. The last feature is typically of more than 80% of patients and it is due to osteoclast-mediated bone destruction, with the consequence of hypercalcemia, osteoporosis, bone pain and fractures [35]. Patients may have vertebral fractures, which are associated with a high impairment of quality of life, morbidity and mortality. Bone resorption is not only a relevant issue for patients' quality of life, but represents also a critical step in the development of this disease, since it supports tumor growth and survival and finally contributes to the development of drug resistance [36, 37].

1.4 The role of bone marrow niche in MM

Bone marrow microenvironment consists of both cellular and non-cellular elements. The cellular compartment of BM microenvironment consists of immune cells, erythrocytes, hematopoietic stem cells (HSCs), BM fibroblast-like stromal cells (BMSCs), vascular endothelial cells, osteoclasts and osteoblasts. The non-cellular compartment is composed by extracellular matrix (ECM) proteins, such as collagen, laminin, fibronectin and osteopontin. The interaction between MM cells and BMSCs plays a key role in the pathogenesis of MM, since this interaction activates several signaling pathways that promote growth, survival, differentiation, drug resistance and homing and adhesion to the bone marrow of MM cells (Figure 1.5 and Figure 1.6) [38]. Moreover, pathways activated by this interaction are able to support angiogenesis, osteoclastogenesis and the secretion of soluble factors such as vascular endothelial growth factor (VEFG), insulin-like growth factor 1 (IGF1), stromal cell-derived factor 1 (SDF-1) and interleukin-6 (IL-6) [38, 39].

MM cells adhesion to BMSCs or ECM is mediated by adhesion molecules such as the very late antigen 4 and 5 (VLA-4 and VLA-5), CD44, the intracellular adhesion molecule (ICAM-1), syndecan 1 and MPC-1. The interaction between MM cells and the non-cellular compartment of BM niche (collagen, laminin and fibronectin) is mediated by integrins, a heterodimeric cells surface receptors that are involved also in the interaction between MM cells and immunoglobulin superfamily molecules [40]. Integrins are expressed by all cell types and both primary MM cells and MM cell lines express several integrins. Also CD138 (known as syndecan-1), one of the specific surface marker of MM cells, is involved in the interaction between MM cells and BM niche [41].

Adhesion molecules are involved in the development of MM cells resistance to chemotherapeutic drugs such as doxorubicin (an anthracycline) and Melphalan (an alkylating agent) leading to treatment failure. Drug resistance mediated by adhesion molecules is known as cell adhesion mediated drug resistance (CAM-DR) and it blocks the apoptosis induced by drugs [42]. Bortezomib, a proteasome inhibitor, is able to overcome CAM-DR since it selectively downregulates VLA-4 on MM cells [43].

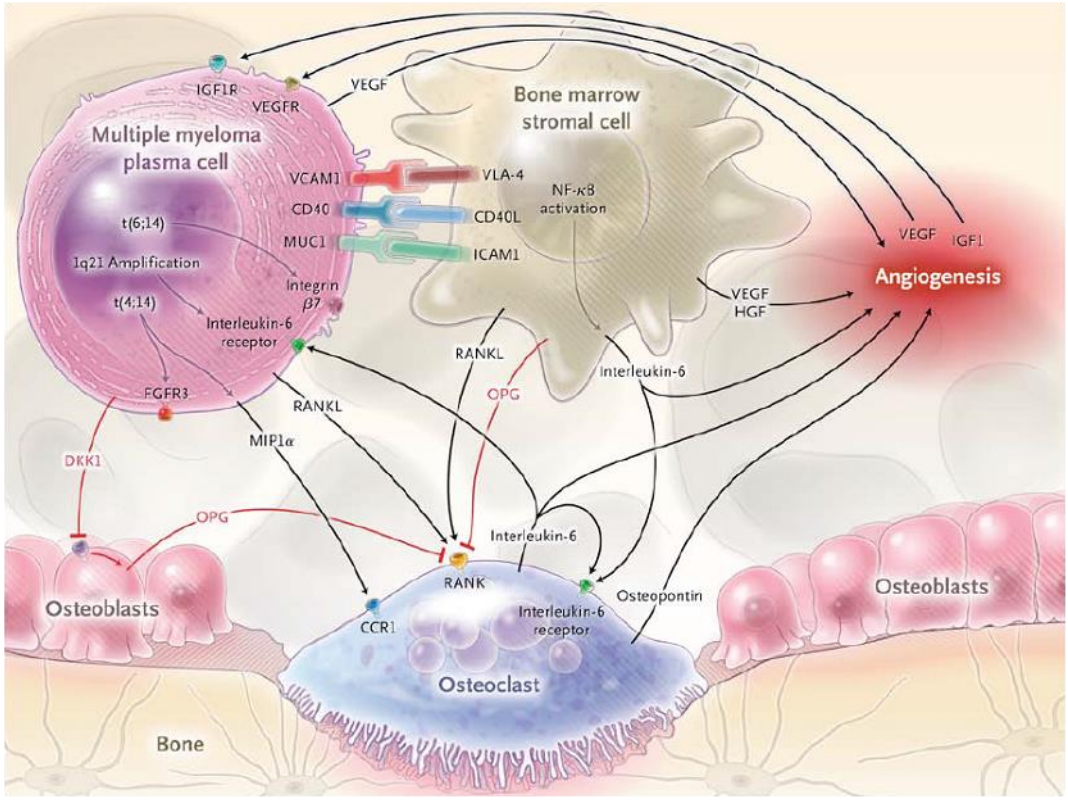

Figure 1.5. Interaction between malignant plasma cells and bone marrow in multiple myeloma *[44].*

As previously reported, MM mainly progresses in the bone marrow and signals provided by the microenvironment play a very important role in the maintenance of plasma cells growth, migration, survival, drug resistance and angiogenesis. An important mechanism that mediates interactions between PCs and BM cells is represented by several cytokines and their receptors produced by both PCs and BM cells.

MM cells are able to secrete transforming growth factor-β (TGF-β), VEGF, Angiopoietin-1, tumor necrosis factor-α (TNFα), FGF-2 and matrix metalloproteases (MMPs) that directly support tumor growth.

The interaction between PCs and BM cells mediated by adhesion molecules induces the transcription and secretion by BM cells of several cytokines such as IL-6, SDF-1, VEGF, Hepatocyte growth factor-scatter factor (HGF-SF) and IGF-1 [39].

IL-6 is the most important cytokine in MM pathogenesis, in fact it is well known that IL-6 induces in vitro growth of freshly isolated MM cells and that IL-6 receptors (IL-6R) is expressed by MM cells. Several studies have highlighted that the major source of IL-6 are the BMSCs since even if all HMCLs express IL-6R mRNA, only a small subset expresses IL-6 mRNA. There are several evidences that MM cells adhesion to the BMSCs results in significant increase of IL-6 production by BMSCs that directly supports tumor growth [45]. Through gene reporter assays, it is also evidentiate that NF-κB pathway is involved in the regulation of IL-6 transcription in BMSCs, such as various soluble factors (i.e. IL-1α; IL-1β; TNFα and VEGF) are able to mediate IL-6 secretion both by BMSCs and MM cells [45].

Considering other soluble factors, VEGF is expressed and secreted by malignant PCs and BMSCs. VEGF plays an important role in the pathogenesis of MM since it favors proliferation of MM cells through the activation of ERK pathway, it sustains migration of MM cells through the activation of a PKC-dependent cascade [46] and it stimulates the expression of IL-6 by microvascular endothelial cells and BMSCs [47]. Talidomide, a MM drug, directly inhibits the secretion of VEGF, (and bFGF and HGF) by endothelial cells and reduces their proliferation and angiogenesis ability [48].

Both MM cells and BMSCs are able to produce TNFα that is an important mediator of inflammation and bone resorption. This soluble factor favors the proliferation of MM cells and induces the expression of several adhesion molecules by MM cells (such as VLA-4, VCAM-1 and ICAM-1). TNFα acts also on the BMSCs, where it improves the secretion of IL-6 and the expression of ICAM-1 and VCAM-1 in BMSCs and it induces NF-κB activation [49].

One of the typical features of MM is represented by bone lesions since MM cells are able to degrade bone matrix through the expression of matrix metalloproteases (MMPs) [50]. MMPs belong to a family of zinc-dependent endopeptidases that have a proteolytic activity on several components of the ECM. These MMPs have both a physiological and pathological role, since they are involved in physiologic ECM turn over, bone remodeling and angiogenesis and they play a role also in the pathological conditions of rheumatoid arthritis and tumor invasion. More in details, MM cells constitutively express and secrete MMP-9, while BMSCs secrete MMP-1 and MMP-2.

One of the most well-known chemokine system that has an important role in MM development and progression is represented by the SDF1α/CXCR4 that is involved in the regulation of MM cell homing, adhesion, motility and growth [51, 52]. This axis seems to be involved also in the mobilization and intravasation of primary cancer cells and in the metastatic invasion of the bone by MM cells, since it seems to be involved also in the extravasation of cancer cells because of its ability to attract lymphocytes and monocytes and to retain these cells in the bone marrow environment [39].

Another chemokine axis involved in MM progression is the CCR1/CCR5 system. This system is composed by the receptors CCR1 and CCR5 and their ligand MIP1α: this system could have a direct effect on MM cells and an indirect effect on stromal cells. It is established that over than 70% of MM patients has a higher production of MIP1α: data from *in vitro* and *in vivo* experiments show that MIP1α blockade reduces tumor growth, bone disease and the ability of MM cells to migrate in the bone marrow [53].

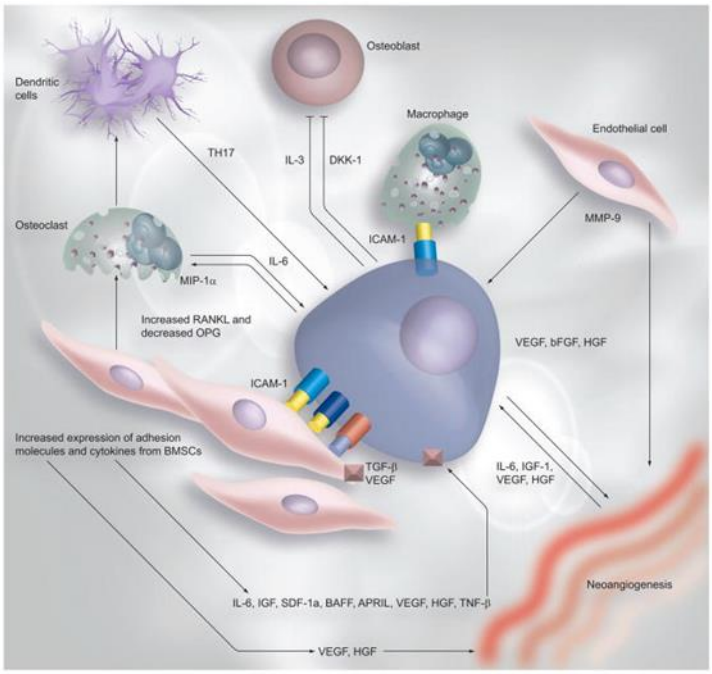

Figure 1.6. Bone marrow microenvironment and multiple myeloma cells [54].

One of the most common clinical features in MM is the presence of bone lesions that is due to the ability of MM cells to alter the osteoclast activity, resulting in lytic lesions and bone resorption. About 80% of MM patients have bone lesions due to the ability of malignant PCs to alter the ratio between osteoclasts (OCLs) and osteoblasts (OBLs) in favor to the first [37, 55]. This effect is mediated by an increase of BM-associated anti-osteoblastogenic factors, such as IL-3, DKK1, TGF-β and IL-7 [55, 56], or pro-osteoclastogenic factors, such as TNFα and RANKL [57-59]. Importantly, MM are able to induce bone disease directly or indirectly. In fact, MM cells may autonomously produce RANKL or stimulate the surrounding BM cells to release RANKL and other soluble factors that promote OCL differentiation [59]. OCLs directly support MM cell proliferation and survival, leading to disease progression [60]. Recent studies from Colombo *et al* [61] show that Notch pathway is involved and has a crucial role in determining osteoclastogenesis in MM. In addition to secrete RANKL, myeloma cells produce MIP1α and TNFα that activate osteoclasts and directly contribute to dysregulation of molecules as DKK1 and RUNX2 that alter the normal differentiation and functional maturation of osteoblasts.

The importance of the relationship between BM niche and MM in disease progression and drug resistance has highlighted the necessity for new drugs and their combinations in order to overcome drug resistance.

1.5 Pharmacological treatments of MM

Initial treatment of MM first depends on patient age and on the presence of comorbidities. For patients under 65 years without comorbidities and organ dysfunction, treatment of choice consists of high dose chemotherapy combined with autologous hematopoietic stem cell transplantation [62, 63]. Before transplantation, patients receive “chemoterapic induction” treatment that is necessary to induce the migration of hematopoietic stem cells from BM to peripheral blood. These cells are then harvested from patient’s blood with leukapheresis and then preserved. After this process, stem cells are re-infused into the patient in order to reconstitute BM. This treatment is not curative, but may extend the survival. It is also possible to make an allogeneic transplant with good results, but this possibility is available only for a small percentage of patients [64].

Multiple myeloma can be also treated with chemoterapic agents. Drug commonly used for treatment of MM patients are alkylating agents (such as melphalan) and immunomodulatory agents (such as lenalidomide and thalidomide), corticosteroids (dexamethasone) and

proteasome inhibitor (bortezomib and carfilzomib) or a combination of them. There are also new drugs with novel mechanism of action. They include monoclonal antibodies and new treatments that are able to target not only MM cells but also the interaction between neoplastic PCs and the BM microenvironment, specific inhibitors of signaling pathways and kinases, deacetylase inhibitors and agents activating the unfolded protein response, especially Hsp90 inhibitors [65].

MM remains still an incurable disease because neoplastic cells are able to develop intrinsic drug resistance (due to *TP53* mutation for example) or through a selection process after pharmacological treatments. Furthermore, localization of tumor cells within the BM may be responsible drug resistance due to the interaction of tumor cells with the surrounding microenvironment. The interaction between MM cells and cells from BM niche (BMSCs, osteoclast, osteoblast and endothelial cells) and the production of growth factors such as TGFβ may induce transcription and secretion of cytokines that improve drug resistance of tumor cells [38]. There are many expectations for new drugs, such as Carfilzomib, because they could overcome intrinsic drug resistance and could induce cytoxicity in MM cells within the BM. Moreover, these drugs could also overcome clinical drug resistance due to conventional high-dose chemotherapy [66].

2 NOTCH pathway

The oncogene NOTCH was discovered for the first time in 1919 in a strain of Drosophila with irregular, "notched" margins wings and lately associated to haploinsufficiency in the NOTCH gene [67]. NOTCH family is composed by four isoforms of transmembrane receptors (NOTCH1-4), with high structural homology, which has maintained sequence homology during evolution from *C. Elegans* to humans [68]. Two family of ligands, Delta-Like ligands (DLL1-3-4) and Serrate like ligands (JAGGED1 and JAGGED2) also contribute to the pathway. NOTCH pathway is essential for the development of complex organisms, because it is involved in the regulation of embryonic development, vasculogenesis, myogenesis, gliogenesis, neurogenesis, angiogenesis and hematopoiesis. It regulates different cellular processes such as proliferation, morphogenesis, differentiation, apoptosis, adhesion and chemotaxis [69, 70]. It is also involved in the regulation of homeostasis of adult tissues through the regulation of cell differentiation [71], in the promotion of stem cells self-renewal [72] and in the determination of cell fate choice in tissue development, including the commitment to T and B cell lineages [73]. To this, deregulation of NOTCH pathway has been described to be involved in different types of cancers, including solid tumors (i.e. breast cancer, melanoma, colorectal cancer, glioblastoma, pancreatic cancer) [74] and hematologic neoplasia (i.e. T-ALL, B-ALL, AML, B-CLL, MM) [39, 69, 75, 76]. Deregulation of NOTCH pathway is also responsible for other diseases such as Alagille syndrome [70], spondylocostal dysostosis, Aortic valve disease [77, 78] and the late-onset disease CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) [79].

2.1 NOTCH components structure

2.1.1 NOTCH receptors structure

NOTCH pathway is composed by four different receptor isoforms called NOTCH1, 2, 3 and 4 that bind to specific ligands presented on adjacent cell surface in order to activate signal transduction. After ligands binding, three different proteolytic cleavages occur in the receptors structure with the final release of an intracellular peptide (intracellular NOTCH, ICN) that translocates into the nucleus where it works as transcriptional factor for NOTCH target genes.

NOTCH receptors are a single-pass transmembrane protein composed by different portions (fig.2.1) [80]:

- The extracellular portion is composed by the epidermal growth factor (EGF)-like repeats that are involved in the binding of the receptor to the ligand. This portion consists of variable number of repeat (29-36): repeats 11-12 are responsible for the receptor-ligand *trans*-interaction (receptor and ligand are expressed on different cells) [81]. This interaction allows receptor activation and its translocation into the nucleus. Repeats 24-29 are involved in ligand-receptor *cis*-interaction (if ligand and receptor are expressed both on the same cell) and are responsible for NOTCH signaling inhibition. Moreover, there is also a negative regulatory region (NRR) that is involved in the prevention of receptor activation in the absence of ligand [70]. NRR blocks the first proteolytic cleavage (in S2 site) through the induction of a specific tridimensional conformation that will change only after ligand binding.
- The transmembrane domain (TMD) divides extracellular and intracellular portion.
- The intracellular portion is formed by RBPj association module (RAM) domain followed by 7 ANK (Ankyrin) repeats, 2 NLSs (Nuclear Localization Signals) and a motif rich in proline, glutamic acid, serine and threonine (PEST) at C-terminal of the protein. RAM domain plays an important role in transcriptional activation of NOTCH target genes. In fact, RAM domain recognizes and binds the transcription factor CSL (CBF-1, Suppressor of Hairless, Lag-2), called RBPj (Recombination Signal Binding Protein For Immunoglobulin Kappa J Region) in mammals, and induces the assembly of transcriptional complex into the nucleus. The role of ANK repeats is to recruit nuclear protein necessary for gene transcription, while NLS is responsible for ICN trafficking into nuclear compartment. PEST domain regulates the stability of NOTCH receptor since it is a target of polyubiquitination signals that induces NOTCH degradation through the proteasome.

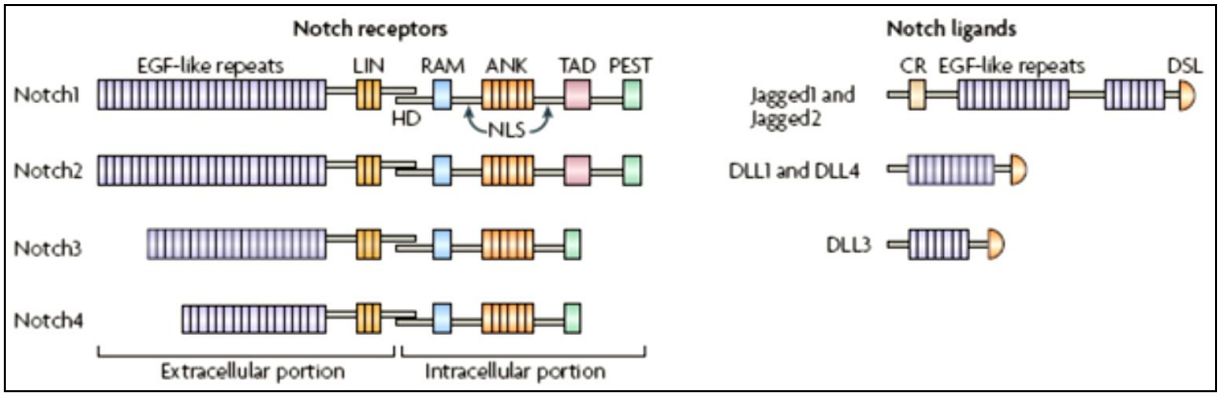

Fig.2.1 Structure of NOTCH receptor and ligands.

2.1.2 NOTCH ligands structure

The *cis*-interaction between NOTCH receptor and ligand is essential for the correct signaling pathway activation. NOTCH ligands are single pass transmembrane proteins and in mammals there are five different ligands: Delta-like protein 1, 3 and 4 (DLL1,3,4) belong to the Delta family, and JAGGED1 and JAGGED2 belong to the Serrate family (Figure 2.1). All ligands present an extracellular portion that is involved in receptor binding and activation and it consists of EGF repeats (6-16), DSL domain and N-terminal portion. The differences between these two families are that in JAGGED ligands the number of EGF repeats is double than that presents in DLL ligands. Moreover, JAGGED ligands present a cysteine rich region (CR) that DLL ligands do not display [82-84].

All ligands have an intracellular region containing multiple lysine residues that are involved in ligand signaling activity. JAGGED1, DLL1 and DLL4 have also a PDZ (PSD-95/Dlg/ZO-1)-ligand motif that is required for cytoskeleton-interaction. PDZ-ligand motif seems to be involved in migration [85, 86]; cell adhesion [87, 88], and oncogenic transformation [89]. In fact, JAGGED1 is able to interact with other PDZ-containing proteins through its PDZ-ligand motif [89] and this interaction could alter gene expression of the JAGGED expressing cells [90].

Among NOTCH ligands, DLL3 is different from the other because it has missed the structural region involved in *trans*-interaction with receptor. Indeed there is a study suggesting a possible inhibition role for DLL3 since it has been demonstrated that its overexpression in mammal cells blocks NOTCH activation [91].

2.2 Activation and intracellular signaling of NOTCH

Signal-sending cell presents ligand, which binds to receptor presents on signal-receiving cell: this interaction induces a conformation change in NRR region that exposes the extracellular S2 site to ADAM10 (A Disintegrin and Metalloproteinase 10). ADAM10 action produces a truncated peptide that remains still anchored to cell membrane. This truncated fragment is then recognized by γ-secretase complex that cleaves the single-pass transmembrane portion of NOTCH. The γ-secretase complex is an integral membrane multi-subunits protease complex composed of Presenilin-1 or 2 (PS1, 2), Nicastrin (NCT), anterior pharynx-defective 1 (Aph1) and Presenilin enhancer 2 (PEN-2). It is able to recognize S3 region, which is localized between TMD and RAM domain of the receptor, and it induces, through proteolytic cleavage,

the release of the active ICN from the membrane [70]. Once cleaved, ICN is able to translocate into the nucleus, because of its NLS, where it recognizes, through RAM domain, the DNA binding protein RBPj, localized on the regulative sequences of NOTCH target genes [70]. RBPj forms a complex with several co-repressor protein that includes KyoT2 [92], histone demethylase KDM5A [93] and histone deacetylase (HDAC) [94]. ICN induces the removal of the co-repressor complex from RBPj since ICN binding site overlaps to that of KyoT2, so the binding of KyoT2 and ICN to RBPj is mutually exclusive. Once RBPj is free from corepressor, it forms a tertiary complex with Master mind-like protein (MAML) and its co-activators, such as histone acetyltransferase, the histone demethylase LSD1 and PHF8, PBAF nucleosome remodeling complex subunit BRG1 and AF4p12 [95], and this big complex could start NOTCH target genes transcription (Figure 2.2). The serine/threonine kinase Glycogen Synthase kinase 3-β (GSK3-β) binds ICN1 (ICN from NOTCH1 receptor) and induces its positive regulation by increasing its stability [96]. On the other hand, CDK8 kinase is able to phosphorylate nuclear ICN at PEST domain: this phosphorylation causes the recognition of PEST domain by the E3 ubiquitin ligase FBW7, which induces degradation of ubiquitinylated ICN through proteasome machine [97].

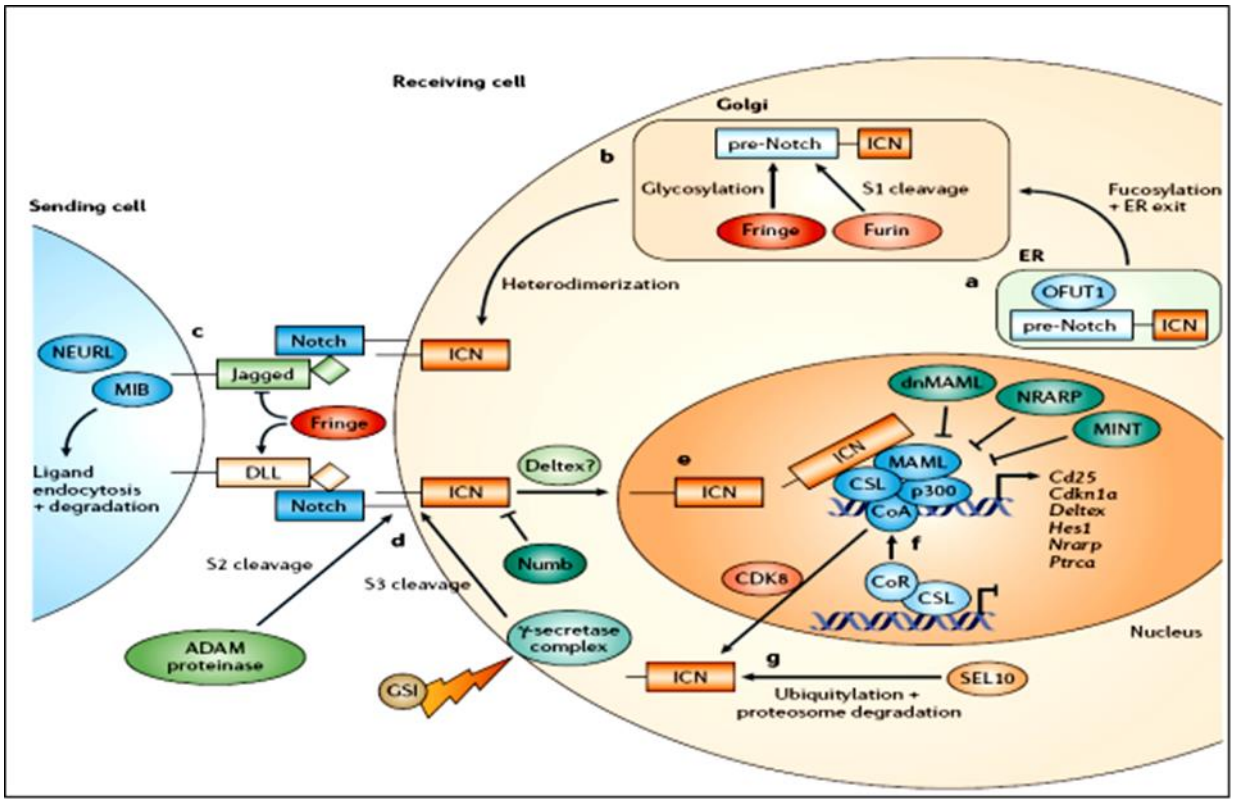

Figure 2.2. NOTCH pathway

NOTCH target genes include important genes involved in the oncogenic process such as the Hairy and enhancer of split (HES) family [98], Hairy-related transcription factor (HRT also known as HEY) [98], MYC [99] and Phosphatase and tensin homolog (PTEN) [100]. HES and HEY family are composed by genes with basic helix-loop-helix (bHLH) motif acting as transcription repressor. They mediate several NOTCH biological effects such as multipotent cell maintenance during prenatal development and in adult life, and cell fate decision [98]. MYC is a transcription factor well known to act as a proto-oncogene and to be overexpressed (or mutated) in several tumors (leukemias and lymphomas) [99]. The phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN) acts as a tumor suppressor and is involved in the regulation of cell cycle. Its inactivation, due to mutations or deletions, cause increased cell proliferation and reduction of cell death [101]. Other NOTCH target genes are CD25 (IL2R and α-chain of pre-T cell receptor) and GATA3 (GATA Binding Protein 3), a transcription factor activated during T cell development. Also, CCND1, p21/WAFL, BCL2, E2A, HOXA5,9,19, NF-κB, IFI-202, IFI-D3 and ADAM19 are NOTCH targets. Additionally, it exists also a negative feedback loop to control NOTCH signaling pathway: for example, NRARP and DELTEX1 are targets of NOTCH and they acts as a negative regulator of NOTCH signaling [102].

2.3 NOTCH pathway regulation

2.3.1 Receptors regulation

NOTCH receptors are a 300kDa protein synthetized as single precursor in endoplasmic reticulum (ER) and then translocated at the cell membrane. Because of the importance of NOTCH pathway in several cellular processes, during the translocation NOTCH receptor undergoes several modifications that regulate its maturation and activity (Figure 2.3).

It is first targeted by O-fucosyltransferase 1 (POFUT1) that catalyzes the addition of an O-fucose to serine and threonine residues in the NOTCH C-terminal extracellular portion. POFUT1 is able to act also as ER chaperone [103] and is involved in the process of translocation of NOTCH receptor from ER to Golgi. It has been reported that POFUT1 loss-of-function causes a complete NOTCH pathway inhibition. The extension of the O-fucose on EGF repeats increases NOTCH-DLL binding and decreases NOTCH-JAGGED binding, directly altering NOTCH reactivity to ligands presented by adjacent cells [103, 104]. PGLUT1 (O-glycosyltransferase1) mediated O-glucose addition to serine residues in the

extracellular portion of NOTCH. It seems that PGLUT1 activity is involved in the regulation of ligand binding and cleavage of NOTCH to generate ICN [105]. The third step of modification occurs in the Golgi reticulum and is mediated by Furin-like convertase, that cleavages NOTCH precursor at site S1 [106]. The generation of a mature NOTCH receptor in cell membrane needs an intracellular cleavage of NOTCH that generates two fragments, the transmembrane N-terminal and the extracellular C-terminal portion that are joined together by non-covalent Ca^{2+} dependent linkage [107]. Several E3 ubiquitin ligases regulate mature NOTCH form. One of this is represented by the CBL family, which is involved in NOTCH degradation after endocytosis. In particular, ITCHY promotes the ubiquitation of the intracellular portion, while c-CBL targets NOTCH1 for degradation through lysosomes [108]. Another protein involved in the ubiquitination of NOTCH is DELTEX, that can have a positive [109] or negative [110] regulation function. DELTEX is a zinc-finger protein that interacts with ICN through the ANK cytoplasmic domain. It seems that DELTEX antagonized p300 by reducing transcription of different genes and by modifying the expression of different transcriptional factors classes, such as E47. Moreover, there are some evidences that support the idea that the same ICN can regulate DELTEX level [111]. DELTEX is able to direct the trafficking of NOTCH to the recycling or the degradation pathway. The last protein involved in this process is NUMB that negatively regulates NOTCH intracellular portion [112, 113]. NUMB is an adapter protein that is involved in the endocytosis processes of NOTCH in combination with α-adaptin and Exp-15. NUMB acts in several ways:

- it interacts with and activates ITCH (a member of ubiquitin E3 ligase family), which promotes poly-ubiquitination and subsequent proteasomal degradation of ICN [113];
- it promotes endocytosis of the cleaved S2 form of NOTCH before its cleaving by γ-secretase and ICN1 release. It works in collaboration with the AP2 domain of α-adaptin and NAK (Numb Associated Kinase) [114];
- it prevents the localization of *SANDOPO* gene product, a transmembrane protein that promotes Notch signaling [113].

Once translocated into the nucleus, NOTCH regulation is operated by Sel-10, MINT and NRARP. Sel-10 is an E3-ubiquitin ligase that binds ICN and recruits the SCF complex (Skp1-Cullina-F-box), that ubiquitinates NOTCH and induces its degradation by proteasome. Sel-10 regulation requires the presence of the PEST domain and its hyper phosphorylation mediated by CDK8, which occurs after transcriptional activation induced by MAML binding to p300 and CDK8 [115]. MINT (MSX2-interacting nuclear target protein) inhibits Notch pathway by

preventing the binding of ICN to CSL and by blocking transcriptional activation mediated by NOTCH [116]. NRARP (NOTCH regulated ankyrin repeat protein) can bind to the ICN-CSL complex through two ANK repeats and can inhibits the complex and/or destabilizes ICN [116].

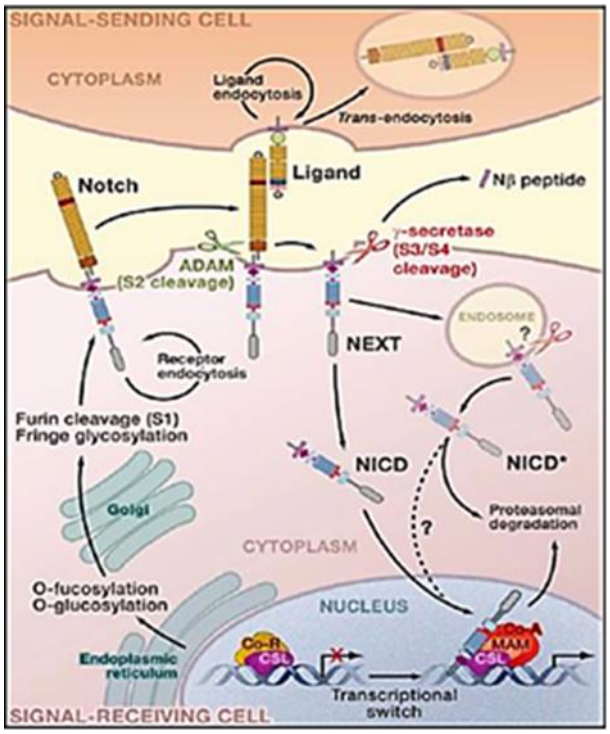

Figure 2.3. NOTCH pathway regulation

The best characterized mechanism for NOTCH degradation is represented by the proteasome-dependent mechanism mediated by the E3-ubiquitin ligases ITCH and Sel-10. However, it has also been reported the involvement of lysosomal pathway, in which acts the c-CBL ubiquitin ligase that interacts with NOTCH and causes its monoubiquitylation, after PEST domain phosphorylation [108].

2.3.2 Ligands regulation

The modulation of NOTCH signaling pathway is mediated also by the regulation of ligands expression and activity, which is controlled by proteolysis, endocytosis, ubiquitination, glycosylation or by other signaling molecules. For example, EGF repeats in the extracellular portion undergo O-fucosylation that could be subsequently modified through the addition of N-acetylglucosamine by Maniac Fringe (MFNG), a specific β 1, 3-N-acetylglucosaminyltransferase [117]. This modification is very important because it regulates

the ability of ligands to bind the receptors, directly increasing or decreasing the receptor affinity for specific class of ligands.

In addition, ligands can undergo proteolytic cleavage by ADAMs, that recognizes a juxtamembrane region, and γ-secretase complex, that acts at the transmembrane region. The action of ADAM has a negative effect, because it reduces the number of ligands available for NOTCH binding [118]. Also for ligands, the combined action of ADAM and γ-secretase generates an intracellular portion of ligands that could translocate to the nucleus [119]. It is yet unclear how this proteolytic mechanism acts, but it seems to be an interesting mechanism regulating ligands availability and their possible intracellular activity.

As for receptors, also ligands have an ubiquitination regulation. In fact, the ubiquitination of multiple lysine residues is involved in ligand endocytosis and degradation. There are two different classes of E3 ubiquitin ligase involved in ligands regulation. The first is represented by MIND BOMB 1 and 2 (MIB1 and MIB2): this family is responsible for ligands ubiquitination and endocytosis and has an important role for NOTCH receptor activation. They are the first player in ligand mono-ubiquitination and their action mediates ligands endocytosis. The second family is composed by Neuralized 1 and 2 (NEUR1 and NEUR2): their ubiquitination activity mediates the degradation of internalized ligands [120]. NEUR is responsible for the degradation of poly-ubiquitinated ligands. Consequently, ligands can undergo to endocytosis or degradation depending on mono or poly-ubiquitination.

Other molecules that can directly interact and influence ligands expression [121] represent the last mechanism for ligands regulation. One of these is represented by the vascular endothelial growth factor (VEGF) that directly induces DLL1 up-regulation on endothelial cells: this specifically stimulates arterial cells to induce angiogenesis. DLL1 is up-regulated in neural stem cells also by fibroblast growth factor (FGF) and this induces the maintenance of spinal cord stem cells [122]. JAGGED1 is regulated by transforming growth factor β (TGF-β) and Platelet derived growth factor (PDGF). The first growth factor increases JAGGED1 expression in epithelial cells favoring the transformation of epithelial cells to mesenchymal cells [123], while the second downregulates JAGGED1 in vascular smooth muscle cells and induces growth retardation [124]. WNT signaling can regulate both DLL1 and JAGGED1 [125].

There are some evidences that in addition to the traditional complexes ligand-receptor typical of *in trans* activation between two different cells, NOTCH receptor can form an *in-cis* inhibition complex if both receptor and ligand are presented on the surface of the same cell.

This mechanism has an important role in the limitation of the NOTCH activity areas and it determines that when the ligands are more abundant the cells provides the signal, conversely when receptors are more abundant the cells receive the signal [126]. Alternatively, it has been reported that ligands and receptors can be segregated into different membrane sub-domains allowing the simultaneous reception and transmission of the signal [127] (Figure 2.4).

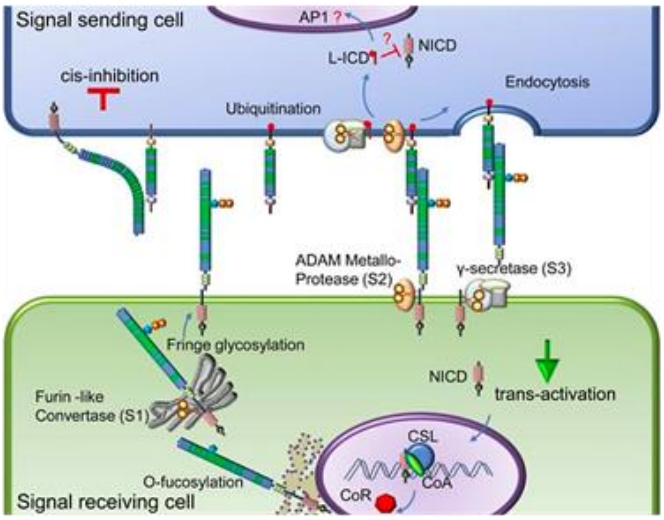

Figure 2.4. NOTCH pathway *cis*- and -*trans* activation.

2.4 NOTCH interaction with other signaling pathway

Because of the importance of NOTCH pathway in the regulation of important cell processes, NOTCH has an important cross talk with fundamental signaling system involved in tissue development and maintenance, normally deregulated in cancer diseases. There is a bidirectional regulation of these pathways, because NOTCH signaling can modulate the activity of these important pathway, as well as these pathways can directly or indirectly regulate NOTCH signaling activity. These important pathways are represented by NF-κB, mTOR, PI3K/AKT, EGFR, WNT and HEDGEHOG pathways.

2.4.1 Nuclear factor-κB

NOTCH signaling cooperates with one of the most important cell fate regulatory network that is involved in development, inflammation, immunity and cancer: the NF-κB signaling [128]. This pathway consists of five different proteins NF-κB1 (p50), NF-κB2 (p52), RelA (p65), c-Rel, and RelB. They form homo or hetero dimers that remain inactive in the cytoplasm because of the presence of NF-κB inhibitors (IκBs). This family includes IκBα, IκBβ, IκBε and NF-κB precursors p100 (p52 precursor) and p105 (p50 precursor) [129, 130]. IκB

kinase (Ikk) complex consists of two catalytic subunits, Ikkα and Ikkβ and the regulatory protein Ikkγ, and it is able to regulate NF-κB activation through the canonical or non-canonical pathway [128].

T-cell receptor, TNFα, Toll-like receptors, IL-1, lipopolysaccharide (LPS) and lipopeptides activate the canonical NF-κB signaling. From a molecular point of view, this activation consists of the phosphorylation of Ikkβ, which in turn phosphorylates IκBs, that undergoes to proteasomal degradation, and induces the activation of NF-κB, composed mainly by p50/RelA and p50/c-Rel dimers that translocate into the nucleus where they direct the transcription of target genes [131]. IκBs act as a negative regulator of NF-κB since they promote the export of NF-κB from the nucleus to the cytoplasm, directly limiting the activation of NF-κB signaling.

TNF family members, including CD40L and RANKL, are able to activate, via the NF-κB inducing kinase (NIK), the non-canonical NF-κB signaling, through the activation of the homodimer Ikkα [132]. Once activated, Ikkα acts on p100, which generates, through proteasome-mediated proteolysis, p52/RelB heterodimer. This dimer can translocate into the nucleus and can induce the transcription of NF-kB responsive genes.

There are several evidences that NOTCH and NF-κB cooperate by directional interaction through different mechanisms during both normal development and pathological conditions. NOTCH regulates NF-κB pathway by different ways. NOTCH can control NF-κB from a transcriptional point of view, through physical binding and through the indirect regulation of Ikk. The transcriptional regulation may be both positive and negative. For example, NOTCH1-RBPj complex is able to induce the activation of the promoter of p100 subunit [133]: because of its inhibitory role, the increase of p100 results in the inhibition of NF-κB non-canonical pathway. However, if an active form of Ikkα is present, high expression levels of p100 could result in increased formation of p52/RelB dimers and consequently higher levels of NF-κB pathway activation. Anyway, it is demonstrated that in hepatic stellate cells ICN1 reduces NF-kB activation through the overexpression of IκBα [134]; moreover, in murine model, it has been shown that NOTCH1 is able to activate NF-κB activity through the transcriptional control of p50, p65, RelB, and c-Rel [135].

There has been described also a physical interaction between NOTCH and NK-κB heterodimers. For example, the inhibition of NF-κB activity in pluripotent human embryonal carcinoma cell line is mediated by nuclear interaction of the N-terminal portion of ICN1 with

p50 subunit [136]. In T-ALL disease, ICN1 acts as antagonist of IκBα for the binding of the p50 subunit in the p50/c-Rel complex and inhibits its nuclear export, directly increasing its transcriptional activity [137]. Furthermore, NOTCH1 acts as cytosolic scaffold and facilitates the assembly of the membrane-tethered complex required for TCR-mediated NF-κB activation in activated T-cells [138]. In breast cancers, a study conducted on MDA-MB-231 cell line shows that NOTCH1 is able to increase p65 translocation and NF-κB DNA-binding activity, with the consequent transcription of target genes such as MMP2/9, VEGF, Survivin, Bcl-X_L, and CCND1 [139]. In T-ALL disease, it is demonstrated that NOTCH is able to activate NF-κB through the binding to Ikk complex [140]. More in detail, NOTCH1 is able to activate the canonical NF-κB pathway [140], while NOTCH3 induce the non-canonical pathway [141]. Another mechanism of action mediated by NOTCH pathway is the ability of HES1 to repress the negative Iκκ complex regulator, CYLD (a de-ubiquitinase) [142]. In BM-endothelial cells, mir155 is downregulated by RBPj and the upregulation of miR-155, due to the lack of RBPj in murine model, induces the activation of NF-κB signaling (through the inhibition of the negative regulator κB-RAS1) and the production of proinflammatory cytokine that promoted a myeloproliferative phenotype [143].

On the other hand, there are evidences that NF-κB can regulate NOTCH pathway. It has been reported that NF-κB pathway is able to induce the transcription of *JAGGED1*, *DLL1* and *HES5* in B-cells [144]. In mouse embryonic fibroblasts, TNFα stimulation induces the recruitment of Ikkα and Ikkβ into the nucleus where they induce the release of IκBa from *HES1* promoter, directly inducing its expression [145]. Also in colorectal carcinoma cells the association of nuclear Ikkα to specific NOTCH target promoters induces the transcription of *HES1*, *HES5* or *Herp2/Hrt1*, through the release of SMRT corepressor [146]. Lastly, recent studies demonstrate that NOTCH and NF-kB pathway can act as co-regulators: in fact, in T-ALL disease they co-activate the transcription of miR-223 that represses the tumor suppressor FBW7 [147].

2.4.2 mTOR

The mammalian target of rapamycin (mTOR) pathway is activated during important cellular processes, such as adipogenesis, T-lymphocytes activation, angiogenesis, insulin resistance and tumorigenesis [148]. It has a key role in the regulation of proliferation, growth, survival and cell metabolism and it can integrate intracellular and extracellular signals. The *mTOR*

gene encodes for a conserved 298kDa serine-threonine kinase that belongs to PI3K-related kinase family. It acts as two different complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [149]. These two complexes differ for the presence of two different scaffold proteins, Raptor and Rictor, in mTORC1 and mTORC2 respectively. They both contribute to the assembly and to the biding to the substrate and they both have a regulatory function. mTORC1 is sensitive to rapamycin and induces translational initiation through the activation of the ribosomal protein S6 kinase (S6K) and the inhibition of 4EBP1 (Eukaryotic Initiation Factor 4E-Binding Protein 1). PI3K and Ras-MAPK (mitogen activated protein kinase) pathways positively regulate mTORC1 activity: they inhibit the formation of TSC1 and TSC2 (tuberous sclerosis complex 1 and 2) complex, which are no longer able to negative regulate mTORC1 activity. mTORC2 is insensitive to rapamycin and it is an upstream kinase for cAMP-dependent, protein kinase G/protein C, including AKT [150].

Concerning the interaction between mTOR and NOTCH pathway, it has already been described that in T-ALL cells NOTCH1 can induce mTOR pathway by PI3K/AKT activation, through HES1 dependent suppression of PTEN [100]. Studies conducted on human gastric cancer have shown that the aberrant activation of PI3K/AKT/mTOR pathway have an important role in tumorigenesis, through its ability to initiate aberrant proliferation and tissue expansion.

More studies are needed to better elucidate the cooperation between NOTCH and mTOR pathway. Until today, it has been described that in gastric stem cells NOTCH activation is associated to increase of mTOR signaling, playing an important role in the regulation of proliferation and differentiation of stem cells. In fact, the inhibition of mTORC1 reduces the proliferative effect of ICN1 [151]. Another aspect that supports the idea that NOTCH may be hyperactivated in tumors with mTOR activation is the finding that the expression of JAGGED1 and HES1 is inhibited by rapamycin in several cell lines from breast, liver, lung, prostate and pancreas tumors [152]. Another evidence that sustains the interaction between mTOR and NOTCH pathway is the reduction of TSC activity due to NOTCH signaling up-regulation. Loss of TSC causes a multisystem syndrome characterized by neurologic disease, pulmonary lymphangioleiomyomatosis (LAM), and hamartomatous tumors of the brain, heart, kidney, and skin. It is very complicated to explain if NOTCH activation is dependent or independent to mTOR, because NOTCH remains active also in the presence of mTOR inhibitor, suggesting that the TSC/NOTCH pathway is TORC1 independent [153]. However, in TSC-dependent tumors, cells respond to TORC1 inhibitor, supporting the idea that TORC1

and NOTCH inhibitors may have synergic effect in the treatment of TSC dependent tumors. The supposition is that NOTCH signaling is involved in the primarily differentiation of TSC-related tumors, while mTOR directly promotes their proliferation [153]. These data need to be confirmed in TSC tumors, because in T-ALL cells it has been already demonstrated that mTOR, proliferation and cell survival are under NOTCH control [99].

2.4.3 Phosphatidylinositol 3-kinase/AKT

PI3K/AKT is a conserved signaling pathway involved in important biological and cellular processes, such as proliferation, survival and differentiation [154]. PI3K catalyzes the phosphorylation of phosphatidylinositol-4,5-diphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3), which can recruit several protein kinases from cellular membrane, such as AKT and PDK1 (phosphatidylinositol-dependent kinase 1) [155]. The activation of AKT, induced by its binding to PIP3, is mediated by its phosphorylation by PDK1 and mTORC2 [156]. Once activated, AKT is able to act on its targets inducing their activation. These targets have an important function in cell proliferation and survival, and in glucose metabolism: they include the FOXO-family of transcription factors; BAD; $p21^{waf}$, $p27^{kip1}$, MDM2, TSC2 and glycogen synthase kinase 3 (GSK3β). Since its important role, AKT action is controlled by the tumor suppressor protein PTEN (phosphatase and tensin homolog deleted on chromosome ten) that controls and reduces the availability of AKT substrates needed for its activation [101].

Several studies, conducted on T-ALL cells, have demonstrated that reciprocal regulation between NOTCH and AKT pathways exists. There are several evidences suggesting that AKT is a downstream target of NOTCH pathway: in normal thymocytes and in leukemic T cells *PTEN* expression is inhibited by HES1 allowing AKT activation and downstream signaling increase [157]. Moreover, T cells carrying oncogenic forms of NOTCH, with inactivated PTEN and consequent hyperactivation of AKT, are insensible to NOTCH1 inhibition. Recent studies have demonstrated that T-ALL cell lines with inactive PTEN are able to increase their level of phosphorylated AKT if treated with GSIs (γ secretase inhibitors): these data suggest that it could exist also a PTEN independent collaboration between NOTCH and PI3K/AKT [158]. Moreover, recent studies evidence the ability of NOTCH to regulate also IGFR1 and IL7Rα, which are up-stream activator of PI3K/AKT and Ras-Raf-MAPK pathways. All these data suggest the existence of a very complex communication and interaction system between

NOTCH and AKT pathway [159]. This collaboration has also been demonstrated in other cancer models, such as breast epithelial tumor and cervical neoplasia [160, 161].

From the opposed point of view, there are several evidences that support the ability of AKT to positive regulates NOTCH signaling. These studies have been conducted on both non-tumor cells (such as neurons, arterial endothelial cells or CD4+ lymphocytes) and tumor cells (T-ALL and neuroblastoma): in both cases, NOTCH signaling is activated by AKT through direct or indirect action. In the first case, the activation of PI3K/AKT is mediated by VEGF and induces the over-expression of *NOTCH1* and *DLL4* [162]. In the second case, AKT directly inhibits the kinase GSK-3β that is no longer able to phosphorylate and inactivate nuclear NOTCH that can prolong its transcriptional activity [163]. Group of Chiaramonte and colleagues [164] have described a novel molecular mechanism in T-ALL cells that underlies NOTCH1 positive regulation dependent on AKT. This study indicates the ability of AKT to maintain high levels of NOTCH receptors on cell surface. It also exists a negative regulation of NOTCH mediated by AKT. In fact, ICN4 has four different phosphorylation sites, targets of AKT, that, once phosphorylated, are binding by 14-3-3 regulatory scaffold proteins, which are involved in the regulation of nucleocytoplasmic shuttling of target proteins. This binding induces longer localization of ICN4 in the cytoplasm, suggesting a negative regulation role for PI3K/AKT pathway in NOTH4 signaling [165].

2.4.4 Epidermal growth factor receptor

Several evidences support the existence of a cross talk between NOTCH and epidermal growth factor receptor (EGFR) tyrosine kinase (HER) pathways that directly controls drug resistance, recurrence and metastatic progression. Indeed, NOTCH and/or HER are overexpressed in several tumors such as lung, ovary, brain and skin cancers directly supporting their action as potent oncogenes [166, 167].

Kinase inhibitors targeting components of EGFR signaling pathway can promote NOTCH activity, sustaining the hypothesis of a communication between these two pathways. However, these cross-talk can have different effects, both synergic or antagonistic, depending on tissues and developmental context, so the mechanism of interaction is still unclear [168].

EGFR is activated by growth factors such as EGF, Heparin binding EGF (HB-EGF), amphiregulin (AREG), or transforming growth factor alpha (TGF-α). HER3 and HER4 are activated by the heregulin (neuregulin) family (Hrg/Nrg1,2,3,4). EGFR acts on AKT/PKB

and MAPK pathways through HER phosphorylation: once activated, HER is able to activate PI3K that phosphorylates AKT that directly activates mTOR. It seems that collaboration between NOTCH and EGFR pathway is involved in the acquisition of resistance to tyrosine kinase inhibitors and in the development of EMT (epithelial to mesenchymal transition) with the consequent over expression of Snai and vimentin (and down regulation of E-cadherin) [169].

There are different types of cancer in which there is the hyperactivation of both NOTCH and EGFR signaling pathway, for example more aggressive breast cancer subtype [170] and lung cancer (where there is NOTCH3 and EGFR overexpression) [171]. In the last case, it has been shown that the inhibition of NOTCH3 makes cells more sensible to EGFR inhibition approach, supporting the idea that NOTCH3 and EGFR pathways promote tumorigenesis through the inhibition of pro-apoptotic genes, such as BIM [172].

In those cells where NOTCH has a role of tumor suppressor, such as in differentiating keratinocytes, EGFR is able to negative regulate NOTCH1 expression and activity causing the suppression of differentiation [173].

2.4.5 Wnt pathway

Wnt pathway controls cell-fate decision during development. It is highly conserved and its signaling may be activated in three different manners: canonical pathway, through β-Catenin-T-cell-specific transcription factor (TCF)–lymphoid enhancer-binding factor (LEF) transactivation complex and two non-canonical pathways (Wnt-calcium pathway and planar cell polarity, that regulates intracellular calcium level and cytoskeleton, respectively) [174].

The canonical Wnt pathway is activated through the simultaneous engagement, by Wnt ligands, of Frizzled (Fz) family of serpentine receptors and synergic low-density lipoprotein receptor-related proteins (LRP5/6) on plasma membrane [175] and it is involved in tumorigenesis. There are 19 different Wnt ligands in vertebrate which undergo very fine spatial and temporally regulation during development. The direct consequence of Wnt activation is the increase of cytosolic β-catenin levels: normally, β-catenin level is controlled by a large cytoplasmic destruction complex consisting of the tumor suppressor APC, scaffolding Axin, constitutively active GSK-3β kinase and casein kinase1 (CK1) [176]. It captures and phosphorylates β-catenin which can become a substrate for ubiquitination and proteasomal degradation. In this way, β-catenin cytoplasmic concentration remains at low

level. Wnt is able to inhibit the activity of the destruction complex through the cytoplasmic protein Disheveled (Dsh): this favors the translocation of β-catenin into the nucleus where it can bind to TCF/LEF transcription factors directly activating Wnt responsive genes transcriptions [177].

Several studies demonstrate that NOTCH and Wnt pathways cooperate during embryonic development and that a bidirectional cross talk exists and plays an important role in several diseases, including cancer. For example, the shaping development of fly wings are induced by the co-activation of these two pathways that activate enhancer elements containing both TCF and RBPj binding sites [178]. There are evidences in Drosophila and in vertebrate multipotent cardiac progenitor cells that NOTCH pathway may negative regulate Wnt signaling through the ability of membrane-bound NOTCH to bind active β-catenin, favoring its lysosomal degradation [179]. In osteoblasts, the ability of ICN to hamper Wnt/ β-catenin signaling, through its ability to decrease β-catenin and increase HES1, blocks Wnt target genes transcriptions and inhibits osteoblastogenesis [180]. This role of NOTCH as negative regulator of Wnt is also present in several cancer conditions such as in human colorectal cancer [181].

From the opposite point of view, there are several evidences that Wnt signaling is able to positively regulate NOTCH signaling, both in healthy and in tumor cells [182]. One of the mechanism proposed to explain how Wnt can regulate NOTCH is represented by the ability of Wnt to increase *JAGGED1* expression both in hair follicle and in cancer development [125]. In fact, leukaemogenesis can be induced by osteoblasts in which inactivating mutations of β-catenin cause the overexpression of JAGGED1 with the consequent activation of NOTCH pathway cascade [183]. There have been described also different mechanisms of action involving the member of destruction complex GSK3β. Wnt activation reduces GSK3β with the consequent increase of NOTCH2 transcriptional activity [163].

2.4.6 Hedgehog pathway

The (Hh) Hedgehog pathway is involved in the regulation of tissue patterning and cell fate during embryonic development. It is highly conserved during evolution and its ligands, a class of 19kDa protein including Sonic, Indian and Desert, interact with heparin presented on cell surface and are tethered to the surface by fatty acyl and cholesterol modification [184]. If inhibited for ligands absence, the Hh receptor, called Patched (PTC), inhibits the seven-transmembrane domain protein Smoothened (SMO), an essential protein for Hh pathways activation [185]. When Hh ligands bind to PTC, the derepression of SMO results in the

activation of the GLI transcription factors, that can translocate into the nucleus and initiate Hh target genes transcription [184].

NOTCH and Hh pathway are involved in the same processes during fetal development and their collaboration can orchestrate proliferation, survival and differentiation ensuring the presence of all cell types needed for tissue-specific functions. To this, several evidences confirm the existence of an interaction between NOTCH and Hh during fetal development. For example, in mouse and zebrafish it has been demonstrated that NOTCH is able to partially compensate the loss of Indian Hh signaling through the increase of the formation of VE-cadherin^{+} cells [186]. In neural-progenitor differentiation, Hh signaling is controlled by the inhibition of transcription factor Gli1 through Numb-dependent ubiquitination [187], as well as neurogenesis of spinal cord is controlled through regulation, mediated by transcription factor downstream of Hh signaling, of JAGGED1 and DLL1 expression [188]. Moreover, Shh is able to regulate HES1 in different cell types, including cerebellar granul neurons, retina progenitor cells and mesodermal cells [189-191].

Since their involvement in important cell processes, the cross talk between these two pathways has been described in several cancers. For example, in hepatic stellate cells the activation of NOTCH pathway is able to stimulate epithelial-mesenchymal transition in combination with an active canonical Hh pathway [192]. Moreover, NOTCH pathway inhibition induced by GSIs treatments alters the expression of Hh target genes, including PTC. The Smo antagonist GDC-0449 reduces NOTCH2, HES1, HEY2 and HEYL expression [193].

2.5 NOTCH involvement in cancer disease

As previously described, NOTCH pathway is a conserved pathway involved in the regulation of very important aspects of cell biology such as differentiation, cell proliferation and apoptosis [71]. This pathway has a role in development, because it is involved in neurogenesis, gliogenesis, myogenesis, vasculogenesis and hematopoiesis due to its ability to regulate cell fate decision [70]. It is able to determine commitment of cells during tissue development (such as the commitment to B or T lineage) [72] and it is involved in the maintenance of adult tissue homeostasis, because of its ability to sustain stem cells population self-renewal [73]. All these processes are altered in cancer, so NOTCH pathway deregulation (both receptors and ligands) has a role in several cancer, such as T acute lymphoblastic

leukemia, B-lineage malignancies (B-cell chronic lymphocytic leukemia and B-cell acute lymphocytic leukemia; classical Hodgkin's lymphoma; diffuse large B-cell lymphoma; mantle cell lymphoma; multiple myeloma), myeloid malignancies (myeloproliferative neoplasia; acute myeloid leukemia) and solid tumors (breast carcinoma; prostate carcinoma; lung carcinoma; brain tumors; liver malignancies).

T-cell acute lymphoblastic leukemia is the first tumor associated to NOTCH deregulation due to translocation (less than 1% of cases) or mutations (more than 60% of cases). The t(7;9)(q34;q34.4) translocation places ICN1 under the transcriptional control of the regulative element of the TCRβ locus with the consequent overexpression of an active form of NOTCH1 [194]. More commons are activating mutations involving NOTCH1, which represent the main oncogenic lesions in T-ALL. There are two major "hot-spots" of mutation in the HD and PEST domains [195] and more than one hundred different mutations have been found in the HD, PEST and TAD domains [196]. These mutations have different effects, because mutations in HD induce ligand independent activation whereas mutations in PEST domain increase ICN stability [197] and mutation in TAD control the correct formation of NOTCH1/RBPj/MAML transcription complex [198]. However, most NOTCH1 mutations are not able to induce the disease alone, but they can accelerate T-ALL through the collaboration with other altered genes such as K-RAS (activating mutation) [199], or the oncosoppressor FBW7 (inactivating mutation) [200]. NOTCH1 has also a prognostic factor in T-ALL disease, in fact it is observed that the association of NOTCH1 and FBW7 activation induce a favorable early treatment response in pediatric trials (with an unclear outcome) [201]. It seems that JAGGED1 expression is associated to adverse prognosis in T-ALL patients [202]. Obviously, NOTCH1 is a promising target for T-ALL treatment, but cells with mutations in NOTCH pathway could overcome anti-NOTCH treatment because of their MYC or AKT up-regulation [157, 203].

The involvement of NOTCH deregulation in B-cell malignancies has been described in B-CLL (B-cell chronic lymphocytic leukemia), an incurable heterogenic leukemia typical of adults [204]. In B-CLL it has been described an overexpression of both receptors, NOTCH1 and NOTCH2, and ligands, JAGGED1 and JAGGED2, that induces a constitutively activation of NOTCH pathway [205]. Mutations in the PEST domain has been found in less than 10% of cases: these mutations generally cause frameshift mutations that induce stop codon and the disruption of the PEST domain. NOTCH1 can not be degradated and the accumulation of ICN1 altered this signaling pathway. It has not been found mutation in the

PEST-domain or HD region of NOTCH2 gene [206]. NOTCH1 mutations in B-CLL is an independent negative prognostic factor (it correlates with poor survival and aggressive form of the disease) and it is still unclear if mutations are primary events or the consequences of clonal selection [204]. Their frequency increase during progression disease (from 8% at diagnosis to ~30% in Richter transformation) and in refractory CLL (~20%). NOTCH1 has an important role in the cross-talk between malignant B-CLL cells and the surrounding microenvironment: the activation of NOTCH signaling in tumor cells, mediated by stromal cells, alters the expression of important genes (such as p53, NF-κB and BCL-2), directly promoting drug resistance to pharmacological treatments, and induces c-MYC expression that alters cells metabolism and represents malignant marker [207]. Targeting NOTCH pathway represents a promising mechanism to reduce cells growth and drug resistance and it can also destroy the protective interaction between neoplastic cells and stromal niche.

In B-ALL the role of NOTCH pathway is still unclear. B-ALL is malignancy occurred in immature B-cells precursor and it is the most common form of acute lymphoblastic leukemia [208]. B-ALL cells express NOTCH receptor but the pathway is not constitutively activate as it occurs in T-ALL disease and there have not been detected mutations in NOTCH pathway members [75]. In B-ALL cells, it has been described a hypermetilation of NOTCH3, JAGGED1 HES2, HES4 and HES5 and the exogenous expression of HES5 induces apoptosis and cell growth inhibition [209]. In these cells, it seems that NOTCH pathway has an oncosoppressive role.

Classical Hodgkin's lymphoma (cHL) represents 10% of all lymphomas and is one of the most curable cancer disease. Hodgkin and Reed-Sternberg (HRS) cells, deriving from mature B-cells and representing the clonal progeny of germinal center B cells, are the feature cells of the disease. These cells are known to express JAGGED1 and DLL1-3-4 and to have high levels of JAGGED2. Moreover, they have an aberrant expression of NOTCH1 and NOTCH2 and the NOTCH target genes HES7 and HEY1 [210].

The most common form of non-Hodgkin lymphoma is represented by diffuse-large B-cell lymphoma (DLBCL): it is characterized by biological and genetic heterogeneity that renders neoplastic cells resistant to common chemotherapic agents [211]. About 8% of patients carries mutations in the PEST domain and in the heterodimerization site of NOTCH2. In these cells, it seems that the activation of MYC induced by NOTCH signaling causes the downregulation of miR30-a, which finally induces the overexpression of NOTCH1 and NOTCH2 [212]. In

vitro experiments demonstrate that cells with constitutively expression of HES1 are sensible to γ-secretase inhibitors [213].

Mantle cell lymphoma represents about 5%-7% of all lymphoma and it is still incurable due the development of drug resistance and the onset of relapse [214]. NOTCH pathway is reported to be deregulated in this disease. In fact, the hypomethylation of NOTCH1 promoter induces the overexpression of *NOTCH1* gene [215]. Moreover, about 12% of patients carries a mutation in NOTCH1 that in the majority of cases are non-sense or frameshift mutations resulting in the truncation of the PEST domain [216]. Mutations in NOTCH1 are a negative prognostic factor associated to poor overall survival. There are some *in vitro* studies that evidentiate that MCL cells are sensible to γ-secretase inhibitors (NOTCH signaling withdrawal reduce cells proliferation and increase apoptosis) [216].

Myeloproliferative neoplasms (MPNs), composed by essential thrombocytosis (ET), polycythemia vera (PV) and myelofibrosis (MF), are non-malignant hematological neoplasms that could evolve to myelodysplastic syndrome or acute myeloid leukemia. MPNs generate from the transformation of hematopoietic stem cells (HSCs) that propagate as leukemic stem cells. There are several evidences that MPNs may be caused by the complete absence of NOTCH signaling pathway due to the deletion of Mindbomb (Mib1) [217], ADAM10 [218], Nicastrin [219]or Pofut1 [220].

In AML (Acute Myeloid Leukemia), NOTCH pathway may have both an oncogenic and an oncosoppressive role depending on the specific genetic landscape. In most cases, NOTCH pathway has an oncosoppressive role even if AML cells express both ligands and receptors on their surface and may autonomously activate NOTCH pathway. In fact, the overexpression of NOTCH1 an NOTCH2 in a mouse model causes apoptosis, disease regression and reduces leukemic-initiating cells. It has been reported that AML cells express both NOTCH1 and JAGGED1 protein, but they do not express HES1 (and so they have not constitutive canonical pathway activation) [75]. Other groups demonstrate that AML cells express also NOTCH2 receptor, without any evidence for the expression of NOTCH target genes indicating that the pathway is silenced [221]. In this disease, no mutations in NOTCH gene pathway have been found, so the deregulation of this pathway may be due to three different mechanisms: aberrant methylation patterns of NOTCH pathway genes such as LFNG, MAML3, and HES5 [222]; loss of MAML1 that causes the inability of ICN1 to induce the transcription of target genes; alternative splicing that produce splicing variants of NOTCH2 associated with low activation of NOTCH signaling [223]. There are some evidences that NOTCH pathway may have also

an oncogenic role in AML, in fact high expression level of NOTCH1 correlates with poor overall survival [224]. Activating mutations in NOTCH1 are rare event in AML occurring in stem cells during the molecular pathogenesis of the disease. It seems that the oncogenic role of NOTCH pathway is associated to specific genetic alteration characterizing AML subset of patients with acute megakaryocytic leukemia originated by the t(1;22)(p13;q13) translocation that gives origin to the OTT-MAL fusion protein that is able to activate RBPj independently of NOTCH signaling activation [225].

2.5.1 NOTCH in Multiple Myeloma

Several studies have highlighted the important role of NOTCH pathway in the biology of MM because of its ability to influence not only tumor cells, but also the surrounding microenvironment. Even if no mutations in NOTCH pathway genes have been found yet, the up-regulation of NOTCH signaling pathway has been described as a consequence of the translocations t(14;20)(q32;q11) and t(14;16)(q32;q23): the activation of the transcription factors MAFB and c-MAF induces the expression of NOTCH2 and the consequent up-regulation of its targets HES1-5-7 [226]. The up-regulation of NOTCH pathway, due to the overexpression of NOTCH1 and JAGGED1, has been described during the evolution from MGUS to MM phase, but the molecular mechanisms responsible for this deregulation are not clear [227]. The overexpression of JAGGED2 occurs during the initial stage of the disease and is caused by epigenetic events, such as the hypomethylation [228] or the deacetylation, due to the loss of the corepressor SMRT/NCoR2 [229], of JAGGED2 promoter. Moreover, JAGGED2 may be deregulated because of the over-expression of the ubiquitin ligase Skeletrophin, which promotes JAGGED2 internalization and the consequent NOTCH pathway activation [230]. The importance of JAGGED2 in the disease is highlighted also by the evidence that the ovexpression of Skeletrophin is associated to bone lesions [230] and that JAGGED2 is involved in the maintenance of self-renewal ability of MM stem cells [231].

The deregulation of both receptors and ligands favors homotypic interaction between MM cells that directly promotes tumor progression. In fact, it has been demonstrated that NOTCH signaling is able to control PCs homing and migration to the BM through the regulation of CXCR4/SDF1α chemochine axis [51]. Moreover, NOTCH pathway plays a key role in the regulation of several important cell processes: in fact, its blockade increases MM cell apoptosis and decreases cells proliferation [232]. Both *in vitro* and *in vivo* studies demonstrate also that NOTCH withdrawal increases tumor cells sensibility to chemoterapic agents (such as

Bcl-2/Bcl-xL inhibitors or standard drugs as Doxorubicin or Melphalan) [233] and reduces the development of drug resistance [232].

As previously described, in MM an important role is played by the ability of tumor cells to interact with the surrounding microenvironment. To this, the over-expression of JAGGED1 and JAGGED2 on MM cell surface induces the activation of NOTCH pathway in the neighboring normal cells, which finally favors the disease progression [234]. The activation of NOTCH pathway in BMSCs, mediated by JAGGED2 presented on MM cells surface, induces the secretion of several soluble factors (such as VEGF, IL-6 and IGF-1) that directly sustain tumor growth and survival [228]. NOTCH pathway is also able to promote the formation of bone lesions typical of the disease, because it induces and promotes osteoclast differentiation. In fact, the activation of NOTCH pathway in MM cells directly increases their production of the osteoclastogenic factor RANKL, whereas the activation of NOTCH2 receptors in the surrounding osteoclast precursors favors their differentiation [61].

All these evidences support the idea that NOTCH pathway plays an important role in the pathogenesis of MM, because of its ability to regulate tumor cell growth and stem cell maintenance, intrinsic drug resistance, and the migration of tumor cells to the bone marrow. More important is the ability of MM cells to influence the surrounding microenvironment: the deregulation of JAGGED ligands on MM cells improve the ability of BM cells to support tumor growth and osteolytic lesions. Even if more studies are necessary to better understand the molecular mechanisms causing NOTCH activation, current knowledge suggests that NOTCH may be an important target in MM, due to its ability to regulate both tumor cells and their interaction with the BM niche.

3 Interleukin-6 pathway

Interleukin-6 (IL-6) is a 26KDa molecular weight protein containing 185 amino acids. IL-6 is a cytokine that is involved in several cell processes, both physiological (such as immune and inflammatory responses and hematopoiesis) and pathological (Figure 3.1). In fact, in several inflammatory conditions or in different type of cancers there are high level of IL-6 in patient's serum. There are some cell types that constitutively produce IL-6 (such as endothelial cells, monocytes and fibroblasts) and other cell types that produce the cytokine if stimulated (such as macrophages, B and T lymphocytes, granulocytes, eosinophils, osteoblasts, keratinocytes and glial cells) [235].

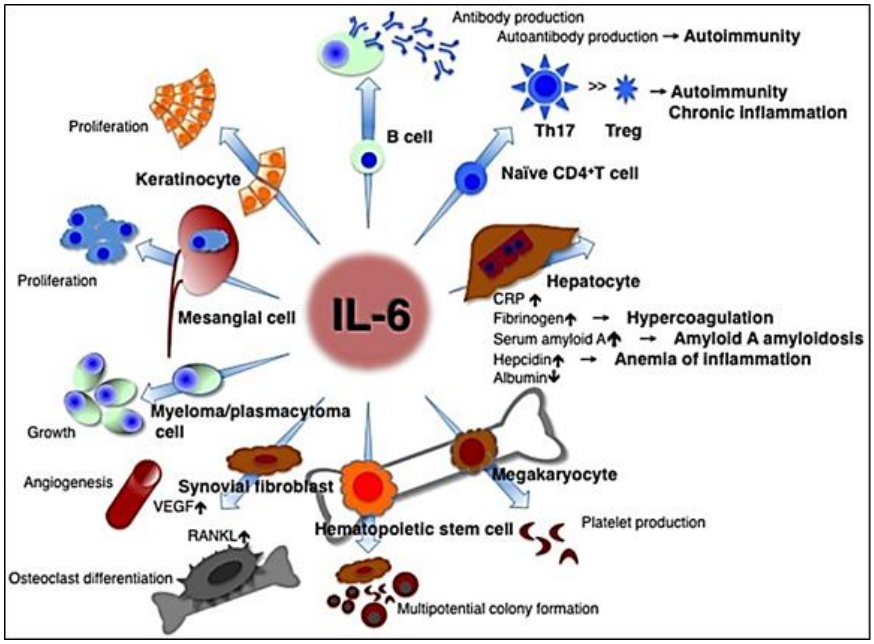

Figure 3.1. **IL-6 has pleiotropic effects and its deregulated and persistent production induce the development of autoimmune and chronic inflammatory diseases.** IL-6 was originally discovered as a stimulatory factor of B cells, it stimulates antibodies production by activated B lymphocytes, inducing their differentiation into PCs. In combination with TGF-β, it supports the differentiation of naïve $CD4^+$ T-lymphocytes toward Th17, inhibiting their differentiation into Treg cells induced by TGF-β. Consequently, the imbalance between Th17/Treg can induce the development of autoimmune and chronic inflammatory diseases. IL-6 also induces the production of acute phase proteins, such as C-reactive protein, fibrinogen, serum amyloid A protein, hepcidin, and reduces the synthesis of albumin in hepatocytes. Persistent high levels of serum amyloid A and hepcidin lead to amyloidosis, and anemia of chronic inflammation, respectively. In the BM, IL-6 induces the maturation of megakaryocytes into platelets and active hematopoietic stem cells; IL-6 also promotes osteoclast differentiation and angiogenesis, keratinocyte proliferation and proliferation of myeloma and plasmacytoma cells.

3.1 IL-6 signaling

IL-6 receptor is composed by two different subunits: α subunit, which determines the specificity for the ligand (called also IL-6R, GP80 or CD126), and β subunit (CD130), or glycoprotein GP130, responsible for binding affinity, which is shared by other cytokines belonging to the same family (eg. IL-11, LIF, oncostatin M). GP130 is ubiquitously express, while some cell types, such as liver cells, neutrophils, monocytes and B and T lymphocytes, specifically express GP80. It also exists a soluble form of IL-6R (called sIL6-R or GP55) that is obtained through GP80 cleavage by metalloproteinases belonging to ADAM family. sIL-6R acts as agonist, since it maintains the ability to bind IL-6 and the complex IL-6/sIL-6R is able to induce signal transduction [236].

IL-6 binding to IL-6R induces a cascade of cellular events that causes the activation of the Janus kinase (JAK) and of the signaling mediated by Ras (Figure 3.2). Once activated, JAK phosphorylates members of the transcription factor family STAT, particularly STAT3 (Signal Transducers and Activators of Transcription-3) and SHP2 (Src Homology-2 domain-containing Tyrosine Phosphatase). Phosphorylated STAT3 forms dimers that translocate to the nucleus where they can activate the transcription of genes containing specific response elements. This is essential for the correct action of GP130 signaling and mediates processes involved in cell survival and the transition from G1 to S phase of the cell cycle. Among transcriptional target of STAT3 it can be cited c-MYC and Pim, both involved in cell survival and proliferation [237].

SHP2 binds the pathway of IL-6 to that of the Ras/MAP kinases (Mitogen-Activated Protein), which acts upstream through SHC, GRB2 (Growth Factor Receptor Bound protein-2) and SOS1 (Son of Sevenless-1) and actives the MAP kinase downstream, with the final positive regulation of the transcription factors Elk1 and NF-IL-6 (C/EBP- β). These and other factors such as AP1 (Activating protein 1) and SRF (Serum Responde Factor) represent a convergence point for different signals activated by IL-6.

IL-6 also acts on the PI3K/AKT signaling pathway, which has the anti-apoptotic function to phosphorylate BAD (Bcl-2 Associated Death Promoter): phosphorylated BAD is captured by protein 14-3-3 that prevents its binding to Bcl-XL, which can perform its anti-apoptotic activity promoting cells survival. Through the action of STAT3, IL-6 is also able to induce the expression of the anti-apoptotic gene Bcl-2 in pro B-cells.

It exists a feedback mechanism to block IL-6 signaling mediated by the action of SOCS (Suppressor of Cytokine Signaling), PIAS (Protein Inhibitors of Activated STATs) and GP130 internalization (Figure 3.2).

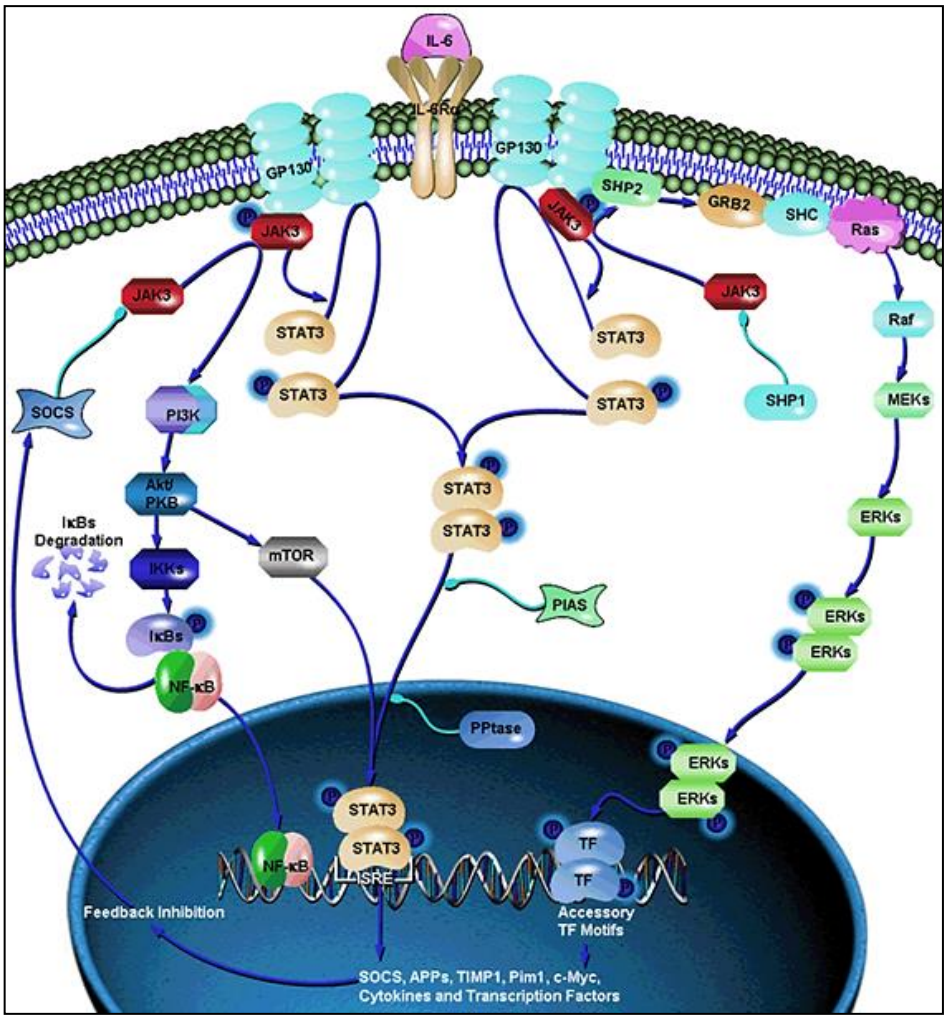

Figure 3.2 IL-6 signaling pathway

3.2 IL-6 and cancers

Several studies demonstrate that there is an increase of IL-6 during the later stages of tumor growth: in fact, elevated IL-6 serum levels are associated with active disease and negative prognosis in many tumors. Moreover, in several tumors it can be found also an increase of IL-6R expression [238]. Tumor microenvironment is composed by different cell types: high level of IL-6 in this microenvironment creates a suitable atmosphere that promotes tumor proliferation, survival, angiogenesis and metastasis since IL-6 can trigger several oncogenic factors.

There are several studies reporting that patients with different cancers (such as colon, breast, pancreatic, gastric, hepatic, esophageal, renal, cervical cancers and multiple myeloma) have significantly high level of IL-6 in the serum [239, 240]. The most studied cancer in which IL-6 plays an important role is breast cancer. In this tumor it is described that the activation of IL-6, IL-6R and GP130 complex (which is higher express in this cancer) induces the up-regulation of Bcl-2 and the down-regulation of BAX: this imbalance between these two

regulators of the apoptotic machine causes an inhibition of the apoptosis [241]. In a subgroup of breast cancer, in particular the most aggressive triple negative form (TNBC), the autocrine production of IL-6 can promote proliferation and growth of neoplastic cells [242]. To further sustain the important role of IL-6 in tumor cells, it has been described that the poorly tumorigenic human mammary MCF-7 cells express very low level of IL-6: benzanthracene (environment pollutant and carcinogen) can induce IL-6 production in these cells making theM highly tumorigenic [243]. It is also reported that the inhibition of IL-6 and its downstream signaling obtained with small molecules as diacerein and apratoxin can induce pro-apoptotic effect in cancer cells [244, 245].

Il-6 is involved also in the promotion of metastatic events. In breast cancer it is reported a positive correlation between the over-expression of IL-6, induced by the hyperactivation of NF-κB p65 members and AP-1 Fra-1/c-Jun transcription factors, and a very aggressive and metastatic behavior of cancer cells [246]. Furthermore, bone marrow mesenchymal stem cells promote bone metastatic invasion of ER positive breast cancers through the activation of STAT3 mediated by IL-6 [247].

Tumor microenvironment is composed also by various nonmalignant components such as fibroblast stromal cells and mesenchymal stem cells that create condition to favor angiogenesis, tumor growth and metastasis. In breast cancer, it seems that pro-tumor activity of fibroblast cells is mediated by IL-6, that is able to activate other oncogenic factors such as component of the NOTCH pathway (NOTCH3 and JAGGED1) and anhydrase IX [248]. In colon cancer, the overexpression of IL-6 induce the production of VEGF that acts on fibroblast cells creating favorable condition for angiogenesis: this finally promotes tumor invasion in colon cancer. The treatment of these cells with an IL-6R antibody significantly reduce stromal expression of IL-6 with the consequent reduction of angiogenesis and tumor growth [249]. The involvement of IL-6 in the development of endometrial cancer is described also in cervical cancer, where IL-6 is considered as one of the major risk factor for poor survival: in this neoplasia IL-6 sustains tumor proliferation and progression through the stimulation of ERK and NF-κB signaling pathways [250, 251]. In HPV-induced cervical cancer, HPV E6 protein activated IL-6/STAT3 signaling in cancers-associated fibroblast cells, inducing senescence. This finally activates an autocrine/paracrine IL-6 secretion mechanism that promotes suitable environment in favor of cervical cancer progression. IL-6 induces proliferation, invasion and adhesion also in ovarian cancer [252]. Also in hematologic cancer IL-6 plays an important role: in AML, IL-6 promotes tumor cells proliferation through the

activation of sonic hedgehog pathway (Shh) [253]. Modus operandi of IL-6 in different types of cancer development, metastasis and invasion has been presented in Table 1.2 [254].

Table 1.2. Role of IL-6 in cancer development, metastasis and invasion.

Type of cancer	Cell line/Cancer model	Molecular action of IL-6
Prostate cancer	LNCaP, C4-2, C81, CWR22Rv1	IL-6 secreted by endothelial cells promotes tumor metastasis through activating IL-6/Androgen receptor/TGF-β/MMP-9
Cervical cancer	Hela	Endothelial cells in tumor milieu secrets IL-6 which aids in tumor progression via STAT3 signaling
Hepatic carcinoma	In vivo mouse model	Obesity induced IL-6 promotes hepatocellular carcinoma through activation of oncogenic transcription factor STAT3
Hepatic carcinoma	Case control study in patient samples	Increased IL-6 serum level is well associated with risk factor of hepatic carcinoma
Endometrial cancer	-	Feedback stimulation of IL-6 induces aromatase expression and promoting endometrial cancer
Non small cell lung cancer	A549 and NCI-H358	IL-6 and TGF-β regulate the paracrine loop in between non small cell lung cancer and fibroblast cells.
Human Esophageal Squamous cell carcinoma	CE81T	IL-6 plays as a tumor promoting mediator in cancer microenvironment and promotes chemoresistance and EMT induction
Colon cancer	Azoxymethane (AOM) induced colon cancer model	IL-6 secreated by lamina propria myeloid cells promotes colitis associated cancer through regulating IL-6/STAT3/NF-kB signaling
Colon cancer	Azoxymethane (AOM) induced colon cancer in transgenic mice model	IL-6 mediated gp130/STAT3 promotes intestinal epithelial cell survival that lead to development of inflammatory colon cancer
Osteosarcoma cancer	Saos-2	IL-6 derived from mesenchymal stem cell (MSC) activates STAT3 inducing cellular proliferation
Gastric cancer	Patient sample	IL-6 mediated over expression of STAT3 involves in cancer progression
Glioblastoma	U251-MG and U87-MG	NF-κB induced IL-6 promotes aggressive behavior of cancer by STAT3 activation
Glioma	Human glioma xenograft cell lines 4910 and 5310	Interaction of MMP-2 and α5β1 activates IL-6 mediated STAT3 signaling in glioma cells
Pancreatic cancer	-	IL-6 promotes progression of pancreatic intraepithelial neoplasia and pancreatic ductal adenocarcinoma
Multiple myeloma	-	Bone marrow stromal cells secrete IL-6 and initiates multiple myeloma through APE1/Ref pathway
Melanoma	MMRU and SK-mel-3	Integrin-linked kinase promotes angiogenesis by activating p65 NF-kB and IL-6/STAT3 signaling
Chronic myelogenous leukemia (CML)	Mice model	BCR/ABL (a deregulated tyrosine kinase) mediated IL-6 promotes CML pathogenesis

3.2.1 IL-6 in Multiple Myeloma

IL-6 is one of the most important factors for MM cells growth and survival *in vivo* [255]. There are two different sources of IL-6 in MM that favor tumor growth and maintenance *in vivo*: one is represented by autocrine IL-6 derived from MM cells [256] and the other one is represented by paracrine IL-6 produced by non-malignant cells of the BM niche [56, 257]. BMSCs produce high levels of IL-6 that directly stimulates survival and proliferation of malignant PCs [255]. The importance of IL-6 in MM is supported by several evidences that show how high level of IL-6 in patient's serum

- correlates with tumor burden and disease severity [258, 259];
- promote MM progression through pleiotropic effects on survival, migration, cell proliferation and resistance to conventional drugs [49, 260];
- could represent a negative prognostic factor [261, 262].

To further enforce the role of IL-6 in the disease, an anti–IL-6 monoclonal antibody treatment is able to transiently reverse MM manifestations [263]. Furthermore, it has been shown that serum from MM patients have high level of sIL-6R [264].

3.3. NOTCH pathway and IL-6

The relationship between NOTCH and IL-6 is well documented in various pathological contexts. There are several data proposing different mechanisms to explain the interaction between these two pathways.

Some studies show direct relationship in which active NOTCH positively regulates the transcription of IL-6. For example, in breast cancer with bone metastases, Jagged1 is overexpressed and stimulates NOTCH signaling in bone marrow stromal cells. The consequent NOTCH activation increases expression and secretion of IL-6 by stromal cells that stimulates tumor growth and resistance to chemotherapy [265]. Recent studies report that active NOTCH directly controls maturation of osteoclasts, thereby increasing bone lesions [61]. Other studies on $CD34^+$ cells reveal that NOTCH signaling contributes to the maintenance of self-renewal ability of hematopoietic stem cells from cord blood through IL-6 production [266]. It has also been described that NOTCH1 is able to bind IL-6 promoter in the murine cell line RAW264.7 [267]. Furthermore, it has been demonstrated that NOTCH activates STAT3, which is involved in the induction of IL-6 and IL-6Rα transcription [268].

Other studies suggest that the interaction between these two pathways is mediated by the involvement of NK-κB pathway. In fact, the over-expression of IL-6 is induced by the activation of the non-canonical NOTCH signaling pathway mediated by IKKα and IKKβ (component of NF-κB cascade) [269].

The last mechanism of interaction between these two pathways has been described in the context of breast cancer in which it is demonstrated that, in MCF-7 cell line, IL-6 is able to induce the overexpression of NOTCH3 and JAGGED1 [270].

MATERIALS and METHODS

1. Cell cultures

Single cultures

The Human Multiple Myeloma Cell Lines (HMCLs) used were:

- CMA-03: this HMCL was obtained from peritoneal effusion from refractory multiple myeloma. Cells were cultured in presence of IL-6 10 ng/ml.
- CMA-03/06: this HMCL was obtained from CMA-03 cultured in the absence of IL-6 for four months.
- XG-1: this HMCL was obtained from patient with PCL. Cells express cytoplasmic Ig, CD38, CD77, CD28, CD28. These cells are IL-6 dependent and they grow with IL-6 1ng/ml.
- U266: this HMCL was established from the peripheral blood of a 53-year-old man with IgE-secreting myeloma (refractory, terminal) in 1968; cells were described to produce IgE; λ. Cells were negative for CD3, CD10, CD19 and CD20 and positive for CD138. They grow partially (loosely) adherent.
- OPM2: this cell line was established from the peripheral blood of a 56-year-old woman with multiple myeloma (IgG λ) in leukemic phase (relapse, terminal) in 1982. Cells were negative for CD3, CD10, CD80, CD19 and CD20 and positive for CD138. They grow in suspension.

Table 2.1. Cytogenetic characterization of HMCLs.

Cell line	t(4;14)_overexpression *MMSET/FGFR3*	t(11;14)_overexpression *CCND1*	t(6;14)_overexpression *CCND3*	t(8;14)_ overexpression of *c-myc*	t(14;16) or t(14;20) or t(20;?)_ overexpression of MAF/MAFB genes --> upregulation of NOTCH pathway due to overexpression of *NOTCH2* gene	del13q
CMA-03	-	-	-	+	+	+
CMA-03/06	-	-	-	+	+	+
XG-1	ND	+	ND	+	ND	+
U266	-	+	-	-	-	-
OPM2	+	-	-	+	+	-

All HMCLs were cultured in RPMI1640 (Gibco, Thermo Fisher) with 10% FBS (Gibco, Thermo fisher). CMA-03 and XG-1 cells were added with IL-6 10ng/ml and 1ng/ml respectively (R&D System, Minneapolis, MN, USA). Cells were cultured in 5% CO_2 at 37°C, maintaining the optimum concentration at $3x10^5$cells/ml with complete change of medium every two days.

It was used fibroblast cell lines as mimetic of bone marrow stromal cell (BMSC). These cell lines were:

- NIH3T3: this is a cell line of mouse embryonic fibroblasts isolated in 1962 at the New York University School of Medicine Department of Pathology. They grow adherent.
- HS5: this is a human fibroblast cell line derived from bone marrow stroma isolated in a Caucasian male of 30 years.

NIH3T3 and HS5 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS (Gibco, USA), 2 mM L-glutamine (Invitrogen Corporation Carlsbad, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells have a doubling time of 18-20h and were divided three times/ week.

HS5 cells were transduced with pGIPZ vector (Thermo Scientific) in order to obtain a GFP-positive cell line (GFP^+ HS5). Lentiviral supernatant was produced following the manufacturer's instruction. 3×10^5 HS5 cells were plated in a 6-well tissue culture plate with 1ml of lentiviral supernatant and 4µg/ml of polybrene (Sigma-Aldrich). After the infection cells were selected using 1µg/ml puromycin (Sigma-Aldrich).

Primary MM cells were isolated from aspirates of BM patient: $CD138^+$ malignant PCs were purified using the Human Whole Blood $CD138^+$ Selection Kit EasySep (StemCell Technologies). Primary BMSCs were isolated as previously reported by Garayoa *et al* [271]. Briefly, mononuclear cells from BM aspirates were obtained after density gradient centrifugation using Ficoll-Paque Premium 1.073 (Sigma Aldrich, USA) and cultured in DMEM supplemented with 10% FBS for 3-4 days. Subsequently non-adherent cells were removed, whereas stromal cells were selected by their adherence to plastic ware.

Co-culture of MM-BMSC cells

For co-culture experiments, MM cell lines were seeded in a 24-well plate at a density of 3×10^5 cells/ml on a monolayer of GFP^+ HS5 or NIH3T3 cells (70-80% confluence) and cultured for 2 days. For primary cell co-cultures, BMSCs, isolated as described above, were stained with the fluorescent dye PKH26 (Sigma-Aldrich) and allowed to adhere for 3 hours. $CD138^+$ cells were seeded on the monolayer of PKH^+ BMSCs (50-60% confluence) and cultured for 4 days in the presence/absence of DAPT (γ-secretase inhibitor).

2. Treatments

2.1 NOTCH pathway inhibition

NOTCH pathway was inhibited with γ-secretase inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester, Chalbiochem), also known as

GSI-IX (Figure 2.1). Cells from CMA-03/06, OPM2 and U266 cell lines were plated at a concentration of 300000/ml and treated for 96h with 50µM DAPT with or without 10ng/ml of IL-6. After 48h cells were counted and re-suspended at a concentration of 300000/ml with the somministration of new DAPT and IL-6; after 96h cells were counted and collected for RNA extraction. DMSO and BSA were used as control. Co-culture of primary cells were treated for 96h with or without 25µM DAPT that was re-somministered after 48h.

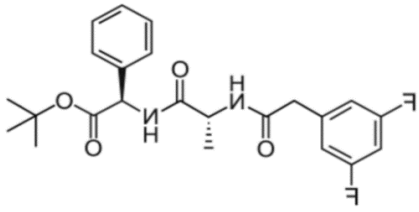

Figure 2.1. Molecular structure of DAPT.

2.2 NOTCH pathway activation

Notch pathway was activated in HMCL cells through the somministration of JAGGED1 peptide (AnaSpec Inc. USA). IL-6 dependent cell lines CMA-03 and XG-1 was treated with 5 µg/ml for 96h, in the presence or absence of IL-6 (10ng/ml or 1ng/ml, respectively). MilliQ water or BSA were used as control. Cells were plated at a concentration of 400000/ml at time 0. After 48h cells were counted and re-suspended at a concentration of 400000/ml with the somministration of new JAGGED1 peptide and IL-6; after 96h cells were counted and collected for RNA extraction.

3. Gene expression analysis

3.1 RNA extraction

Total RNA was isolated using TRIZOL reagent (Thermo Fisher). Protocol is the following:

- Harvest cells by centrifugation and remove media.
- Add 1mL of TRIzol® Reagent per 5–10 × 106 cells
- Lyse cells in sample by pipetting up and down several times.
- Incubate the homogenized sample for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex.
- Add 0.2mL of chloroform per 1 mL of TRIzol® Reagent used for homogenization.
- Shake tube vigorously by hand for 15 seconds.
- Incubate for 2–3 minutes at room temperature.
- Centrifuge the sample at 12000g for 15 minutes at 4°C. Note: The mixture separates into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA

remains exclusively in the aqueous phase. The upper aqueous phase is ~50% of the total volume.

- Remove the aqueous phase of the sample.
- Place the aqueous phase into a new tube.
- Add 0.5mL of 100% isopropanol to the aqueous phase, per 1mL of TRIzol® Reagent used for homogenization. -Incubate at room temperature for 10 minutes.
- Centrifuge at 12000g for 10 minutes at 4°C.
- Remove the supernatant from the tube, leaving only the RNA pellet.
- Wash the pellet, with 1mL of 75% ethanol per 1mL of TRIzol® Reagent used in the initial homogenization.
- Vortex the sample briefly, then centrifuge the tube at 7500g for 5 minutes at 4°C. Discard the wash.
- Vacuum or air dry the RNA pellet for 5–10 minutes. Do not dry the pellet by vacuum centrifuge.
- Re-suspend the RNA pellet in RNase-free water by passing the solution up and down several times through a pipette tip.
- Incubate in a water bath or heat block set at 55–60°C for 10–15 minutes.
- Proceed to downstream application, or store at –70°C.

3.2 RNA quantification

RNA was quantified by Nanodrop (spectrophotometric measure), using 1 µl of RNA. Absorbance was reading at two different wavelengths, at 260 nm (A1) and 280nm (A2). High quality RNA was used (A1/A2 ratio closed to 2).

3.3 Reverse transcription

cDNA was obtained by reverse transcription with RevertAid M-MuLV Reverse Transcriptase (ThermoScientific).

The reaction (20µl) was prepared as follow:

- 2µg RNA
- 1µl of Random primers (250ng/µl)
- H2O DEPC up to 10µl
- Sample was heated at 65°C for 5’.

The mix was prepared as follow:

- 4µl of 5x RT Buffer
- 4µl dNTPs 10mM (2.5mM each)

- 1µl of RevertAid M-MuLV Reverse Transcriptase (200U/µl)
- 1µl of H2O DEPC

The mix was added to the reaction composed by RNA and Random primers.

Sample was incubated 42°C for 60 minutes and finally stored at –20°C.

3.4 Quantitative RT-PCR

Quantitative PCR reactions were carried out on a Step-One Plus PCR system (Applied Biosystems, Life Technologies Italia, Italy) using the Maxima SYBR Green/ROX qPCR Master Mix (2X) (ThermoFisher Scientific).

The reaction, for a final volume of 15µl for a 96-well plate, is the following:

- 7,5µl Maxima SYBR Green Master Mix (2X)
- 2µl Primer Mix (final concentration 0,45µM)
- 2µl cDNA 5ng/µl (10ng total)
- 3,5µl H2O RNasi-free

The following program was used:

- Hot start activation: 95°C for 10’
- Denaturation
- Annealing } 40 cycles
- Dissociation

95°C for 15”
60°C for 60”

95°C for 15”
60°C for 15”
85°C for 15”

Each sample was analysed in triplicate with no template controls. Results were calculated with $2^{-\Delta\Delta Ct}$ method, and the copy numbers of the analysed mRNA were normalized using GAPDH mRNA levels. Primer sequences used for cDNA amplification are displayed in Table 2.2.

RT-qPCR primers	**Forward Primer 5’-3’**	**Reverse Primer 5’-3’**
mGAPDH	TTGGCCGTATTGGGCGCCTG	CACCCTTCAAGTGGGCCCCG
mHES5	GGCTCACCCCAGCCCGTAGA	TCGTGCCCACATGCACCCAC

mIL-6	TGAACAACGATGATGCACTTGCAGA	TCTCTGAAGGACTCTGGCTTTGTCT
hGAPDH	ACAGTCAGCCGCATCTTCTT	AATGGAGGGGTCATTGATGG
hNOTCH1	GGCGGGAAGTGTGAAGCGGC	GTGGCATGTCCCGGCGTTCT
hNOTCH2	AGACCATTTTGCCAATCGAG	GTGCTTCAGGCTGAGGAAAG
hNOTCH3	GTTCATGCATTGACCTCGTG	AGCGCAAACCAGTGTATCCT
hNOTCH4	GAGGAAGAAGAGGGGCAGTG	ACAGGGTTCTGGGAAACTCC
hHES1	GATGCTCTGAAGAAAGATAGC	GTGCGCACCTCGGTATTAAC
hHES6	ATGAGGACGGCTGGGAGA	ACCGTCAGCTCCAGCACTT
hHES5	GTGGAGAAGATGCGCCGCGA	AGCGTCAGGAACTGCACGGC
hJAGGED1	TTCGCCTGGCCGAGGTCCTAT	GCCCGTGTTCTGCTTCAGCGT
hJAGGED2	CCGGCCCCGCAACGACTTTT	CCTCCCTTGCCAGCCGTAGC
hDLL1	GGTGGGCAGGTACAGGAGTA	TATCCGCTATCCAGGCTGTC
hDLL3	TCACCTCCAATCTGGTCTCC	TCCCAGAATTTCAAACCCAA
hDLL4	CCTGTCCACTTTCTTCTCGC	ACTACTGCACCCACCACTCC
hIL-6	TTCAATGAGGAGACTTGCCTGGTGA	TCTGCACAGCTCTGGCTTGGTTCC

Table 2.2. Primer for qRT-PCR sequences.

3.5 Gene Expression Profiling (GEP) analysis

Highly purified PC samples (CD138 ≥ 90%) from the BM of 129 MM and 24 primary plasma cell leukemia (pPCL) patients at onset, together with 4 healthy donors (N) samples, were previously profiled on the GeneChip Human Gene 1.0 ST array (Affymetrix, Santa Clara, CA) [28]. Main molecular genomic aberrations (IGH translocations, hyperdiploidy, del(13q), del(17p), and 1q gain) were investigated in all samples by fluorescence in situ hybridization (FISH), as previously described [272] and already reported for the entire sample dataset [28]. Multiple myeloma samples were stratified in five groups according to the translocation/cyclin D (TC) classification [17, 273]. Gene expression profiling data were generated as described [274], using Brainarray annotation procedure [275, 276]. The GEP data have been deposited in the NCBI Gene Expression Omnibus database (GEO; http//www.ncbi.nlm.nih.gov/geo; accession No. GSE66293, GSE73452). The Institutional Review Board of Fondazione IRCCS

Policlinico Ca' Granda, Milano, Italy, approved the design of this study. Written informed consent was obtained in accordance with the Declaration of Helsinki.

4. Western Blot

Whole cell extracts were prepared using a RIPA lysis buffer (containing 50mM Tris-HCl, pH 7.4, 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1mM EGTA, 1mM EDTA) added with added with proteases and phosphatases inhibitors (ThermoScientific) and 50 mM NaF. After incubation on ice for 15 min, the lysate was centrifugated for 10 min at 4°C. Protein concentration was determined using the Bradford assay (Bio-Rad Laboratories). Protein samples (40 µg) were run on a 4-12% NuPAGE gel (ThermoScientific), transferred onto a nitrocellulose membrane (Biorad), and blocked with 5% non-fat milk in TBS-T (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% Tween-20). Membranes were incubated overnight at 4 °C with rabbit anti-NOTCH2 (1:1000, Cell Signaling), rabbit anti-HES1 (1:1000, Abcam) or rabbit anti-β-actin (1:5000, Sigma), and then with the appropriated HRP-conjugated species-specific secondary antibody (Pierce Protein Biology). Detection was performed by ECL (Biorad) according to the manufacturer's instructions. The signal was detected and analyzed by ChemiDoc MP System (Biorad).

5. RNA interference

In order to selectively inhibit NOTCH signaling in the HMCLs XG-1, OPM2 and U266 there were designed a specific siRNA for the NOTCH ligands JAGGED1 and JAGGED2. In order to discount any change in gene expression profile due to delivery method, a "scrambled" siRNA negative control was used (SCR). Positive control is represented by cells treated with fluorescent siRNA "BLOCK-IT" (Life Technologies). To this issue, Stealth Select RNAi^{TM} siRNA system (Life Technologies Italia, Milan, Italy) was used according to the Manufacturer's guidelines.

Specific anti-JAG siRNAs were delivered following these steps:

- Cells were plated at $3x10^5$/ml in RPMI1640 without antibiotics;
- 24h later, cells were diluted to $3,6x10^5$/ml in medium without antibiotics and plated in 0,5 ml of final volume;

- siRNAs (25nM anti-JAG1+25nM anti-JAG2/or 50nM scrambled siRNA/or 50nM fluorescent dsRNA) were diluted in 50µl of Opti-MEM medium (Invitrogen, Life Technologies Italia, Milan, Italy) without serum and antibiotics;
- 1µl of RNAi-MAX lipofectamine transfecting reagent (Invitrogen, Life Technologies Italia, Milan, Italy) was diluted in 50µl of Opti-MEM medium without serum and antibiotics;
- solutions (siRNA/lipofectamine) were mixed and incubated for 20' at room temperature;
- 100µl of lipofectamine/siRNA mix was added to the cells (final cells concentration $3x10^5$/ml);
- After 48h cells were diluted 1:1 with medium antibiotics-free and treated again with JAG1/JAG2 siRNA
- Cells were maintained in RPMI 1640 medium (Sigma-Aldrich Co., St Louis, MO) supplemented with 10% (v/v) FBS (Gibco, Rockville, MD) and 2 mM L-glutamine (Invitrogen Corporation Carlsbad, CA, USA) without antibiotics and incubated in 5% CO_2 at 37°C for 96h.

In order to confirm that the transfection occurred, the percentage of BLOCK-IT positive cells were analyzed through flow cytometry at each time point. 10000 cells were acquired with Beckman Coulter analyzer using FL2 bandpass filter (λex=488nm; λem=575nm) for BLOCK-IT fluorophore conjugated sdRNA. JAG1 and JAG2 effective silencing was confirmed both at RNA level (by quantitative PCR) and protein level (by Western blot), compared to scrambled siRNA-receiving cells.

The same protocol was used for silencing of NOTCH1 (in the HS5 cell line) and IL-6 (in HS5 and U266 cell lines).

6. Luciferase reporter assay

The pIL6-651 plasmid carrying *IL-6* promoter [277, 278] was a kind gift of Dr. Norifumi Takeda. The thymidine kinase promoter-driven Renilla luciferase (TK-pRL; Promega Italia s.r.l., Milano, Italy) was used for normalization; pcDNA3.1 mock plasmid was from Invitrogen (Invitrogen Life Technologies Italia, Italy). The plasmids carrying ICN1 and ICN2 were as previously described [73, 279]. U266 cells were harvested and re-suspended in RPMI1640 without antibiotics at a concentration of 10^7/ml and 10µg of DNA were used for electroporation in U266 cells. Electroporation was performed using 250 V and 950 µF. 10µg of total DNA were transfected in NIH3T3 cells using TurboFect Transfection Reagents (Thermo Fisher Scientific). All analyses were performed 48h after transfection using Dual-

Luciferase Reporter Assay System (Promega Italia s.r.l., Milano, Italy) according to the manufacturer's directions.

7. Flow cytometry analysis

7.1 Apoptosis assay

Apoptosis assay was performed with BD FACSVerse™ System (BD Biosciences, USA). 300000 cells were washed with cold PBS 1X and were re-suspended in binding buffer 1X. Cells were incubated with Annexin V-FITC (Immunotools) and Propidium Iodide (2.5 mg / ml final, Sigma-Aldrich Co.) for 15' at room temperature in the dark; after this incubation 400µL of binding buffer were added to the samples. 10000 events were acquired using FL1 and FL3 bandpass filter for Annexin V-FITC (λex=488nm; λem=520nm) and propidium iodide (λex=488nm; λem=617nm). Data were analyzed using flow cytometry analysis program "Cytomics FC500" (Beckman Coulter).

Binding Buffer 1X

- 0,01M HEPES
- 0,14M NaCl
- 2,5mM CaCl2
- H2O

7.2 Cell cycle assay

For cell cycle detection, cells were washed with cold PBS 1X and resuspended in "GM Buffer" 1X (glucose 1mg/ml, EDTA 0,2mg/ml, 2% FBS in PBS). Cells were then fixed by adding Ethanol (70% final) and incubated O.N. at 4°C. Then, samples were washed in PBS 5% FBS and incubated in Staining Buffer (RNAse 25µg/ml (Applichem), Propidium Iodide (Sigma Aldrich) 25µg/ml, NP-40 (Sigma Aldrich) 0,004% in PBS) O.N. at 4°C. Cells were processed and analyzed using the BD FACSVerse™ System (BD Biosciences).

7.3 Intracellular IL-6 detection

To quantitative evaluate IL-6 at intracellular level, cells were stained with specific anti human IL-6 antibody (eBioscience Inc, USA) or isotype matched control.

100000 cells were labeled in the following way:

- pellet cells and discard surnatant
- re-suspend pellet in 100µL of PBS 1% BSA 0.1% NaN3

- add 100µL of 4% formaldehyde in PBS and incubate 20 'at RT
- centrifuge at 1500g for 5' and discard the supernatant
- resuspend pellet in 100µL of PBS 0.5% BSA 0.5% saponin and incubate 10'at RT
- centrifuge at 1500g for 5' and discard the supernatant
- re-suspend pellet in 30µL of PBS 0.5% BSA 0.5% saponin
- divided sample into two tubes of 15µl each: add in the first tube the anti-human IL-6 antibody (4µl of the stock solution) and in the second tube the same amount of isotype control
- incubate 1hour in the dark at 4°C
- wash pellet with 100µL of PBS 0.1% NaN3
- re-suspend pellet into 300µL of PBS 0.1% NaN3 and read by flow cytometry

8. Immunohistochemistry (IHC)

BM biopsies from 21 MM patients were analyzed. All cases under study had a κ light chain phenotype of the neoplastic component; histopathological diagnosis was carried out according to the WHO classification; tumour stage (extent of BM infiltration by myeloma cells) was evaluated as follows: stage I: less than 20% (7 cases), stage II: 20-50% (7 cases), stage III: >50% (7 cases). Briefly, three-µm thick tissue sections were used for immunohistochemically (IHC) studies using a standard avidin-biotin-peroxidase complex technique. Sections were dewaxed, rehydrated in xylene-alcohol; antigen retrieval was performed with buffered citrate (pH 6) at 97C° for 35 min and endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. Serial sections were incubated with the primary antibodies against the following targets: HES6 (polyclonal Abcam; 1:300), IL-6 (monoclonal mouse Ab, Santacruz; 1:300). Monoclonal neoplastic component was visualized by double IHC, with κ light chain (polyclonal rabbit, DAKO Cytomation; 1: 20000). HES6 and IL-6 reaction was detected in brown with the autostainer (480 Bioptical instruments Thermoscinetific) by Novolink Max polymer detection system (Leica Microsystem, Italy), after incubation with diaminobenzidine (Novolink-NOVOCASTRA); κ-light chain was visualized in red (Ultravision Quanto Detection System AP polymer, Thermoscientific); sections were then counterstained with hematoxylin. Negative control slides were processed without primary antibody. IHC examination of the slides was performed independently by two pathologists; in case of discordant grouping attribution, slides were re-evaluated and discussed till inter-observer concordance. Images were acquired with the NanoZoomer-XR C12000 series (HAMAMATSU PHOTONICS K.K.).

9. Statistical analysis

Data are represented as means ± SD from at least 3 independent experiments. Statistical analyses were performed as follows: for single culture and co-culture experiments using MM and BMSC cell lines we used two-tailed Student's t-test to compare the means of normally distributed values and analysis of variance was performed by one-way ANOVA with Tukey's post-test. For co-culture experiments on human primary cells one-way ANOVA with Bonferroni post-test was performed.

In GEP experiments Kruskal-Wallis test was applied to measure the differential expression of the selected genes between groups, Dunn's test was exploited to perform nonparametric pairwise multiple comparisons between the independent groups and Benjamini-Hochberg correction was applied to adjust significance of multiple testing.

RESULTS

1. Gene expression profiling (GEP) analysis on a cohort of MM patients

Transcript expression level of NOTCH signaling members have been analyzed in highly purified $CD138^+$ PCs from MM patients. To this end, we investigated a proprietary data set composed by 129 MM, 24 pPCL patients and 4 normal controls profiled on gene expression microarray chip 1.0 Affymetrix. This analysis reveals that *JAGGED1* and the NOTCH target gene *HES5* are overexpressed in the different types of PC dyscrasias compared to normal controls, highlighting a higher level in primary PCLs (Figure 3.1).

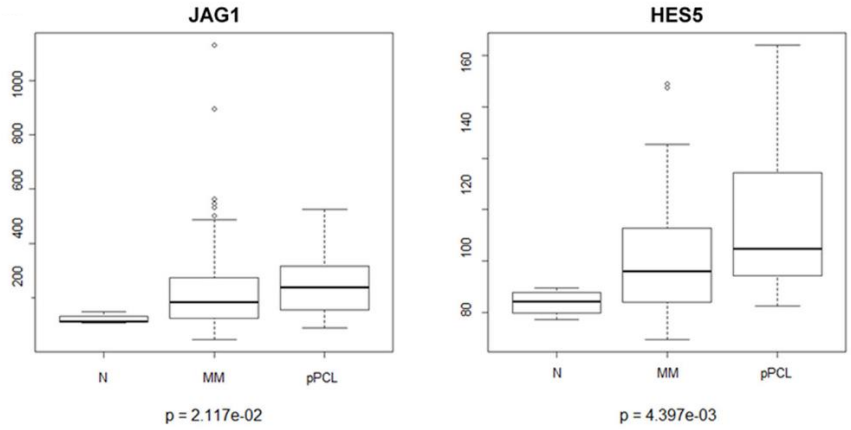

Figure 3.1. *JAGGED1* and *HES5* are overexpressed during MM progression. Microarray expression levels of genes belonging to the NOTCH pathway in purified plasma cells (PCs) from normal controls (N), multiple myeloma (MM), and primary plasma cell leukemia (pPCL) samples. The absolute expression levels (linear scale) of *JAGGED1* and *HES5* transcripts have been assessed by means of microarray analysis. Box plot represents messenger RNA expression levels in PC samples from 4 healthy donors (N), 129 MM and 24 pPCL patients. Kruskal-Wallis test has been applied to assess statistical significance of differential gene expression profiles between all PC dyscrasias. Dunn's test has been performed for nonparametric pairwise multiple comparisons between all analyzed groups in PC dyscrasia and the Benjamini-Hochberg correction has been applied for multiple comparisons. Statistical analysis indicates a significant p-value between N and pPCL (p= 0.0211) for *JAGGED1*; N and pPCL (p= 0.0071), MM and pPCL (p= 0.0043) for *HES5*.

Furthermore, the expression patterns of NOTCH signaling members have been analyzed in MM cases classified according to TC classification. They included 33 TC1, 28 TC2, 38 TC3, 19 TC4 and 6 TC5 cases. Notably, the six TC5 patients had a significantly increased expression of *NOTCH2* gene (Figure 3.2). TC5 patients also showed a significantly higher expression of *HES5* and *HES6* compared to other TC classes (Figure 3.2). All GEP data have been validated by qRT-PCR (correlation plots are represented in Figure 3.3).

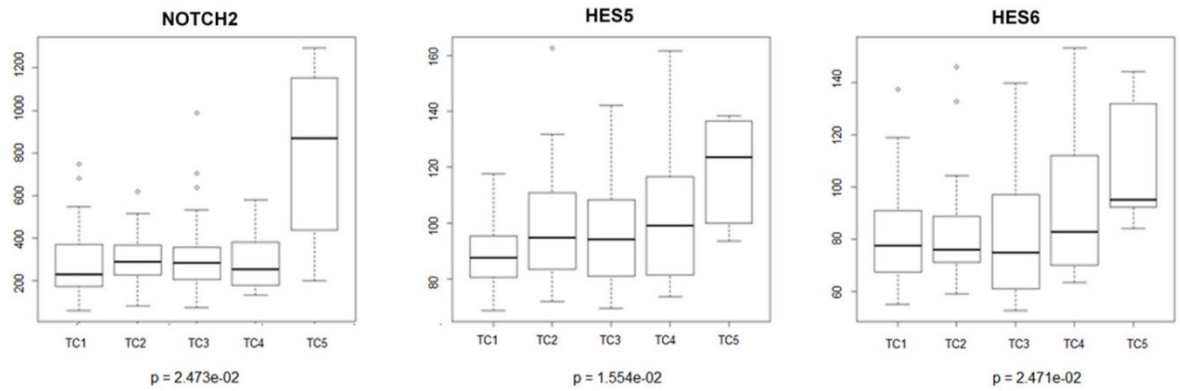

Figure 3.2. *NOTCH2, HES5* and *HES6* are overexpressed in the high-risk group TC5 multiple myeloma patients. The absolute expression levels (linear scale) of *NOTCH2*, *HES5* and *HES6* transcripts have been assessed by means of microarray analysis. Box plot representations of messenger RNA expression levels in 129 MM samples stratified according to TC classification (33 TC1, 28 TC2, 38 TC3,19 TC4 and 6 TC5). Kruskal-Wallis test has been applied to assess statistical significance of differential gene expression profiles between MM TC classes, respectively. Dunn's test has been performed for nonparametric pairwise multiple comparisons between all analyzed MM samples datasets and the Benjamini-Hochberg correction has been applied for multiple comparisons. Statistical analysis indicates a significant p-value between TC5 and the other TC classes (TC1 p=0.0068, TC2 p=0.0215; TC3 p=0.0150; TC4 p=0.0112) for *NOTCH2*; TC5 and TC1(p=0.0056) for *HES5*; TC5 and TC3 (p=0.0239) for *HES6*, respectively.

Since patients belonging to the TC5 subgroup or affected by pPCL are characterized by the presence of highly proliferative and aggressive tumor cells and they show less dependency from signal provided by the BM, such as IL-6, it could be hypothesized that hyperactive NOTCH signaling may participate in MM progression by compensating or activating IL-6 proliferative signaling.

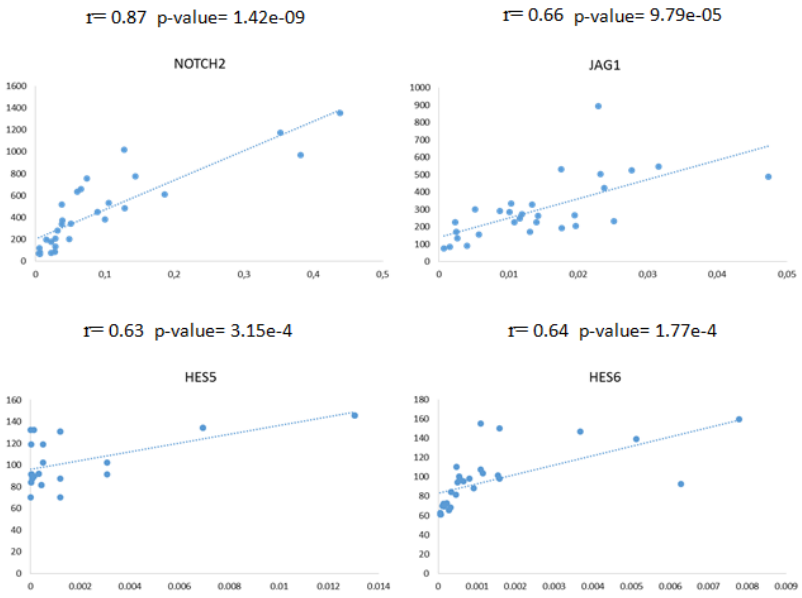

Figure 3.3. Quantitative RT-PCR validation of GEP data for NOTCH genes pathway in 30 MM cases. *NOTCH2*, *JAG1*, *HES5* and *HES6* expression validation; Pearson's correlation coefficient (r) was calculated between GEP data (y-axis) and quantitative RT-PCR results (x-axis) expressed as $2^{-\Delta\Delta Ct}$. Significant positive correlation between GEP and qRT-PCR data are evidenced by Pearson's coefficient r>0.60 with a p-value<0.05.

2. IL-6 independence is a consequence of NOTCH pathway activity

In order to confirm the hypothesis that MM cells can develop compensatory mechanisms to acquire IL-6 independence, the expression level of NOTCH signaling pathway genes has been analyzed in the HMCLs CMA-03 and CMA-03/06 previously established in our laboratory that may represent a model of IL-6 independence acquisition in MM [280]. This model is composed by the parental cell line CMA-03, which needs IL-6 for its growth, and CMA-03/06 cell line, which is IL-6 independent and un-responsive to the cytokine. CMA-03/06 cells were obtained from CMA-03 after 4 months of culture with decreasing concentration of IL-6.

We reasoned that the acquisition of IL-6 independence could be due to the activation of NOTCH signaling pathway. More specifically, the progression from CMA-03 to CMA-03/06 should be caused by NOTCH signaling activation and should be reverted by NOTCH inhibition. On the other hand, CMA-03 dependence to IL-6 could be reverted by NOTCH pathway upregulation.

Firstly, the expression level and activity of NOTCH signaling members in CMA-03 and CMA-03/06 has been analyzed by qRT-PCR. Notably, there are significant differences between CMA-03 and CMA-03/06 in the expression level of both NOTCH receptors (NOTCH1-2-3-4), ligands (JAGGED1-2 and DLL1,3,4) and transcriptional target genes (HES1 and HES6), which are used as a measure of NOTCH activation (Figure 3.4), since the IL-6 independent cells express higher level of all NOTCH signaling members.

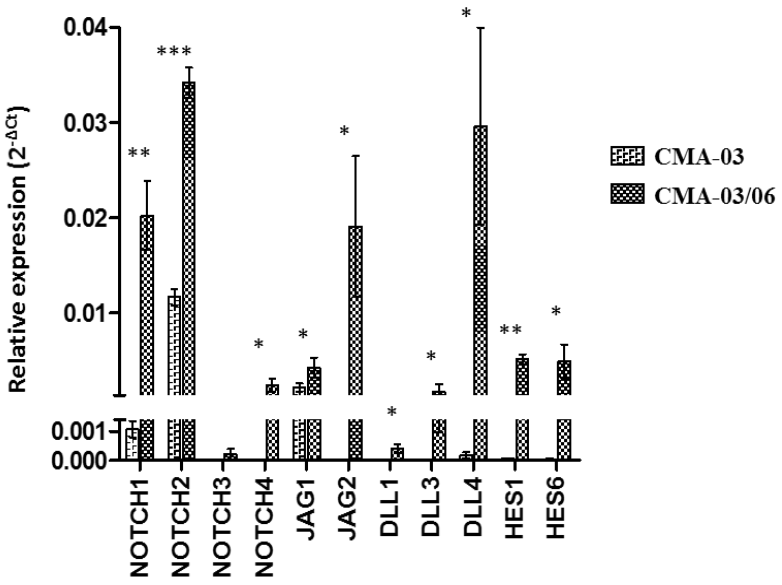

Figure 3.4. NOTCH pathways members are up-regulated during the acquisition of IL-6 independence in MM cells. The expression levels of NOTCH receptors (*NOTCH1-4*), NOTCH ligands (*JAGGED1-2* and *DLL1,3,4*) and NOTCH transcriptional target genes, *HES1* and *HES6*, have been assessed in the two cell lines, CMA-03 and CMA-03/06, by qRT-PCR. Results have been calculated by the $2^{-\Delta Ct}$ formula and SDs were calculated from 3 independent experiments. Statistical analysis has been performed using Student's t-test: *=p<0.05;**= p<0.01;***= p<0.001.

NOTCH signaling activation has been confirmed also at protein level. We investigated by Western Blot (Figure 3.5) the expression of NOTCH2, both the transmembrane (TM) and the active intracellular (IC) form, and the NOTCH target gene HES1. As shown in the figure, these results indicate that the acquisition of IL-6 independency is associated with a significant increase in NOTCH pathway activity.

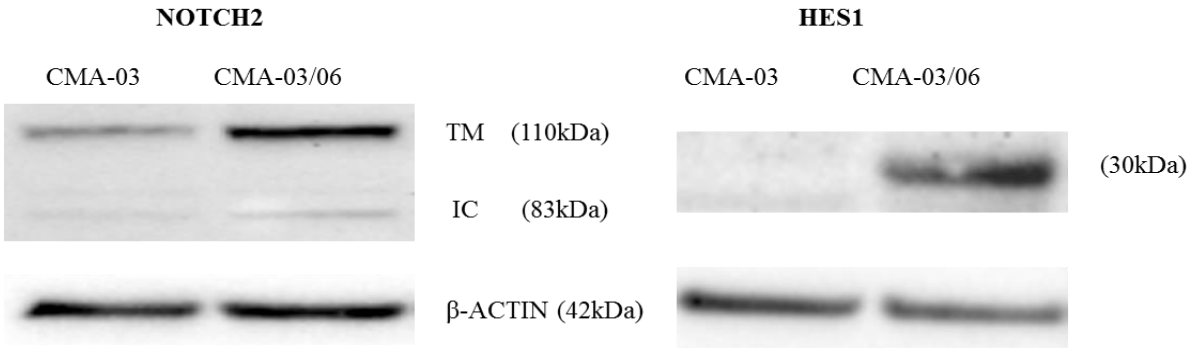

Figure 3.5. NOTCH2 and HES1 are overexpressed during the progression from the IL-6 dependent cell line CMA-03 to the IL-6 independent CMA-03/06 cell line. Protein expression level of transmembrane (TM) and active intracellular (IC) NOTCH2 and HES1 in CMA-03 and CMA-03/06. NOTCH2 and HES1 are overexpressed also at protein level in CMA-03/06 compared to CMA-03. Representative results of three independent experiments.

In order to further confirm the hypothesis that IL-6 independent MM cells need active NOTCH pathway to maintain their independence, NOTCH pathway has been inhibited in three different HMCLs using the γ-Secretase inhibitor DAPT. To this aim, CMA-03/06, OPM2 and U266 cells have been treated for 96h with 50μM DAPT, 10ng/ml IL-6, or a combination of both compounds; the respective vehicles, DMSO and BSA, have been used as controls. NOTCH pathway blockade was confirmed by analyzing the expression level of the NOTCH target gene *HES6* by qRT-PCR (Figure 3.6).

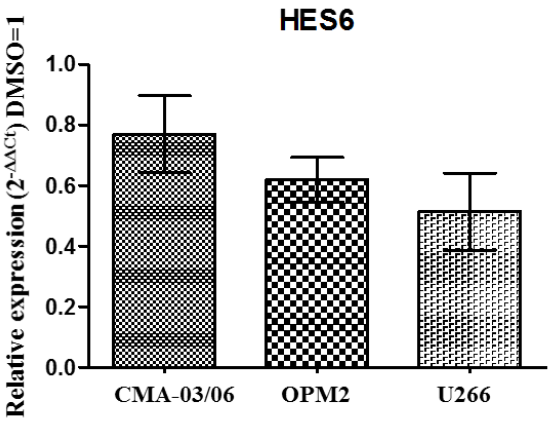

Figure 3.6. DAPT inhibits Notch activity in CMA-03/06, OPM2 and U266 cell lines. The relative gene expression of *HES6* (normalized to GAPDH) in CMA-03/06, OPM2 and U266 cells treated with 50μM DAPT has been assessed by qRT-PCR. Results are obtained by comparing DAPT-treated cells to DMSO by the $2^{-\Delta\Delta Ct}$ formula. Graph shows the mean values ± SD.

As shown in figure 3.7, NOTCH withdrawal induces a significant reduction in cell proliferation that has been largely recovered by IL-6 stimulation. It is interesting to note that IL-6 is able to stimulate CMA-03/06 cells growth only if NOTCH pathway is inhibited: in fact, IL-6 has no effect when NOTCH signaling is active. Since CMA-03/06 cells become responsive and dependent on IL-6 in the absence of an active NOTCH signaling, it seems that NOTCH pathway is able to compensate IL-6 activity. These results were also confirmed in other two IL-6 independent HMCLs, OPM2 and U266: also in these cell lines, NOTCH blockade due to DAPT treatment, reduced cells growth which was reverted by IL-6 stimulation (Figure 3.7).

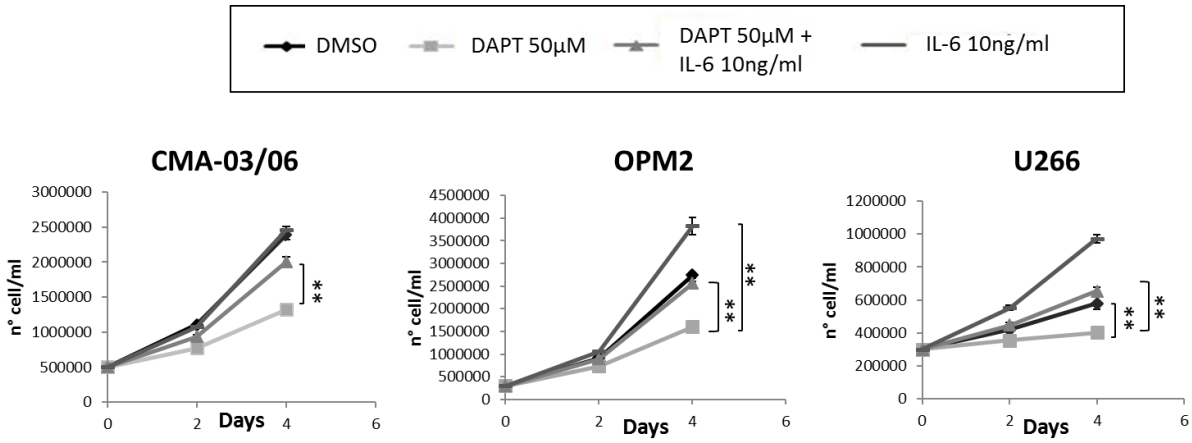

Figure 3.7. The lack of an active NOTCH pathway reverted the IL-6 independency in those cells able to grow in the absence of the cytokine. The contribution of Notch and IL-6 to MM cells growth has been evaluated. Cell growth analysis of CMA-03/06, OPM2 and U266 cells treated with or without DAPT 50µM and/or IL-6 for 96h. Mean values ± SD are shown. Statistical analysis was performed using ANOVA and Tukey test: **= $p<0.01$.

We also supposed that NOTCH signaling is sufficient to induce IL-6 independence in CMA-03 cells. To confirm this, NOTCH signaling has been activated through the stimulation with soluble JAGGED1. To this end, CMA-03 cells have been treated with 5µg/ml soluble JAGGED1 ligand in the presence or absence of IL-6 (10µg/ml). As shown in figure 3.8, JAGGED1 stimulation was able to compensate IL-6 withdrawal inducing a complete recovery of cells growth.

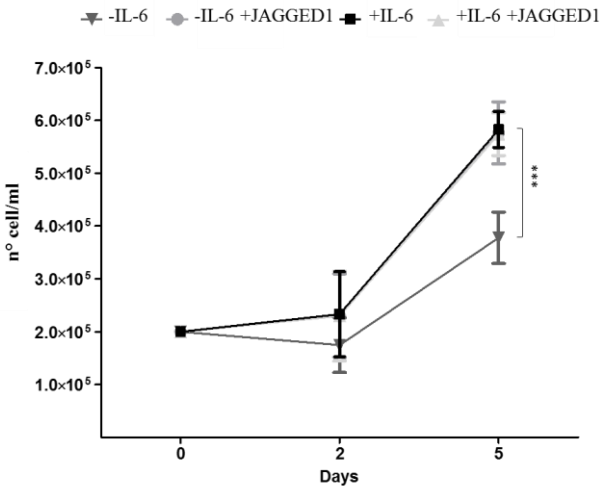

Figure 3.8. Notch signaling reverts IL-6 dependency in the IL-6 dependent cell line CMA-03. CMA-03 cells have been treated with 5µg/ml soluble JAGGED1 ligand for 5 days: JAGGED1 stimulation is able to compensate for IL-6 withdrawal. SD are calculated from 3 independent experiments and statistical analysis has been performed using ANOVA and Tukey test: ***= $p<0.001$.

These data have been confirmed in the IL-6 dependent cell line XG-1: in these cells, JAGGED1 stimulation can revert about 45% of cell reduction due to the absence of IL-6. These results indicated that NOTCH signaling could compensate the lack of IL-6 stimulus in IL-6 dependent MM cell lines (Figure 3.9A). The confirmation of NOTCH pathway activation was obtained through qRT-PCR analysis of HES1 overexpression (Figure 3.9B).

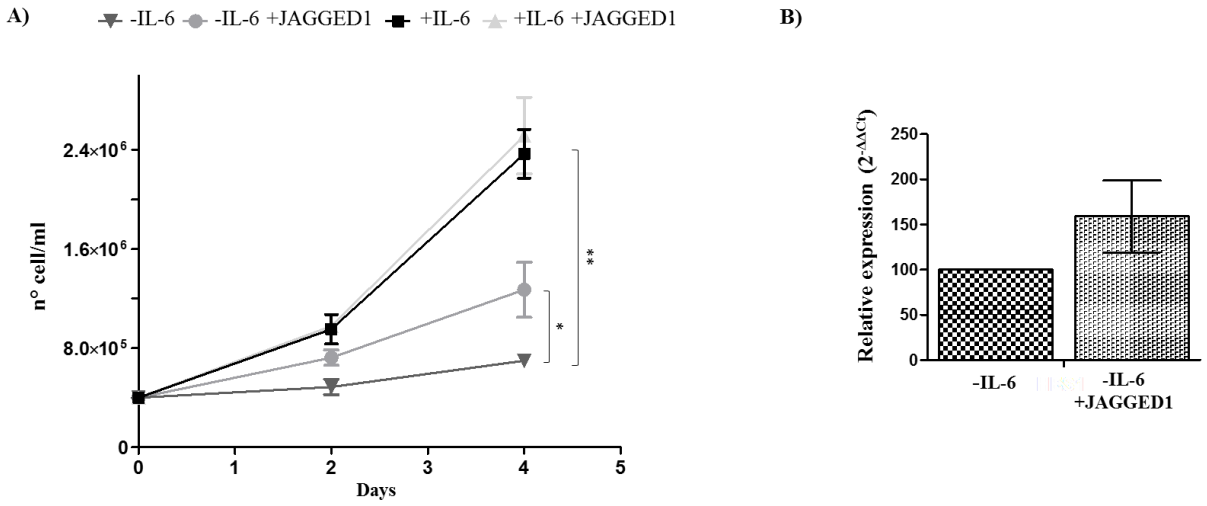

Figure 3.9. Notch signaling partially reverts IL-6 dependency in the IL-6 dependent cell line XG-1. XG-1 cells have been treated with 5µg/ml soluble JAGGED1 ligand for 4 days: JAGGED1 stimulation is able to compensate for IL-6 withdrawal. SD were calculated from 3 independent experiments and statistical analysis was performed using ANOVA and Tukey test: *= $p<0.05$;**= $p<0.01$. B) Confirmation of JAGGED1 stimulation effectiveness has been obtained by qPCR measure of relative *HES1* gene expression variation in absence of IL-6 in JAGGED1 stimulated cells compared to untreated, calculated by the $2^{\wedge-\Delta\Delta Ct}$ formula. SD were calculated from 3 independent experiments. Mean value ± SD are shown. Statistical analysis by t-test: *= $p<0.05$.

3. IL-6 production is induced by NOTCH signaling in MM cells

Since it has been described that NOTCH signaling is able to induce *IL-6* expression in different cells type [265, 267], our work wished to demonstrate that *IL-6* expression is under NOTCH pathway control also in MM cells. To confirm this, we used U266 HMCL since it represents a cellular model characterized by IL-6 independence due to autocrine production of this cytokine [281]. We inhibited NOTCH signaling by using two different siRNAs against JAGGED1 and JAGGED2 [61]. JAGGED1 and JAGGED2 silencing was evaluated by assessing the decrease in *JAGGED1*, *JAGGED2*, *HES1* and *HES6* gene expression levels by qRT-PCR (Figure 3.10) and confirmed at protein level, as shown in figure 3.10.

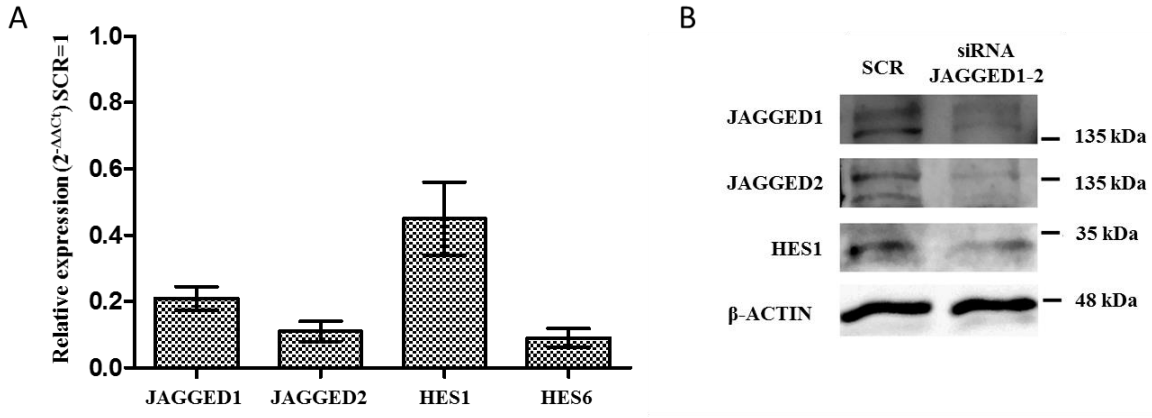

Figure 3.10. NOTCH pathway downregulation through two different siRNAS against JAGGED1 and JAGGED2 is confirmed at transcriptional and protein level. U266 cells have been transfected with two specific siRNAs targeting *JAGGED1* and *JAGGED2* or the corresponding scrambled control (SCR). **A)** Confirmation of JAGGED-2 silencing efficiency in U266 cells has been obtained by qPCR measurements of relative gene expression variation of *JAGGED1* and *JAGGED2* and NOTCH target genes *HES1* and *HES6* in cells transfected with siRNA against JAGGED1-2 compared to cells transfected with scrambled control, calculated by the $2^{-\Delta\Delta Ct}$ formula. *IL-6* expression levels were also analyzed. SDs were calculated from 3 independent experiments. Two-tailed t-test confirmed statistically significant downregulation of the genes tested; **B)** Western blot confirmed that JAGGED1, JAGGED2 and HES1 are downregulated in silenced U266.

Once demonstrated that RNA interference against JAGGED1 and JAGGED2 correctly worked, we evaluated the consequence of NOTCH pathway inhibition on IL-6 production. JAGGED1 and JAGGED2 silencing induced a significant reduction of IL-6 of about 50% both at transcriptional and protein level, as demonstrated by qRT-PCR and flow cytometry respectevely (Figure 3.11).

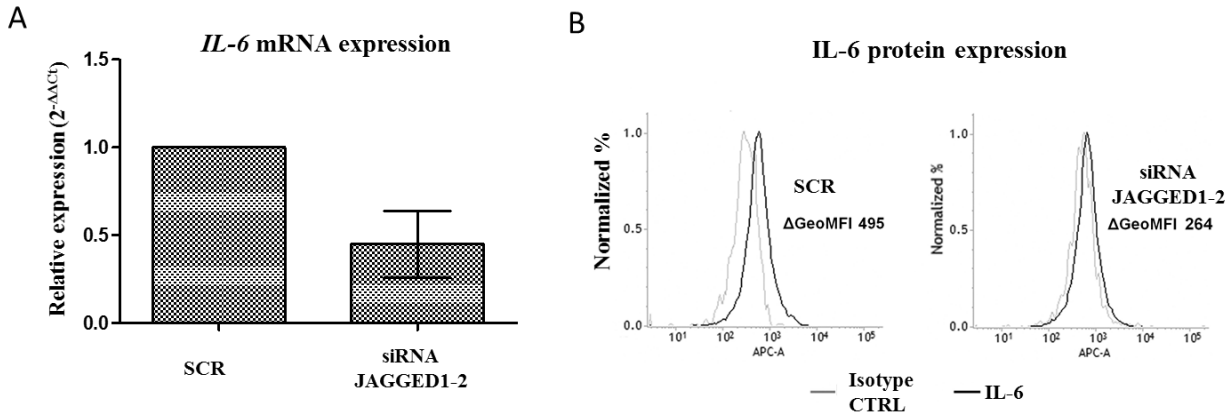

Figure 3.11. JAGGED1 and JAGGED2 silencing, and the consequent NOTCH pathway withdrawal, reduces IL-6 expression level both at RNA and protein level. A) *IL-6* expression levels have been analyzed in U266 cells treated with siRNA against JAGGED1 and JAGGED2 compared to SCR cells. SDs were calculated from 3 independent experiments. **B)** Histograms display the levels of intracellular IL-6 analyzed by flow cytometry in U266-siRNA JAGGED1-2 or U266-SCR in single culture (black lines), and an isotype-matched control (gray line). ΔGeoMFI have been obtained by subtracting the appropriate isotype control from the positive signal. Histograms are representative of 3 experiments with similar results.

From a biological point of view, NOTCH withdrawal, obtained through ligands knowdown, reduced U266 cells growth of about 30% (as shown in Figure 3.12A), increasing the apoptotic rate (Figure 3.12B) and inducing partial cell cycle arrest in G0/G1 phase (Figure 3.12C).

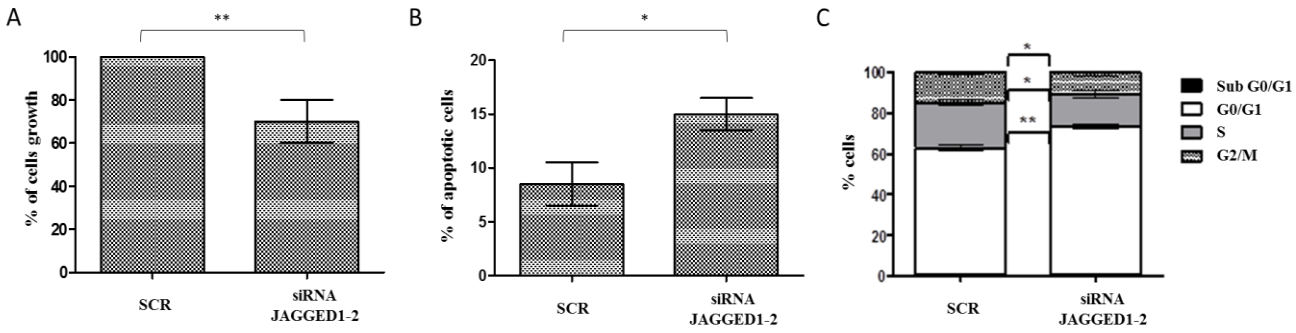

Figure 3.12. JAGGED1 and JAGGED2 silencing reduces U266 cells growth through the increase in apoptosis rate and partially cell cycle arrest. A) JAGGED1 and JAGGED2 silencing in U266 cells caused a significant decrease in cell proliferation. Mean values ± SD are shown. Statistical analysis has been performed using two-tailed t-test: **= p<0.01. **B)** Annexin-V and PI double staining has been performed to measure the apoptotic rate of MM cells treated with anti-JAGGED1-2 siRNAs. Statistical analysis has been performed by two-tailed t-test (*=p<0.05). **C)** Cell cycle analysis. A standard PI staining has been used to measure cell cycle distribution based on cell DNA content. Means and standard deviations have been calculated on three independent experiments and statistical analysis has been performed by two-tailed t-test (*=p<0.05; **=p<0.01).

To further confirm that IL-6 is a transcriptional target of NOTCH pathway in MM cells, a dual luciferase assay, composed by IL-6 reporter pIL-6-luc651, has been performed in U266 cells in order to evaluate if IL-6 promoter could be transactivated by plasmids carrying the active forms of NOTCH1 (ICN1) and NOTCH2 (ICN2). As shown in Figure 3.13, both ICN1 and ICN2 are able to activate the *IL-6* responsive element.

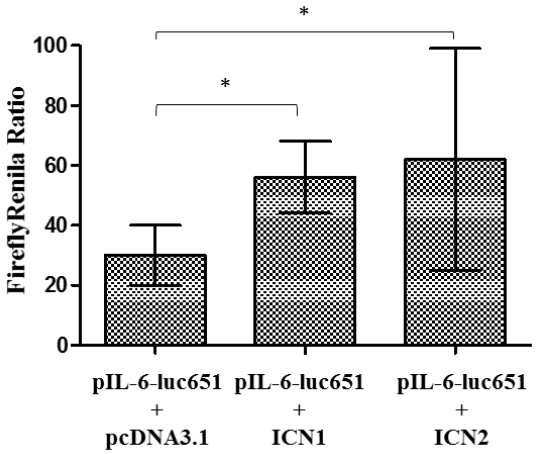

Figure 3.13. NOTCH drives MM cell-autonomous production of IL-6. Luciferase reporter assay showing that both ICN1 and ICN2 are able to boost the activity of IL-6 promoter. Mean values ± SD are shown. Statistical analysis has been performed using one-tailed, unpaired t-test *= $p<0.05$.

To validate the correlation between NOTCH and IL-6 found in HMCLs also in primary MM cells, we investigated gene expression data in our proprietary microarray dataset. Even if no significant correlation between *IL-6* and *HES6* was obtained when the whole dataset has been analyzed, a positive, albeit not statistically significative, correlation has been found in six patients with the highest IL-6 expression, when stratified according to *IL-6* GEP expression levels (with index $r=0.54$ and $p=0.1358$) using IL-6 expression level of U266 cell line as a cutoff. This finding was confirmed by qRT-PCR ($r=0.71$ and $p=0.0573$) (Figure 3.14). Most likely, the lack of a significant statistically correlation could be due the small sample size of patients with high IL-6 level.

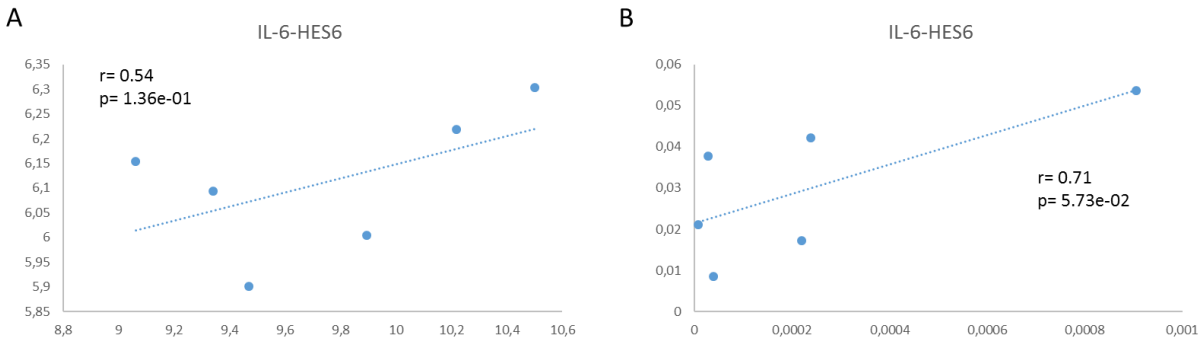

Figure 3.14. Correlation of *IL-6* and Notch activity in MM patients with high IL-6 expression levels. A) Correlation plot of *IL-6* (y-axis) and *HES6* (x-axis) expression levels (log2 scale) measured by microarray gene expression profile in 6 MM patients of the proprietary GEO dataset No. GSE66293, showing the highest *IL-6* gene expression levels. One-tailed Pearson's correlation coefficient (r) and the corresponding p-value are reported. **B)** Correlation plot of *IL-6* (y-axis) and *HES6* (x-axis) expression levels ($2^{-\Delta Ct}$ method) measured by qRT-PCR in the same 6 MM patients of panel A. One-tailed Pearson's correlation coefficient (r) and the corresponding p-value are reported.

4. BMSCs are able to support MM tumor growth through IL-6 production induced by NOTCH signaling pathway

BM microenvironment plays a very important role in MM, since BMSCs are the most important source of IL-6 and other growth factors necessary for tumor survival and proliferation [56, 257]. Due to its role in tumor progression and sustenance, we tried to evaluate the MM JAGGED ligands ability to activate NOTCH signaling in BMSCs [51] and boost IL-6 production.

To this end, we firstly verified if also in BMSCs, *IL-6* expression is under NOTCH signaling control. NOTCH signaling pathway has been inhibited in the human BMSC line HS5 through a silencing approach using a NOTCH1-targeting siRNA (HS5-N1KD). Ninety-six hours after transfection of siRNA, NOTCH pathway inhibition has been evaluated in HS5-N1KD through qRT-PCR for the evaluation of *HES6* gene expression. As shown in Figure 3.15, NOTCH pathway silencing induces a significant reduction in *IL-6* gene expression (Figure 3.15A) confirmed also at the protein level by flow cytometry (Figure 3.15B).

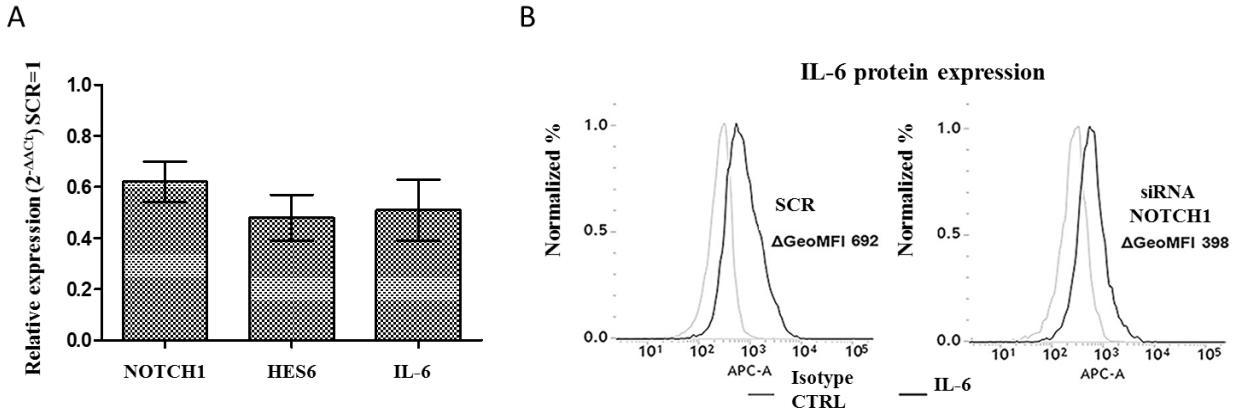

Figure 3.15. IL-6 is under NOTCH signaling control in human BMSCs. A) Confirmation of NOTCH1 inhibition in HS5-N1KD by qPCR measurements of *NOTCH1*, *HES6* and *IL-6* gene expression. HS5-N1KD cells are compared to HS5-SCR control cells. Fold changes are calculated by the $2^{-\Delta\Delta Ct}$ formula as reported above. SD were calculated from 3 independent experiments. **B)** Histograms display the levels of intracellular IL-6 (black line) analyzed by flow cytometry in HS5-SCR or HS5-N1KD cells, and an isotype-matched control (gray line). ΔGeoMFI are obtained by subtracting the appropriate isotype control from the positive signal. Histograms are representative of 3 independent experiments with similar results.

As previously demonstrated by a dual luciferase assay, we further confirmed that NOTCH pathway is able to directly activate *IL-6* promoter also in the murine NIH3T3 cell line that could be considered as BMSCs mimic (Figure 3.16).

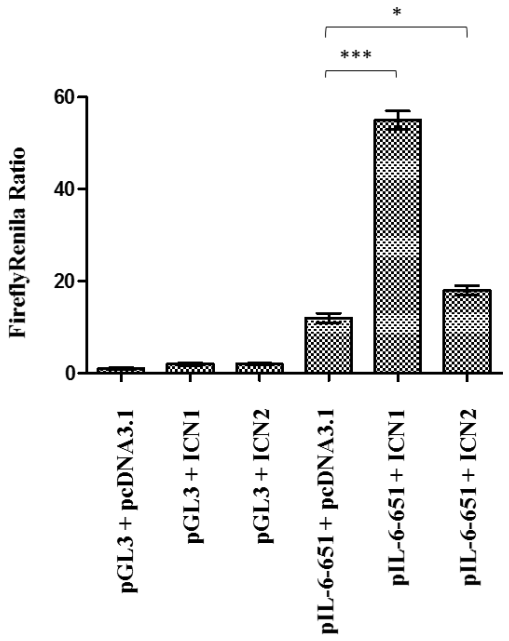

Figure 3.16. NOTCH signaling directly activate IL-6 promoter. Luciferase assay on the stromal-mimetic cell line NIH3T3 showed that both ICN1 and ICN2 promotes the activation of IL6 promoter. Mean values ± SD are shown. Statistical analysis was performed using one-way ANOVA and Tukey test: *=p<0.05; ***=p<0.001.

Once demonstrated that *IL-6* expression is under NOTCH control also in BMSCs, we evaluated if BMSCs deprived of NOTCH pathway, maintained their ability to support the proliferation of the IL-6 dependent cell line XG-1. To this end, we utilized a co-culture system in which XG-1 cells are cultured for 48h in the absence of IL-6 on a layer of GFP^+ HS5 silenced for NOTCH1 or transfected with scrambled siRNA control (SCR). As shown in Figure 3.17, HS5-N1KD cells lack their ability to support XG-1 cells growth. In fact, XG-1 cells growth, enumerated by absolute counts of GFP^- cells by flow cytometry on a layer of HS5-N1KD cells, was comparable to that obtained in a single culture of these MM cells in the absence of IL-6.

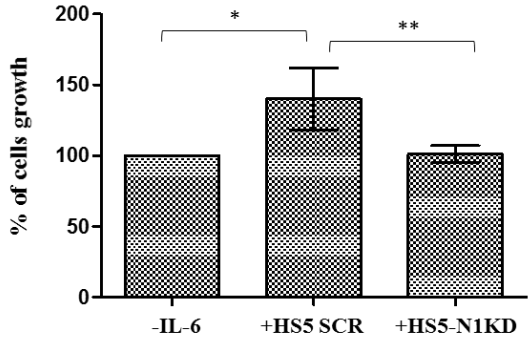

Figure 3.17. BMSCs without active NOTCH signaling are no longer able to support MM cells growth. Cell growth analysis of XG-1 cells cultured alone or co-cultured with HS5-Scr or HS5-N1KD. Mean values ± SD are shown. Statistical analysis was performed using one-way ANOVA and Tukey test: *= p<0.05, **=p<0.01.

This reduction in tumor growth is due to the increase in the apoptotic rate, without significant changes in the distribution of cell cycle phases (Figure 3.18).

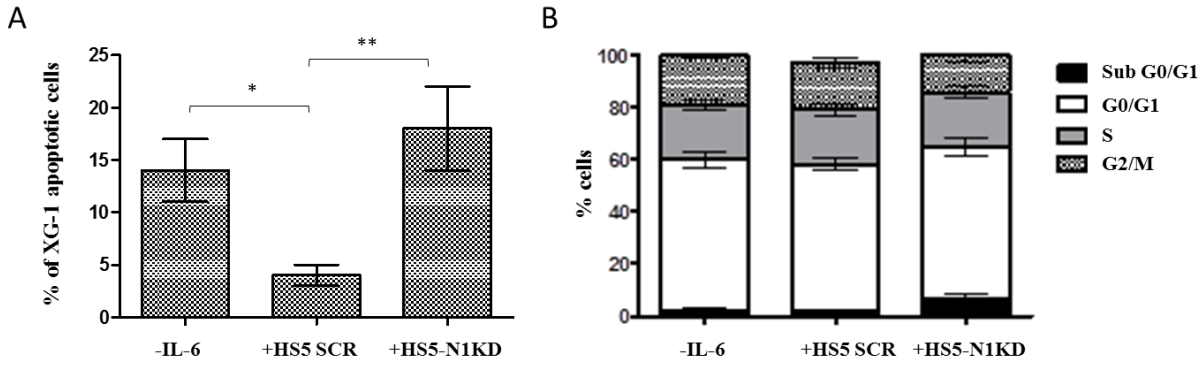

Figure 3.18. NOTCH1 silencing in HS5 cells reduce the ability of stromal cells to support XG-1 cells growth and survival. A) Annexin-V-APC staining has been performed to measure the apoptotic rate of MM cells co-cultured with SCR or N1KD HS5. The presence of GFP in HS5 cells allow to discriminate the two populations in flow cytometry. Results are normalized on apoptosis in XG-1 cultured alone in presence of IL-6. Statistical analysis has been performed with one-way ANOVA (*=p<0.05; **=p<0.01). **B)** Cell cycle analysis. DRAQ5 is used to measure cell cycle distribution of GFP-negative cells based on cell DNA content. Means and standard deviations are calculated on three independent experiments. Statistical analysis, performed with one-way ANOVA, fails to detect statistically significant changes in the cell cycle of XG-1 cells.

To confirm that the impaired ability to HS5-N1KD cells to support XG-1 cells growth is due to the lack of IL-6 production, IL-6 expression was blocked in HS5 cells (HS5-IL-6KD) through an anti-IL-6 siRNA. We used a co-culture system in which XG-1 cells are cultured on a monolayer of HS5-IL-6KD or with the respective control HS5-SCR. As shown in Figure 3.19, *IL-6* silencing was confirmed both at mRNA level (Figure 3.19A), by qRT-PCR, and protein level (Figure 3.19B), by flow cytometry.

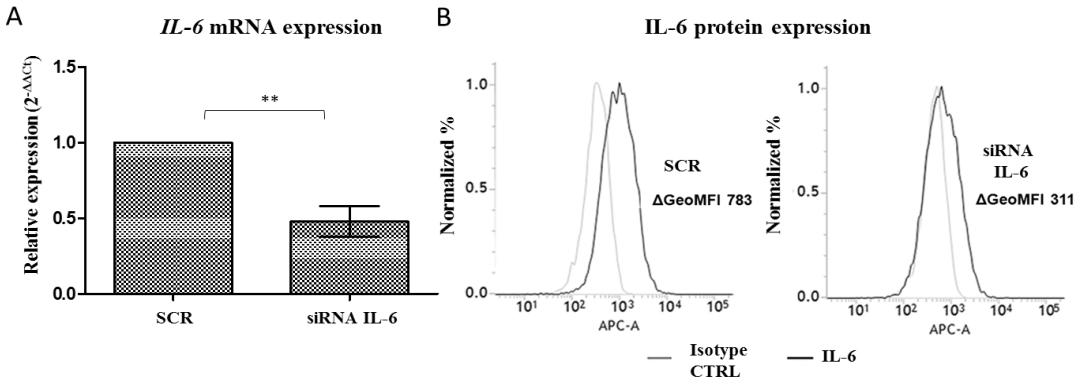

Figure 3.19. IL-6 silencing confirmation. A) Confirmation of *IL-6* silencing has been obtained by qPCR of *IL-6* gene expression; fold change has been calculated by the $2^{-\Delta\Delta Ct}$ formula as reported above. SD has been calculated from 3 independent experiments. **B)** Histograms display the levels of intracellular IL-6 (black line) analyzed by flow cytometry in HS5-SCR or HS5-IL-6KD, and an isotype-matched control (gray line). ΔGeoMFI are obtained by subtracting the appropriate isotype control from the positive signal. Histograms are representative of 5 independent experiments with similar results.

As shown in figure 3.20, the biological consequence of the reduced IL-6 produced by BMSCs is the significant cells growth decrease of co-cultured XG-1 cells.

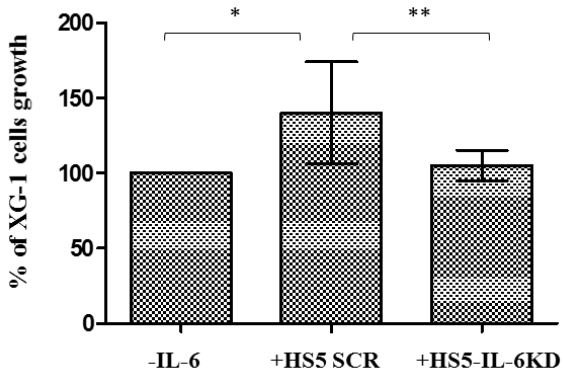

Figure 3.20. IL-6 silenced BMSCs loss the ability to support MM cells growth. Cell growth analysis of XG-1 cells cultured alone or co-cultured with HS5-SCR or HS5-IL-6KD. Mean values ± SD are shown. Statistical analysis has been performed using one-way ANOVA and Tukey test: *= $p<0.05$, **=$p<0.01$.

5. NOTCH pathway activation in BMSCs mediated by JAGGED ligands presented on MM cells promotes IL-6 production by BMSCs and their ability to support tumor growth

Since there are several evidences that JAGGED1 and JAGGED2 are dysregulated in multiple myeloma, we focused our attention on the ability of the overexpressed NOTCH ligands in MM cells to shape the BM microenvironment. To this end, NOTCH pathway has been inhibited in the IL-6-sensitive HMCLs XG-1, OPM2 and U266 through specific siRNAs against JAGGED1 and JAGGED2 (HMCLs-J1/2KD) and a scrambled siRNA was used as negative control (HMCLs-SCR). A co-culture system in which GFP^+ HS5 are co-cultured with silenced HMCLs was used; in such a system, the two cell types can be distinguished through the presence of GFP^+ HS5. Flow cytometry analysis allows to evaluate the percentage of IL-6 produced by stromal cells HS5. As shown in Figure 3.21A, the percentage of IL-6 produced by stromal cells HS5 is increased of about 60% in the presence of XG-1-SCR and it is significantly reduced in the co-culture with XG-1-J1/2KD. Consequently, HS5 cells co-cultured with XG-1-J1/2KD cells were no longer able to support tumor cell growth (Figure 3.21B).

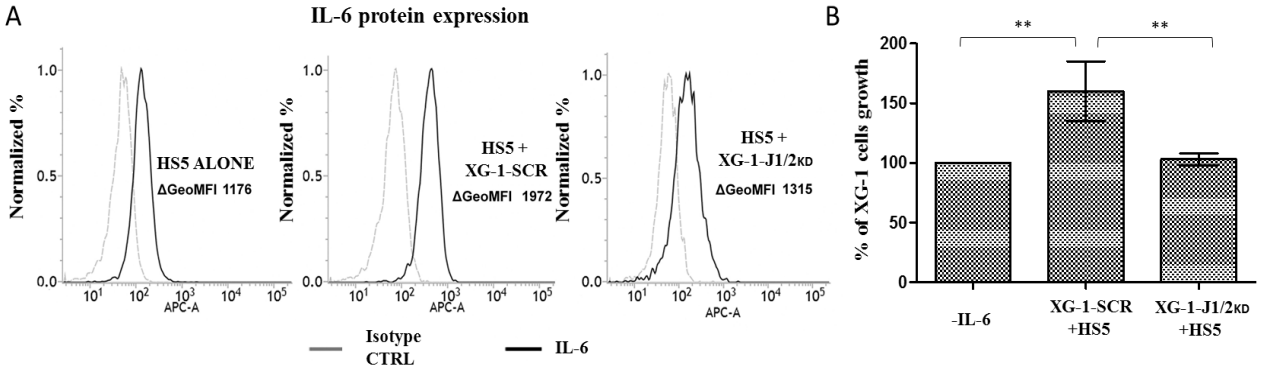

Figure 3.21. MM cells induce IL-6 production by BMSCs through JAGGED ligands. MM cells are able to shape the BM microenvironment and increase IL-6 production. A co-culture system of XG-1 MM cells with the BM stromal cell line HS5 has been established. **A)** Histograms display the levels of intracellular IL-6 analyzed by flow cytometry in HS5 cells in single culture or after co-culture with XG-1-SCR or XG-1-J1/2KD (black lines) and an isotype-matched control (gray line). ΔGeoMFI are obtained by subtracting the appropriate isotype control from the positive signal. Histograms are representative of 3 independent experiments with similar results. **B)** Cell growth analysis of XG-1-J1/2KD or XG-1-SCR cultured alone or co-cultured with HS5 cells. Mean values ± SD are shown. Statistical analysis has been performed using one-way ANOVA and Tukey test: **= $p<0.01$.

This represents a reliable cell model for MM since their growth depends completely on the support of BM stroma (represented by HS5 cells) or IL-6. Results indicated that the reduction of XG-1 cell number is due the apoptosis without any alteration in cell cycle phases (Figure 3.22).

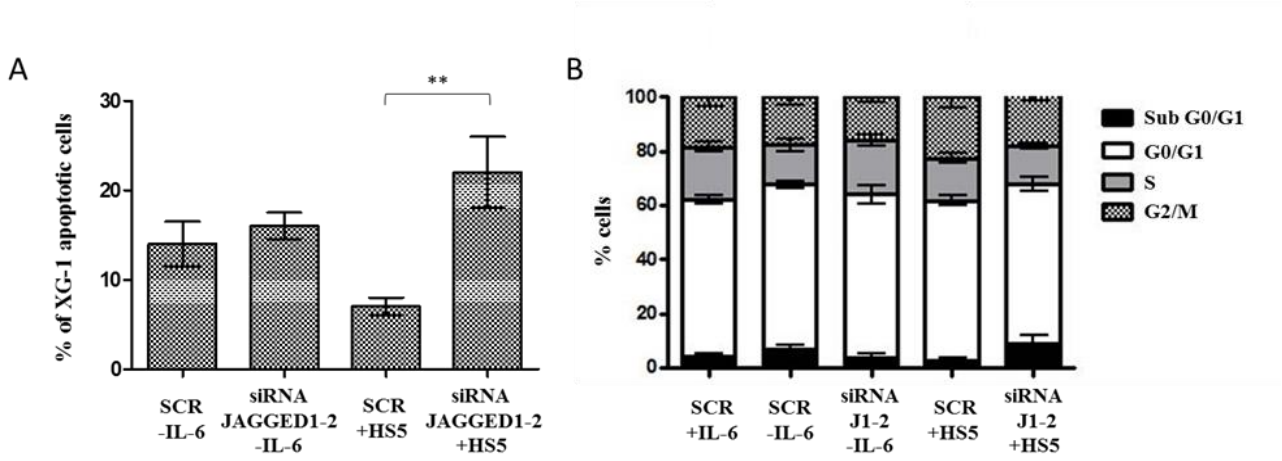

Figure 3.22. JAGGED1 and JAGGED2 silencing does not alter XG-1 apoptosis and cell cycle. A) Annexin-V-APC staining has been performed to measure the apoptotic rate of XG1-J1/2KD or XG1-SCR cells co-cultured with GFP^+ HS5 cells. Results of flow cytometric analysis are normalized on apoptosis in XG-1-SCR cultured alone in presence of IL-6. Statistical analysis has been performed with one-way ANOVA (**=$p<0.01$). **B)** DRAQ5 has been used to measure cell cycle distribution of GFP^- cells based on cell DNA content. Means and standard deviations are calculated on three independent experiments. Statistical analysis, performed with one-way ANOVA, failed to detect statistically significant changes in the cell cycle of XG-1 cells.

Data obtained on XG-1 cells were confirmed also in OPM2 (Figure 3.23A-B) and U266 cells (Figure 3.23C-D).

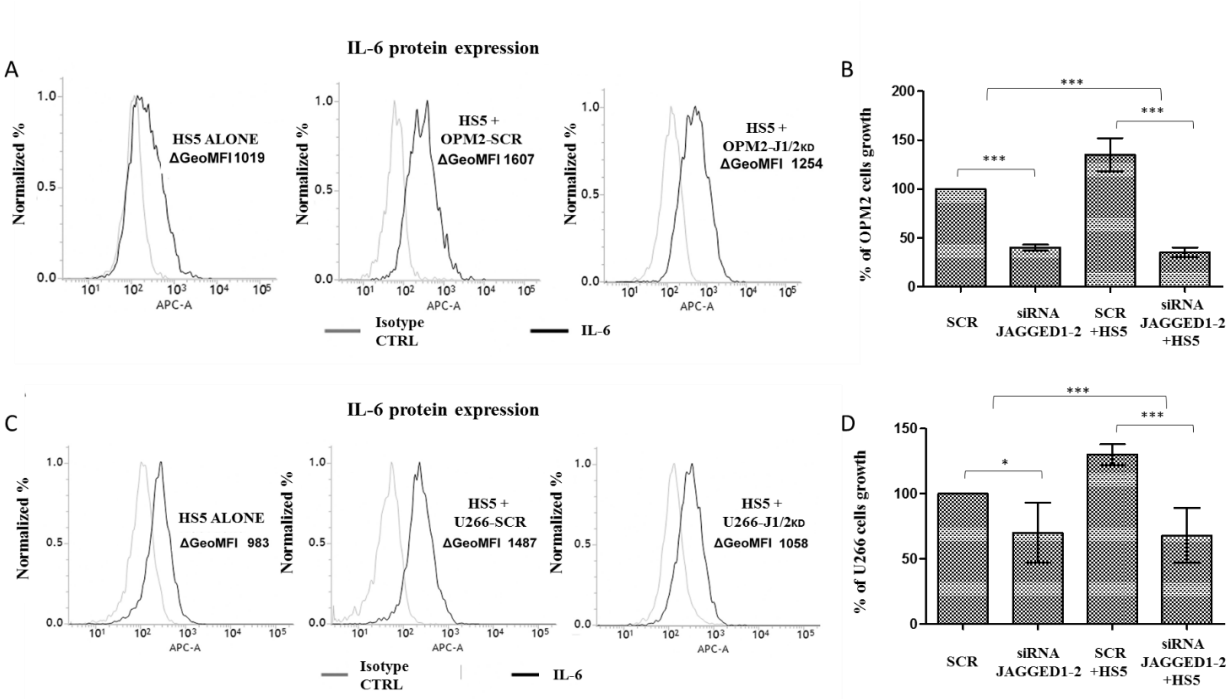

Figure 3.23. MM cells induce IL-6 production by BMSCs through JAGGED ligands. A,C) Histograms display the levels of intracellular IL-6 analyzed by flow cytometry in HS5 cells in single culture or after co-culture with OPM2-SCR/U266-SCR or OPM2-J1/2KD/U266-J1/2KD (black lines) and an isotype-matched control (gray line). ΔGeoMFI are obtained by subtracting the appropriate isotype control from the positive signal. Histograms are representative of 3 independent experiments with similar results. **B,D)** Cell growth analysis of OPM2-J1/2KD/ U266-J1/2KD or OPM2-SCR/U266-SCR cultured alone or co-cultured with HS5 cells. Mean values ± SD are shown. Statistical analysis has been performed using one-way ANOVA and Tukey test: **= $p<0.01$.

Finally, we also confirmed that variation of IL-6 at protein levels directly reflects the variations of *IL-6* at transcriptional level. We used a co-culture system of human OPM2 cells and murine fibroblast NIH3T3 cells, and identified the origin of mRNAs by qRT-PCR analysis with species-specific primers. As shown in Figure 3.24, MM cells are able to induce NOTCH pathway activation in NIH3T3 cells, as demonstrated through the increase of *hes5* gene expression, and this activation is accompanied by an increase in murine *il-6* gene expression. As previously demonstrated in the human co-culture system, also in this case JAGGED1 and JAGGED2 silencing reduces the ability of MM cells to activate the NOTCH pathway in stromal cells, directly decreasing the production of *il-6* (Figure 3.24).

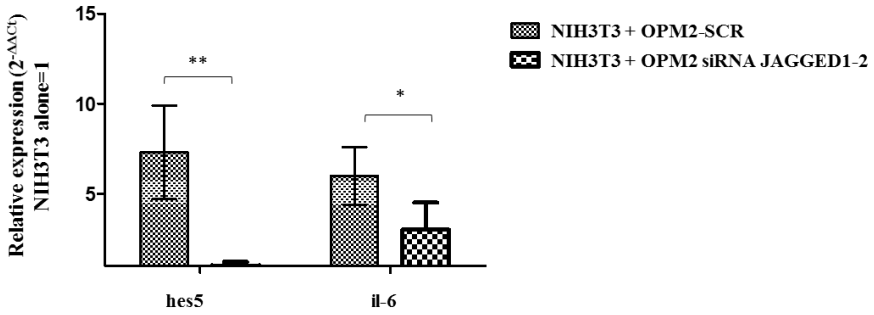

Figure 3.24. MM cells induce IL-6 expression by BMSCs through JAGGED ligands. qRT-PCR for *il-6* and *hes5* gene expression in NIH3T3 cells co-cultured with OPM2-J1/2KD or OPM2-SCR. compared NIH3T3 coltured alone (=1), calculated by the $2^{-\Delta\Delta Ct}$ formula. *hes5* has been used as a control for pathway activity. Mean values ± SD are shown. Statistical analysis has been performed using two-tailed t-test: **= p<0.01; *=p<0.001.

The same results were obtained when U266 cells were used (Figure 3.25).

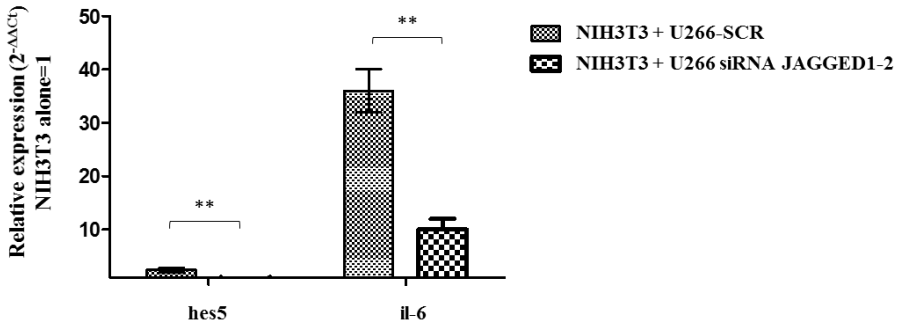

Figure 3.25. MM cells induce IL-6 expression by BMSCs through JAGGED ligands. qRT-PCR for *il-6* and *hes5* gene expression in NIH3T3 cells co-cultured with U266-J1/2KD or U266-SCR. compared NIH3T3 coltured alone (=1), calculated by the $2^{-\Delta\Delta Ct}$ formula. *hes5* has been used as a control for pathway activity. Mean values ± SD are shown. Statistical analysis has been performed using two-tailed t-test: **= p<0.01; *=p<0.001.

6. NOTCH signaling withdrawal reduces the release of IL-6 in co-cultures of primary MM cells and BMSCs

Since in this work we investigated the ability of BMSCs to release IL-6 upon MM cell-mediated NOTCH signaling activation, we tried to validate these data also in primary tumors. To this end, we used primary co-culture systems of highly purified $CD138^+$ MM cells and BMSCs from BM aspirates of 7 MM patients, purified as described by Garayoa *et al.* [271]. Co-cultures have been maintained for 96 hours in presence of 25μM DAPT in order to inhibit the NOTCH signaling (the same amount of DMSO was used in control cultures) and the BMSCs-mediated IL-6 production was evaluated by flow cytometry. As shown in Figure 3.26, BMSCs significantly increase IL-6 production when stimulated by MM cells (from 12% to 41% $IL\text{-}6^+$ BMSCs) and this effect is reverted in the presence of NOTCH pathway inhibitor DAPT (15% $IL\text{-}6^+$ BMSCs), confirming previously *in vitro* findings in HS5/HMCLs co-cultures.

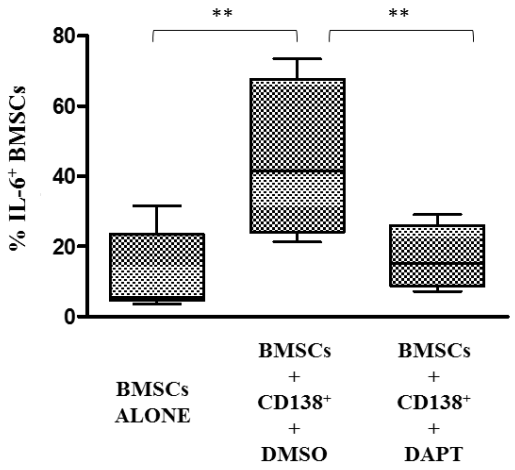

Figure 3.26. NOTCH pathway inhibition reduces the *ex-vivo* ability of MM cells to induce IL-6 production by BMSCs. Levels of intracellular IL-6 are analyzed by flow cytometry in primary BMSCs co-cultured with $CD138^+$ cells from MM patients in the presence or absence of 25μM DAPT. Seven independent experiments have been performed and the percentage of $IL\text{-}6^+$ BMSCs is shown in the bar graph. Statistical analysis is performed using one-way ANOVA and Bonferroni post-test. (*= $p<0.05$).

We were also interested in the evaluation of IL-6 level changes in MM cells. Unfortunately, we were not able to evaluate IL-6 expression in MM cells single culture since they did not survive in the absence of IL-6 source (that could be represented by BMSCs or soluble IL-6 addiction).

However, it is possible to evaluate IL-6 positive MM cells when they are co-cultured with BMSCs: 3 out of 7 patients presents appreciable levels of IL-6 (22.81%, 33.45%, 49.80% of

IL-6$^+$ cells) in their CD138$^+$ cells. Results presented in Figure 3.27, showed that DAPT treatment in co-culture system significantly reduced IL-6 expression level in each of the three IL-6-positive MM patient samples (from 36±13% to 14±5% positive cells).

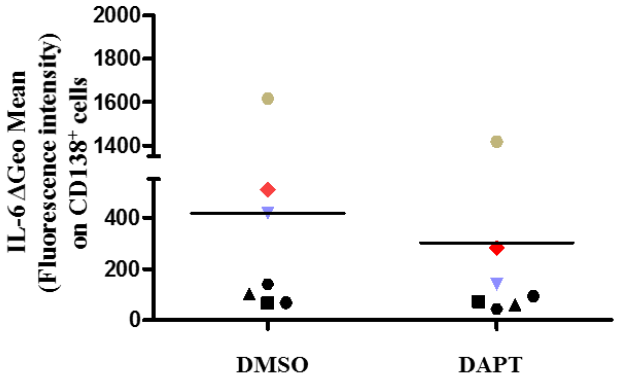

Figure 3.27. DAPT reduces IL-6 expression in MM cells also in the presence of BMSCs. Analysis of intracellular IL-6 levels by flow cytometry in primary CD138$^+$ cells co-cultured with BMSCs from MM patients in the presence/absence of 25μM DAPT. Scatter plot shows the ΔGeoMFI of all the analyzed IL-6$^+$ (colored dots) and IL-6$^-$ (black dots) samples.

In order to confirm these *ex-vivo* data, it was analyzed the correlation between *IL-6* and *HES1* gene expression in the dataset reported by Garcia Gomez *et al*. (REF [282]). This dataset includes 37 mesenchymal cells sample from MM patients or healthy donors that were cultured alone or co-cultured with HMCL MM1S. As shown in Figure 3.27A, there is a positive correlation between expression level of *IL-6* and *HES1* (r=0.57 and p=2.28x10-4) and there is an up-regulation of gene expression level of both *IL-6* and *HES1* upon mesenchymal cells stimulation with MM1S tumor cells (Figure 3.27B).

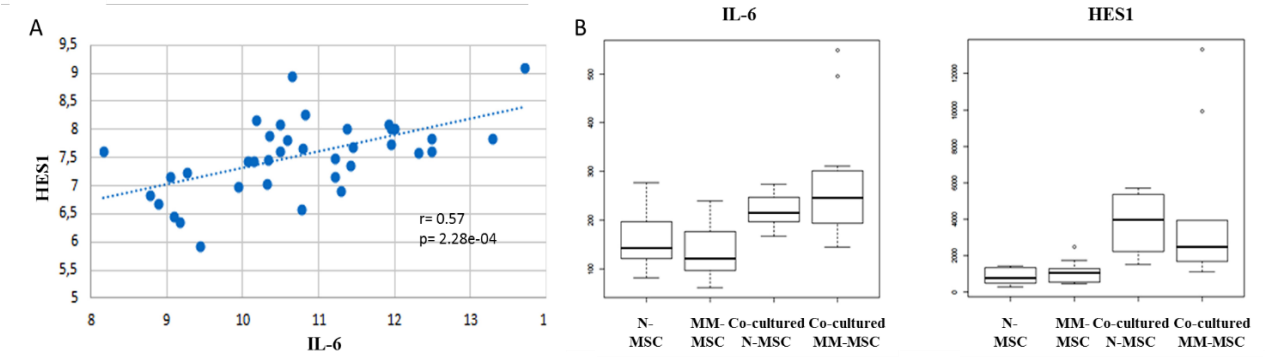

Figure 3.27. Primary mesenchymal stromal cells increase their expression level of *IL-6* and *HES1* upon MM cells stimulation. A) Pearson's correlation plots of *IL-6* and *HES1* expression levels (log2 scale) in 37 mesenchymal stromal cell (MSC) samples from 16 healthy donors and 21 MM cases, alone (8 N-MSC, 9 MM-MSC) or in co-culture with MM1S cell line (8 N-MSC, 12 MM-MSC), extracted from an external database (GSE46053) profiled on Affymetrix HG-U133 Plus 2.0 array. **B)** Gene expression profiles of *IL-6* and *HES1* in the same dataset of panel A. Kruskal-Wallis test has been performed to assess statistical significance of differential gene expression profiles between all four MSC groups. Dunn's test and the Benjamini-Hochberg correction are applied for nonparametric pairwise multiple comparisons. Statistical analysis indicates a significant p-value for *IL-6* in N-MSC and MM-MSC alone groups compared to both co-cultured N-MSC and MM-MSC samples (p=0.0008, p=0.0018 for N-MSC alone; p=0.0012, p=0.0038 for MM-MSC alone), respectively; similarly, for *HES1* in N-MSC and MM-MSC alone compared to both co-cultured N and MM groups, respectively (p=0.0485, p=0.0101 for N-MSC alone; p=0.0135, p=0.0017 for MM-MSC alone).

Finally, BM biopsies from 21 MM patients have been analyzed to evaluate the correlation between NOTCH activity and IL-6 in the endogenous MM bone marrow niche. Figure 3.38 shows the immunoreactivity of IL-6 and HES6 analyzed by immunohistochemistry. In all cases analysed, HES6 is present both in malignant PCs and in surrounding stromal cells. Interestingly, HES6 immunoreactivity intensity increases with multiple myeloma infiltration within the BM. Concerning IL-6, its signal increases in bone marrow MM cells in tumour progression. In fact, as shown in Figure 3.38, in the case of BM biopsies with low infiltration of tumour cells, IL-6 localizes almost exclusively in non-myelomatous cells. These non-neoplastic cells are surrounded by small groups of MM cells that only in sporadic examples displayed a faint cytoplasmic IL-6 immunoreactivity. Considering an intermediate stage of BM infiltration (in which there is up to 30% of MM cells), an increase of the IL-6 immunopositive neoplastic cells localized principally near the bone spicule, has been observed. With the progression of the disease, in which the infiltrate of MM cells is more that 30%, most of neoplastic cells expressed IL-6 with less than 5% of malignant PCs negative for the cytokine. In all investigated cases that are positive for IL-6 immunoreactivity, there are not significant differences in the level of cytokine.

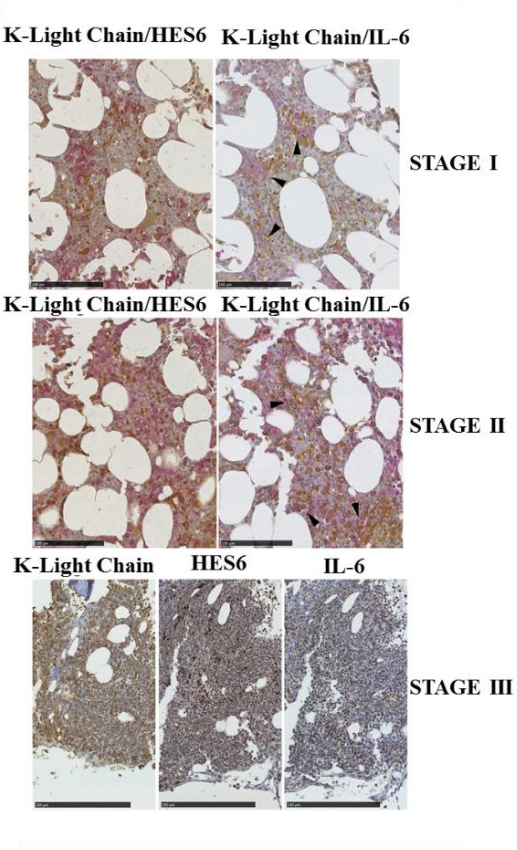

Figure 3.28. Representative images of IHC staining for HES6 and IL-6 (brown immunoreactivity) in human BOM infiltration by κ light chain myeloma (MM) (red immunostaining) at different stages (for each tumor stage, consecutive histological sections were stained with different antigens) Stage I MM: HES6 immunoreactivity can be easily detected both in neoplastic and in stromal cells; IL-6 immunostaining is detectable in non-neoplastic cells surrounding myelomatous nests (arrows). Stage II MM: a more intense HES6 immunoreactivity can be detected in both BM components; IL-6 immunoreactivity can be observed also in some MM cells. Stage III MM: in late stages of tumor infiltration, intense HES6 and IL-6 immunoreactivity is appreciable in κ light chain immunoreactive MM cells. In this case, to better visualize HES6 and IL-6 immunoreactivity and distribution, single antigens immunoreactivity was evaluated individually on each consecutive slide; the same BM areas of the case under study are here compared.

CONCLUSIONS and DISCUSSION

MM is the second most common hematological cancer; about 600,000 people have been affected every year in western countries [283]. Even if new treatments have been developed and introduced, MM still remains an incurable disease with a survival of 45% after 5 years from diagnosis [284]. This is due to the high rate of recurrence that remains more than 40% after 24 months due to the ability of malignant PCs to develop drug resistance. Therefore, novel therapeutic approaches able to overcome drug resistance must be investigated.

NOTCH pathway has been proposed as a therapeutic target in multiple myeloma due to its involvement in several key aspects of the disease such as bone disease [61, 285], stemness [231], drug resistance [232, 286, 287] and migration to the BM [51]. Nowadays, there are different strategies to target the NOTCH pathway. On the base of NOCTH receptors and ligands structure, regulation and function it is possible to identify several steps that can be targeted to inhibit NOTCH signaling pathway. The most common way to inhibit NOTCH pathway is represented by the use of γ-secretase inhibitors. These class of drugs are well known to have cytostatic or cytotoxic activities in several cancer types and are now on clinical trial in a variety of cancer conditions [288-291]. Other approach is to regulate the ligand-receptor binding that could be obtained through the use of blocking monoclonal antibodies directed against NOTCH receptors. Nowadays, there are two different classes of blocking anti-NOTCH antibodies. The first class comprises antibody directed to the extracellular negative regulator region (NRR) of NOTCH: the presence of antibody blocks the conformational change of the receptor that can not be cleavage by ADAM protease [292]. Antibodies against the NRR of NOTCH1 (NRR1), NOTCH2 (NRR2) and NOTCH3 (NRR3) are nowadays in preclinical or *in vitro* studies [292-294]. There are several evidences that NRR1 antibodies have also anti-angiogenic effect, are able to inhibit blood circulation to the tumor and significantly reduce tumor growth. The second class consists of ligand-competitors directed against the EGF-repeat region of NOTCH receptors that represents the ligand blocking domain (LBD). In fact, there are anti-DLL4 mAb [295] or soluble DLL4-Fc fusion proteins [296, 297] that are able to bind NOTCH receptors preventing their activation by endogenous DLL4. These antibodies inhibited NOTCH signaling in endothelial cells inducing disorganized angiogenesis and inhibition of tumor growth [295]. Other mechanism to inhibit NOTCH pathway is represented by the use of soluble forms of the extracellular domain of NOTCH receptors or NOTCH ligands. These soluble decoys act in this way: they compete with their endogenous cell surface-bound counterparts and abrogate NOTCH signaling due to the lack of a transmembrane region necessary for receptor activation. For example, a NOTCH decoy that acts as a ligand-dependent NOTCH antagonist blocks NOTCH signaling in

endothelial cells, reducing tumor neoangiogenesis and growth. The last approach to target NOTCH pathway is to block the transcriptional nuclear complex formed by active NOTCH (ICN), CSL and coactivator. There were developed dominant negative peptides derived from MAML1 that are able to form a transcriptionally inert complex with NOTCH1 and CSL [298, 299]. The most recent of them is SAHM [299] that has been tested in T-ALL cell lines where it induced a direct transcriptional repression that had an antiproliferative effects on tumor cells. The use of these stabilized, cell-permeable peptides to interfere with protein complex formation possesses several attractive features; these molecules have relatively small size, they have a high structural compatibility with target proteins, and have the ability to disrupt protein-protein interfaces.

The use of these anti-NOTCH molecules in multiple myeloma may be a promising therapy in combination with commons chemoterapic agents since they are able to act not only on tumor cells but are also able to reduce the interaction between neoplastic cells and the bone marrow niche, resulting in a major efficacy of chemoterapic drugs.

In fact, there are several evidences that NOTCH pathway is deregulated in multiple myeloma due to different mechanisms. For example, Skrtic and collegues [227] showed that expression of NOTCH1 and JAGGED1 increase during the progression from MGUS to MM; it has also been reported that NOTCH2 is a target of the transcription factors c-MAF and MAFB that are translocated in about 7% of MM patients [226]. Other evidences show that JAGGED2 is deregulated early both at transcriptional and post-transcriptional levels [228-230] and that its expression correlates with clinical stages [228]. Since MM cells simultaneously express NOTCH receptors and ligands, both homotypic activation of NOTCH signaling within MM cells and heterotypic activation in the surrounding cells of BM niche may occur. In agreement with these observations, the inhibition of NOTCH signaling in MM cells reduces their proliferation, increases apoptosis and inhibits the pharmacological resistance [39, 51]. Moreover, the overexpression of JAGGED ligands allows MM cells to activate NOTCH signaling in the surrounding bone marrow cells, directly favoring osteoclastogenesis that contributes to the bone damage associated with myeloma [61]. In addition, the overexpression of JAGGED2 is able to stimulate stromal cells in order to produce important growth factor for MM growth, such as VEGF, IGF-1 and IL-6 [228]. Overall, JAGGED ligands deregulation could determine the pathological interaction between neoplastic cells and stromal cells underlying the key role of the latter in the maintenance of malignancy and in the promotion of drug resistance.

Based on these considerations, the aim of this work was to clarify the role of the up-regulation of NOTCH signaling pathway in MM [227-230, 276]. In particular, we investigated its cross-talk with IL-6, the most important chemokine for malignant plasma cell survival and disease progression [49, 260, 261].

Our study started with the analysis of GEP data showing a positive correlation between the activation of NOTCH signaling (evaluated by *HES5* expression level) and the acquisition of a highly malignant phenotype: in fact, this activation occurs principally in TC5 MM patients and PCL patients, which represent the worst prognosis groups. There is a similar trend also for gene expression level of *JAGGED1*, in accordance to previously immunohistochemical data [227], that confirms the important role of this ligand in the autonomous NOTCH pathway activation as already reported [61]. When MM patients were stratified according to the TC molecular classification, we found that MAF-translocated group (TC5) has the highest *NOTCH2* expression levels associated with an increased NOTCH activity (evaluated as *HES5* and *HES6* expression levels). This finding confirms previous data showing that *NOTCH2* is a transcriptional target of MAF and MAFB factors [226]. Since translocations involving MAF factors are frequently associated with aggressive forms of *de novo* MM or with high risk PCLs [23], these results suggest that the activation of NOTCH pathway is typical of the most aggressive form of multiple myeloma and is associated with high-risk disease onset or evolution.

MM cells need IL-6 to proliferate [300]. As previously mentioned, the main source of the cytochine is represented by the BM niche [257], which could be further stimulated by MM cells to produce IL-6 through cell to cell contacts [56]. In some case MM cells develop the ability to autonomously produce IL-6 [301].

IL-6 signaling in multiple myeloma has a pleiotropic effect on proliferation, survival, drug resistance and cell migration directly favoring disease progression. In fact, IL-6 provided important survival signals by activating STAT3 signaling cascade whit the final effect to positively regulate the transcription of some BCL-2 family members [260] leading the way of the Ras/MAPK and PI3K/AKT signals [49].

From a clinical point of view, the relevance of IL-6 in MM is confirmed by the evidence that plasma from the bone marrow and peripheral blood of MM patients frequently present high levels of IL-6 and sIL-6R, which represent poor prognosis factors [259]. Furthermore, all HMCLs require IL-6 for their initial stabilization and only after several passages become independent from the cytokine, even if pathological cells derive from patients with extramedullary myeloma [280, 302]. To further underline the important role of IL-6 in MM,

there are several clinical trials in which the IL-6 monoclonal antibody Siltuximab (CNTO-328, Janssen Biotech, Inc) is used in combination with other chemoterapeutic agents. Indeed, there is a phase II clinical trial that compare bortezomib-melphalan-prednisone regimen with and without siltuximab in 106 transplant-ineligible patients with newly diagnosed MM [303]. Another study evaluates the double treatment siltuximab and bortezomib vs bortezomib alone in patients with relapsed/refractory MM [304]. Unfortunately, both studies failed to show that IL-6 inhibition may improve progression free survival or median overall survival of MM patients and now siltuximab is being studied in high risk smouldering multiple myeloma in which it may have a future [305].

Based on this evidence, we investigated in our study if the up-regulation of NOTCH pathway could have a role in the promotion of malignant progression of multiple myeloma cells by reducing their dependence on signals provided by the BM microenvironment, particularly IL-6. In fact, there are several evidences showing that highly malignant PCs from MM patients are less dependent by the BM, partially due to their ability to autonomously produce IL-6 [256, 306].

We tested this hypothesis in a progression MM model represented by the parental IL-6 dependent cell line CMA-03 and the derived IL-6 independent CMA-03/06 cell line. In this model, we demonstrated that:

- CMA-03/06 cells show an up-regulation of NOTCH receptors and ligands compared to IL-6 dependent CMA-03 cells, that is associated with an increased in NOTCH target genes expression level;
- CMA-03 cells acquire the ability to grow independently from IL-6 if stimulated with JAGGED1 soluble ligand;
- CMA-03/06 cells, that are independent and unresponsiveness to IL-6, recover their dependence when NOTCH pathway is inhibited in the presence of DAPT.

These findings were confirmed also in other HMCLs and indicate that clonal selection leading to the development of IL-6 independent MM clones might involve the up-regulation of NOTCH pathway genes. These data strongly suggest that NOTCH pathway activation could have a role in myelomagenesis by directly inducing the acquisition of IL-6 independency.

As reported above, MM cells may be able to autonomously produce IL-6. In the contest of the HMCLs, U266 cells are able to produce the cytokine [258, 307]. In these cells, we demonstrated through a luciferase reporter assay, that the acquisition of IL-6 independence could be due to the ability of NOTCH pathway (both ICN1 and ICN2 forms) to transactivate

IL-6 promoter directly. Moreover, this cell line needs an active NOTCH pathway to maintain its ability to produce IL-6: in fact, when NOTCH pathway is inhibited through RNA interference against JAGGED1 and JAGGE2 in U266 cells, IL-6 production decreases significantly. Interestingly, these results obtained *in vitro* are confirmed also in *ex-vivo*. In fact, in primary MM cells that express IL-6, we were able to demonstrate that if NOTCH pathway is inhibited through DAPT treatment, also IL-6 expression decreases significantly (from 36% to 14% IL-6 positive cells on average). Moreover, based on immunohistochemical analysis of BM biopsies, we observed that MM cells increase their ability to produce IL-6 during disease progression as found in cases with different levels of BM infiltration and that this increase is positively associated with NOTCH pathway activation in malignant PCs.

Considering primary cells from MM patients, we found a positive correlation by GEP and qRT-PCR analyses, between *IL-6* and *HES6* expression (the last gene has been used as a marker for NOTCH pathway activity) only in those patients with the higher levels of *IL-6*. Unfortunately, this correlation does not reach a statistical significance and this can be partially explained because the limited sample size or the effect of other inflammatory stimuli from the BM, such as NF-κB, that influence *IL-6* expression in primary malignant PCs. This last observation opens another chapter since it has already been demonstrated that the induction of *IL-6* expression mediated by NOTCH pathway is subordinated to the NF-κB signaling pathway [267]. More elucidations are necessary for understanding the interplay between NOTCH pathway and inflammatory stimuli derived from bone marrow and involved in IL-6 production in MM cells. Generally, all these findings sustain the hypothesis that NOTCH pathway is able to favor the acquisition of IL-6 independence from BM niche or directly from IL-6 stimulation through the promotion of autonomous production of the cytokine by MM cells. Furthermore, since NOTCH activation is able to compensate IL-6 proliferative signal also in those MM cells that do not produce IL-6 autonomously (such as cell lines used in this work as OPM2, CMA-03/06 and XG-1) it is possible that other mechanisms are involved in the cooperation between IL-6 and NOTCH pathways.

As previously discussed, the most important source of IL-6 in MM is represented by bone marrow stromal cells [56, 257]. In this regard, the other aspect that we evaluated in this work was the ability of NOTCH signaling to influence the production and release of IL-6 in BMSCs. We have demonstrated that IL-6 expression is under NOTCH signaling control also in BMSCs. In fact, in the NIH3T3 murine fibroblast cell line, used as a mimic of BMSCs, *IL-6* expression is increased upon NOTCH pathway stimulation, since *IL-6* promoter is transactived by both ICN1 and ICN2. With a different approach, we demonstrated that also in

the human BMSC line HS5 the IL-6 production is under NOTCH signaling control since the silencing of NOTCH1 by means of RNA interference causes the decrease of IL-6 expression, both at mRNA and protein levels. As a consequence of this, we observed a reduced ability of silenced stromal cells to sustain MM cells cell growth, as demonstrated in the co-culture system of HS5-N1KD cells and IL-6 dependent XG-1 MM cells.

This evidence prompted us to investigate the ability of myeloma cells to activate NOTCH pathway in the surrounding stromal cells through the overexpression of JAGGED1 and JAGGED2 ligands. We found that MM cells are able to improve the ability of murine (NHI3T3) and human (HS5) BMSCs to produce IL-6. This ability is mediated and dependent on the presence of JAGGED ligands on their cell surface, since in the absence of the two ligands, MM cells are no longer able to improve IL-6 production by BMSCs that consequently lose their ability to support tumor growth. As demonstrated in the cell lines co-culture systems, MM cells with silenced JAGGED ligands are no longer able to activate NOTCH pathway in the BMSCs (represented by HS5 cell line) leading to a reduced production of IL-6 by HS5 cells that are no longer able to sustain MM cells growth. These data obtained in *in vitro* systems were also confirmed in *ex-vivo* co-culture systems in which $CD138^+$ MM cells were able to stimulate primary MM BMSCs to produce IL-6. These data are consistent with data reported in the literature, demonstrating that JAGGED peptides are able to stimulate MRC5 lung fibroblast cell line to produce IL-6 [228], as well as in breast cancer in which tumor-derived JAGGED ligands are able to induce the production of IL-6 in BMSCs [265]. Moreover, by *ex-vivo* experiments we demonstrated that NOTCH pathway is responsible for the ability of malignant PCs to induce IL-6 production in BMSCs since MM cells loss their ability when co-culture is treated with a γ-secretase inhibitor.

These findings are further confirmed by immunohistochemical studies and GEP analysis in which a positive correlation between NOTCH activation and IL-6 expression has been found. In fact, immunohistochemical analysis on BM biopsies from MM patients indicated that in the low burden disease there are only a few non-myelomatous cells, localized nearby MM cells, that show IL-6 immunoreactivity. Also considering NOTCH pathway activation, it has been shown that HES6 immunoreactivity is localized both in malignant PCs and in the surrounding non-myelomatous cells and interestingly its intensity increases with bone marrow infiltration of MM cells. These protein data are confirmed also at RNA level. Analysis of gene expression profiling data from mesenchymal stromal cells from healthy donors and MM patients [282] showed the existence of a positive correlation between *IL-6* and NOTCH pathway activity

(evaluated as *HES1* expression level) and the up-regulation of *IL-6* and *HES1* in mesenchymal stromal cells when they are stimulated with MM cells line MM1S.

Overall, our data suggest a key role to NOTCH pathway in MM pathogenesis since it is involved in the important step of communication between MM cells and BMSCs. NOTCH signaling pathway helps MM cells to influence BM niche in order to further support tumor growth in particular through the increase of the production of the most important cytokine for multiple myeloma biology, IL-6.

This evidence along with the finding that NOTCH signaling inhibition reduces IL-6 production makes the NOTCH pathway a promising therapeutic target to suppress tumor cells growth and survival. In particular, the evidence that the inhibition of JAGGED ligands in MM cells strongly reduces the ability of BMSCs to support MM cells growth suggests that the selective inhibition of JAGGED ligands is promising as a NOTCH-direct therapeutic approach in order to reduce increase tumor burden, angiogenesis, inhibition of anti-tumor immune response and drug resistance. This selective approach could be very important since treatments with common γ-secretase inhibitors have several side effects [69, 308, 309] due to contemporary inhibition of the activation of all the 4 NOTCH receptors isoforms. The possibility to specifically inhibiting the dysregulated JAGGED1 and JAGGED2 may provide the rational for a safer and effective NOTCH-directed approach in MM therapy. Considering future perspectives, it could be very interesting to validate the JAGGED1-2 directed therapeutic strategy also on an *in vivo* animal model of MM.

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