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THE ROLE OF NOTCH PATHWAY IN MULTIPLE MYELOMA-ASSOCIATED DRUG RESISTANCE

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SOMMARIO

INTRODUZIONE: Il mieloma multiplo (MM) è una neoplasia ematologica incurabile caratterizzata dallo sviluppo di farmacoresistenza intrinsica o mediata dalle cellule stromali midollari (BMSCs). Nelle cellule di MM, il pathway di Notch può essere iper-attivato a causa dell'iper-espressione dei ligandi Jagged oppure di una trans-attivazione di Notch2 dovuta ad una deregolazione dei fattori trascrizionali MAF. Il microambiente midollare, dove si localizzano le cellule di MM, può contribuire all'aberrante attivazione della via di Notch grazie alla presenza dei ligandi, espressi dalle BMSCs, oppure attraverso diversi stimoli, tra cui l'ipossia. Diverse evidenze in letteratura dimostrano come l'ipossia e il pathway di Notch siano in grado di supportare le cellule staminali tumorali (CSCs), che sono una popolazione cellulare intrinsicamente farmacoresistente e sono spesso causa di sviluppo di recidive nei pazienti. Nel MM sono state descritte le cellule staminali tumorali di MM (MM-SCs) come cellule aventi fenotipo CD138$^-$.

SCOPO: Lo scopo di questo lavoro è stato quello di valutare l'effetto dell'iper-attivazione del pathway di Notch sulla farmacoresistenza intrinseca e mediata dalle BMSCs nel MM; inoltre ho valutato l'effetto dell'ipossia sul pathway di Notch nelle MM-SCs.

METODI: Per gli esperimenti, ho utilizzato due linee cellulari umane di MM, OPM2 e U266, che sono state coltivate in singolo o in co-coltura con la linea umana HS5 o la linea murina NIH3T3 per mimare le interazioni tra le cellule tumorali e le cellule stromali. Nelle co-colture, ho utilizzato la linea HS5-pGIPZ che esprime stabilmente la GFP per poter distinguere al citofluorimetro (FC) le due popolazioni cellulari. Per valutare il ruolo del pathway di Notch nella farmacoresistenza associata al MM, le linee cellulari di MM sono state silenziate per i ligandi Jagged1 e 2 utilizzando due

specifici siRNA (HMCL^{-JAG}) o il controllo scrambled (HMCL^{-SCR}) e trattate con Bortezomib, un farmaco usato comunemente nella terapia del MM; il tasso di apoptosi è stato misurato al FC come percentuale di cellule Annessina-V$^+$. L'analisi dell'espressione genica è stata effettuata tramite RT-PCR quantitativa con primers specie-specifici per meglio caratterizzare il contributo delle cellule di MM (primers umani) e il contributo delle BMSCs (primers murini). Variazioni nell'espressione proteica di fattori anti-apoptotici sono stati analizzati tramite FC. Gli studi ex vivo sono stati eseguiti su campioni primari di pazienti di MM: le cellule primarie di MM CD138$^+$ sono state isolate immunomagneticamente dall'aspirato midollare, mentre le BMSCs primarie sono state isolate dalla frazione negativa, cambiando il terreno dopo 24h dalla semina. Le cellule primarie di MM CD138$^+$ sono state trasdotte con un vettore lentivirale specifico per il silenziamento dei ligandi Jagged1 e 2, mentre le BMSCs primarie sono state colorate con un colorante lipofilico (PKH26) e poi co-coltivate con le cellule primarie di MM CD138$^+$; le co-colture sono state trattate con i farmaci e al FC è stato misurato il tasso di apoptosi come cellule Annessina-V$^+$. Per mimare una condizione di ipossia le cellule di MM sono state trattate con il cloruro di cobalto (CoCl$_2$), per bloccare il pathway di Notch ho usato il DAPT che è un inibitore della γ-secretasi e inibisce il pathway di Notch. Per studiare le MM-SCs ho utilizzato come modello la linea cellulare umana di MM H929, che è stata caratterizzata fenotipicamente tramite FC per valutare variazioni nell'espressione di CD138 a seguito dei trattamenti con CoCl$_2$, DAPT o la combinazione di entrambi. Per verificare l'effetto dell'ipossia sull'attivazione trascrizionale di Notch, ho utilizzato un sistema reporter in cui la linea cellulare umana HEK293 è stata trasfettata con un plasmide contentente una sequenza Notch-responsiva a monte della luciferasi e con plasmidi che codificano per le forme costitutivamente attive di Notch1 (ICN1) e Notch2 (ICN2); ho

misurato le variazioni dell'attività della luciferasi a seguito del trattamento con $CoCl_2$.

RISULTATI: I risultati ottenuti hanno dimostrato che il silenziamento dei ligandi Jagged1 e 2 influisce sulla biologia della cellula di MM, in particolare sulla farmacoresistenza intrinseca, causando una riduzione dell'espressione genica di proteine anti-apoptotiche quali BCL2, Survivin and ABCC1, BCLXL, SDF-1α, CXCR4 e quindi una maggiore sensibilità delle cellule di MM ai farmaci. Per quanto riguarda l'interazione delle cellule di MM con le BMSCs, ho potuto osservare che essi si attivano reciprocamente il pathway di Notch causando un'aumentata farmacoresistenza delle cellule neoplastiche dovuta a: i) un aumento dell'espressione genica di proteine anti-apoptotiche nelle cellule di MM; ii) il rilascio di fattori solubili da parte delle BMSCs, quali SDF-1α e VEGF che sono importanti per la crescita e sopravvivenza delle cellule di MM. Con i miei risultati ho dimostrato che il silenziamento dei ligandi Jagged1 e 2 nelle cellule di MM co-coltivate con le BMSCs, causa da un lato la diminuzione dell'espressione sia genica che proteica di fattori anti-apoptotici, e dall'altro riduce la capacità protettiva delle BMSCs, aumentato l'apoptosi delle cellule di MM trattate con farmaci. Gli stessi risultati sono stati confermati su campioni primari di pazienti di MM da cui sono state isolate sia le cellule di MM primarie CD138^{+} che le BMSCs primarie. Infine ho dimostrato, con un saggio reporter e trattamento con $CoCl_2$, chel'ipossia è in grado di attivare il pathway di Notch; a seguito del trattamento con $CoCl_2$, vi è un aumento delle MM-SCs e l'attivazione del signalling di Notch, stabilizzato da HIF-1α, è necessario per il mantenimento e l'amplificazione dell MM-SCs.

CONCLUSIONI: I miei risultati ottenuti dimostrano come il silenziamento dei ligandi Jagged 1 e 2 sia in grado di ridurre la farmacoresistenza intrinseca delle cellule di MM e quella mediata dalle BMSCs; inoltre l'attivazione del pathway di Notch, indotta da HIF-1α, sia in grado di

sostenere le MM-SCs. Questi risultati dimostrano come il target di Notch possa essere un buon target terapeutico per contrastare l'insorgere di recidive nei pazienti di MM.

ABSTRACT

BACKGROUND: Multiple myeloma (MM) is an incurable hematological malignancy characterized by drug resistance, intrinsic or induced by bone marrow stromal cells (BMSCs). In MM cells, Notch pathway may be aberrantly activated due to the hyperexpression of Notch1, Notch2 or Jagged1 and 2 ligands. This effect may be attributed to genetic mutation only in part (i.e. translocations involving the MAF transcription factors may increase the transcriptional activity on their target gene Notch2). MM cells settle in the bone marrow (BM) and the BM microenvironment may be another player contributing to Notch signaling activation by triggering Notch receptors through BMSC-derived ligands or other stimuli including hypoxia. Indeed, recent evidences indicate that both hypoxic stimuli and Notch signaling activation are involved in cancer stem cell maintenance and self-renewal, thereby contributing to drug resistance due to the resilience of this cancer subpopulation. MM stem cells (MM-SCs) have been characterized as a CD138$^-$ subpopulation.

AIM: The aim of this study was to investigate the outcome of Notch signaling hyper-activation in intrinsic and BMSC-mediated drug resistance in MM cells and MM-SCs.

METHODS: I assessed the effect of Jagged ligands by an inhibitory approach on MM cells. This was carried out by silencing Jagged1 and 2 through specific siRNAs or lentivirally expressed shRNAs. The study got advantage of OPM2 and U266 cell lines and MM primary cells from 10 patients. MM cells were cultured alone, to assess the effect of Jagged

silencing on intrinsic drug resistance, or co-cultured with BMSCs, to investigate the effect of Jagged inhibition on BMSC-mediated drug resistance. The BMSC models used were: i) the human HS5^{GFP+} cell line that, when cultured with MM cell lines, enabled a flow cytometric analysis of variations in drug resistance and anti-apoptotic proteins expressed by MM cells, along with changes in BMSC-production of pro-tumor cytokines (i.e. IL-6 and SDF-1a); ii) the murine fibroblasts NIH3T3. These cell lines mimic BMSCs and, when cultured with MM cell lines, enabled to confirm changes in key proteins by gene expression analysis through RT-PCR using species-specific primers to distinguish the contribution of MM cells (human) or NIH3T3 cells (murine); iii) experiments on primary CD138$^-$ MM cells were carried out using primary BMSCs stained with PKH26 as feeder cells. Intrinsic and BMSC-induced drug resistance was analyzed by challenging MM cells cultured alone or in co-culture systems with standard-of care drugs, i.e. Bortezomib. Apoptosis was assessed by detection of the percentage of AnnexinV$^+$ cells through flow cytometry. Hypoxic BM microenvironment was mimicked by using cobalt chloride (CoCl$_2$), while Notch pathway activation was inhibited using DAPT (a γ-secretase inhibitor). MM-SCs were analyzed in H929 cell line by flow cytometric analysis of the CD138$^-$ subpopulation. The effect of hypoxia on protein expression changes of Notch pathway members (i.e. Notch2 and Jagged1) was assessed by Western blot assay, while changes of Notch transactivation activity were assessed by dual luciferase Notch reporter assay in OPM2 cells and HEK293 cells. The high transfectability level of HEK293 cell line also enabled its transfection with multiple plasmids to assess the specific effect of CoCl$_2$ treatment on the transcriptional activity of Notch1 and Notch2.

RESULTS: The results of this work demonstrate that Jagged1 and 2 increased expression levels affect MM cell biology maintaining high levels

of intrinsic drug resistance through the expression of anti-apoptotic genes, i.e. BCL2, Survivin and ABCC1, BCLXL, SDF-1α, CXCR4, with the consequent increase of MM cell sensitivity to standard-of-care drugs. Concerning the interaction of MM cells and BMSCs, MM cells stimulate the protective behavior of BMSCs, by inducing Notch activation through tumor-derived Jagged1 and 2, with a consequent increase of drug resistance due to: i) release of pro-tumor soluble factors by BMSCs, i.e. SDF-1α and VEGF; ii) the induction of an elevated anti-apoptotic background in MM cells due to an increased expression of anti-apoptotic genes such as BCL2, Survivin and ABCC1. In vitro results were confirmed by co-cultures of primary MM cells. Finally, I verified that an hypoxic stimulus, mimicked by $CoCl_2$, may be a cause of Notch activation in MM cells by increasing the transcriptional activity of Notch1 and Notch2, supposedly through interaction with HIF-1α that prevents ICN proteosomal degradation. Notch signaling activated by $CoCl_2$ positively regulates the MM-SC population. The resilience that characterizes MM-SCs suggests that hypoxia-mediated activation of Notch signaling may be a further mechanism by which the BM microenvironment may induce the acquisition of drug resistance in MM.

CONCLUSION: The evidences that Jagged1 and 2 silencing affects the intrinsic and acquired drug resistance in MM cells support the rationale for a Notch-tailored approach to overcome MM patients relapse.

INTRODUCTION

1. NOTCH PATHWAY

1.1 Introduction

In 1919 T.H. Mohr identified an haploinsufficient Drosophila Melanogaster strain that was characterized by "notched" wings. The gene responsible for that phenotype was named as Notch and was cloned for the first time in 1985 (1). In humans, like other mammals, Notch family is formed by four homologue sequences named Notch1, Notch2, Notch3 and Notch4 which map, respectively, on chromosome 9, 1, 19 and 6,

In 1991, Ellisen and colleagues identified the first ortholog Notch gene, Notch1 in patients with T-cell acute lymphoblastic leukemia (T-ALL) carrying traslocation t(9;7) (q34;q34.3) and it was called trans location-associated Notch homologue-1 (TAN-1) (2). Notch family is formed by four receptor isoforms, Notch1-4, and two ligands family, Jagged ligands (Jagged1 and 2) and Delta ligands (Dll1-3-4) (3).

Further studies demonstrated that Notch regulates many different biological processes such as morphogenesis, proliferation, apoptosis and cellular differentiation and it is fundamental for embryonic development of multicellular organisms (4). In mammals, Notch has a key role in regulating neurogenesis, gliogenesis, myogenesis, angiogenesis, haematopoiesis and in epidermal development (3). Notch is involved in adult tissue homeostasis promoting stem cell self-renewal, regulating cell fate (e.g. commitment in T cell or B cell lineage).

Due to its important in regulating different cellular processes, Notch receptors or ligands mutation or deregulation are often associated to cancer burden, i.e. breast cancer, ovary, prostate, skin (5) and haematological malignancies: Notch1 deregulation is found in T-ALL, while Jagged2 deregulation in multiple myeloma (MM) (6) (7); Notch3 deregulation correlates with CADASIL (cerebral autosomal dominant

arteriopathy with subcortical infarcts and leukoencephalopathy) onset (8) and Jagged1 with Alagille syndrome (3).

1.2 Notch molecular structure (9)

1.2.1 Notch receptor structure

Notch is a protein with a molecular weight of 300 kDa. Notch is a single-pass transmembrane protein formed by an extracellular subunity, a trans-membrane domain and an intra-cellular subunity that are composed by (figure 1.2.1):

- The extracellular domain is responsible for ligands binding thanks to Epidermal Growth Factor (EGF)-like repeats formed by a changeable number of repeats (29-36); Repeats 11-12 mediate receptor-ligand trans-interaction (receptor and ligands are expressed by two diverse cells), thus causing pathway activation; while repeats 24-29 are responsible for cis-interaction (receptor and ligands are expressed by the cells itself) and leads to Notch pathway blocking. Furthermore, there is a negative regulatory region (NRR) which prevents receptor activation without ligand interaction: NRR inhibits the first cleavage in S2 site thanks to its conformation structure that changes only after receptor-ligands interaction.

- The transmembrane domain (TMD) which separate extracellular and intracellular portion

- The intracellular domain is made by Recombination Signal Binding Protein for Immunoglobulin Kappa J Region (RBPj) association module (RAM) domain, which is fundamental for Notch-mediated transcriptional activation because is able to bind to the transcription factor CBF-1, Suppressor of Hairless, Lag-2 (CSL) and RBPj in mammals, promote the transcriptional complex assembly into the nucleus; one nuclear localization signal (NLS) followed by 7 Ankyrin

(ANK) repeats, that recruits nuclear proteins fundamental for transcription complex; two additional NLSs, responsible for nuclear trafficking and a motif rich in proline, glutamin acid, serine and threonine (PEST) at C-terminal of the protein. PEST domain regulates Notch receptor stability thanks to polyubiquitination signals that promote Notch proteosomal degradation.
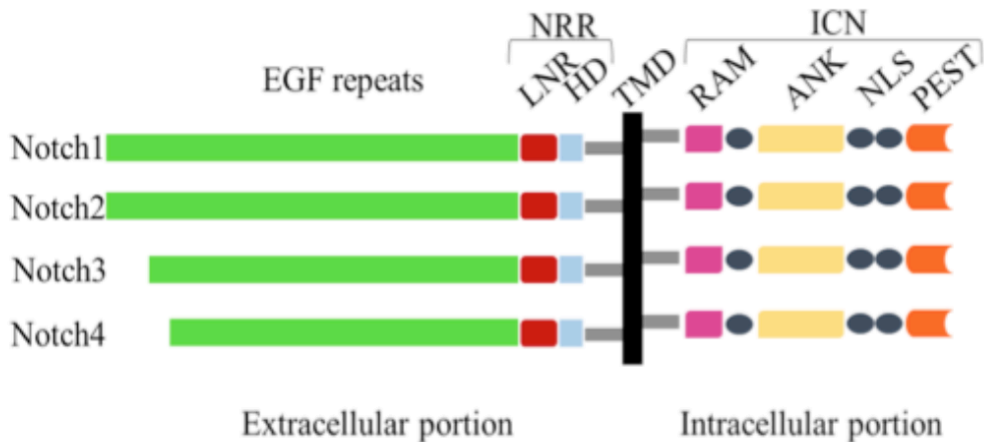
Figure 1.2.1 Notch receptors molecular structures (9).

1.2.2 Notch ligands structure

Notch ligands have a structure similar to those of receptors; they are classified in two families closely related: Delta ligands (Dll-1, 3, 4) and Serrate family (Jagged1, 2). Both ligands families are single-pass transmembrane proteins made by (figure 1.2.2):

- Extracellular domain contains EFG-like repeats (6-16) and the N-terminus (DSL) portion which is responsible for receptor binding and activation; Jagged family, if compared to Delta family, have twice repeats and a cysteine-rich (CR) region.
- Intracellular domain (expect Dll3) contains multiple lysine residues that have a role in ligand activity; PDZ domain in included in

Jagged1, Dll1 and Dll4 ligands and is important for ligands interaction with cellular cytoskeleton and cellular adhesion.
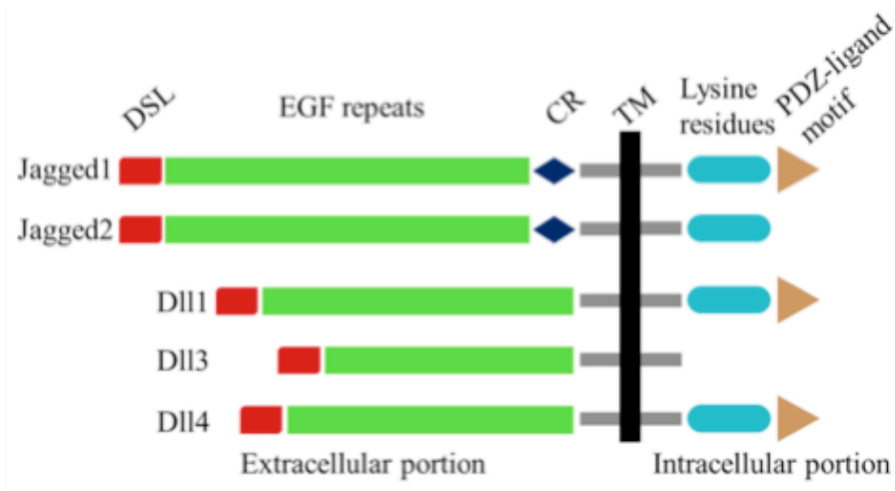
Figure 1.2.2 Notch ligands molecular structures (9).

Among Notch ligands, Dll3 has a different structures because it has missed the region for receptor interaction and trans-activation: Ladi and colleagues demonstrated a possible inhibiting role for Dll3, indeed they demonstrate that, in mammals, Dll3 overexpression causes Notch signalling blocking (10).

1.3 Notch signalling activation

Notch is synthetized in the endoplasmic reticulum (ER) as single polypeptide (pre-NOTCH) formed by extracellular and intracellular domain. The O-fucosyltransferase (OFT1) is necessary for transporting pre-NOTCH from endoplasmic reticulum to Golgi apparatus, where the extracellular domain undergo fucosylation on Serine and Threonine residues (11). Pre-NOTCH is cutted by a furin-like convertase in S1 site leading to the formation of a heterodimer composed by extracellular domain and TMD bound by non covalent bonds (12). Before reaching the cellular membrane,

Notch extracellular domain is glycosylated thus allowing receptor-ligands interaction and influencing ligand specificity (13).
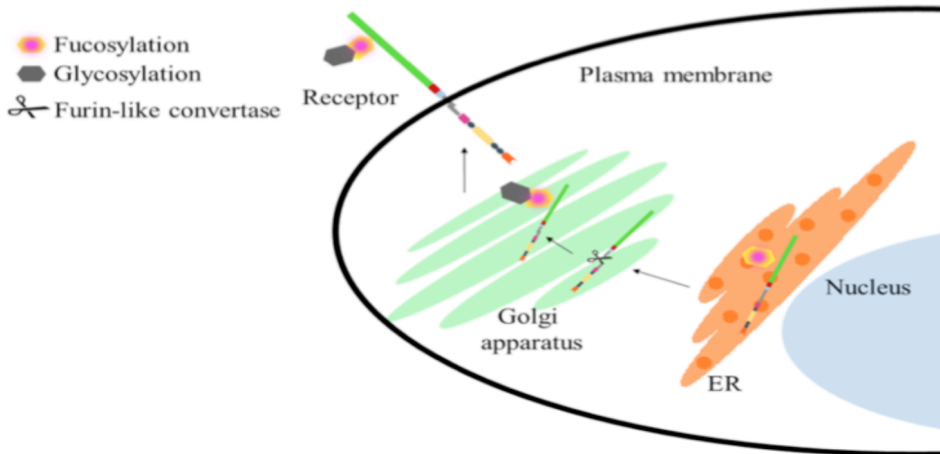
Figure 1.3 Notch receptors synthesis (9).

Upon ligands interaction, there is a conformational change in NRR domain that allow to a A Disintegrin and Metalloproteinase 10 (ADAM10) to exert its proteolytic activity in site S2: the result is a truncated peptide that is still anchored to membrane and recognized by γ-secretase complex which cleaves the single-pass transmembrane portion of Notch. The γ-secretase complex is a multi-complex protease formed by three enzymes: Presenilin-1 or 2 (PS1, 2), Nicastrin (NCT), anterior pharynx-defective 1 (Aph1) and Presenilin enhancer 2 (PEN-2); it recognizes the S3 region, between TMD and RAM domain and causes the release of active Notch intracellular domain (ICN) from the membrane (3). Active ICN migrates into the nucleus where, thanks to RAM domain, it interacts with CSL-RBPj transcriptional factor. If Notch signaling is switched off, the transcription factor CSL inhibit genic transcription in cooperation with co-repressor proteins like SMRT, SHARP, CIR1 and histone-deacetylases (14). Active ICN is able to

uncouple the complex that work as a co-repressor from RBPj and once RBPj is free, the formation of a tertiary complex with Master mind-like protein (MAML) and its co-activators happens (15); finally this big complex induce Notch target genes transcription. Notch1 intracellular domain (ICN1) is bound by a serine/threonine kinase Glycogen Synthase kinase 3-β (GSK3-β) that increase its stability (16); while, CDK8 kinase can phosphorylate nuclear ICN at PEST domain, thus causing the recognition of PEST domain by a E3 ubiquitin ligase, which mediate degradation of ubiquitinylated ICN by proteasomal machinery (17).
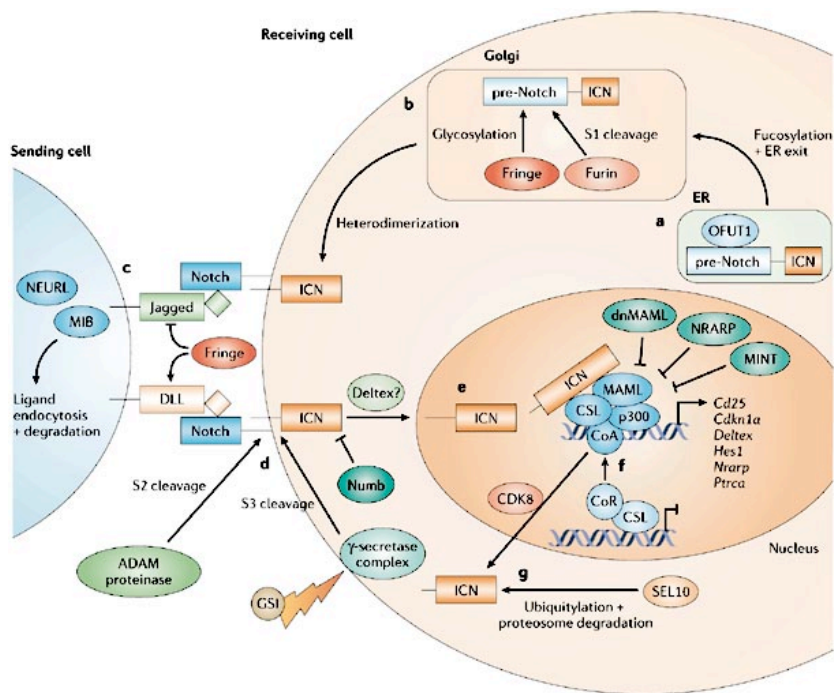
Figure 1.3.1 Notch transcriptional complex (18).

The transcription CSL-dependent is responsible for most of the effects mediated by Notch, but there are evidences in literature that there is also a CSL-independent Notch pathway activation (19). Deltex, that is a zync-finger protein, regulates genic transcription in the CSL-independent

pathway and it interacts with the ANK domain in the ICN. In Drosophila Melanogaster models, but not in mammals, Deltex is a positive regulator of Notch pathway and the hypothesis is that it antagonizes p300 and it reduces genic expression and regulates the expression of particular transcription factors like E47. It seems that ICN itself regulates Deltex expression level (19).

1.4 Notch pathway regulation

Notch signalling is fine-tuned: both receptors and ligands are in dynamic equilibrium between "membrane pool" and "intracellular pool" included in vescicles. Because its relevance, Notch undergoes different modification during its maturation process (figure 1.4).

In the ER, the O-fucosyltransferase 1 (POFUT1) catalyzes the addition of an O-fucose to serine and threonine residues in Notch extracellular domain. POFUT1 can act also as ER chaperone (11) and mediates Notch receptor translocation from ER to Golgi. The adding of the O-fucose on EGF repeats increases Notch-Dll binding and decreases Notch-Jagged binding, by altering Notch affinity to ligands presented by adjacent cells (11, 20).

In the cytoplasm, Notch pathway is regulated by Numb and Deltex: Numb is an adapter protein involved in the endocytosis process of Notch in combination with α-adaptin and Exp-15 and, as shown in different Drosophila and human models, it can inhibit Notch with different mechanisms:

- By binding and activating Itch, which belongs to ubiquitin E3 ligase family and mediated ICN poly-ubiquitination and proteasomal degradation (21).

- By promoting the S2-cleaved Notch endocytosis before the γ-secretase cut and ICN1 release. It works in collaboration with the AP2 domain of α-adaptin and NAK (Numb Associated Kinase) (22).
- By preventing SANPODO gene product localization, that promotes Notch signaling (21).

The role of Deltex has been discussed above.

In the nucleus, Sel-10, Notch regulated ankyrin repeat protein (Nrap) and MSX2-interacting nucleat target protein (Mint) regulate Notch pathway.

Sel-10 is a E3 ubiquitin ligase which binds ICN and recruits Skp1-Cullina-F-Box (SCF) complex, thus causing Notch ubiquitination and its degradation by proteasomal machinery; Sel-10 regulation requires PEST domain to be hyper-phosphorilated by Cyclin-dependent kinase 8 (CDK8); CDK8 hyper-phosphorilates PEST domain after transcriptional activation by binding of MAML to p300 (23).

Nrap can bind CSL-ICN complex to two ANK domains and can either inhibits the complex or destabilizes ICN; Mint inhibits Notch pathway by blocking ICN bound to CSL and the ability of ICN to active transcription (24). The best characterized mechanism by which Notch is degraded is the one mediated by proteasome and by two E3-ubiquitin ligase (Itch and Sel-10), but often the lysosome pathway is preferred: c-Cbl, an ubiquitin ligase, is the key mediator of lysosomal Notch degradation, indeed it binds Notch after a phosphorylation on tyrosine residue in the PEST domain (25).

Notch signaling regulation involves also ligands expression and activity modulation, which is controlled by different mechanisms: EGF repeats in the extracellular domain undergo O-fucosylation that is subsequently modified by Maniac Fringe (MFNG) (26), thus regulating directly the receptor affinity for specific ligands family.

In addition, ADAM can cut ligands in juxamembrane region thus causing a reduction in the number of ligands available for receptor binding (27). Even for ligands, the combined action of ADAM and γ-secretase complex cause the release of ligands intracellular portion that can translocate to the nucleus (28): it is not yet well dissect the mechanism but it seems to regulate ligands availability and their possible intracellular activity.

Ligands have a ubiquitination regulation, as for receptors, and the ubiquitination of multiple lysine residues is involved in this process (29). There are other molecules that can directly interact and influence ligands expression (30): Vascular endothelial growth factor (VEGF) (31); transforming growth factor β (TGF-β) and platelet derived growth factor (PDGF) (32) (33); WNT signaling (34).

Some evidences demonstrate that the formation of an in-cis inhibition complex occurs if both receptor and ligand are in the membrane of the same cell. This mechanism is fundamental in limiting Notch activity area and it determines if the cell can send signal (ligands are more abundant) or if the cell can receive the signal (receptors are more abundant) (35). It has been reported that ligands and receptors can be segregated into different membrane sub-domains inducing either reception or signal transmission (36).
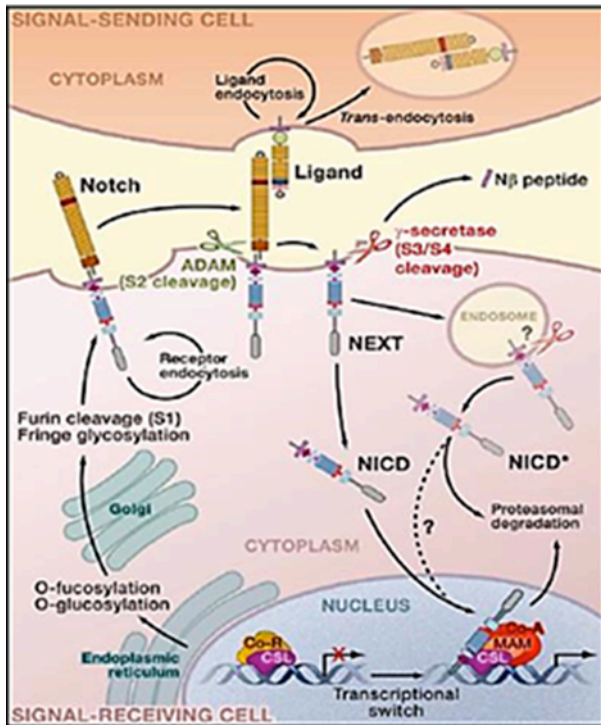
Figure 1.4 Notch pathway regulation.

1.5 NOTCH TARGET GENES

Despite signals mediated by Notch pathway has different results, it has been identified only few Notch target genes in a variety of cellular context. Hairy and enhancer of split (HES) family genes are the most studied and well characterized Notch target in Drosophila; the HES family is conserved during evolution and, in mammals, correspond to HES (1-7) genes and Hey (1, 2, L) family genes that are transcriptional factors with a helix-loop-helix conformation that act as transcriptional repressors. Among Notch target genes there are:

- Negative regulators of the pathway itself (NRARP and Deltex-1).
- Oncogenes (c-myc, cyclinD1, p21/Waf1).

- Genes involved in T-cell maturation and in Th1/2 lineage differentiation (IL2-R, preTa, GATA3).
- Transcription factors (HoxA5, 9, 10 and NFkB2)
- Anti-apoptotic protein coding-genes (bcl-2)
- NOTCH1 and NOTCH3.

1.6 THE ROLE OF NOTCH SIGNALLING IN CANCER

Notch pathway, as previously said, is involved in different biological processes and controls many cellular function, like cell cycle (37), cell differentiation and metabolism (38). Because of its relevance, pathway alteration or deregulation result in cell transformation and tumor burden. Interestingly, Notch can't be defined as an oncogene or a tumor suppressor because its outcome is tissue-specific or depends on the cellular type.

Tumor type	Notch/ligand	Function
T-cell acute lymphoblastic leukemia (T-ALL)	Notch1	Oncogenic
Acute myeloid leukemia (AML)	Jagged1	Oncogenic
B-chronic lymphocytic leukemia (B-CLL)	Notch1, Notch2/Jagged1, Jagged2	Oncogenic
Diffuse large B-cell lymphoma	Notch2	Oncogenic
Marginal zone lymphoma	Notch2	Oncogenic
Multiple myeloma (MM)	Notch1, Notch2/Jagged1	Oncogenic
Precursor B-cell acute lymphoblastic leukemia (pre-B-ALL)	Notch1–4	Tumor suppressive
Cutaneous squamous cell carcinoma (SCC)	Notch1	Tumor suppressive
Melanoma	Notch1	Oncogenic
Breast cancer	Notch4, Notch1,	Oncogenic
Human breast cancer	Notch2	Tumor suppressive
Human breast cancer	Notch1/Jagged1	Oncogenic
Non-small cell lung cancer (NSCLC)	Notch3	Oncogenic
Adenocarcinoma of the lung (ACL, a type of NSCLC)	Notch1/Jagged1, Dll1, Dll4	Tumor suppressive
Small cell lung cancer (SCLC)	Notch1/2	Tumor suppressive
Colorectal cancer (CRC)	Notch1/Jagged1, Jagged2, Dll4	Oncogenic
Pancreatic cancer	Notch1, Notch3/Jagged2, Dll4	Oncogenic
Glioblastoma	Notch2	Oncogenic

Table 1.6 Notch as oncogene or tumor soppressor in many cancers (5).

1.6.1 Notch as oncogene

Notch pathway deregulations occur either in solid tumor (breast cancer, melanoma, neuroblastoma, prostate cancer (39)) or haematological malignancies (leukemia (40), multiple myeloma (41)). From 90s up-to-date, Notch signalling aberration correlate with haematological malignancies like T-ALL, acute myeloid leukemia (AML) and multiple myeloma (MM). In T-ALL, that is an aggressive neoplasia of immature T cells, it has been

demonstrated that 60% patients are carrying Notch pathway mutations that are able to accelerate disease progression (40). In AML, the role of Notch pathway is not well characterized, but my group published a work demonstrating that in AML primary sample, Jagged1 expression was high even if Notch pathway was partially active (42): these findings suggest that may occur a pathway activation ligands-mediated and Notch independent (43). The role of Notch pathway in MM will be discuss in the next chapter. In breast cancer, is evident how Notch pathway has an oncogenic role: indeed the overexpression of Notch4 activates transforming growth factor-β (TFG-β) and hepatocytes growth factor (HGH) signalling pathway, promoting tumor invasion in ductal breast carcinoma (44). In human cervical cancer, Notch is aberrantly activated by either the accumulation of Notch1 and Notch2 or by Jagged1 expression: the final result is activation of PI3K/AKT pathway and Myc upregulation (45).

1.6.2 Notch as tumor soppressor gene

There are evidences showing that Notch signaling can act as a tumor suppressor; but the mechanism is still not well defined and new data demonstrate that it is probably tissue and time specific. Rangarajan and colleges well demonstrate that in keratinocytes, Notch signaling controls cell cycle and its activation causes a block in cell proliferation and cell cycle arrest: this effect is mediated by p21 and p27 upregulation and inhibition of Wnt pathway by β-catenin (46).

2 MULTIPLE MYELOMA

2.1 INTRODUCTION

Multiple myeloma (MM) is a malignant disorder of plasma cells that accounts for approximately 11% of hematologic malignant disorders. The annual age-adjusted incidence is 4,3/100000/year, with 30330 estimated new cases in 2016 in the US (47). This type of cancer is characteristic of the elderly, and the average age at diagnosis is 66 years, with only 2% of patients that are younger than 40 years (48). Despite the introduction of new drugs and the development of new clinical protocols, MM is still an incurable disease, with a survival rate at 5 years of 49% (http://seer.cancer.gov/csr/1975_2013/). The most common symptoms in MM are fatigue and bone disease (bone pain and osteolytic skeletal lesions), which are present in 80% of patients. Other recurrent symptoms are anemia, immunodeficiency and decreased renal function due to Ig precipitation. Multiple myeloma is now considered as the evolution of Monoclonal gammopathy of undetermined significance (MGUS), an asymptomatic condition that is estimated to be present in 3-4% of the general population older than 50 years (49) (50), from which develops due to pathogenic events at a rate of 1%/year (51). It represents a phase of asymptomatic expansion of clonal plasma cell that can be clinically defined by the presence of a percentage of clonal plasma cells ($CD19^-/CD56^+/CD45^-/CD38^+$) <10% of all bone marrow mononuclear cells, by the absence of end organ damage and by a concentration of Ig in the serum lower than 3 g/dl. The passage from MGUS to MM is clinically defined by an increase in the percentage of malignant plasma cells, that exceeds 10%. MM, depending on the presence or not of any other characteristic symptoms, can be further classified in smoldering (asymptomatic) MM and symptomatic MM. Early stages MM cells present a high dependency on the supportiveness of the bone marrow microenvironment, in particular of the bone marrow stromal cells (BMSCs).

MM cells interact directly with these cells, or can benefit of the release of pro-tumor soluble factor (i.e. IL-6). During disease progression, however, tumor cells acquire independency from the surrounding microenvironment, being now able to migrate outside the bone marrow, generating extra-medullary lesions (plasmacytoma) or becoming leukemic (plasma cell leukemia, PCL) at terminal stages.

2.2 MM PATHOGENESIS

MM is a neoplasia with different stages and each one is characterized by an acquired genetic alteration within MM cells, thus causing alteration of important signalling pathways. These steps can be distinguished in: 1) transformation of normal plasma cells to MGUS; 2) progression of MGUS to MM; 3) final evolution to extra-medullary diseases (figure 1) (52).
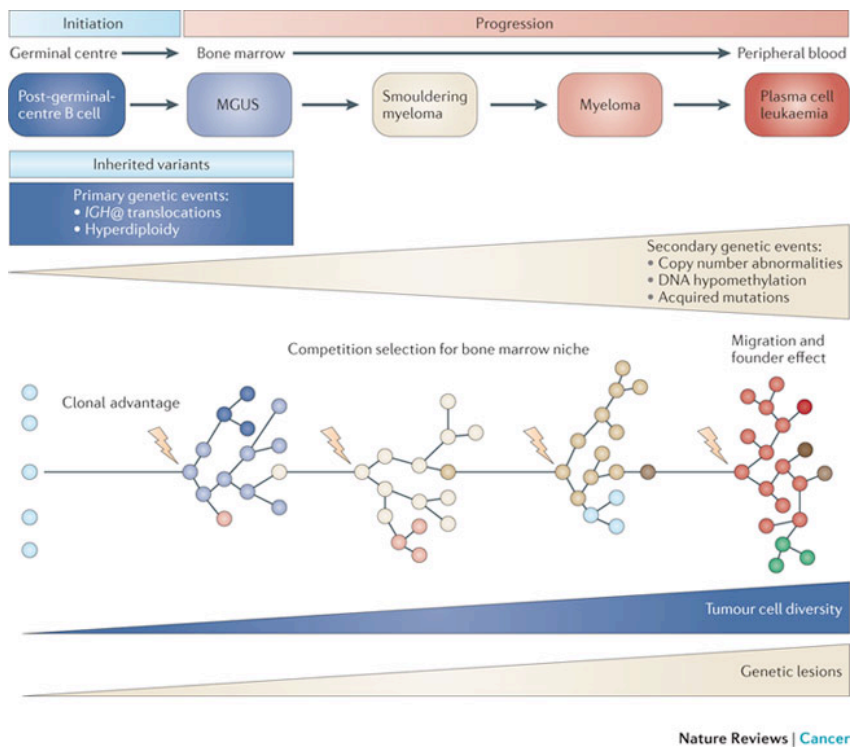
Figure 1: Progression of multiple myeloma (52).

Recent data obtained from analyses conducted with advanced genetic techniques have shown that disease progression from MGUS to MM is not necessarily direct, since in certain cases MM originates as a de novo condition.

B-cells, during their maturation process, go through two different rounds of genomic rearrangement regarding light and heavy chains. In the germinal centre of the secondary lymphoid organs, B cells rearrange heavy chains by interacting with dendritic or T cells in an antigen-dependent manner and becoming plasmacells secreting immunoglobulins upon antigens presentation (53) (54). Plasmacells arrest their cell cycle in the G0/G1 phase and either remain in the lymph nodes as memory B-cells or return to the BM as long-lived plasmacells. The first genetic alteration which gives rise to MM, should regard cell cycle machinery, because plasmacells within

the BM have a really low proliferative rate. The hypothesis is about the fact that there could be two distinct mechanism regulating cell cycle abnormalities inducing plasmacells proliferation, in particular cyclin D family. The first mechanism comprises the arise of recurrent chromosomal translocations in the immunoglobulin heavy chain (IgH) locus at 14q32 (55). The most frequent chromosomal translocation is t(11;14)(q13;q32), observed in 15–20 % of MM cases (56), followed by t(4;14)(p16;q32), with a 12–15 % prevalence (57, 58). Other more rare translocations, including t(14;16)(q32;q23), t(14;20)(q32;q11), and t(6;14) (p21;q32), have a frequency <5 % (59). Each of these translocations induce the regulation of the cyclin D1:

- t(11;14)(q13;q32) translocation causes cyclin D1 overexpression gene located in 11q13 because it is controlled by (60).
- t(6;14)(p21;q32) translocation induces cyclin D3 gene at 6p21 overexpression (61).
- t(4;14)(p16;q32) translocation induces the upregulation of the histone methyltransferase MMSET (also known as WHSC1/NSD2/KMT3G), which modify the methylation profile of the genome, increasing the levels of H3K36me2, thus causing aberrant expression of cyclin D2 (62) (63).
- t(14;16)(q32;q23) and t(14;20)(q32;q11) Translocations upregulate Maf family transcription factors C-MAF and MAFB, two transcription factors that can increase transcription levels of the cyclin D2 (63) (62).

The second mechanism, that happens in the stage of malignant transformation of plasmacells to MGUS, is hyperdiploidy and is observed in up to 55 % of MM patients, and is overlapping with the presence of translocation of the 14q locus in 10 % of MM cases (64). For still unknown

reasons, hyperdiploidy induce a gain of the odd-numbered chromosomes 3, 5, 7, 9, 11, 15, 19, and 21, but it is known that in trisomy 11, hyperdiploidy (approx. 30 %), may derive from an overexpression of the cyclin D1 due to an increase in gene expression. In the stage from MGUS to MM, there is an increased percentage of malignant cells resident in the BM, because they have accumulate genetic alteration, causing an increased proliferation rate; the two major pathways involved in this process are the oncogenic Ras and Myc, as indicated by an increased frequency of the point mutations of K-Ras and N-Ras (7 % in MGUS to 24–27 % in MM) (65, 66). Evidences from literature demonstrated that, during MM progression from MGUS to MM, MM cells carrying t(11;14) translocation, carry the deletion of chromosome 13, causing an haploinsufficiency of the retino-blastoma (RB) tumor suppressor gene at 13q14, inducing cell proliferation and disease progression (67). In the final stage of the disease, named as plasmacells leukemia (PCL), MM cells become independent from the BM microenvironment: MM cells reach blood circulation and inducing lesions in other organs. PCL stage occurs even because NFkB pathway is constitutively activated (68) and another feature is the presence of chromosomal abnormalities, involving e.g. deletion of 17p13 locus inducing p53 inactivation.

2.3 MM THERAPY & DRUG RESISTANCE

MM is still an incurable disease despite the development in recent year of many new drugs and therapeutic protocols. MM patients at diagnosis are divided into different groups based on their age, their category of risk, the presence of eventual comorbidities or other limiting factors that could interfere with the treatment. For newly discovered patients the most diffused approach consists of an initial period (4 months) of treatment with three different classes of compounds (proteasome inhibitors, immunomodulatory drugs, corticosteroid) and to which follow the

autologous hematopoietic stem cell transplantation. Due to the risk of toxic and/or fatal complications related to the transplantation, this approach is offered only to patient younger than 65 years. These patients, after an initial treatment with the "VRd" protocol (bortezomib, lenalidomide, dexamethasone), undergo the "induction chemotherapy" to release the stem cells into the blood flow. After that stem cells have been collected, a new more potent high dose round of chemotherapy is executed in order to kill most, but not all, the tumor cells. Once the treatment has been complete, stem cells are injected back to the patient to allow bone marrow regeneration. This approach does not cure multiple myeloma, but increase overall survival. However, only a small percentage of patients at diagnosis can be treated with this approach. These non-transplantable patients are usually treated with the same regimen "VRd" for longer periods.

As previously said, different classes of drugs have been released in recent years:

- Chemotherapics (Melpahalan)
- Corticosteroids (Dexamethasone);
- Immunomodulating agents (lenalidomide, thalidomide, pomalidomide)
- Proteasome inhibitors (Bortezomib, Carfilzomib, Ixazomib).

Despite these innovations in MM therapy, MM patients will develop resistance to treatment at a certain point due to the presence of both intrinsic and environmental mechanisms that induce drug resistance. Indeed, together with mechanisms activated by the presence of genetic abnormalities, i.e. p53 mutations or deregulation of the NF-κB pathway, interactions of multiple myeloma cells with the surrounding microenvironment can induce drug resistance. Soluble factor-mediated

drug resistance (SFM-DR) and cell-adhesion mediated drug resistance (CAM-DR) are the two complementary mechanisms induced by cell adhesion that have been shown to prevent cell death (69) (70). SFM-DR can be explained by involvement of IL-6, the main cytokine in MM, that, through macrophage inflammatory protein-1α (MIP-1α) can foster cell survival by enhancing adhesion of myeloma cells to bone marrow and fibronectin. On the other hand, adhesion of MM cells to BMSCs or ECM proteins by β1 integrins mediate CAM-DR. When myeloma cells adhere to fibronectin, they become resistant to multiple drugs, including doxorubicin and melphalan (71). However, different types of ECM components were altered in different drug resistant MM cells. For example, CAM-DR to doxorubicin, melphalan, vincristine, bortezomib and mitoxantrone has been induced in MM through adhesion to FN or BMSCs by VLA4 integrin (α4β1) and IEA-1 (72) (73).

2.4 ROLE OF NOTCH PATHWAY IN MM

Notch signaling is one of the key signaling pathway that results to be dysregulated in MM due to different mechanisms, some of them are still not completely elucidated:

- NOTCH1 and JAGGED1 result to be overexpressed during disease progression, even if no known mechanism was reported;
- A group of MM patients (approximately 6%) carrying the translocations t(14;16)(q32;q23) and t(14;20)(q32;q11) showed higher levels of transcription of the gene coding for NOTCH2, that result in an increased activity of the Notch pathway. This effect was due to the activation of the C-MAF and MAFB transcription factors induced by the translocation themselves (74).
- An early complex event, that probably occur during the MGUS phase, involves different possible mechanisms and induces JAGGED2 deregulation. Three different mechanisms have been

proposed to explain JAGGED2 overexpression: hypomethylation of the promoter of JAGGED2; alterations in the expression of Skeletrophin, an Ubiquitin-ligase necessary for JAGGED2 activity; loss of SMRT/NCoR2 corepressor, resulting in JAGGED2 promoter acetylation and increased transcription (75).

Overexpression of Notch related proteins in MM has as a first result the abnormal activation of the Notch signaling inside tumor cells themselves. This may derive from both homotypic interactions between nearby myeloma cells, as well as from the contact with surrounding stromal cells. The importance of the activation of Notch signaling in tumor cells is demonstrated by the effects resulting from the inhibition of the pathway: increase rate of apoptotic cells, decreased proliferation rate and higher sensibility to compounds that increase apoptosis (i.e. Bcl-2/Bcl-XL inhibitors) and to standard chemotherapics. An important result obtained by our laboratory, and confirmed both in vitro and in vivo, allowed us to link Notch pathway with the well-known CXCR4/SDF1α axis, that has been reported to play a crucial role in MM migration toward the bone marrow, as well as in promoting tumor survival and growth (76). For this reason, high activation levels of the Notch signaling in MM cells could be connected to an increased ability to migrate inside the bone, generating new lesions. In the last years, another crucial role for Notch signaling has been reported in cancer stem cell self-renewal. A role for the Notch pathway in the MM stem cells (MMSCs)-mediated effects has also been proposed on the basis of functional assays. Indeed MMSCs, for which there are not univocal marker since their origin is still a matter of debate, seems to play a role in drug resistance, tumor dormancy and relapse. Chiron and colleagues, on the other side, reported that JAGGED2 expression positively correlates with the spontaneous clonogenic growth of MM cells, while Jagged silencing in vivo impaired tumor growth (77).

Homotypic interactions between tumor cells are not the only interactions mediated by Notch receptors/ligands. As reported by Xu and colleagues, BMSCs express DLL1 that can engage with the NOTCH2 receptor present on the membrane of MM cells. Activation of the signaling cascade by NOTCH2 is then responsible for the upregulation of the gene coding for the CYP1A1 (cytochrome P450, family 1, subfamily A, polypeptide 1), a cytochrome involved in the development of resistance to treatment with bortezomib. An important outcome derived from the JAGGED2-mediated interaction between MM cells and BMSC is the increased secretion of soluble factors by the latter, including interleukin 6 (IL-6), vascular endothelial growth factor (VEGF) and insulin-like growth factor 1 (IGF1). These soluble factors act as pro-tumor soluble factors, exerting their function on tumor cell:

- IL-6 is the major growth factor for MM cells, involved also in the development of resistance to dexamethasone in vitro;
- VEGF has a double effect, by promoting MM cell growth and stimulating neo-angiogenesis;
- IGF1 promotes survival in MM cell and development of bortezomib resistance.

A paper recently published by our group revealed the effects of Notch signaling activation in the context of bone disease development, showing that Notch signaling activation in both MM cells and BMSCs stimulates the release of the major osteoclastogenic soluble factor, RANKL. Once released, RANKL engages RANK receptor in OCL progenitors that activate the osteoclastogenic NF-kB pathway; this in turn result in the increased expression of NOTCH2 receptor, that can interact with the Jagged ligands expressed by MM cells, activating Notch pathway in OCL progenitors too (78). Since osteoclast formation and the insurgence of bone lesions represent two processes in which are involved also OBLs, Zanotti and

colleagues analyzed the effects of Notch activation in OBL, demonstrating that it inhibits OBL differentiation. These results were then confirmed by an in vivo model, in which treatment with γ-secretase inhibitor reverted the inhibition of OBL maturation (79).

3. THE BONE MARROW MICROENVIRONMENT IN MULTPLE MYELOMA

3.1 INTRODUCTION

Different evidences find out in recent years, have shown that either different types of solid tumors or haematological malignancies depend from the surrounding microenvironment. MM localizes within the bone marrow (BM) and is strictly dependent from its microenvironment which is composed by different cell types and non-cellular molecules. The different cell types resident within the BM are: BM fibroblast-like stromal cells (BMSCs), vascular endothelial cells, hematopoietic stem cells (HSCs), progenitor cells, immune cells, erythrocytes, osteoclasts and osteoblasts. The non-cellular components, that is named as extracellular matrix (ECM) gives support to the cellular compartment and is composed by a variety of different proteins, i.e. fibronectin, collagen and laminin.
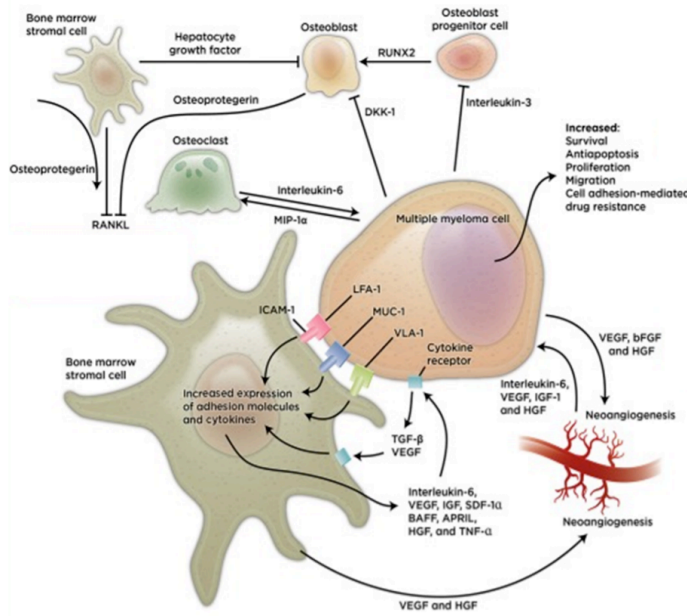

Figure 2: MM bone marrow microenvironment (80).

MM cells can directly interact with the BM microenvironment, thus causing the activation of different pathways and, as a consequence, influencing MM cells biology in term of cell growth, survival, migration, invasion and drug resistance. Furthermore, it is known that MM-associated comorbidities, such as osteclastogenesis, or the release of soluble factors crucial for MM cells are triggered by the interaction of neoplastic cells with either cellular and non-cellular compartments (7).

3.2 ADHESION MOLECULES

MM cells express on their surface different classes of integrins, that are adhesion molecules involved in MM cells contact with components of ECM, laminin, collagens and fibronectin; the integrin responsible for this interaction is the β1 integrin (81). MM cells express other types of surface proteins mediating their interactions with the BM microenvironment, among which is remarkable CD138, also known ad Syndecan-1. Adhesion molecules have a role, not only in mediating MM cells interaction with other cells, but also in drug resistance because they induced a phenomenon known as cell adhesion mediated drug resistance (CAM-DR), preventing MM cells drug-induced apoptosis (69). Bortezomib, a proteasome inhibitor and a commonly used drugs in therapy, overcame CAM-DR by selectively downregulating the very late antigen-4 (VLA-4) expression in MM cells (72).

3.3 SOLUBLE FACTORS

All the signals that MM cells receive and send within bone marrow niche, can be either secreted cytokines or proteins presented on cellular membrane of different cells resident in the BM: in both cases, they play a fundamental role in sustaining disease progression and in mediating several biological processes described above. Cellular interaction happen in both direction and, for this reason, MM cells can release soluble factors or express receptors stimulate cells of the BM, thus causing the establishment of a "self-sustaining loop" in which, MM cells "shape" BM

cells to release cytokines that sustain malignant cells progression. The main cytokines released by MM in the BM microenvironment are:

- Tumor necrosis factor-α (TNFα)
- Transforming growth factor-β (TGF-β).
- VEGF,
- Angiopoietin-1,
- FGF-2
- Matrix metalloproteases (MMPs).

Moreover, "shaped" BM microenvironment cells secrete different cytokines such as IL-6, VEGF, Stromal derived factor 1 (SDF-1) (CXCL12), Hepatocyte growth factor-scatter factor (HGF-SF) and Insuline-like growth factor-1 (IGF-1). IL-6 is a secreted cytokine by BMSCs that has fundamental role in regulating MM cells biology; MM cells also express IL-6 receptor (IL-6R) on the cellular surface, hypothesizing that can be an autocrine regulation of IL-6 production and uptake by MM cells. In the past, many published articles demonstrate that BMSCs were the major producer of IL-6 and in 1996, Chauhan and colleagues found out that MM cells-BMSCs interaction can boost the secretion of IL-6 by BMSCs and IL-6 transcription were regulated by NFkB pathway; they also demonstrated that different cytokines classes, e.g. IL-1α, IL-1β, TNFα and VEGF, can activate IL-6 transcription and secretion in bot MM or BMSCs cells (82).

VEGF, a secreted growth factors by BMSCs and MM cells, induces malignant cells proliferation by activating ERK signalling and promotes MM cells migration by triggering protein kinase C (PKC)-dependent response (83), finally, it stimulates the expression of IL-6 microvascular endothelial cells and BMSCs (84). TNFα is a pro-infiammatory cytokine involved in bone resorption processes and is expressed by BMSCs and by MM cells.

TNFα effects dependd on the cellular context: in MM it promotes proliferation, expression of adhesion molecules (ICAM-1, VCAM-1 and VLA-4) and activation of the MAPK/ERK pathway; while in BMSC it induces IL-6 secretion, activation of the NF-κB pathway and expression of adhesion proteins (ICAM-1 and VCAM-1) (85). MM cells express metalloproteases (MMPs), that are zinc-dependent protease able to cleaved protein in the ECM, increasing the ability of neoplastic cells to migrate and inducing the releasing of growth factors trapped inside the ECM (86).

3.4 SDF1α/CXCR4 AXIS IN MULTIPLE MYELOMA

Chemokine family is a superfamily of proteins (6-14kDa) released by different cell types during the inflammatory processes and have overlapping functions. Chemokines have a role in different contexts, either pathological or physiological and are classified in two groups: the CXC family and the family CC. Secreted chemokines can bind to specific receptor, that is a G-protein 7-span transmembrane receptors (GPCRs), inducing their specific effects. The peculiar feature of chemokines family is that a receptor can bind different types of chemokines and a chemokine can bind to a variety of receptors. In MM, chemokines have a fundamental role in disease progression and, in particular, the SDF1α/CXCR4 axis is fundamental for MM cell homing in the BM, adhesion, growth and motility: BMSCs release SDF-1α that binds with CXCR4 receptor expressed by MM cells in the membrane. In literature, there are several evidences of the importance of this chemokine system in vitro and ex-vivo models: it has been observed that SDF-1α levels correlates with disease progression and that it was significantly increased within different areas of the BM where MM cells are more concentrated; furthermore, in in vitro systems, SDF-1α inhibition in BM-mesenchymal stem cells (BM-MSCs) caused a a downregulation of pathways associated with adhesion, migration and survival. When, in MM cells, SDF-1α binds to CXCR4 induces the

activation of P13K and ERK/MAPK pathways, and cytoskeleton modifications helping MM cells to migrate: regarding this Parmo-Cabañas and colleagues demonstrated that, in two different murine MM cell lines, SDF-1α acts as chemoattractant inducing an increased expression of MMP-9 that helps malignant cell migration and invasion (87). The integrin α4β1, known as VLA-4, has a role in promoting transendothelial migration of MM cells induced by SDF-1α stimulus, thus causing an increased MM cells adhesion CS-1/fibronectin and VCAM-1, thanks to sphingosine-1-phosphate and the activation of the GTPase RhoA: RacI and RhoA, that belong to GTPase RhoA family, promote malignant cells migration or adhesion thanks to the presence of ROCK, a Rac1 and RhoA effector, thus causing actin polymerization and activation of LIMK, SRC, FAK and cofilin. Data from in vitro experiments as well as correlation studies on patients affected either by MGUS or by MM showed that SDF-1α have a role in angiogenesis. The hypoxia-inducible factor-2 (HIF-2) binds to SDF-1α promoter, increasing the levels of SDF-1α, as well as the expression of CXCR4 in MM primary cells and cell lines. Another SDF-1α receptor is CXCR7, that is expressed on the cellular surface of angiogenic mononuclear cells (AMCs): it is involved in trafficking and homing of AMCs to BM areas of MM growth and contribute to neo-angiogenesis; recent studies indicate that neo-angiogenesis can be a new therapeutical target to inhibit tumor growth at very early stages. SDF-1α has also a role in regulating osteoclastogenesis and bone resorption in MM. There are different findings supporting this hypothesys: first, SDF-1α levels positively correlated with bone resorption in MM patients; second, if an osteoclasts precursors culture is supplemented with SDF-1α, there in a motility and activation, as well as a positive variation in the number and the size of resorption pits. SDF-1α ability to control osteoclast differentiation might be mediated by Bruton tyrosine kinase (BTK). BTK, a protein involved in B-

lymphocyte development and in osteoclastogenesis, may induce migration toward SDF-1 with the consequently homing in the BM.

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4. HYPOXIA

Hypoxia is described as a state in which oxygen pressure is below a critical threshold ($pO_2 < 5$ mmHg), thus reducing organs functions; it can be caused by different conditions: increased altitude, localized ischemia or in solid tumor the core is hypoxic. Hypoxia plays also physiological roles in mammals, regulating embryogenesis, erythropoiesis, angiogenesis and, at cellular level, switching aerobic to anaerobic metabolism and inducing the expression of proteins involved in cell death and apoptosis processes (88).

4.1 HIF-1α PATHWAY

Hypoxia inducible factor-1α is the master regulator of hypoxic response: it is a transcription factor formed by two subunits: HIF-α subunit which expression is regulated and HIF-β that is costitutively expressed (89). It has been described three different HIFα isoforms: HIF-1α, HIF-2α and HIF-3α; HIF-1α expression is ubiquitous while HIF-2α is tissue speficic (90). HIF-3α has double effect: it can bind HIF-1β to active transcription or it can act as HIF-1α dominant-negative inhibitor because it has a truncated inhibitory PAS domain that is a result of a splice variant (91).

The well-characterized HIF-1α regulation is the mechanism mediated by ubiquitin-proteasome degradation and oxygen level is the sensor switching on this process.

In aerobic state, HIF-1α is hydroxylated by specific prolyl hydroxylases (PDH1, PDH2 and PDH3) on two conserved proline residues located within the oxygen-dependent degradation (ODD) domain and the reaction requires oxygen to occur (92); under hypoxic condition, PDH activity is blocked, causing HIF-1α stabilization.

HIF-1α hydroxylation causes binding of Von Hippel Lindau protein (pVHL) to ODD domain that creates the binding site of an E3 ubiquitin ligase complex (elongin C, elongin B, cullin-2 and ring-box 1) that poly-

ubiquitinate HIF-1α inducing its degradation by the proteasomal machinery (93) (figure 4.1).

The central role of pVHL is underlined in the Von Hippel Lindau (VHL) disease, in which VHL gene is inactivated, causing the development of highly vascularized tumours in the kidneys, retina and central nervous system (94).
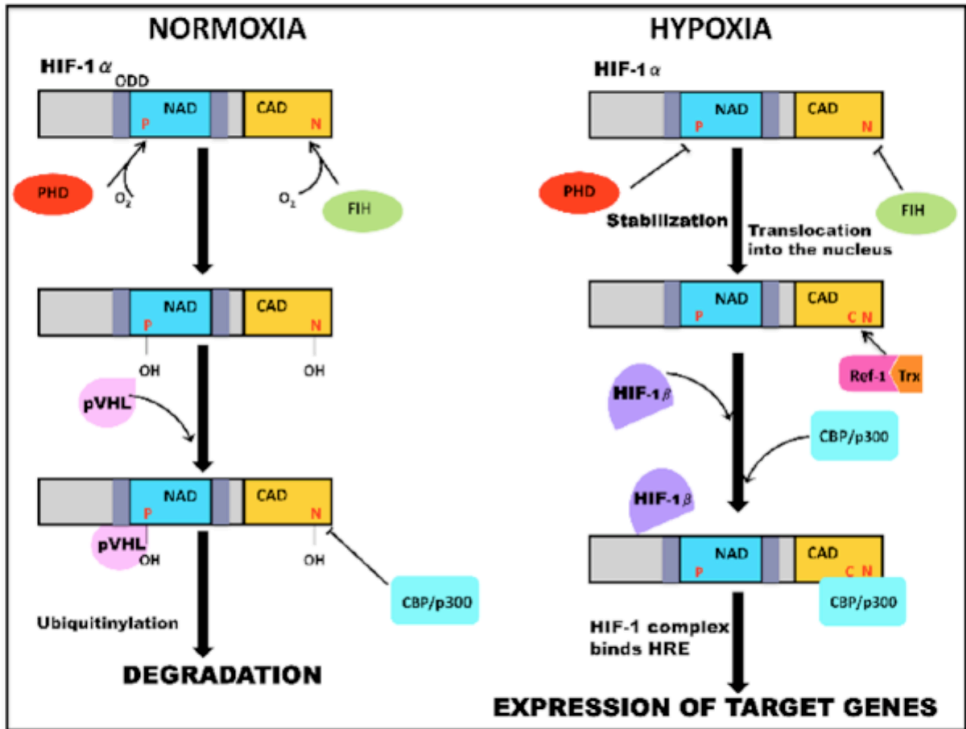
Figure 4.1. Hypoxia pathway (94).

4.2 HIF-1α TARGET GENES (88)

HIF-1α binds to specific Hypoxia rensponsive elements (HRE) and activates transcription of more than 100 genes that are involved in adapting cell to hypoxia and regulate a variety of cellular processes:

- Anaerobic metabolism: HIF-1α promotes switch metabolism to anaerobic condition; it promotes anaerobic glycolysis and upregulates the expression of glucose transporter (GLUT1 and GLUT3) and glycolytic enzimes. HIF-1α activation also stops Kreb's cycle by upregulating pyruvate dehydrogenase kinase I.

- pH regulation: the increased glycolysis causes a toxic intracellular acidosis due to the increased production of lactic acid and CO_2. HIF-1is able to upregulate the expression of monocarboxylate transporter 4, which mediates lactic acid efflux, and of membrane-

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bound carbonic anhydrase IX, which catalyses the conversion of extracellular CO_2 to carbonic acid (H_2CO_3).

- Angiogenesis: HIF-1α target genes include several pro-angiogenic factors, among which there is vascular endothelial growth factor (VEGF); the major consequence is the formation of new blood vessels that can supply nutrients and oxygen.

- Other responses: HIF-1α induces an increased erythropoiesis and has a role in changes in cell proliferation because it affects c-Myc expression; in solid tumor it promotes metastasis and its expression correlates with poor prognosis.

4.3 HIF-1α & NOTCH PATHWAY (95)

It is known from literature that Notch pathway and HIF-1α signalling interact but the mechanism of how this happens is not well characterized. Gustaffson and colleagues tried to elucidate how Notch and HIF-1α interact. They started to evaluate if hypoxia could upregulate the expression of Notch target genes and they find out that Hey2 and HES1 mRNA was increased respectively in C2C12 cells and in neural stem cells; the activation of Notch target genes by hypoxia was a mechanism Notch mediated because, in hypoxic condition, there is the activation of reporter gene controlled by a Notch-responsive promoter. They hypothesized two mechanisms by which HIF-1α interacts with Notch pathway: on one hand, HIF-1α binds ICN thus preventing Notch from degradation and stabilizing Notch transcriptional activity (Figure 4.3). On the other hand, the authors proposed also that HRE sequences are contained in the promoter region of Notch target genes, Hey2, which directly binds HIF-1α only if Notch pathway is active.
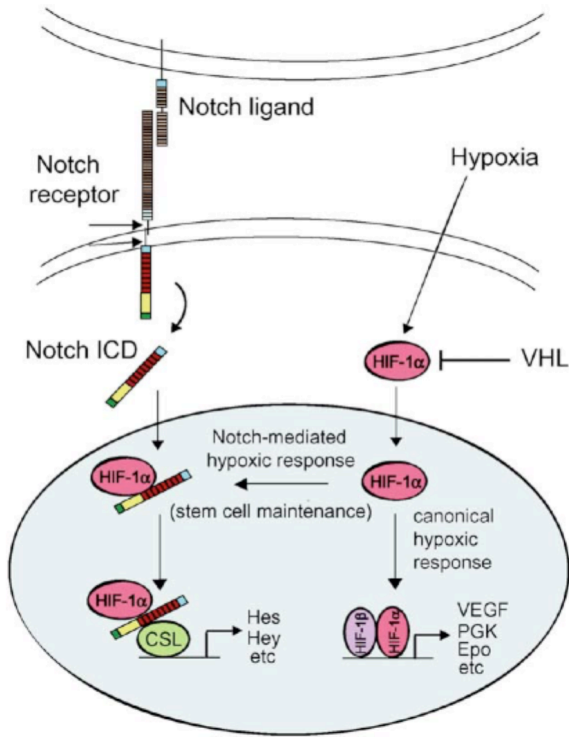
Figure 4.3 ICN and HIF-1α interaction.

5. CANCER STEM CELLS

5.1 INTRODUCTION

In 1994, Lapidot et al. first described cancer stem cells (CSCs) in a AML study: they identified a population enriched with $CD34^+/CD38^-$ cells by transplanting it in severe-combined immune deficient (SCID) mice (96). Nowadays, CSCs were described in different solid tumors, breast, brain, prostate, melanoma; and notably, researches find out that about 100 CSCs are sufficient to create tumor in non-obese diabetic/severe-combined immune deficient (NOD/SCID) mouse model (97).

CSCs have the characteristic asymmetric division, which consist in generating one SC and one cell able to differentiate after division. Furthermore CSCs have self-renewal capacity, express specific surface markers and are intrinsically drug resistance thus causing patients relapse (98). There are two alternative theories about CSCs generation: the first hypothesized that CSCs originate from normal stem/progenitor cells that gain the ability to generate tumor by genetic mutations or environmental alteration (98); the other theory suggests that CSCs may arise from somatic cells which gain stemness-like property thanks to genetic/heterotypic alteration, i.e. epithelial-to-mesenchymal transition (EMT) (99)

5.2 MULTIPLE MYELOMA CANCER STEM CELLS

In recent years, several debates has been made whether MM stem cells (MM-SCs) really exist. Early studies in MM models demonstrated that only a minority percentage of MM cells are able of clonogenic growth (100).

Matsui et al. first described MM-SCs as a population $CD138^-$; treatment of $CD138^-$ population with drugs, Bortezomib, Dexamethasone and Lenalidomide, doesn't affect their ability to form colonies, if compared to $CD138^+$ counterpart. MM-SCs have an higher expression of ABCG2, a membrane pump involved in drugs efflux, thus explaining their intrinsic drug

resistance feature; finally if CD138$^-$ population were injected into NOD/SCID mouse model, they engrafted and CD138$^+$ plasmacells were detectable (101, 102).

AIMS

Multiple myeloma (MM) is the second haematological malignancy and despite new therapies and new drugs, such as, immunomodulator agents (Lenalidomide, Pomalidomide) it is still incurable with a 5-years survival fare of 49% (https://seer.cancer.gov/archive/csr/1975_2013/) and diagnosis occurs at a median age of 69 (103). MM cells localize within the bone marrow, where they interact with different cell types, among which bone marrow stromal cells (BMSCs) sustain malignant cell survival and mediate drug resistance: drug resistance is the major cause of patients' relapse. Notch pathway is a highly conserved signalling that is involved in different biological processes and in cell-cell interactions; because its importance, Notch pathway deregulation are often associated to cancer burden (7). Evidences in the literature have shown that Notch pathway is involved in MM progression: MM cells overexpress Jagged ligands, thus causing an aberrant Notch pathway activation in surrounding cells, that can be either other MM cells (homotypic interaction), or, e.g. BMSCs or osteoclasts precursors (heterotypic interaction). The axis CXCR4-SDF1α has a crucial role in MM progression, regulating cells proliferation, drug resistance and bone marrow (BM) homing and correlates with poor prognosis (104, 105); furthermore, my group has previously demonstrated that CXCR4 is a direct Notch target gene and, by blocking Notch pathway with GSIs they observed a decreased CXCR4 expression at gene and protein level (76).

Regarding MM, few genetic alterations at the basis of Notch pathway deregulation, but the importance of microenvironment for disease progression suggests that, there could be other factors affecting Notch pathway activation or receptors/ligands overexpression: among this, hypoxia is known to positively regulate Notch signalling (95). The BM microenvironment is hypoxic and both Notch pathway and HIF-1α signalling pathway sustain cancer stem cells. This population is intrinsically drug resistant and therefore responsible for disease relapse.

The aim of this work is to evaluate the role of Notch pathway in MM drug resistance. The first part of this research aims to investigate the outcome of Jagged ligands silencing in MM cell intrinsic drug resistance by analysing changes in:

- MM cells sensitivity to drugs

- Genic and protein expression of anti-apoptotic factors

In the second part, I have analysed the effects of Jagged silencing in MM cells interaction with BMSCs. More specifically the following aspects have been assessed: i) the ability of MM cells to increase BMSCs-induced drug resistance; ii) changes in released soluble factors crucial for malignant cell survival and drug resistance and confirmation of their involvement, and iii) changes in the expression of anti-apoptotic factors in MM cells.

The in vitro results have been confirmed in primary MM cells isolated from BM aspirates of MM patients.

Finally, I have investigated the role of microenvironment in activating Notch signalling in MM cells, focusing on hypoxia and on its ability to increase multiple myeloma stem cell populations.

RESULTS

1. *IN VITRO* STUDY OF THE EFFECT OF JAGGED SILENCING ON INTRINSIC DRUG RESISTANCE OF MM CELLS

MM cells show an overexpression of Jagged ligands that cause an aberrant activation of Notch receptors in malignant cells with a consequent effect on MM cells biology, for example drug resistance. In order to extend the investigation to the outcome of Jagged ligands overexpression on drug resistance, I carried out a RNA interference approach to knock-down Jagged ligands expression. Two different human MM cell lines (HMCLs), OPM2 and U266, were transfected with two siRNA specific for Jagged1 and 2 every 48h and after 96h I analyzed the biological effect on. drug resistance, along with the variations in gene expression variation of Notch pathway members and others target genes involved in sensibility to apoptosis.

1.1 Outcome on Notch signalling activity

U266 and OPM2 cell lines were silenced twice every 48 h with 25 nM anti-Jagged1 and 25 nM anti-Jagged2 siRNA (HMCL^{-JAG}) or the respective scrambled control (HMCL^{-SCR}). At 96h, I tested the effect on gene expression levels of Notch pathway members by quantitative real time PCR (qRT-PCR):
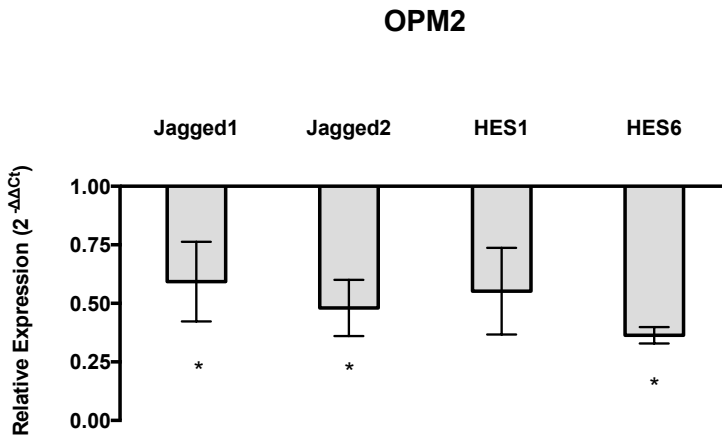
A.
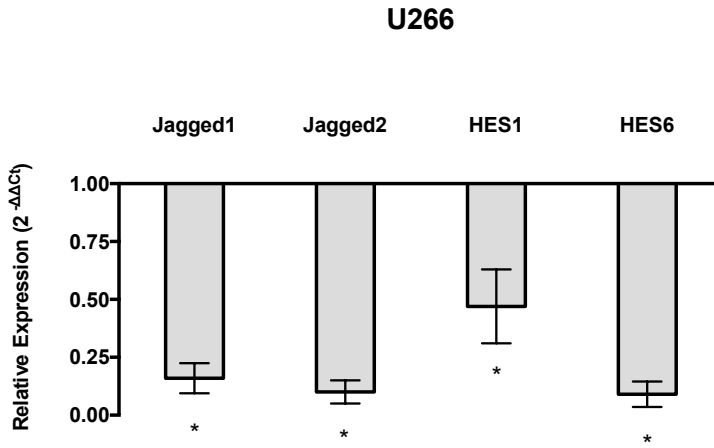
Figure 1.1. Analysis of gene expression after Jagged ligands silencing.
Jagged 1/2 were silenced in U266 (A) or OPM2 (B) cell line for 96h and
gene expression were analysed by qRT-PCR; HMCL^{-JAG} were compared to
HMCL^{-SCR} and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)
gene was used as housekeeping; HMCL^{-SCR} =1.

Fold change is calculated by the $2^{-\Delta\Delta Ct}$ formula (for details see "Material and Methods" section), error bars show standard deviation of three independent experiments. Statistical analysis performed by one tail t test; *=p<0.05.

As shown in figure 1.1, Jagged 1 and 2 expression was specifically knock-down by siRNAs transfection and Notch pathway activity is inhibited as shown by the down-regulation of two Notch target genes, HES1 and HES6 (HES1=Hairy enhancer of Split 1; HES6=Hairy enhancer of Split 6).

1.2 Effect on Bortezomib drug resistance

Once assessed the efficacy of Jagged1/2 silencing in U266 and OPM2 cells, I evaluated its biological effect on intrinsic Bortezomib (Bor)-drug resistance: Bor is a proteasome inhibitor used as front-line therapy in MM treatment. A set up of drug concentration was previously carried out and indicate the most appropriate concentration of 8 nM for Bor. To assess the outcome of Jagged1 and 2 silencing on MM cell drug sensibility, following 96h of transfection, cells were treated for 24h with the reported drugs. At the end of the treatment, apoptosis levels were assessed by flow cytometric analysis using coloration with Annexin-V.

A.
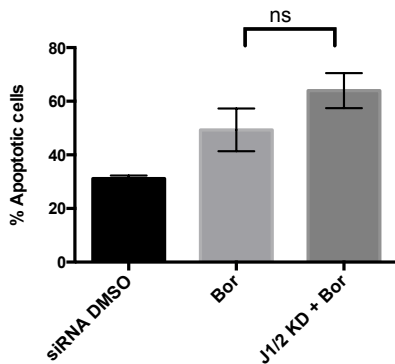
B.
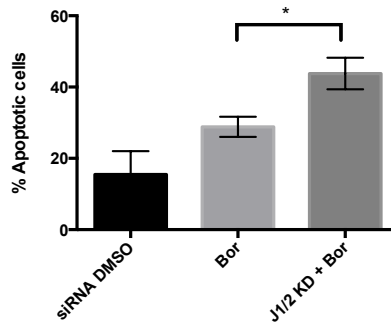
*Figure 1.2. Jagged silencing affects intrinsic HMCLs drug resistance. U266 (A) or OPM2 (B) cells. HMCL^{-SCR} or HMCL^{-JAG} were treated for 24h with 8 nM Bortezomib. Apoptotic cell were measured by flow cytometry as Annexin-V⁺cells. Graph shows the mean values of 3 indipendent experiments and error bars show standard deviation. Statistical analysis was performed using ANOVA and Tukey's post test: *=p<0.5; **= p<0.01. HMCL^{-JAG} =J1/2 KD.*

As shown in figure 1.2, Jagged1 and 2 silencing affects MM cell sensibility to Bor: indeed, the apoptosis levels in U266 (A) or OPM2 cells (B) when Jagged ligands are silenced is significantly increased if both HMCL were treated with with Bor. This result suggests that Notch pathway may have a role in mediating intrinsic Bor drug resistance of MM cells and targeting Jagged ligands may increase the sensibility of MM cells to Bor.

1.3 Effect on gene expression of anti-apoptotic factors

To investigate the molecular effectors underlying Notch pathway ability to mediate intrinsic drug resistance of MM cells, I evaluated the effect of Jagged silencing on gene and protein expression of anti-apoptotic effectors including CXCR4 (CXCR4=C-X-C chemokine receptor type 4), BCL_{XL} (BCL-XL=B-cell lymphoma-extra large), BCL2 (BCL2=B-cell lymphoma 2)

and ABCC1 (ABCC1=ATP-binding cassette subfamily C member 1) that have been demonstrated to be under Notch transcriptional control in different cellular settings. Indeed, Chiaramonte's group previously demonstrated that chemokine receptor CXCR4, involved in the regulation of MM cell proliferation, apoptosis and migration, is also a direct transcriptional target of Notch (76). BCLXL, BCL2 and Survivin are anti-apoptotic genes whose expression has been demonstrated to be regulated by Notch in different cells (106); finally ABCC1 (ATP Binding Cassette Subfamily C Member 1), also known as MRP-1 (multi-drug associated protein 1) is a multispecific efflux pump that plays a key role in cancer cell drug resistance due to its ability to transport many chemotherapeutic drugs out of the cells (107). Also ABCC1 and other efflux pumps are Notch transcription targets.

Gene expression analysis is shown in figure 1.3:

A.
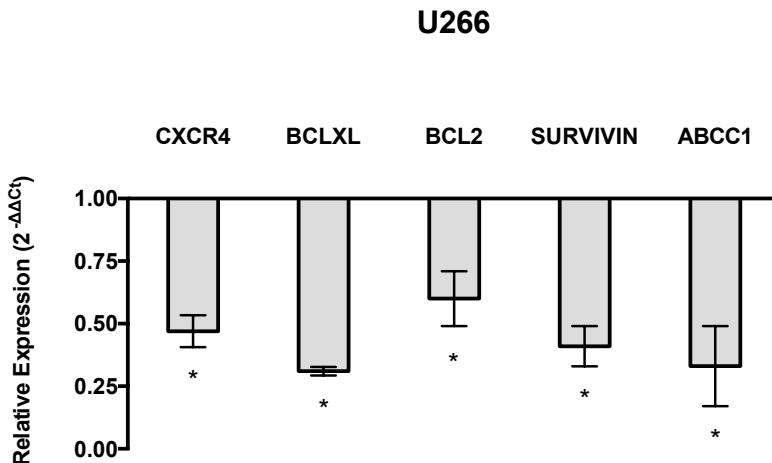
B.

OPM2
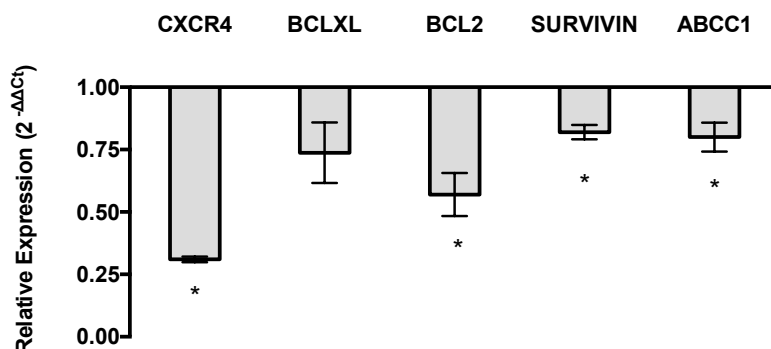
1.3 Effect of Jagged silencing on gene expression of anti-apoptotic factors. Upon Jagged silencing, U266 (A) or OPM2 (B) cell lines the expression of anti-apoptotic genes was analysed by qRT-PCR. HMCL^{-JAG} were compared to HMCL^{-SCR}. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) gene was used as housekeeping gene; HMCL^{-SCR}=1. Fold changes were calculated by the $2^{-\Delta\Delta Ct}$ formula (for details see "Material and Methods" section), error bars show standard deviation (SD) of three independent experiments.

As shown, Jagged silencing is able to decrease the gene expression of all the anti-apoptotic factors investigated, thereby explaining at least in part the mechanism underlying Notch pathway involvement in MM cell drug resistance.

1.4 Effect on protein expression of anti-apoptotic factors

The effect of knock down of Jagged ligands on anti-apoptotic proteins was also confirmed by assessing Survivin, ABCC1 and BCL2 protein variation by flow cytometry (figure 1.4):

ABCC1
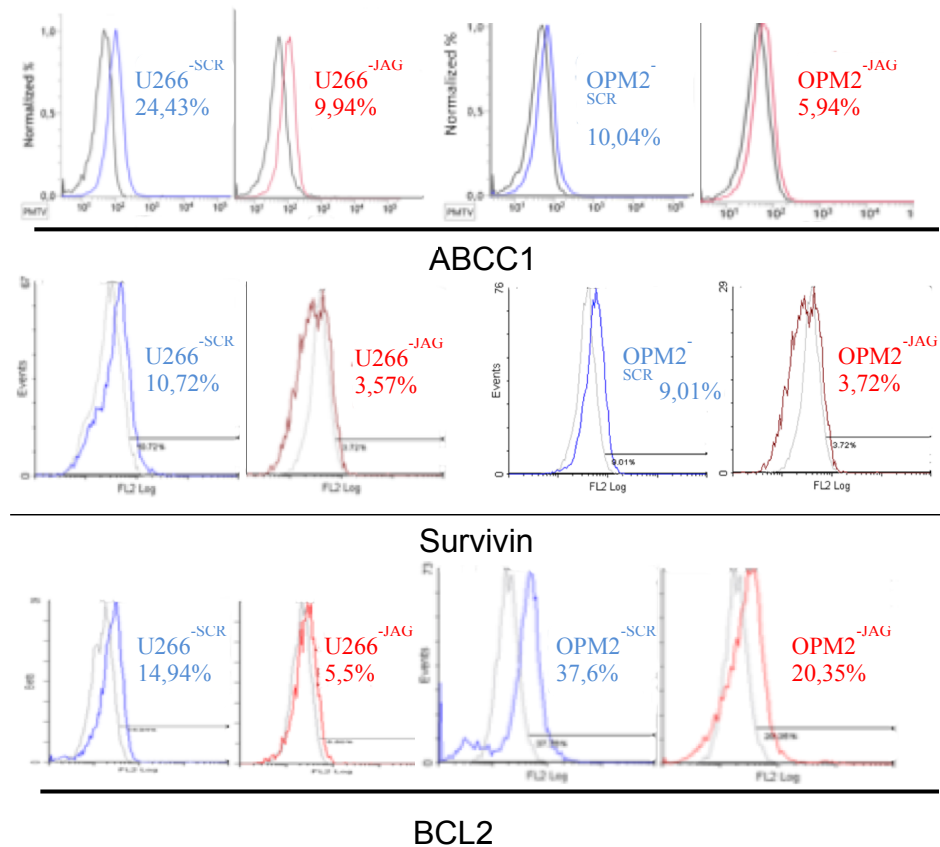
Survivin

BCL2

Figure 1.4 Protein expression of anti-apoptotic factors in U266 or OPM2 cells silenced for Jagged ligands. Histograms display the levels of BCL2, Survivin and ABCC1 analyzed by flow cytometry in HMCL^{-SCR} (green lines) or in HMCL^{-JAG} (red lines), and an isotype-matched control (gray line). Histograms are representative of 3 experiments with similar results.

As shown in figure 1.4, Jagged inhibition is able to reduce the anti-apoptotic proteins Survivin, ABCC1 and BCL2.

2. IN VITRO STUDY OF THE EFFECT OF JAGGED SILENCING IN MYELOMA CELLS ON THE INTERACTION WITH BMSC.

MM cells are established within the bone marrow where they interact with different cell types, among these BMSCs play a crucial role in sustaining malignant cell survival. My purpose is to investigate if the levels of Jagged ligands expressed by myeloma cell may affect its ability to interact with BMSCs and induce a pro-tumor behavior. The hypothesis is that MM cell-derived Jagged might trigger Notch signaling in the same tumor cell resulting in the release of soluble factors that stimulate BMSC pro-tumor behaviour, or that, upon cell-cell contact, MM cell-derived Jagged may activate Notch signalling in the nearby BMSCs, and these in turn may stimulate the release of pro-tumor factors. To answer this question, I set up a co-culture system of MM cells and BMSCs to measure the ability of BMSCs to support MM cell drug resistance and verify if silencing Jagged1 and 2 in MM cell may reduce the supportive ability of BMSCs. For this study, presented in section 2.1 and 2.2, I took advantage of two different co-culture systems: the first co-culture system was composed by HMCL^{-SCR} or HMCL^{-JAG} and the human BMSCs, HS5. HS5 cells were engineered to stably express GFP (HS5-GFP) and help the detection of the two cell populations by flow cytometry. The second co-culture system consisted of HMCL^{-SCR} or HMCL^{-JAG} (U266 or OPM2) and NIH3T3 cells to mimic the mesenchymal stromal cell component. This culture system was specifically used to carry out the analysis of the changes induced on gene expression by Jagged1/2 knock down. Indeed, the presence of human myeloma cells and murine stromal cells enabled to detect the cellular origin of the investigated transcript by qRT-PCR using species-specific primers.

2.1 Effect of Jagged-silenced MM cells on BMSC-induced Bortezomib drug resistance

I analysed the effect of Jagged silencing in MM cells on BMSC-mediated drug resistance. MM cells were silenced for 96h, co-cultured with HS5-GFP for last 48h and treated for last 24h with 8 nM Bor. Apoptotic MM cells were measured by flow cytometry as Annexin-V$^+$cells:
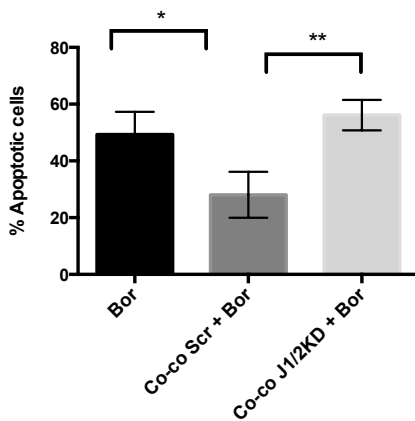
A.   B.
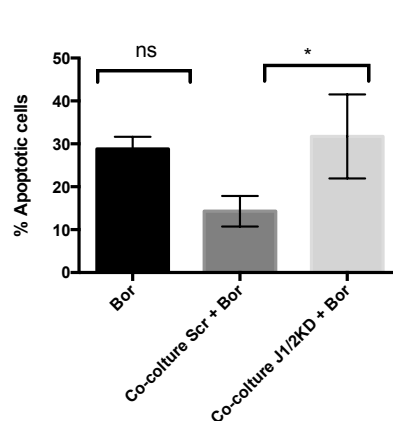
Figure 2.1 Effect of Jagged silencing on drug resistance induced by BMSCs. U266 (A) or OPM2 (B). HMCL^{-SCR} or HMCL^{-JAG} were co-cultured with HS5-GFP for 48h and treated with 8 nM Bor for last 24h. Apoptosis rate of MM cells were assessed by flow cytometry. Graph shows the mean values of 3 independent experiments and error bars show standard deviation. Statistical analysis was performed using ANOVA and Tukey's post test: *=p<0.5; **= p<0.01.

The results, shown in figure 2.1, demonstrate that HS5 cells can significantly protect co-cultured U266 cells from apoptosis induced by Bor if compared to MM cells in single culture. If Jagged ligands are knocked-down, MM cells become again sensible to Bor, resulting in the loss of protection by BMSCs. These results indicate that Notch pathway has a role in BMSC-mediated Bor drug resistance. It is important to underline the fact

that Bor does not affect BMSCs viability: indeed BMSCs treated with 8 nM Bor do not show an increased apoptosis if compared to vehicle-treated cells (data not shown).

2.2 Molecular and biological changes induced by Jagged-silenced MM cells in BMSCs

2.2.1 Outcome on Notch signalling activity in BMSCs

In this section, I will investigate the molecular mechanisms underlying the ability of MM-derived Jagged ligands to promote BMSCs pro-tumor behaviour. My hypothesis is that Jagged ligands my trigger a pro-tumor Notch signaling in BMSCs, To address this issue, I will measure the variation of Notch transcriptional activity analysing changes in the level of a acknowledged transcriptional target gene.

U266 (A) or OPM2 (B) cells were silenced for 96h and co-cultured with murine NIH3T3 for last 48h. RNA was extracted and gene expression variation in NIH3T3 cells analysed by qRT-PCR. The analysis of the gene expression changes of the Notch target gene HES5 (HES5=Hairy enhance of split 5) indicated that Jagged ligands expressed on MM cells surface can trigger Notch pathway activation in BMSCs (figure 2.1.1):

A.   B.
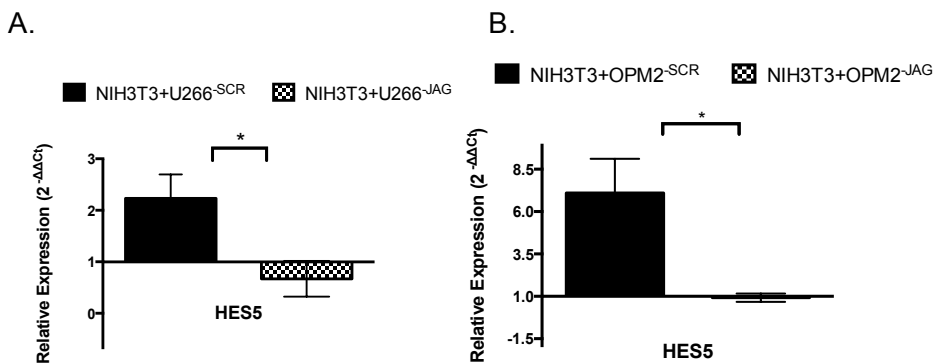
Figure 2.2.1. Activation of Notch signalling in NIH3T3 cells by MM cell-derived Jagged1/2; The expression of HES5 on NIH3T3 cells co-cultured

with HMCL^{-SCR} or HMCL^{-JAG} was analysed by qRT-PCR. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) gene was used as housekeeping gene; NIH3T3 cells=1. Fold changes were calculated by the $2^{-\Delta\Delta Ct}$ formula (for details see "Material and Methods" section), error bars show SD of three independent experiments. Statistical analysis performed by one tail t test; *=p<0.5.

Indeed, as shown in figure 2.2.1, the HMCLs^{-SCR} are able to activate Notch pathway in NIH3T3 cells. On the contrary, Jagged ligands were silenced in HMCLs, there was a significant reduction in HES5 gene expression levels demonstrating that MM cells were no longer able to activate Notch pathway in NIH3T3 cells.

2.2.2 MM cell-derived Jagged positively regulates the ability of BMSCs to secrete growth factors by activating Notch signalling

MM cells may induce BMSCs to secrete pro-survival factors fundamental for MM cells survival. We used the same co-cultures used for experiments in point 2.2.1 to investigate the outcome of silencing MM-derived Jagged1/2 on the ability of BMSCs to release IL-6 and SDF1α, two important cytokines in MM with important effects on drug resistance, tumor cells migration and osteoclasts maturation.

A.
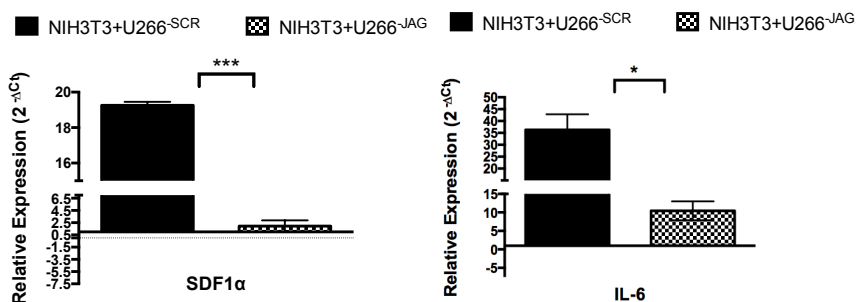
B.
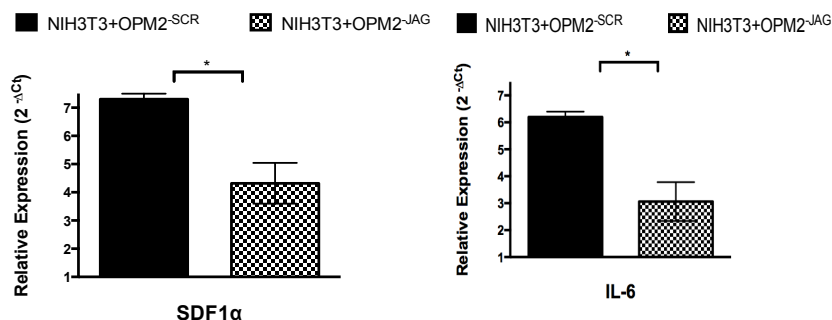
Figure 2.2.2 Jagged silencing in MM cells impairs the ability of co-cultured BMSCs to express the genes encoding for SDF1α and IL-6. U266 (A) or OPM2 (B) cells were silenced for 96h and co-culture with murine NIH3T3 for last 48h. RNA was extracted and gene expression variation in NIH3T3 cells analysed by qRT-PCR; GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) gene was used as housekeeping gene; NIH3T3 cells=1. Fold changes are calculated by the $2^{-\Delta\Delta Ct}$ formula (for details see "Material and Methods" section), error bars show standard deviation of three independent experiments. Statistical analysis performed by one tail t test; *=p<0.5.
SDF1a=Stromal derived factor 1a; IL-6=Interleukin-6.

Results reported in figure 2.2.1, I demonstrate that HMCL[-SCR] can induce an upregulation of both SDF1A and IL-6 gene expression in NIH3T3 cell, on the contrary the absence of MM cell-derived Jagged results in a significant reduction of the two genes expression, suggesting that both SDF1A and IL-6 are direct Notch target genes or are indirectly regulated by Notch signalling.

The results obtained were confirmed at protein level by fluo-cytometric staining as shown in figure 2.2.2:

A.

SDF1α

IL-6

B.

SDF1α
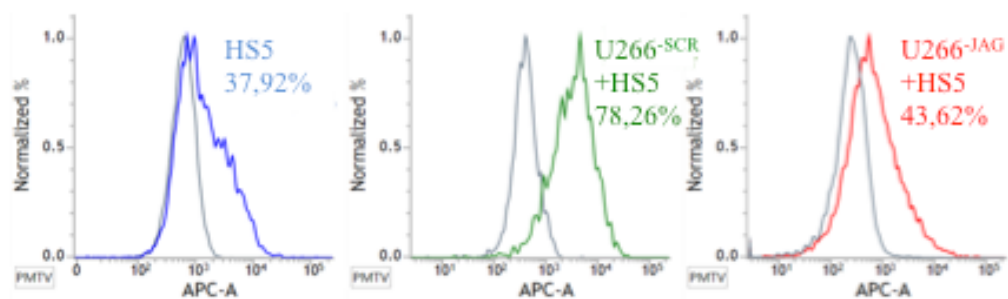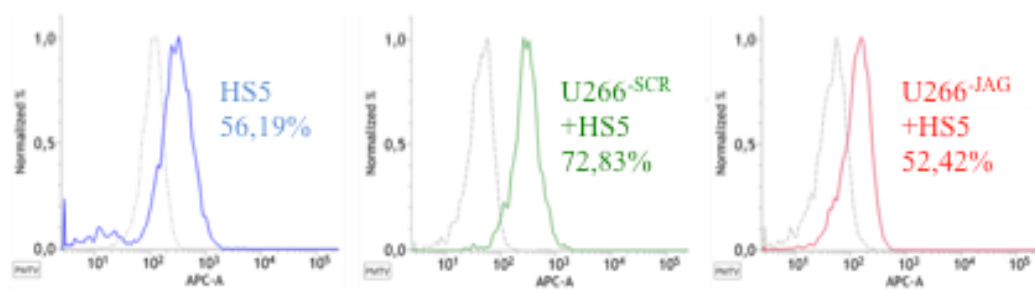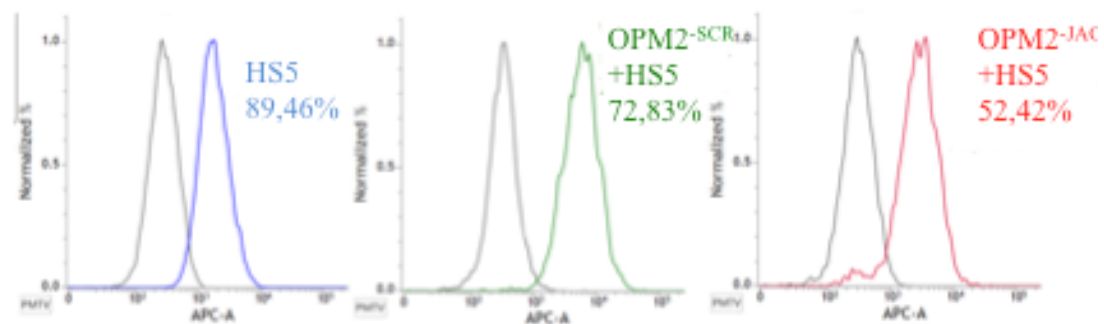
IL-6
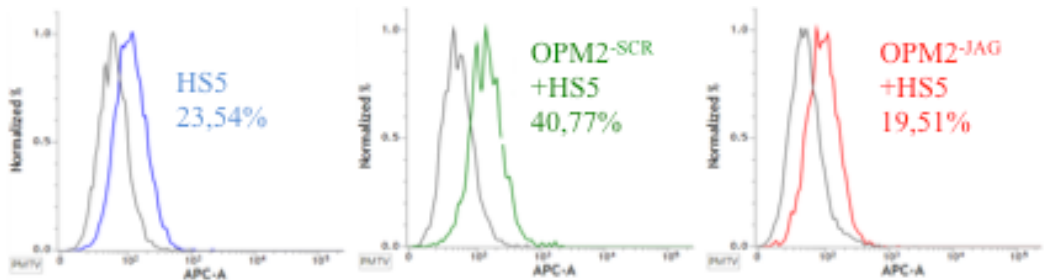
Figure 2.2.2 *Jagged silencing in MM cells impairs the ability of co-cultured BMSCs to secrete growth factors. U266 (A) or OPM2 (B) cells were silenced for 96h and co-cultured with human HS5-GFP cells for further 48h. Histograms represent the level of intracellular SDF1α or IL-6 or isotype matching control (gray lines) analyzed by flow cytometry in HS5-GFP cells. Histograms are representative of 3 independent experiments with similar results.*

As shown in figure 2.2.2, BMSCs co-cultured with MM cells expressed increased SDF1α and IL-6 proteins if compared to BMSCs cultured alone; Jagged ligands inhibition reduces the secretion of SDF1α and IL-6 by BMSCs.

2.2.3 Confirming the role of Notch1 signalling in promoting BMSC ability to secrete pro-tumorigenic factors.

The results obtained suggested that HMCL[-JAG] induce BMSCs to secrete SDF1α and IL-6 by activating Notch signalling. To verify this hypothesis, I evaluated if Notch receptors activation in BMSCs could promote the secretion of SDF1α and IL-6.

At this purpose, I specifically knocked down Notch1 receptor in HS5 cell due to its high levels of expression; HS5 cell line was treated twice, every 48h, with 50 nM anti-Notch1 siRNA (HS5[-N1]) or scrambled control (HS5[-SCR]).

Variations of SDF1α and IL-6 expression were assessed by flow cytometry (figure 2.1.3):

SDF-1α
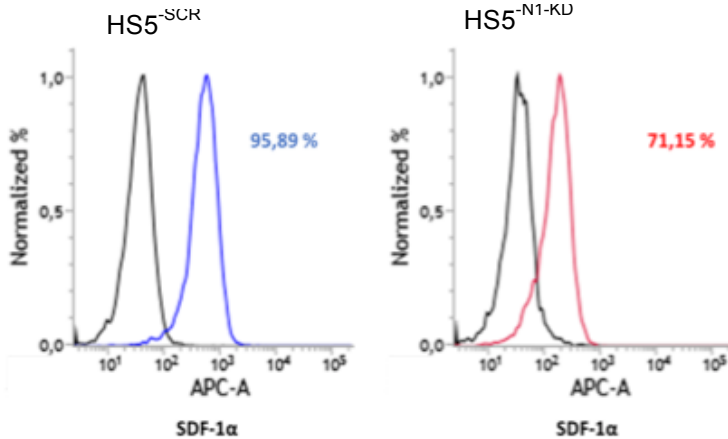
IL-6
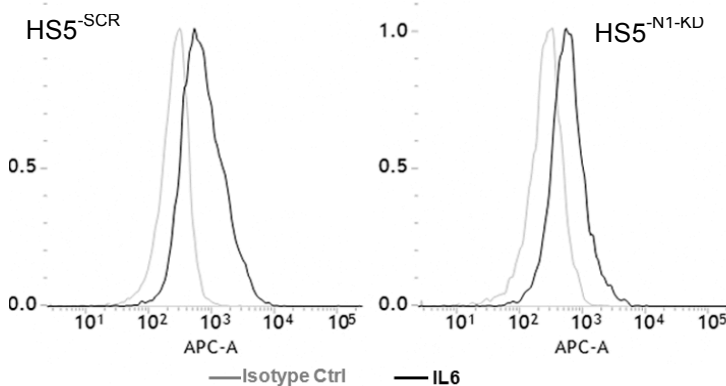
Figure 2.2.3 Notch1 knock down decreases the expression of the anti-apoptotic citokines SDF1α and IL-6, in BMSCs. Histograms display the levels of intracellular SDF1α and IL-6 in HS5 Scr or HS5 N1-KD, along with an isotype-matched control (gray line). Histograms are representative of 3 independent experiments with similar results.

As reported in figure 2.1.3, specific Notch1 knock down is able to reduce the protein expression of SDF1α and IL-6 in HS5 treated cells.

2.2.4 Effect of MM cell-derived Jagged1/2 on BMSC ability to support the anti-apoptotic background of MM cells

In the previous section, I demonstrated that Notch signalling is crucial to induce BMSCs to support MM cells survival and increase their pharmacological resistance. Results obtained indicate that the molecular mechanism involved stems from the ability of MM cell-derived Jagged to trigger Notch signalling in BMSCs, that in turn induce the secretion of anti-apoptotic factors, e.g. SDF1α and IL-6. In this paragraph, I will show the effect of inhibiting MM cells-derived Jagged in terms of gene expression and secretion of anti-apoptotic factors by BMSCs.

To clarify the mechanism of rescue from apoptosis activated by "educated" BMSCs (stimulated by MM cells), I studied if it may be induced by variation in gene expression of anti-apoptotic proteins. At this purpose, I analyzed if HMCLs co-cultured with BMSCs expressed different levels of key factors such as, BCL-$_{XL}$, BCL2, Survivin and ABCC1. Moreover, I verified if Jagged KD reduced their expression (figure 2.2.4). I took advantage of the two different co-culture systems reported in section 2.

A.
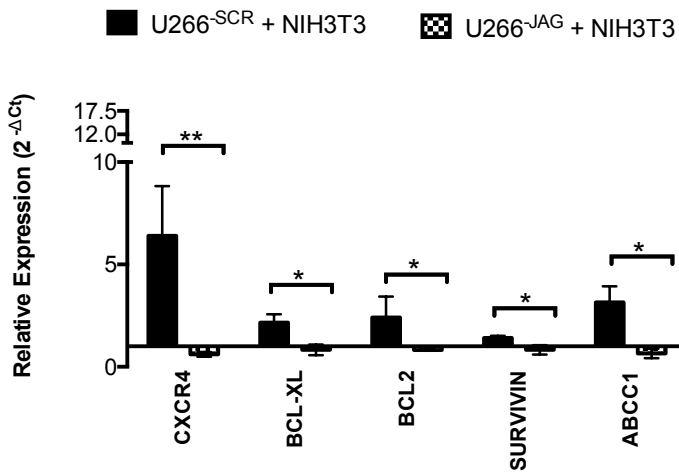
B.
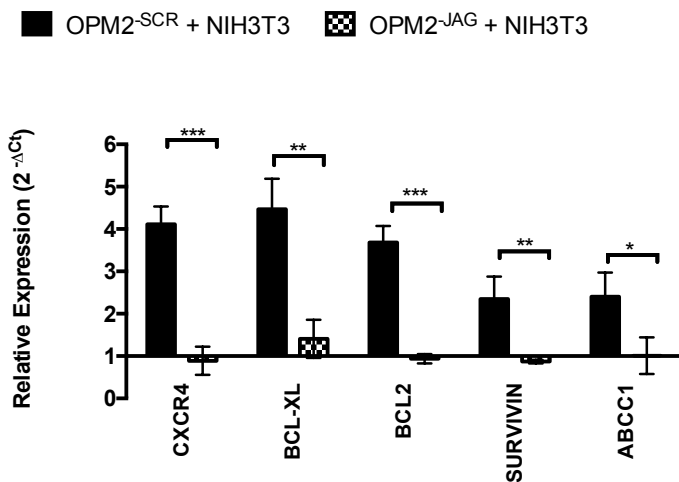
Figure 2.2.4 Gene expression of MM cells anti-apoptotic factors induced by BMSCs: outcome of Jagged1/2 silencing. The expression of anti-apoptotic genes were analysed by qRT-PCR. The expression in HMCL^{-JAG} + NIH3T3 cells was compared to that obtained in HMCL^{-SCR} + NIH3T3 cells and normalized on HMLCs scr alone =1. GAPDH was used as housekeeping gene. Fold changes were calculated by the 2$^{-\Delta\Delta Ct}$ formula (for details see "Materials&Methods" section), error bars show SD of three independent

experiments. Statistical analysis performed by one tail t test; *=p<0.05;
**=p<0.01; p<0.001.

The results obtained demonstrated that HMCLs co-cultured with NIH3T3 cells upregulated the expression of anti-apoptotic and pro-survival genes such as CXCR4, BCL-$_{XL}$, BCL2, Survivin and ABCC1, by contrast in HMCL$^-$^{JAG} anti-apoptotic genes were significantly downregulated. This result suggests that the release of pro-tumor factors by BMSCs induced by MM cell-derived Jagged1/2 may enhance tumor cell drug resistance by inducing the overexpression of anti-apoptotic genes.

2.2.5 Effect of MM cell-derived Jagged1/2 on BMSC ability to increase the protein expression of anti-apoptotic factors in MM cells

I validate previously obtained gene expression results analysing protein variations through a similar experiment, including only one variation: U266 or OPM2 cells were silenced for Jagged1 and 2, co-cultured with HS5-GFP cells. Protein expression of BCL2, Survivin and ABCC1 was assessed by using specific primary antibodies and subsequent analysis by flow cytometry.
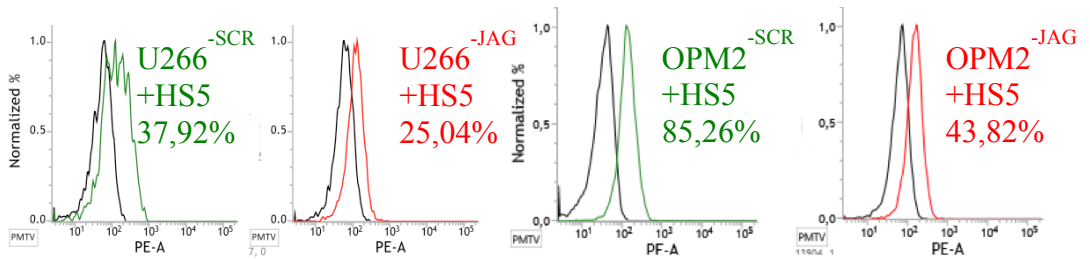
ABCC1
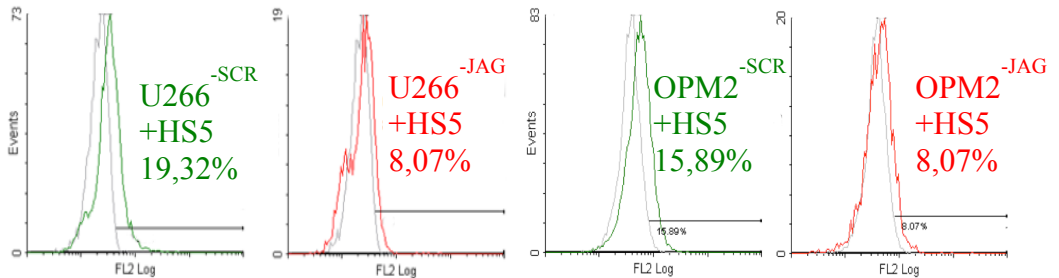
Survivin
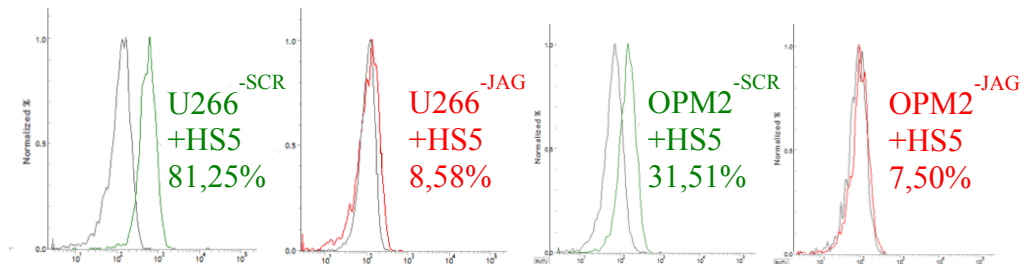
BCL2

Figure 2.2.5 Protein expression of anti-apoptotic factors. U266 or OPM2 were silenced for Jagged ligands and co-cultured with HS5-GFP. Histograms display the levels of CXCR4, BCL2, Survivin and ABCC1 analyzed by flow cytometry in HMCL^{-SCR} (green lines) or in HMCL^{-JAG} (red lines), and an isotype-matched control (gray line). Histograms are representative of 3 experiments with similar results.
*CXCR4=C-X-C chemokine receptor type 4; BCL2=B-cell lymphoma 2; ABCC1=ATP-binding cassette subfamily C member 1.*

As shown in figure 2.2.5, HMCLs^{-SCR} co-cultured with HS5 human BMSCs were able to upregulate the protein expression of anti-apoptotic factors (BCL2, Survivin and ABCC1), oppositely, HMCLs^{-JAG} express decreased levels of CXCR4, BCL2, Survivin and ABCC1 protein expression.

On the whole, these results indicate that MM cells may educate BMSCs to promote pharmacological resistance by i) releasing pro-survival factors such as IL6 and SDF-1a and ii) stimulating the expression of anti-apoptotic factors in the same MM cell.

The possibility that IL6 and SDF1a may be involved in the upregulation of the anti-apoptotic background of MM cell still need to be verified.

Our results further indicate that MM cells-mediated "education" of BMSCs is carried out by triggering Notch activation through tumor-derived Jagged ligands.

3. STUDY ON THE OUTCOME OF CXCR4 SIGNALLING DOWNSTREAM NOTCH PATHWAY IN BMSC-MEDIATED BORTEZOMIB DRUG RESISTANCE

The axis CXCR4-SDF-1α has a fundamental role in MM because it is involved in several process such as migration, immunosuppression, proliferation, drug resistance (104) and it is correlated with poor prognosis (105). A recent work carried out in MM cells, published by my research group has demonstrated that, the axis CXCR4-SDF-1α is transcriptionally controlled by Notch signalling and blocking Notch pathway activation with gamma secretase inhibitor XII (GSI-XII) inhibits the expression of both CXCR4 and SDF-1α at gene and protein levels (76).

These evidences prompted me to verify if SDF-1α secreted by BMSCs might support tumor cell resistance to chemotherapeutic drugs. To address this issue, I verified if the inhibition of CXCR4 signalling by the antagonist, AMD3100, may increase HMCL drug sensibility.

I tested my hypothesis on U266 cells co-cultured with HS5-GFP cells and treated with AMD3100 and 8 nM Bor. The percentage of apoptotic MM cells, analysed by flow cytometry, is shown in figure 3.
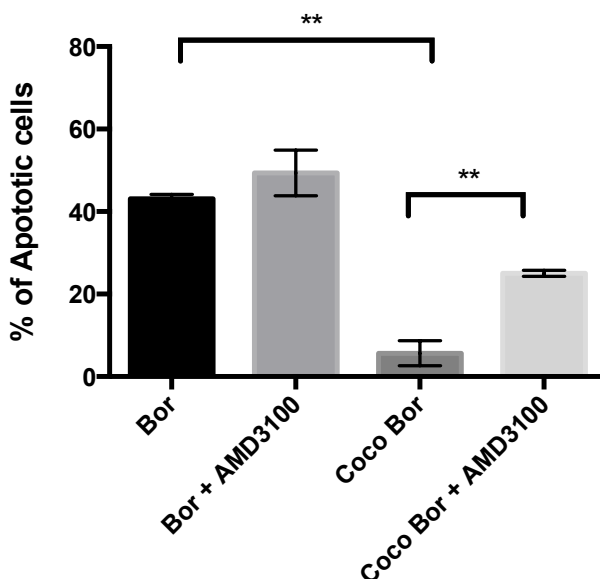
Figure 3. CXCR4 inhibition affects BMSC-mediated drug resistance in U266 cell. U266 cells were treated for 48h with 100 µM AMD3100, a CXCR4 inhibitor, co-cultured for 48h with HS5-GFP and treated for the last 24h with 8 nM Bor or DMSO as vehicle. Apoptotic MM cells were measured by flow cytometry as Annexin-V$^+$cells. Graph shows the mean values of 3 independent experiments and error bars show SD. Statistical analysis was performed using ANOVA and Tukey's post test: **= p<0.01.

The results obtained demonstrated that, as expected, HS5 cells can significantly protect MM cells from drug-induced apoptosis; by contrast, if CXCR4 signaling is blocked by AMD3100 treatment, HS5 cells are no longer able to protect U266 cells from Bortezomib induced apoptosis, indicating that CXCR4-SDF-1α axis could mediate Bortezomib resistance in MM cells.

4. VALIDATION OF JAGGED LIGANDS ROLE IN BORTEZOMIB DRUG RESISTANCE OF PRIMARY MM CELLS.

To confirm the biological relevance of Jagged mediated drug resistance in MM cells, *ex vivo* experiments were carried out on primary MM cells from patients.

Primary MM cells and primary BMSCs were isolated from BM aspirates of MM patients. Primary CD138 positive MM cells were separated by immunomagnetic sorting, while BMSCs were isolated from the negative fraction adherent cells (see "Material&Methods" section for details). CD138$^+$ cells were analysed by flow cytometry to verify the purity after positive selection, figure 4.1, and as expected, more than 90% of cells were positive for CD138 expression.
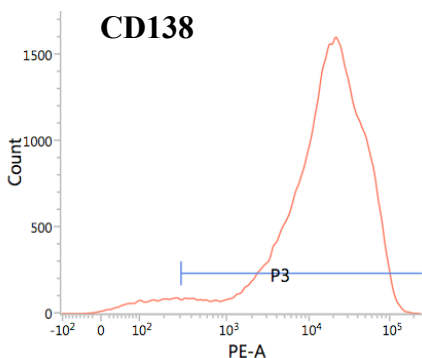
Figure 4.1 Primary MM cells CD138 expression. After positive selection, primary MM cells were analysed for CD138 expression by flow cytometry. Histograms are representative of 3 experiments with similar results.

Primary BMSCs were characterized either by optical imaging and by flow cytometry (figure 4.2): BMSCs are adherent big cells with a fibroblast-like shape which are positive for the expression of CD105 and CD90 and are negative for CD45, a pan-leucocyte marker, and for CD14, a monocyte marker.
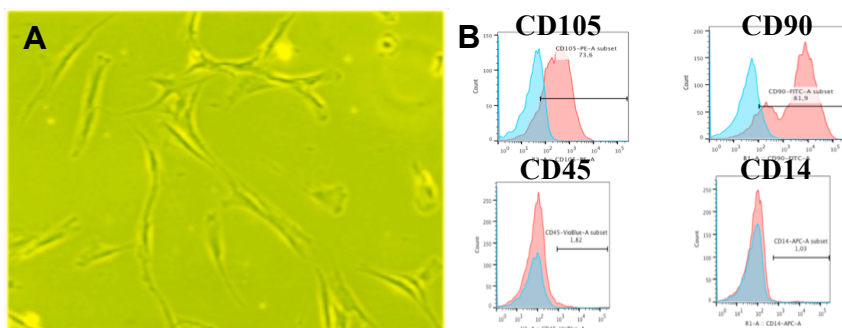
Figure 4.2 Morphological and flow cytometric characterization of primary BMSCs. Primary BMSCs were isolated from BM aspirates of MM patients. In panel A is shown morphological evaluation by optical imaging, picture was taken by Zeiss PrimoVERT Microscope; in panel B is shown flow cytometric characterization, the expression of surface proteins (red histograms), i.e. CD105, CD90, CD45 and CD14, and isotype-matched controls (blue histograms) were analysed by flow cytometry.

Co-culture experiments of MM cells and BMSCs were performed in order to verify Jagged ligands ability to promote Bor drug resistance even in primary MM cells. The experiments were carried out only in co-culture system because primary MM cells could not survive without feeder primary BMSCs.

Primary CD138^{+} MM cells from 10 MM patients were transduced with a lentiviral vector pLL3.7, carrying Jagged1 and 2 shRNA or Scr control o.n., then they were co-cultured for 72h with primary BMSCs previously stained with PKH26, a fluorescent dye that enables an easy detection of the two different populations by flow cytometry. Co-cultures were treated with 8nM Bor or the corresponding vehicle and the percentage of apoptotic MM cells was detected by flow cytometry, as shown in figure 4.3.
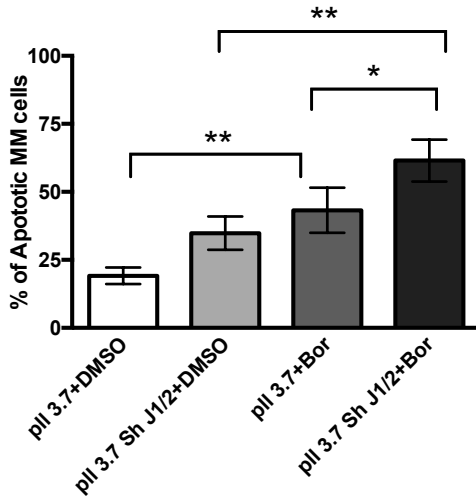
Figure 4.3 Jagged1 and 2 silencing affects the ability of BMSCs to promote drug resistance ex vivo. Apoptosis of primary MM CD138^{+} cells were assessed by flow cytometry by Annexin-V^{+} staining. Primary MM CD138^{+} cells were treated for 24 h with 8nM Bortezomib (10 patients). Apoptosis analysis was performed by on primary CD138^{+} MM cells; Statistical analysis was performed by using one-way ANOVA and Tukey's post test: *=p<0.05; **= p<0.01; ***=p<0.001.

The results obtained demonstrated that Jagged ligands knocked-down significantly increases the apoptosis of primary MM cells and their sensibility to pharmacological treatment with Bor, overcoming the protection of BMSCs.

5. EFFECT OF HYPOXIA ON NOTCH PATHWAY ACTIVATION

Previous results, and evidences from this and other research groups outlined a key role of Notch signalling deregulation in determining MM cell aggressiveness and the ability to shape the microenvironment. This section of the work aims to investigate if the microenvironment surrounding the MM cell may induce the expression of Notch ligands or receptors or their activation.

Genetic mutations at the basis of Notch pathway members deregulation in MM involve only 5% patients with a dysregulation of MAFB and c-MAF genes due to t(14;16)(q32;q23) and t(14;20)(q32;q11); Notch2 is a direct target of these transcription factors and, thereby results expressed at higher levels (74).

The general sensibility of MM cells to Notch signaling inhibition and the general increase of Notch receptors and ligands during MM progression suggest that Notch signaling activation may be induced also by other aspects. The importance of the microenvironment in MM progression, prompted me to investigate its characteristics, moreover the evidences that the BM microenvironment is hypoxic (108) and hypoxia is reported to positively regulate Notch signaling activity (95) prompted me to evaluate the effect of BM hypoxia on Notch signalling activation in MM cells and the possible biological effects.

5.1 Effect of hypoxia on the protein expression of Notch pathway members

This preliminary work addressing the effect of hypoxia on HMCLs was carried out through an inhibitory approach performed by in vitro administration of a chemical compound, Cobalt Chloride ($CoCl_2$). $CoCl_2$ mimics hypoxia thanks to its ability to inhibits HIF-1α degradation by blocking PDH enzyme, thus leading to the accumulation of HIF-1α; as a

result, HIF-1α can migrate into the nucleus and activate the transcription of hypoxia-responsive genes.

As a first step I evaluated if $CoCl_2$ treatment could activate HIF-1α in OPM2 cell line.

OPM2 cells were treated with 100 µM $CoCl_2$ and analyzed at 2h, 6h, 12h, 24h to determine by Western Blot analysis if variation in HIF-1α expression were associated to variation in Notch signaling members.
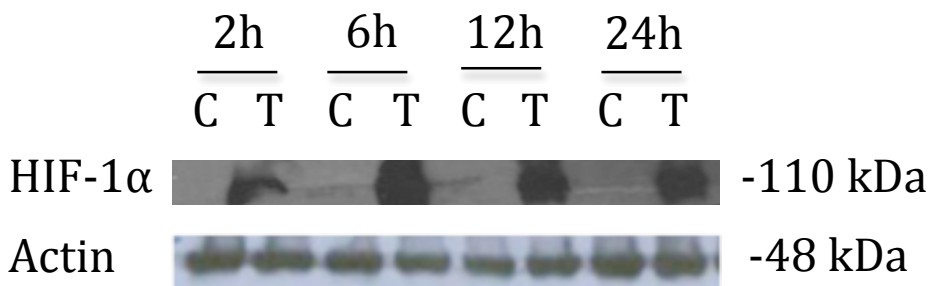
5.1.1 Expression of HIF-1α by CoCl$_2$ treatment in OPM2 cells.Western blot shows the expression level of HIF-1α in OPM2 cells after CoCl$_2$ treatment for the reported time points. 100 mg of whole cell lysate were loaded in each lane. Actin was used as loading control. Results are representative of three independent experiments.

HIF-1α=Hypoxia inducible factor-1α; C=Vehicle treated cells; T= CoCl$_2$ treated cells.

Figure 5.1.1 confirms that $CoCl_2$ induces an accumulation of HIF-1α in OPM2 cells after 2h of treatment and the expression of HIF-1α that increases in the subsequent timepoints in comparison to control cells. The next step was to evaluate if the activation of HIF-1α, by $CoCl_2$ treatment, is associated to the increased expression of Notch pathway members. At this purpose the analysis was initially focused on Jagged1

and the intracellular domain of Notch2 (ICN2) that are specifically overexpressed in OPM2 cell line and are relevant in MM progression.

	2h		6h		12h		24h		
	C	T	C	T	C	T	C	T	
Jag1									-180 kDa
Actin									-48 kDa
ICN2									-83 kDa
Actin									-48 kDa
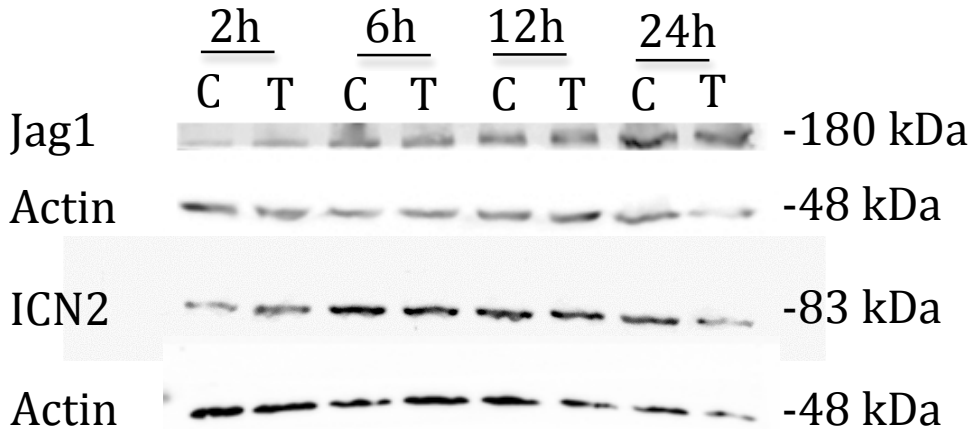
5.1.2 Expression of Notch pathway members after HIF-1α activation in OPM2 cells. OPM2 whole cells lysates were analyzed by western blot at the reported time points during treatment with CoCl$_2$ for the expression of Jagged1 and ICN2. 100 mg of whole cell lysate were loaded in each lane. Actin was used as loading control.Results are representative of three independent experiments.
C=Vehicle treated cells; T= CoCl$_2$ treated cells.

Results of western blot analysis reported in figure 5.1.2 show no modulation of Jagged1 and ICN2 protein.

5.2 Effect of hypoxia on Notch transcriptional activity

Although experiments in section 5.1 should be confirmed by a complete analysis of the effect of CoCl$_2$ on all the Notch members, the obtained results suggested that it could not be played at a transcriptional level. Thereby, we investigated if CoCl$_2$ might directly affect Notch receptor

transcriptional activity as reported for HIF-1α in different cellular context (95).

To address this issue we analyzed the outcome of $CoCl_2$ on Notch transcriptional activity by a Notch responsive dual luciferase gene reporter assay. OPM2 cell line was transfected for 48h with a reporter plasmid carrying Firefly luciferase controlled by a Notch-responsive promoter named 13XCSL and a second plasmid constitutively expressing Renilla luciferase for normalization of transfection efficiency (for details see "Materials & Methods"). Transfected cells were treated with100 μM $CoCl_2$ for last 24h and then luciferase activities were sequentially assessed by measuring their luminescence (figure 5.2.1):
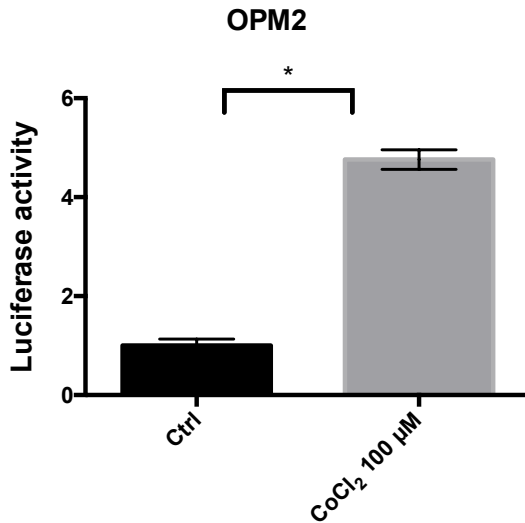
5.2.1 $CoCl_2$ treatment can modulate Notch transcriptional activity in OPM2 cell line. OPM2 cells were transfected for 48h with a Notch reporter and treated for last 24h with 100 μM $CoCl_2$ or with vehicle (Ctrl). Experiments were normalized using Renilla firefly; graph shows the mean values of 3 independent experiments and error bars show SD. Statistical analysis was performed using one tail t test: p<0.05.

The results of dual luciferase assay shown in figure 5.2.1 demonstrate that CoCl$_2$ treatment can induce a 5-fold increase of Notch transcriptional activity.

To confirm and better characterize the molecular mechanism by which HIF-1α accumulation enhances Notch transcriptional activity, I used a different *in vitro* model characterized by a higher transfection efficiency, the HEK293 cell line.

HEK293 cells were transiently transfected with the 13XCSL reporter and a plasmid that constitutively express high levels of ICN1 or ICN2, the most relevant isoforms in MM (see Materials and Methods for plasmids details); HEK293 cells were treated with CoCl$_2$ as previously reported and luciferase activity triggered by ICN1 or ICN2 was measured by dual luciferase assay (figure 5.2.2).
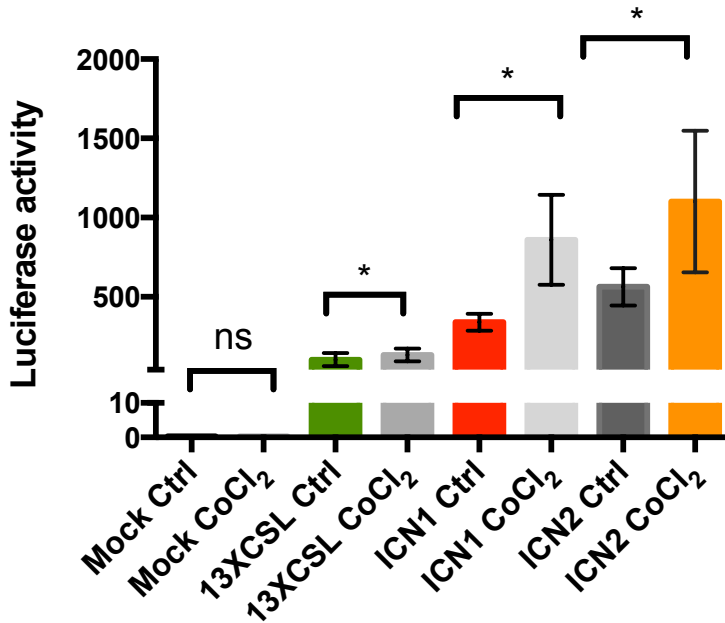
5.2.2 HIF-1α activation positively modulates ICN1 and ICN2 transcriptional activity in HEK293 cells. HEK293 cell line were transfected for 48h with 13XCSL plasmid and either plasmid expressing ICN1 or ICN2 or empty plasmid (Mock) and treated for last 24h with 100 µM $CoCl_2$ or with vehicle (Ctrl). Transfection efficiency was normalized using Renilla firefly; graph shows the mean values of 3 independent experiments and error bars show SD. Statistical analysis was performed using one tail t test and comparing for each set cells treated with vehicle to $CoCl_2$ treated cells: $p < 0.05$.

Results shown in figure 5.2.2 confirm that $CoCl_2$ treatment increases the luciferase activity triggered by the endogenous Notch receptors in HEK293 cells and indicate that $CoCl_2$ positively regulates ICN1 and ICN2 transcriptional activity.

This result indicates that a hypoxic microenvironment may activate Notch signalling by a positive regulation of ICN1 and ICNs activity operated by HIF-1a and suggest that this may occur also in MM cells.

5.3 Study on the role of Notch pathway as a downstream mediator of HIF-1α supportive effect on MM stem cell population

Cancer stem cells (CSC) have been described as a subpopulation characterized by high level of rdug resistance, ability to give raise to the bulk population and the ability to metastatize recreating further tumors masses in distant sites (109). CSCs have been found in different cancer types (97, 110).

Matsui et al. described the MM-SCs as a population characterized by a CD138⁻ phenotype, clonogenic ability and intrinsic drug resistance (101, 102).

From literature it is known that both hypoxia and Notch pathway can sustain and maintain the stem cell niche (111, 112). This induced me to evaluate if hypoxia positively regulates the amplification of MM-SCs and this effect required Notch signalling activation. To address this issue I induced hypoxia with $CoCl_2$, analyzed the outcome on MM-SC population size (expected an increase), and verified if it could be reversed by inhibiting Notch signaling.

I choose, as a model, the MM cell line H929 due to a higher percentage of CD138⁻ MM-SCs in comparison to other HMCLs. To mimic a hypoxic-like condition, H929 cell line was treated for 96h 20 μM $CoCl_2$ and to block Notch signalling cells were treated with 50 μM DAPT, a γ-secretase inhibitor, or a combination of the two drugs. At the end of the experiment, cells were analysed by flow cytometry to detect variations in CD138⁻ population (figure 5.3).
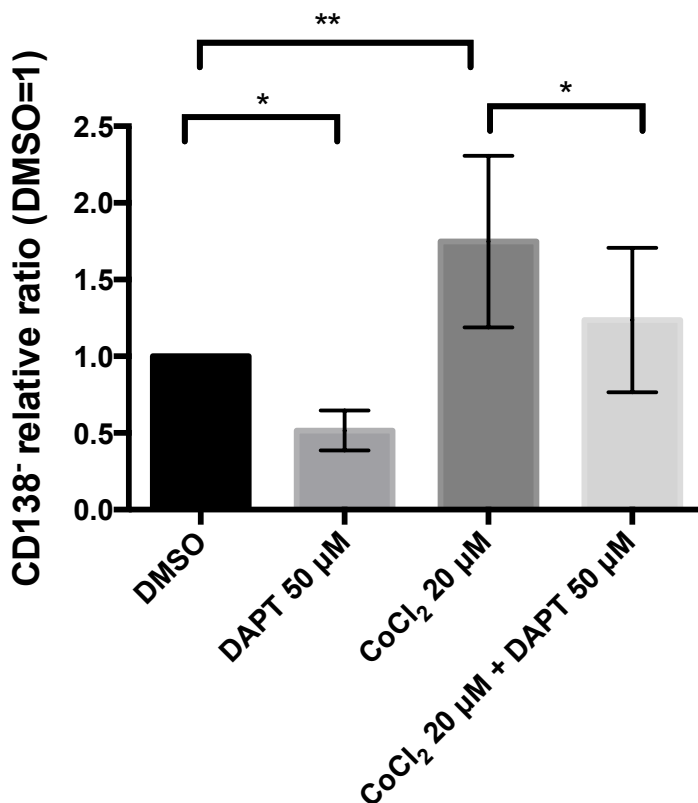
Figure 5.3 HIF-1α activation expands MM-SC subpopulation in H929 cells. Variation of CD138$^-$ population was assessed by flow cytometry staining of CD138. Graph shows the mean values of 3 independent experiments and error bars show SD. Statistical analysis was performed by two-way ANOVA and Holm-Sidak post's test: *=p<0.05; **=p<0.01.

The results shown in figure 5.3 demonstrate that $CoCl_2$ treatment increases CD138$^-$ cell population, while DAPT treatment significantly decreases it, as expected. The double treatment demonstrates that the positive effect of HIF-1α activation on CD138$^-$ population requires active Notch signalling, indeed the effect of $CoCl_2$ is significantly dampened by DAPT.

DISCUSSION&CONCLUSION

MM represents 10% of all haematological malignancies. Despite new drugs development, it is still incurable with an overall survival of 8 years at diagnosis. Neoplastic cells accumulate within the BM microenvironment, where they interact with different cell types, among which the BMSCs that support cancer cell growth and mediate drug resistance. MM cells display an intrinsic resistance to a specific drug after treatment and this process is known as "acquired drug resistance", while they can become drug resistant also stimulated by interactions with other different cell types, i.e. BMSCs or OCLs. In this process, cell interactions can trigger the secretion of cytokines which are able to mediate MM cells drug resistance (85).

Notch receptors are members of a highly conserved family during evolution; the family is composed by 4 receptor isoforms, Notch1-4, and two ligands families, Jagged (Jagged1-2) and Delta (Dll1-3-4) ligands. Upon receptor-ligand interaction, two proteolitic cleavages enable the release of the intracellular form of Notch into the nucleus, where it is able to activate the transcription of the target genes. Notch has a role in different cellular process, i.e. proliferation, apoptosis, cellular differentiation, adult tissue homeostasis and maintenance of stem cells. Because of its relevance, Notch receptor or ligands mutations or deregulation are often associated to cancer onset and progression (3).

In MM, Notch deregulation can be found at both receptors and ligands level and the deregulation positively correlates with disease stage and progression (41, 75, 113-116). MM cell express both Notch receptors and ligands, thus inducing either homotypic activation of Notch pathway in other MM cells, or heterotypic interactions with the other cells within the BM microenvironment. According to this, Notch signalling is able to affect the biology of MM cell and its pathological interaction with other cells, for example BMSCs. Previous published work from Chiaramonte's group and others teams demonstrated how Notch inhibition, by GSIs treatment,

results in increased apoptosis, decreased cell proliferation (6, 76, 114, 117) and increased drug sensitivity of MM cells (106, 118).

Besides genetic alterations that cause Notch pathway hyperactivation, there are other factors that can modulate Notch signalling activity among which hypoxia. The BM environment is, by definition, hypoxic (119); HIF-1α is the master regulator of hypoxic response and it is a transcription factor that induces the expression of genes involved in different processes such as angiogenesis, metabolism shift towards anaerobic glycolysis, cell cycle arrest and maintenance of stem cell population (88). Recent evidences indicate that Notch pathway and hypoxia pathway physically interact: Gustaffson and colleagues demonstrated that HIF-1α and Notch interact together; the mechanism is still unclear but different mechanisms have been proposed based on as many evidences: i) HIF-1α can positively regulates Notch pathway members; ii) HIF-1α has been proposed to bind the ICN and stabilize it preventing ICN degradation via proteasome machinery; iii) HIF-1 α induces Notch pathway members by binding hypoxia responsive elements (HRE) in Notch gene promoter (95).

Hypoxic condition and Notch signalling synergistically cooperate to sustain the cancer stem cells (CSCs) population that is intrinsically drug resistant and responsible for tumour relapse in patients (70); Matsui and colleagues first described MM-stem cells (MM-SCs) as a population characterized by a CD138^{-} phenotype, clonogenic ability, drug resistance and able to generate a primary tumour mass if injected in mice (101, 102).

The aim of my work is to evaluate the role of Notch pathway in mediating intrinsic and BMSC-mediated drug resistance in MM cells; furthermore I have studied the role of hypoxia on Notch signalling to better characterize the mechanism by which they interact and the effect on MM-SCs.

In the first part of my thesis, I focused my attention on the effect of Jagged silencing on MM cell biology in terms of drug resistance.

Since MM cells overexpress Jagged ligands, I decided to choose a Jagged-directed approach to specifically inhibit Notch pathway activation. At this purpose, I treated MM cells with two specific siRNAs targeting Jagged1 and 2 because (75, 116). MM cell lines, OPM2 and U266, were treated with anti-Jagged1/2 siRNAs (HMCL[-JAG]) or scrambled control (HMCL[-SCR]) and the effect of silencing was assessed by qRT-PCR on Notch pathway activation by measuring gene expression of Jagged1, Jagged2 and of two Notch-target genes, HES1 and HES6. My results show that, after silencing, Jagged ligands expression was impaired and the expression of two Notch target genes, HES1 and HES6 (76, 106), was inhibited demonstrating that Notch pathway was knocked down.

The next step was to evaluate the biological effect of Jagged silencing on intrinsic Bortezomib drug resistance. HMCL[-SCR] or HMCL[-JAG] were treated with Bortezomib and apoptosis measured; the results indicate that drugs are able to induce apoptosis in HMCL[-SCR], and if Notch pathway is silenced, MM cells are more sensitive to Bortezomib, confirming results obtained by Nefedova and colleagues and Chen and colleagues who demonstrated that MM cell are more sensitive to drugs if Notch pathway is blocked by GSI treatment (106, 118).

To better characterize the mechanism by which Notch pathway mediates MM cell drug resistance, I studied the expression of anti-apoptotic proteins, both at gene and protein level, such as CXCR4 (CXCR4=C-X-C chemokine receptor type 4), BCLXL (BCL-XL=B-cell lymphoma-extra large), BCL2 (BCL2=B-cell lymphoma 2) and ABCC1 (ABCC1=ATP-binding cassette subfamily C member 1) that are known Notch transcriptional target genes in different cellular context. Regarding CXCR4, Chiaramonte's group has

previously demonstrated that it is regulated by Notch and regulates apoptosis, migration, BM homing and proliferation of MM cells (76); ABCC1 encodes for a transmembrane efflux pump which transports out of the cells many chemical compounds and therefore has a role in drug resistance (107). My results show that Jagged ligands silencing decreases the expression of anti-apoptotic proteins at both gene and expression level. Taken together these findings are consistent with a previously published work in which the authors demonstrated that if paclitaxel-resistant ovarian cancer cells were treated with GSI, they become more sensitive to drugs and show a reduction in anti-apoptotic proteins expression background (120).

The results obtained in the first part of my thesis concern "intrinsic" drug resistance, but MM cell localize within the bone marrow where they interact either with other MM cells (homotypic interaction) or other types of cell (heterotypic interaction), e.g. monocytes, osteoclasts and BMSCs. This communication among cells can be mediated by direct contact or soluble factors. Chiaramonte's group has demonstrated that MM-associated osteoclastogenesis is due to either the release of soluble RANKL or the direct contact of MM cells with osteoclasts progenitors resulting in Notch signalling activation (78).

BMSCs mediate MM cells survival and drug resistance, for this reason in the second section of my thesis, I analysed the role of Notch pathway in the interaction between MM cells and BMSCs and the effect on BMSC-mediated drug resistance.

The first step was to analyse the outcome of Jagged silencing on drug resistance induced by MM cell-BMSCs interaction. MM cells were silenced for Jagged expression and co-cultured with HS5, a human stromal cell line used to mimic BMSCs, and treated with drugs used in MM therapy. The

results obtained show that BMSCs are able to protect MM cell from drug-induced apoptosis; but BMSCs are no longer able to protect MM cells silenced for Jagged ligands, suggesting that Notch pathway activated by MM-derived Jagged has a role in BMSC-mediated drug resistance.

To clarify the mechanism by which BMSCs are able to mediate MM cells drug resistance, I analysed the outcome BMSCs gene and protein expression upon their interaction with MM cells carrying high or low levels of Jagged ligands.

I first verified that variations in the levels of Jagged expressed by MM cells upon silencing resulted in a corresponding change in Notch signalling activation in co-cultured BMSCs, assessed as variations in the expression in the Notch target genes belonging to the HES family of genes.

I further verified that Notch activation was associated to the increased release of important cytokines, i.e. IL-6 and SDF-1α. It is known that BMSCs have a supportive role for MM cells in terms of secretion of cytokines or growth factors among which IL-6 and SDF-1α. IL-6 and SDF-1α are important for MM cells because they mediate different processes crucial for MM proliferation, drug resistance, BM homing and osteoclasts maturation (76).

To elucidate how Jagged silenced-MM cells can modulate the release of IL-6 and SDF-1α by BMSCs, HMCL^{-SCR} or HMCL^{-JAG} were co-cultured with NIH3T3 or HS5 cell line to mimic BMSCs and I evaluate the expression of IL-6 and SDF-1α at both genic and protein level by BMSCs. The results showed that HMCL^{-SCR} are able to upregulate the expression of the two cytokines while if BMSCs are co-cultured with HMCL^{-JAG} there is a decrease in the expression of IL-6 and SDF-1α, demonstrating how their expression is regulated by Notch pathway. These results are in accordance

of previous works published by Chiaramonte's research group in which they demonstrate that both IL-6 and SDF-1α are positively regulated by Notch signalling (76) (121).

To confirm the involvement of Notch pathway in mediating cytokines release (IL-6 and SDF-1α) by BMSCs, I specifically knock-down Notch1 receptor expression by using specific siRNA in HS5 cell line. The results obtained demonstrate that if expression of Notch1 is blocked, the protein expression of IL-6 and SDF-1α is reduced thus confirming that Notch pathway needs to be active to induce the secretion of IL-6 and SDF-1α by HS5 cells.

To clarify the molecular outcome of BMSC-induced drug resistance in MM cells, I investigated changes in the anti-apoptotic background of MM cells. In particular, I analyzed the expression of anti-apoptotic proteins, BCL$_{XL}$, BCL2 and ABCC1 involved in MM cells drug resistance. HMCL^{-SCR} or HMCL^{-JAG} were co-cultured with HS5 cells and variations of anti-apoptotic factors at gene or protein level were assessed: results show that, upon co-culturing, the expression of anti-apoptotic factors is significantly increased in HMCL^{-SCR} if compared to HMCL^{-JAG}; these findings are supported by the fact that MM cell-derived Jagged ligands are able to activate Notch pathway in HS5 cells, that in turn induces the upregulation of BCLXL, BCL2 and ABCC1 in MM cells; furthermore, the activaction of Notch pathway in BMSCs induce the release of soluble factors which are able to promote the expression of BCLXL, BCL2 and ABCC1 as well in MM cells, thus demonstrating that MM-derived Jagged ligands are necessary to induce the expression of anti-apoptotic proteins.

These results, taken together, indicate that MM cells are able to "shape" BMSCs thanks to the expression of Jagged ligands, in turn educated BMSCs secrete pro-tumor soluble factors such as IL-6 and SDF-1α and to

support an increased anti-apoptotic background in MM cells thus mediating drug resistance.

As mentioned above, the chemokinic axis CXCR4-SDF-1α has a fundamental role in MM cell biology because it is involved in different biological processes like proliferation, migration (76), drug resistance (104) and correlates with poor prognosis (105). Furthermore, an article published by Dr. Chiaramonte's lab demonstrates that the axis CXCR4-SDF-1α is controlled at transcriptional level by Notch, and blocking Notch pathway activation by GSI-XII treatment results in reduced expression of both CXCR4 and its ligand SDF-1α (76).

The next step in my thesis was to test if SDF-1α secreted by BMSCs could have a role in mediating MM cells resistance to drugs: U266 cell line in single culture or in co-culture with HS5 cell line was treated with AMD3100, a compound that antagonize CXCR4 receptor, and with commonly used drugs such as Bortezomib and Melphalan. The results indicate that HS5 cells can protect MM cells from drug-induced apoptosis but if CXCR4 is blocked by AMD3100, HS5 cells loose their protective effect and U266 significantly increses sensibility to Bortezomib; the results are no significant for Melphalan-treated cells indicating that CXCR4 may have a role in Bortezomib resistance.

These findings are in accordance to previous work published in literature by Waldschmidt et al. in which demonstrates that MM cells co-cultured with stromal cells and treated with AMD3100 are more sensitive to Bortezomib and Pomalidomide; the authors also hypothesize that CXCR4 is responsible for adhesion-mediated drug resistance (122).

To confirm the biological relevance of Jagged ligands induced drug resistance, I validate the results in ex vivo experiments on primary CD138^{+}

MM cells. Primary CD138$^+$ MM cells and primary BMSCs were isolated from bone marrow aspirates of MM patients at the onset of the disease. Specific Jagged ligands silencing was obtained in primary CD138$^+$ MM cells by lentiviral infection and the results demonstrated that Jagged knock-down increase the sensibility of primary CD138$^+$ MM cells to Bortezomib by reducing the protective effect of primary BMSCs.

The last part of my thesis investigated if bone marrow microenvironment could trigger the expression of Notch receptor or ligands and activate the pathway. As reported above, genetic alteration are at the basis of Notch pathway deregulation: for example, 5% patients, which carry t(14;16)(q32;q23) and t(14;20)(q32;q11), show a dysregulation in MAFB and c-MAF genes that cause Notch2 overexpression (74). Evidences demonstrate that Notch pathway members expression increase during MM progression thus suggesting that there are other factors that influence Notch receptors or ligands expression: the bone marrow in an hypoxic microenvironment and, since Gustaffsson et al. demonstrate that HIF-1α positively regulates Notch signalling (95), I decide to evaluate the role of hypoxia on Notch pathway on MM cell biology.

To mimic hypoxia, I treated OPM2 cells with CoCl$_2$, which is a chemical compound that cause the accumulation of HIF-1α and, after CoCl$_2$ treatment there is an accumulation of HIF-1α at every timepoint. The next step was to evaluate if the accumulation of HIF-1α correspond to an increased expression of two Notch pathway members at protein level: Jagged1 and the intracellular domain of Notch2 (ICN2); my analysis was initially focused on Jagged1 and ICN2 because they are highly expressed by OPM2 cell line and the results show that there is no modulation in the protein expression of both Jagged1 and ICN2 indicating that HIF-1α cannot modulate the transcription of Notch pathway members. To better elucidate the mechanism by which HIF-1α modulates Notch pathway activity, I

wondered if hypoxia can affect the transcriptional activity of Notch intracellular domain (ICN). To verify my hypothesis, thanks to a reporter system, I demonstrate that $CoCl_2$ is able to increase the transcriptional activity of Notch intracellular domain in OPM2 cell line; to better dissect the mechanism and to determine if a specific Notch isoforms is involved in this process, I set up an in vitro reporter system model characterized by high transfection efficiency; I forced the expression of ICN1 and ICN2: I chose these two Notch isoforms because are the most relevant for MM disease (41). The results indicate that $CoCl_2$ treatment enhances the transcriptional activity of both Notch isoforms. These data are in accordance with a previously published work in which the authors proposed a mechanism by which HIF-1α modulates Notch pathway activity: basing on the obtained results, the authors hypothesisez that HIF-1α binds the ICN thus protecting it from degradation and increasing its half-life and transcriptional activity (95).

From literature it is known that hypoxia and Notch signalling cooperate to sustain the CSC niche: CSC are intrinsically drug resistance and display clonogenic ability; Matsui and colleagues first described MM-SCs and demonstrate that they have CD138^{-} phenotype, are drug resistant, have clonogenic potential and if injected are able to generate a tumour mass (101, 102). All these evidences, prompted me to analyse the effect of Notch pathway and hypoxia on MM-SC population. H929 cell line was choosen due to their higher reservoir of CD138^{-} MM-SCs if compared to other MM cell lines; hypoxia was mimicked with $CoCl_2$ treatment and Notch signalling was blocked after treatment DAPT, a γ-secretase inhibitor.

The results showed that, as expected, HIF-1α accumulation increased the CD138^{-} population and oppositely blocking Notch pathway significantly decrease it; while the double treatment demonstrated that the effect of

hypoxia on CD138^{-} MM-SCs population requires an active Notch pathway as demonstrated by DAPT ability to weaken $CoCl_2$ treatment.

Further experiments are needed to better characterize the mechanism by which hypoxia and Notch pathway interacts.

The future directions of this work include the validation *in vivo* in Zebrafish animal model. MM cells can be injected in Zebrafish either by injection in the perivitelline space (123) or by intracardiac injection (124). Sacco et al. demonstrate that MM cells xenografts migrate in the caudal haematopoietic tissue (CHT) which mimic mammalian BM function supporting MM cells growth and engraftment (104). I will inject Zebrafish with MM cells that conditionally express anti-Jagged1 and 2 siRNAs upon doxacycline treatment; fishes with xenografts will be treated with drugs and apoptosis rate of MM cells was assessed.

The results obtained in this thesis provide the rational for considering Jagged ligand as a new therapeutic target to overcame, on one hand MM-associated drug resistance and, on the other hand, gut toxicities due to GSI treatment (125) (126).

MATERIALS & METHODS

1. CELL CULTURES

1.1 SINGLE CULTURES

Multiple myeloma cell lines (HMCL) used were:

- OPM2: established in 1982 from the peripheral blood of a 56-year-old woman with multiple myeloma (IgG lambda) in leukemic phase

(relapse, terminal); cells negative for CD3, CD10, CD19, CD80 and CD20, cells positive for CD138. They grow in suspension.

- U266: established in 1968 from the peripheral blood of a 53-year-old man with IgE- secreting myeloma (refractory, terminal); cells were described to produce IgE lambda; cells negative for CD3, CD10, CD19 and CD20 and positive for CD138. They grow partially adherent.
- NCI-H929: established from the pleural effusion of a 62-year-old woman with myeloma (IgA kappa) at relapse; cells synthesize high amounts of immunoglobulin; cells are neagtive for HLA DR and markers of early B cell development; cells are positive for CD38. They grow in suspension.

All MM cell lines were maintained in 75 cm^2 flask in RPMI 1640 medium (Sigma-Aldrich Co., St Louis, MO), supplemented with 10% (v/v) FBS (Gibco, Rockville, MD), 2 mM L-glutamine (Invitrogen Corporation Carlsbad, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. The serum was de-complemented for 30' at 56°C before use. Cells were cultured in 5% CO2 at 37°C, maintaining the optimum concentration at 3x10^5 cells/ml with complete change of medium every two days.

The fibroblast cell line used as mimetic of bone marrow stromal cell (BMSC) was:
- NIH3T3: cell line of mouse embryonic fibroblasts isolated in 1962 at the New York University School of Medicine Department of Pathology. They grow adherent.
- HS5-pGIPZ: Stable cell line derived from HS5 cells that were stably transfected with the lentivirale vector pGIPZ to express green

fluorescent protein (GFP). Cells were maintained in culture adding 1 µg/mL puromycin for selection.

The cell line used as a model for luciferase assay was:

- HEK293: cells were isolated from an human aborted fetus in 1973.

Both BMSCs or HEK293 cell lines were maintained in 10 cm^2 plate dishes, in DMEM medium (Sigma-Aldrich Co., St Louis, MO, USA), supplemented with 10% (v/v) FBS (Gibco, Rockville, MD, USA), 2 mM L-glutamine (Invitrogen Corporation Carlsbad, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. The serum was de-complemented for 30' at 56°C before use. Cells were cultured in 5% CO2 at 37°C, with complete change of medium every two days.

1.2 CO-CULTURE SYSTEMS

NIH3T3 or HS5-pGIPZ were plated in 24-well plate at different concentration: $1,5x10^5$ cell/mL (OPM2) or $7,5x10^4$ cell/mL (U266). After 24h MM cell line, OPM2 or U266, were plated on BMSCs monolayer at concentration of $3x10^5$ cell/mL. Co-cultures were maintained in RPMI full for 48h and analysed by flow cytometry or for gene expression analysis.

2. TREATMENTS

2.1 NOTCH PATHWAY INHIBITION BY DAPT

Notch pathway inhibition was obtained by DAPT (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester, Sigma-Aldrich), also known as GSI-IX, that is a γ-secretase inhibitor. It is dissolved in DMSO. The cells were treated with 50 µM DAPT and controls with the same amount of DMSO.
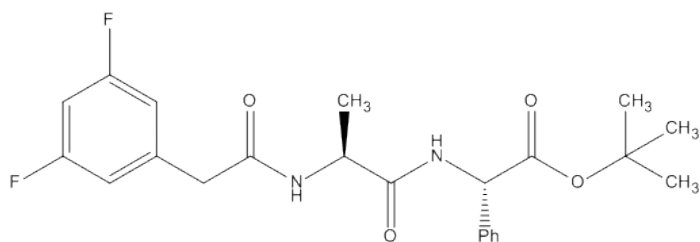
Figure 2.1 DAPT molecular structure.

2.2 HIF-1α ACTIVATION BY CoCl₂ TREATMENT

CoCl$_2$ (Cobalt chloride) is a chemical compound that blocks the degradation of HIF-1α by inhibiting PDH (Proline hydroxylase) enzyme thus causing an accumulation of active HIF-1α in the cytoplasm. It is dissolved in water. Cells were treated with 100 µM or 20 µM CoCl$_2$ and controls were treated with the same amount of water.

2.3 DRUG

To test MM cells Bortezomib drug resistance, cells were treated with 8 nM Bortezomib (Sigma-Aldrich) for 24h. Controls were treated with the same amount of DMSO.
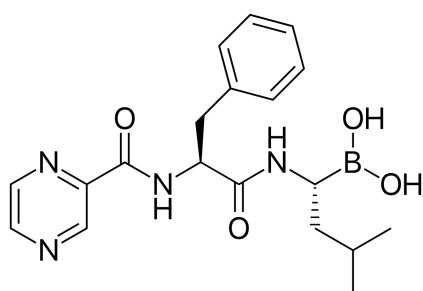
Figure 2.3.1 Bortezomib molecular structure.

3. GENE EXPRESSION ANALYSIS

3.1 RNA EXTRACTION

Total RNA was extracted by TRIzoL® reagent (Sigma-Aldrich). Protocol is optimized for 10^6 cells:

- Add 1 mL of TRIzol® Reagent.
- Lyse cells in sample by vortexing.
- Incubate the homogenized sample for 5 minutes at room temperature
- Add 0.2 mL of chloroform and vortex sample for 10 seconds.
- Incubate for 15 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.
- Remove the aqueous phase of the sample into a new tube.
- Add 0.5 mL of 100% isopropanol to the aqueous phase, and vortex sample for 10 seconds.
- 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 75% ethanol.
- Centrifuge the tube at 7500g for 5 minutes at 4°C and discard the supernatant.
- Air dry the RNA pellet for 5–10 minutes at room temperature.
- Re-suspend the RNA pellet in RNase-free water.
- Incubate at room temperature for 10–15 minutes.
- Proceed to downstream application, or store at –70°C.

3.2 RNA QUANTIFICATION

RNA was quantified by Nanodrop (ThermoFisher) using 1 µl of RNA and following manufacture's instruction.

3.3 REVERSE TRANSCRIPTION

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

The reaction was settled as follow:

- 1µg RNA
- 1µl of Random primers (25 0ng/µl)
- H_2O 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 10 mM (2.5 mM each)
- 1µl of RevertAid M-MuLV Reverse Transcriptase (200 U/µl)
- 1µl of H2O DEPC

The mix was added to the reaction composed by RNA and Random primers and incubated at 42°C for 1 hour. Samples were stored at -20°C or used for downstream application.

3.4 QUANTITATIVE REAL-TIME PCR

Quantitative PCR is an amplification technique that allow for simultaneous quantification of PCR products. It is based on the presence in the reaction mix of a fluorescent reporter

(SYBR Green) that is able to intercalate in dsDNA and to reveal the presence of PCR amplicons; fluorescence signal is directly proportional to the specific amplification products.

Gene target expression was obtained using the ΔCt method; GAPDH was used as housekeeping gene. Differences in gene expression between two

different conditions, e.g. treated vs control, were determined using the ΔΔCt method. In detail, was applied the following formula $2^{-\Delta\Delta Ct}$:

- Ct (cycle threshold): represents the number of cycles at which the detector started to reveal the presence of PCR products.

- ΔCt: represents the difference between the Ct of the target gene and the Ct of the housekeeping gene.

- ΔΔCt: represents the difference between the ΔCt of the treated sample and the ΔCt of the control sample.

Quantitative PCR (qRT-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 mix for a 96 well plate is the following (final volume of 15 µl):

- 7,5µl Maxima SYBR Green Master Mix (2X)
- 2 µl Primer Mix (final concentration 0,45 µM)
- 2 µl cDNA 5ng/µl (10 ng total)
- 3,5 µl H_2O RNasi-free

The following program was used:

- Hot start activation: 95°C for 10'
- Denaturation
- Annealing   40 cycles   95°C for 15"
- Dissociation   60°C for 60"

95°C for 15'

60°C for 15"

85°C for 15"

RT-qPCR primer	Forward primer 5'-3'	Reverse primer 5'-3'
hGAPDH	ACAGTCAGCCGCATCTTCTT	AATGGAGGGGTCATTGATGG
hJAGGED1	TTCGCCTGGCCGAGGTCCTAT	GCCCGTGTTCTGCTTCAGCGT
hJAGGED2	CCGGCCCCGCAACGACTTTT	CCTCCCTTGCCAGCCGTAGC
hHES1	GATGCTCTGAAGAAAGATAGC	GTGCGCACCTCGGTATTAAC
hHES6	ATGAGGACGGCTGGGAGA	ACCGTCAGCTCCAGCACTT
hCXCR4	GGCCTTATCCTGCCTGGTAT	TCGATGCTGATCCCAATGTA
hBCL-XL	CGTAGACAAGGAGATGCAGGTA	CTGCTGCATTGTTCCCATAGAG
hBCL2	GTCATGTGTGTGGAGAGCGT	GCCGTACAGTTCCACAAGG
hSURVIVIN	AGCCAGATGACGACCCCAT	CTTGGCTCTTTCTCTGTCCA
hABCC1	TAATCCCTGCCCAGAGTCCA	ACTTGTTCGGACGTGTCCTC
mGAPDH	TTGGCCGTATTGGGCGCCTG	CACCCTTCAAGTGGGCCCCG
mHES5	GGCTCACCCCAGCCCGTAGA	TCGTGCCCACATGCACCCAC
mSDF-1α	CAGCTCTGCAGCCTCCGGC	AAGAACCGGCAGGGGCATCG
mIL-6	TGAACAACGATGATGCACTTGCAGA	TCTCTGAAGGACTCTGGCTTTGTCT

Table 3.4 qRT-PCR primers sequence.

4. RNA INTERFERENCE

To specifically block Notch pathway, OPM2 and U266 cell lines were treated with two specific anti-Jagged1 and 2 siRNAs or with scrambled control in order to verify that any gene expression change occurs upon siRNAs delivery.

Cells treated with fluorescent sdRNA "BLOCK-IT" (Life Technologies Italia, Milan, Italy) were used as positive control.

Stealth Select RNAiTM siRNA system (Life Technologies Italia, Milan, Italy) was used according to the manufacturer's instructions. The protocol includes the following steps:

- OPM2 or U266 cells were plated at $3x10^5$/ml in medium without antibiotics in 24-well plate in 500 µl final volume.
- siRNAs (25 nM anti-Jagged1 + 25 nM anti-Jagged2 / or 50 nM scrambled siRNA/ or 50 nM fluorescent dsRNA) were diluted in 50 µl 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 50 µl of Opti-MEM medium without serum and antibiotics.
- The two solutions (siRNA/lipofectamine) were mixed and incubated for 20' at room temperature; 100 µl of lipofectamine/siRNA mix was added to the cells.
- Every 48h cells were diluted 1:1 with medium antibiotics-free and treated again with Jag1/Jag2 siRNA up to 96h.

To confirm that the transfection occurred successfully, the percentage of BLOCK-IT positive cells were checked trough flow cytometry analysis at each time point. 10000 cells were acquire with BD FACSVerse and analysed with FACSuite Software (BD, San Jose, CA).

To test the specific Jagged1 and Jagged2 knock-down, silenced MM cells were analysed by qRT-PCR and compared to scrambled control.

5. FLOW CYTOMETRY

To carry out flow cytometry experiments, BD FACSVerse was used and experiments analysed with FACSuite Software (BD, San Jose, CA).

5.1 APOPTOSIS

To detect apoptosis rate, was used the following protocol (staining for $5x10^5$ cells):

- Cells were washed with PBS ice-cold.
- Centrifuge at 3000 rpm for 4 min.

- Resuspend cells in 93 µl Binding Buffer 1X (HEPES 0,01M, NaCl 0,14M, CaCl2 2,5mM) and add 1 µl Annexin-V APC conjugated
- Incubate 10 min at room temperature in the dark
- Add 400 µl Binding Buffer 1X and acquire 10.000 events

5.2 INTRACELLULAR STAINING

To stain intracellular proteins, I used the following protocol (staining for $5x10^5$ cells):

- Centrifuge cells at 3000 rpm for 5 min and resuspend the pellet in 100 µl PBS plus 1% BSA and 0,1% NaN_3
- Add 100 µl formaldehyde 4% in PBS and incubate 20 min at room temperature
- Centrifuge cells at 3000 rpm for 5 min
- Resuspend the pellet in 100 µl PBS plus 0,5% BSA and 0,5% Saponin and incubate for 10 min at room temperature
- Centrifuge cells cells at 3000 rpm for 5 min and resuspend in 40 µl PBS plus 1%BSA and 0,1% NaN_3
- Divide the sample in two tubes and add primary antibody (Survivin, BCL2, IL-6 and SDF-1α) or matched isotype control.
- Incubate 1h at 4°C in the dark
- Add 300 µl PBS plus 0,1% NaN_3 and acquire 10.000 events

5.3 SURFACE MARKER STAINING

To stain surface marker proteins, I used the following protocol (staining for $2x10^5$ cells):

- Wash cells with 700 µl PBS plus 2% FBS and 0,1% NaN_3
- Centrifuge cells at 2000 rpm for 4 min
- Resuspend cell pellet in 40 µl and divide the sample into two tubes
- Incubate for 10 min at room temperature
- Add primary antibody (ABCC1) or matched isotype control

- Incubate for 45 min in the dark at 4°C
- Add 300 μl PBS plus 0,1% NaN$_3$ and acquire 10.000 events

6.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 and the protease inhibitors, 50mM NaF, 1mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na3VO4, 2μg/ml aprotinin, 2μg/ml leupeptin:

- Cells were resuspended in RIPA buffer and incubated for 15 min.
- The lysate was clarified by centrifugation for 15 min at 4°C.
- Protein concentration was determined using the Bradford assay (Bio-Rad Laboratories).
- 100 μg whole cell lysate was loaded and run on 8% denaturing SDS-PAGE gels.
- The proteins are transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Bioscience), and blocked with 5% screamed milk in TBS-T (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20).
- The membrane was then incubated o.n. at 4°C with the indicated primary antibodies as follows: Actin 1:5000 (ThermoFisher), Notch 2 intracellular domain antibody-cleaved (Asp1733) 1:500 (Sigma-Aldrich) and Jagged1 1:500 (Santa Cruz Biotechnology).
- Following washes, the membrane was incubated with HRP-conjugated species-specific secondary antibodies (Santa-Cruz Biotechnology).
- Proteins were visualized with ECL reagents (Advansta) according to the manufacturer's instructions.

7. TRANSFECTION AND LUCIFERASE ASSAY

Intracellular Notch1 (ICN1), also named as ICN1-ΔE because it is lacking PEST domain, was previously described by Prof. W.S. (127) (Pear et al.,

1996). To overexpress ICN2, I used the pADTrack-ICN2 plasmid. For the reporter assays, TK-pRL was from Promega Italia s.r.l. (Milano, Italy). The pGL3-based plasmid encoding the firefly luciferase under the control of 13 repeats of the CSL-responsive element (13XCSL) was as described by Shawber C., et al. (32).

Experiments were carried out as follow:

- HEK293 were plated in 48-well plate, 60.000/cell per well in 250 µl complete DMEM.
- After 24h cells were transfected with TurboFect (ThermoFisher) according to manufacturer's instruction and with the following amount of plasmids:
    - 0,4 µg 13XCSL
    - 0,02 µg TK-pRL
    - 0,044 µg ICN1-ΔE
    - 0,055 µg ICN2
- 24h post transfection cells were treated with 100 µM $CoCl_2$.
- The dual luciferase assay was performed according to the manufacturer's directions (Dual-Luciferase® Reporter Assay System, Promega). TK-pRL plasmid was used to control transfection efficiency

8. PRIMARY SAMPLES

8.1 PRIMARY CD138^{+} MM CELLS ISOLATION

Primary CD138^{+} MM cells were isolated from bone marrow (BM) aspirates of MM patients and immunomagnetically sorted using EasySep™ Human CD138 Positive Selection Kit (Stem Cell Technology) according to manufacturer's instruction.

8.2 PRIMARY CD138^{+} MM CELLS LENTIVIRAL TRANSFECTION

Lentiviral infection of primary CD138^{+} MM cells were carried out as follow:

- Primary CD138$^+$ MM cells were resuspended either in lentiviral surnatant pLL3.7-empty or pLL3.7-shRNA anti-Jagged1/2 and add:
    - 12 µg/mL Polybrene
    - 20 ng/mL IL-6 in 0,1% BSA
    - 20 ng/mL GMCSF in 0,1% BSA
    - 100 ng/mL IGF-1 in 0,1% BSA
- After 24h infection, cells were centrifugated, resuspended in RPMI1640 full and plated on BMSCs monolayer

8.3 PRIMARY BMSC ISOLATION, CHARACTERIZATION AND STAINING

Primary BMSCs were isolated starting from the negative fraction of primary CD138$^+$ MM cells isolation. Negative fraction was plated in T75 flask and after 24-48h the medium was changed and the first population of cells that are attached to the flask are primary BMSCs.

BMSCs were characterized both by flow cytometry for CD45, CD14, CD90 and CD105 expression and by morphological shape by optical microscopy. Primary BMSCs growth adherent in complete DMEM medium (Sigma-Aldrich Co., St Louis, MO, USA), supplemented with 10% (v/v) FBS (Gibco, Rockville, MD, USA), 2 mM L-glutamine (Invitrogen Corporation Carlsbad, CA, USA), 100 U/ml penicillin   and 100 µg/ml streptomycin. The serum was de-complemented for 30' at 56°C before use. Cells were cultured in 5% CO_2 at 37°C, with complete change of medium every two days.

For co-cultures set-up, primary BMSCs were stained with PKH26 (Sigma-Aldrich St Louis, MO), which is a lipophilic dye, to allow the detection of BMSCs and primary CD138$^+$ MM cells by flow cytometry.

The protocol for PKH26 is as follow (staining for 10^7 cells):

- Wash cells with serum-free media and centrifuge at 1000 rpm for 5 min.

- The pellet was resuspended in 1 ml of "Diluent C" (Sigma-Aldrich Co., St Louis, MO) + 1 µl of PKH26 dye solution (Sigma-Aldrich Co., St Louis, MO).
- Cells were stained for 5 min at RT and the reaction was blocked by adding 1 ml of FBS.
- Cells were washed one times with complete culture medium and plated at desidered concentration.

8.4 CO-CULTURES SET UP

Transduced primary CD138$^+$ MM cells were plated on BMSCs monolayer in 48-well plate and treated with 8 nM Bortezomib or vehicle (DMSO) for 24h hours. At the end of the treatment apoptosis rate of primary CD138$^+$ cells were assessed by FC as Annexin-V$^+$ cells.

9. STATISTICAL ANALYSIS

Data are represented as mean ± SD of at least 3 independent experiments. Statistical analysis on gene expression analysis and luciferare experiments were performed using two-tailed Student's t-test to compare the means of normally distributed values and in drug resistance co-culture experiments of both in vitro and ex vivo systems and in CD138 expression variation, I performed analysis of variance by a one-way ANOVA or two-way ANOVA with Tukey's post-test.

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