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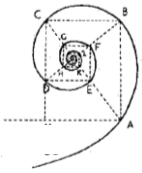

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Rationale for a Notch-directed therapy to prevent multiple myeloma crosstalk with the osteoclastogenic niche

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ABSTRACT

Multiple myeloma (MM) is an incurable hematological tumor stemming from malignant plasma cells. Similarly to normal plasma cells, MM cells accumulate in the bone marrow (BM) where they establish complex interactions with normal BM stroma, which promote tumor survival and bone disease due to unbalanced bone deposition and resorption.

The Notch family of receptors consists of 4 isoforms that, once activated, act as transcription factors. The activation is triggered by membrane-bound ligands (Jagged1-2 and DLL1-3-4). Notch has a key role in the regulation of proliferation, survival, differentiation and stemness in various tissues and tumors. Notch receptors and ligands are deregulated in MM and this signaling system may play a role in the pathogenesis of MM by modulating tumor cell biology, as well as their pathological interactions with the BM niche. Specifically, the myeloma-associated alteration of Notch signaling mainly consists in the aberrant expression of two Notch ligands, Jag1 and Jag2, by tumor cells resulting in Notch signaling activation in both tumor cells and the surrounding cells of the bone niche.

Notch mediated signals have been reported to play a role in MM-induced osteoclasts activity and the release of tumor promoting factors by stromal cells.

This work unequivocally demonstrates that Notch signaling drives MM cell-induced osteoclastogenesis. The underlying molecular mechanisms is based on: 1) Notch signaling-dependent MM cell ability to promote the release of the osteoclastogenic receptor activator of NF-κB ligand (RANKL), 2) Notch2-mediated transcription of osteoclastogenic master genes, such as *Tartrate-resistant acid phosphatase* (TRAP) and *Receptor Activator of Nuclear Factor κ B (*RANK), within osteoclast precursors.

Notch2 signaling activation, occurring upon RANKL stimulation, results to be necessary for osteoclastogenesis completion, and further boosts the differentiation by promoting an autonomous secretion of RANKL by osteoclasts.

Finally, the most crucial finding of this study is that MM-induced osteoclastogenesis could be disrupted by silencing in MM cells two of the Notch ligands, Jag1 and 2,. These results make Jag1 and Jag2 new promising therapeutic targets to hamper MM-associated bone disease and co-morbidities, lacking the toxicity of the currently used drugs which affects the whole Notch pathway.

RIASSUNTO

Il mieloma multiplo (MM) è un tumore ematologico incurabile che ha origine da plasmacellule maligne. Analogamente alle plasmacellule sane, le cellule di MM si accumulano nel midollo osseo, dove stabiliscono una serie di complesse interazioni con lo stroma midollare. Queste interazioni promuovono la sopravvivenza delle cellule maligne e lo sviluppo di un'osteoporosi diffusa, causata dallo sbilanciamento tra il riassorbimento e la deposizione ossea.

La famiglia dei recettori Notch comprende 4 diverse isoforme che, una volta attivate, agiscono da fattori di trascrizione. L'attivazione di questi recettori è mediate da ligandi di membrana (Jag1-2 e DLL1-3-4). Notch ha un ruolo chiave nella regolazione di proliferazione, sopravvivenza, differenziamento e staminalità in diversi tessuti normali e neoplastici. Nel mieloma il pathway di Notch è deregolato sia a livello dei ligandi che dei recettori.

Il signaling di Notch può avere un ruolo nella patogenesi del MM non solo influenzando le funzioni della cellula tumorale, ma anche le interazioni patologiche tra questa ed il microambiente midollare. Nello specifico, le

alterazioni del pathway di Notch associate al mieloma consistono nell'iperespressione di due dei ligandi di Notch, Jag1 e Jag2, da parte delle cellule tumorali. Questo causa un'alterazione nell'attivazione di Notch sia a livello delle cellule tumorali che delle altre componenti cellulari del midollo osseo.

Recentemente è stato riportato un coinvolgimento di Notch nell'incremento dell'attività osteolitica associata al mieloma e nel rilascio da parte delle cellule stromali di fattori solubili che supportano il tumore.

Questo lavoro di tesi dimostra in modo inequivocabile che Notch guida l'osteoclastogenesi indotta dalle cellule di mieloma. Il meccanismo alla base di questo processo si fonda su: 1) l'induzione da parte della via di Notch della capacità delle cellule di mieloma di rilasciare *Receptor Activator of NF-κB Ligand* (RANKL), 2) il ruolo di Notch2 nel promuovere la trascrizione da parte dei precursori degli osteoclasti di geni chiave nel processo differenziativo quali *Tartrate-Resistant Acid Phosphatase* (TRAP) e *Receptor Activator of Nuclear Factor-κB* (RANK). In particolare l'attivazione di Notch2 in seguito alla stimolazione con RANKL è necessaria per una completa osteoclastogenesi e promuove ulteriormente il differenziamento stimolando la secrezione autonoma di RANKL da parte degli stessi osteoclasti.

Ma la scoperta più rilevante di questo studio è che l'osteoclastogenesi indotta dal MM può essere inibita silenziando due ligandi di Notch, Jag1 e Jag2. I risultati che ho prodotto suggeriscono come Jag1 e Jag2 siano due nuovi e promettenti target terapeutici nel trattamento del danno osseo causato dal MM e della comorbidità ad esso associata. Questo tipo di approccio, inoltre, presenterebbe una ridotta tossicità rispetto ai farmaci correntemente usati che agiscono bloccando contemporaneamente tutte le isoforme di Notch.

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CHAPTER 1

THE NOTCH PATHWAY

1.1 INTRODUCTION

The first studies on the Notch oncogene date back to 1919, when T. H. Mohr identified a strain of haploinsufficient Drosophila characterized by wings with irregular margins (Notched). The gene responsible for this phenotype was cloned in 1985 and is known as Notch *(Grimwade et al., 1985).*

In humans, as well as other mammals, the Notch gene family includes a total of four highly homologous sequences Notch-*1,* Notch-*2,* Notch-*3,* Notch-*4,* mapping respectively on human chromosomes 9, 1, 19 and 6. The first human ortholog of Notch*,* Notch-*1,* was found in 1991 by LW Ellisen in T-ALL patients, carriers of the translocation t (7; 9) (q34; q34.3) and it was initially called *TAN-1* (translocation-associated Notch homologue-1)(Ellisen et al., 1991).

Subsequent studies demonstrating that the oncogene Notch regulates morphogenesis, proliferation, apoptosis and cellular differentiation and it is essential for embryonic development of multicellular organisms *(Lai et al, 2004).* In mammals Notch seems to have a key role in the regulation of various processes such as neurogenesis, gliogenesis, myogenesis, vasculogenesis, hematopoiesis and development of the epidermis *(Kopan and Ilagan, 2009).* Notch, however, is also involved in the homeostasis of adult tissues promoting self-renewal of stem cells, determining cell fate (such as the commission towards the T or B lineage) and regulating the differentiation of many cell types. Because of its extensive involvement in

all these process, mutations or deregulation of Notch receptors and/or ligands are associated with the onset of various diseases, many types of cancer (breast, ovarian, prostate, cervical, skin, pancreas and liver cancer, neuroblastoma) including the T-cell leukemia (Notch-1)and multiple myeloma (Jag-2), in developmental disorders such as cerebral artery disease CADASIL (Notch-3) and in Alagille syndrome (Jag1) (Kopan and Ilagan, 2009).

1.2 NOTCH RECEPTORS

The Notch gene encodes for a transmembrane receptor whose structure is highly conserved during evolution *(Lardelli et al., 1995; Maine et al., 1995)* (Figure1.1).

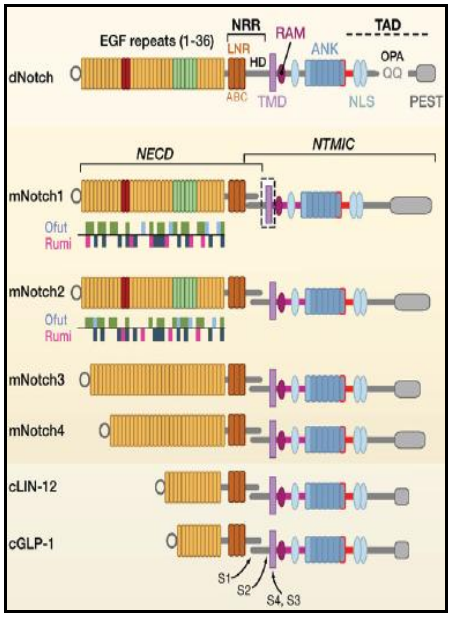

Figure 1.1 The oncogene Notch in invertebrates and vertebrates *(Kopan and Ilagan, 2009).*

The prototype of the *Notch* gene, the dNotch of Drosophila, encodes for a protein of 2703 amino acids with a molecular weight of 300 kDa. The mature form, expressed at the cell membrane, is an heterodimer consisting of two subunits associated by calcium-mediated covalent bonds: an extracellular subunit, involved in the interaction with the ligands, and an intracellular subunit, necessary for signal transduction *(Blaumueller et al., 1997; Logeat et al., 1998).*

NOTCH proteins extracellular domain (ED) contains (Kopan and Ilagan, 2009):

- 29–36 tandem epidermal growth factor repeats (EGF-like repeats, ELR), required for the interactions with ligands;
- a negative regulatory region (NRR), composed of three cysteine-rich Lin12-NOTCH repeats (LNR), that prevents autonomous receptor activation;
- a highly conserved heterodimerization domain of 100 amino acids, containing two cysteine, important for maintaining the receptor in an inactive conformation required for the dimerization.

The citoplasmatic region consisted of*:*

- 6 repeats of the cdc10/ankirina (ANK) domain, important for the interaction with several proteins (like Deltex and Mastermind);
- a high affinity binding module called RAM [RBPjκ (recombination signal sequence-binding protein Jk) association module] formed by 12–20 amino acids. The RAM domain, together with the ANK repeats, interacts with the transcriptional complex CSL and mediates the signaling transduction;
- two sequences for the nuclear localization (NLSs);
- a transactivation domain (TAD), important for the transcriptional activation;
- a PEST domain (proline/glutamic acid/serine/threonine-rich motif), that regulates the receptor degradation.

Notch is synthesized in the endoplasmic reticulum as a single polypeptide (pre-Notch) with one extracellular and one intracellular domain. An unknown glucosyltransferase and the POFUT1 O-fucosiltransferase are necessary for the transport of pre-Notch from the endoplasmic reticulum to the Golgi apparatus where the serine and threonine residues in the EGF repeats are modified (Okajima et al., 2005).

The first cleaved of the pre-Notch protein in the Golgi apparatus is mediated by a furin-like convertase and occurs within the HD domain, in the S1 cleavage site (70 amino acids from the transmembrane domain). This cleavage converts the pre-Notch polypeptide into the heterodimer NECD/NTMIC (Notch-extracellular domain/Notch transmembrane and intracellular domain) (Blaumueller et al., 1997; Logeat et al., 1998).

The two subunits resulting from this process are brought to the plasma membrane as one heterodimer, held together by non-covalent calcium-dependent interactions.

1.3 NOTCH LIGANDS

Notch activation is stimulated by the interaction with the ligands, which induce the proteolytic cleavages, the release of the cytoplasmic portion, and its translocation to the nucleus *(Artavanis-Tsakonas et al., 1999).*

In vertebrates two closely related families of ligands are capable of interacting with Notch receptors: the Delta-like ligands (DLL-1, -3, -4 and DLL-2 described only in Xenopus) and the Serrate-like ligands (Jagged1 and 2) (Kopan and Ilagan, 2009) (Figure 1.2).

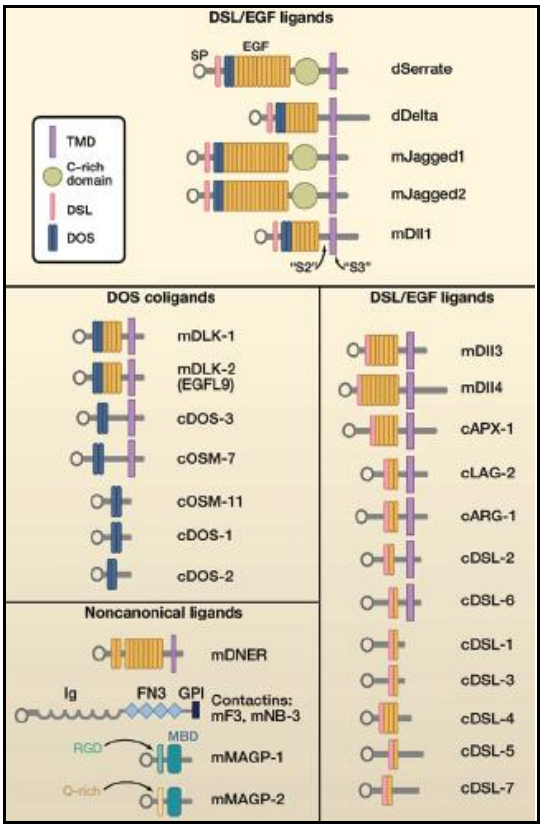

Figure 1.2 . Schematic representation of Notch ligands *((Kopan and Ilagan, 2009).*

The structure of all DSL ligands is very similar to the one of Notch receptors and consists of:

• an extracellular domain containing from 2 to 16 EGFR in tandem;

• a cysteine rich domain (CR) located between EGFR and the transmembrane domain *(Lindsell et al., 1995; Shawber et al., 1996))*. Experimental data suggest that this domain reduce ligand-receptor interaction (Fehon et al., 1990; Rebay et al., 1991)*;*

• a characteristic EGF degenerate sequence at the N-terminal domain known as DSL, which is essential for the activation of Notch (Muskavitch, 1994).

• a cytoplasmic domain that plays a central role in the process of dimerization with the receptor.

The interactions between Notch and its ligands can be complex.

Indeed, some cells simultaneously express both the receptor and the ligands (Muskavitch, 1994). *In vitro* studies have shown that Notch and Delta can interact also when placed in cis *(Fehon et al., 1991).* The ability of Delta to mediate an homotypic cell adhesion represents another level of complexity. In fact, based on the expression levels, the ligands might have an agonist or an antagonist action on Notch (Jacobsen et al., 1998).

1.4 ACTIVATION AND SIGNAL TRANSDUCTION

Upon ligand binding , Notch undergoes conformational modifications in the receptor which cause a sequence of proteolytic cleavages that result in Notch trans-activation (Mumm and Kopan, 2000).

This process is characterize by two steps:

- Following ligand binding, the endocytosis of the ligand–NECD complex induces the unfolding of the juxtamembrane negative control region (NRR). The resulting conformational change in NRR exposes the site 2 (S2) allowing access of the ADAM/TACE metalloprotease (Mumm and Kopan, 2000). ADAM proteases leaves a short-lived fragment called NEXT (Notch extracellular truncation) anchored to the plasma membrane.
- NEXT becomes the substrate for the last cleavage: Notch intracellular fragment is recognized by the inactive aminopeptidase domain of

Nicastrin (NCT), which transfers NEXT to the active site of the enzyme γ-secretase, that operates the cleavage within the transmembrane domain. γ-secretase is an aspartyl-protease presenilin(s) complex composed by four core proteins: presenilin 1 or 2, anterior pharynx defective 1 (APH1), nicastrin and presenilin enhancer 2 (PEN2) (Francis et al., 2002). The γ-secretase cleavage may occur at the cell surface or in endosomal compartments, perhaps following mono-ubiquitination (Kopan and Ilagan, 2009).

This γ-secretase-mediated processing, releases the Nβ peptide and various forms of ICN (Intracellular Notch): only those with a valine residue at the amino terminus (V1744) are able to escape the N-end-rule degradation pathway (Tagami, 2008) and translocate into the nucleus, where ICN interacts with the DNA-binding protein complex CSL [CBF-1 (Cp-binding factor 1)/RBP-Jk] to activate the transcription of several target genes.

Into the nucleus, in absence of activation by Notch, CSL acts as a transcriptional repressor due to its ability to bind several transcriptional co-repressor complexes including SMRT (silencing mediator of retinoic acid and thyroid hormone receptor), histone deacetylase 1 (HDAC1), HDAC2, SHARP (SMRT/HDAC1-associated repressor protein), CIR1 (CBF1-interacting co-repressor) and SKIP (ski-related protein) (Mumm and Kopan, 2000). In the nucleus, ICN binds CSL through the RAM domain and the ANK repetitions and displaces the co-repressor complex by recruiting Mastermind-like proteins (MAMLs). After that, MAMLs recruit some transcriptional co-activators (CoA) as GCN5 and histone acetyltransferase such as SKIP and p300. These proteins form an high molecular weight transcriptional activation complex that converts CSL from repressor to activator (Mumm and Kopan, 2000).

At the end of signal transduction, ICN is degraded and the promoter comes back to an inactive state.

The CSL-dependent signal seems to be responsible of most of Notch-mediated effects, but biochemical data support the existence of a CSL-independent pathway, whose function remains to be determined *(Heitzler, 2010).*

The CSL-independent pathway involves the regulation of the transcription of some genes by Deltex, a zinc-finger protein that interacts with the cytoplasmic ICN through the ANK repeats. Deltex is a positive regulator of the Notch pathway in Drosophila, but not in mammals. Although the exact mechanism of its action is unclear, it seems that Deltex antagonizes p300, reducing the transcription of particular genes and regulating different classes of transcription factors, including E47. It also appears that the expression levels of Deltex are self-regulated by ICN (Heitzler, 2010).

Notch transcriptional activity can be further regulated by direct inhibitors of CSL such as KyoT2 and Hairless. Their interaction prevents ICN action leaving the promoter accessible to other transcriptional activators and thus leading to a different transcriptional activity *(Kao et al., 1998)*.

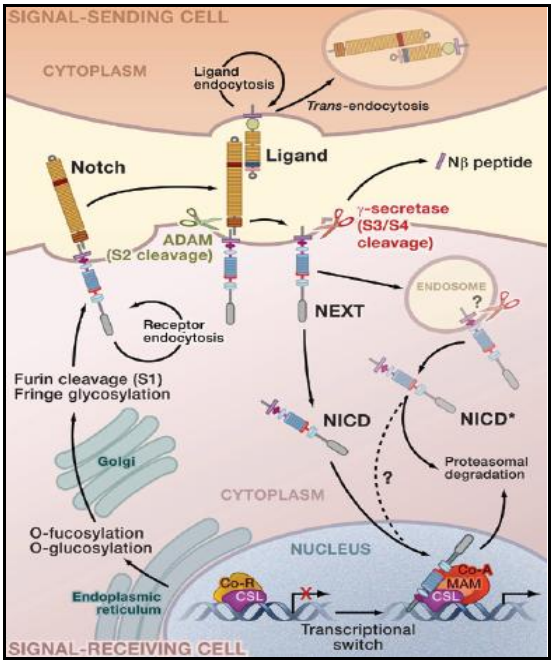

Figure 1.3 Notch's activation and signal transduction (Kopan and Ilagan, 2009).

1.5 REGULATION OF THE NOTCH PATHWAY

The Notch signaling is the result of a fine-tuned balance; both Notch and Notch ligands are in a dynamic equilibrium between a membrane pool and an intracellular vesicle pool, with a transition to an internalized pool upon interaction of adjacent cells.

In the endoplasmic reticulum Notch, modified in the EGF domains by the addition of fucose to serine (Ser) or threonine residues (Thr) (O-fucose), may be further modified at the O-fucose by N-acetyl-glucosaminyltransferase Fringe thus altering the ligand-receptor specificity.

These modification promotes the activation via Delta ligands and limits the Jag-mediated activation *(Okajima et al., 2005).*

In the cytoplasm the Notch pathway is regulated by Numb and Deltex. Numb is an adapting protein that participates at the cellular endocytosis in association with α-adapting and Exp-15. According to numerous studies in Drosophila and mammals, Numb may inhibit Notch function through several mechanisms: by interacting and activating Itch (a member of the Nedd4 family of E3 ubiquitin-ligase), that promotes the polyubiquitination and the subsequent proteasomal degradation of ICN *(Qiu et al., 2000)*; by promoting endocytosis of the S2-cleaved Notch before the release of ICN; by preventing membrane localization of the gene product Sanpodo, a transmembrane protein that promotes the Notch signaling. The role of Deltex in Notch regulation was discussed above.

In the nucleus the Notch pathway is regulated by Sel-10, Mint and Nrap. Sel-10 is an E3-ubiquitin ligase which binds ICN and recruits the SCF complex (Skp1-Cullin-F-box), this complex ubiquitinates Notch and promotes its proteasomal degradation. The regulation by Sel-10 requires the presence of the PEST domain *(Lai, 2002),* which is hyperphosphorylated by the binding of MAML to p300 and to the cyclin-dependent kinase 8 (CDK8) (Lai, 2002). Nrap (Notch regulated ankyrin repeat protein) can bind the ICN-CLS complex through two AKN repeats and may inhibit the complex and/or destabilize ICN *(Lamar et al., 2001).* Mint (MSX2-interacting nuclear target protein) inhibits the Notch pathway by preventing the binding of ICN to CSL and by blocking the transcriptional activation mediated by Notch (Lamar et al., 2001).

The best known mechanism of Notch degradation is proteasome-dependent and mediated by two E3 ubiquitin ligase, Itch and Sel-10, but the lysosomal way appears to be often preferred *(Jehn et al., 2002)* In the

lysosomal degradation pathway, a key role is played by the ubiquitin ligase c-Cbl, which interacts with NOTCH after the tyrosine-phosphorylation of the PEST domain and directs the receptors to lysosomal degradation *(Jehn et al., 2002).*

Also, the endocytosis of the Notch receptor is tightly controlled in time and space: Numb, acts as a Notch inhibitor upstream of the γ-secretase cleavage, in cooperation with the AP2 component α-adaptin and NAK (Numb associated kinase).

Notch ligands are also finely regulated. Probably, ubiquitynation allows their traffic into an endocytic compartment where ligands are modified, activated or re-inserted into specific membrane domains. Indeed, the localization of Notch ligands is important for an effective signaling and may be modulated (Kopan and Ilagan, 2009).

In addition to trans-activating Notch–ligand complexes, when binding occurs between Notch and ligand expressed on the same cell surface the receptor can form cis-inhibitory complexes. This cis-inhibition limits the areas of Notch activity and determines whether a cell will signal (the ligand is more abundant than Notch) or receive (Notch is more abundant than the ligand) (Sprinzak et al., 2010). Alternatively, in some cases ligands and receptors can be segregated into different subdomains to allow simultaneous transmission and reception of signals (Luty et al., 2007)(fig.1.4).

Some recent reports show that ligands also undergo proteolysis and release ligand intracellular domain (LICD) which antagonizes Notch signaling by mechanisms as yet unclear (LaVoie and Selkoe, 2003).

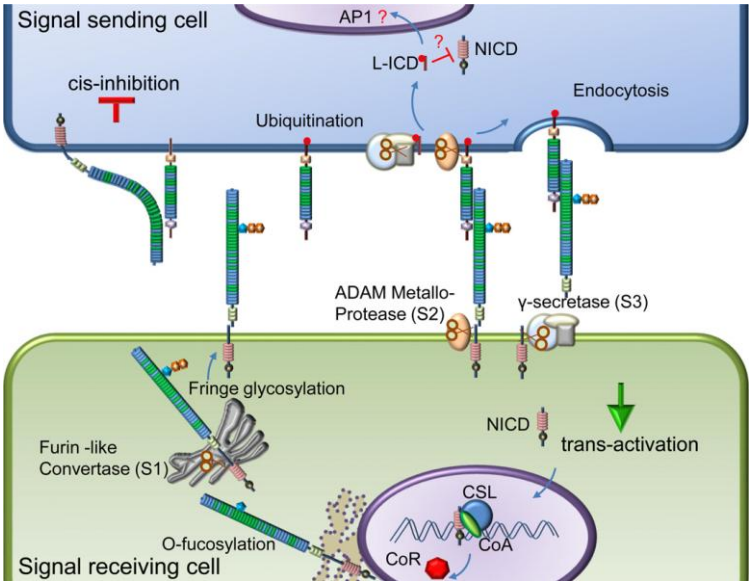

Figure 1.4 Trans- and Cis-activation of Notch.

1.5 NOTCH TARGET GENES

Although signals mediated by Notch receptors may have several outcomes, only a fairly limited set of Notch target genes have been identified in various cellular and developmental contexts.

The most extensively studied and best understood targets are *Hairy and Enancer of Spleet* in *Drosofila* and the related genes Hes and Hey in mammals. In mammals genome seven Hes (Hes 1-7) and three Hey (Hey 1, 2, L) genes have been identified. HES and HEY are helix-loop-helix transcription factors that act as transcriptional repressors and play an important role in development.

CD25 (IL2-R and preTα, pre-T-cell receptor alphachain) and the transcription factor GATA3 are direct Notch target genes activated in T-cell development. Further Notch targets are Myc, CyclinD1, p21/Waf1, Bcl2, E2A, HoxA-5 -9 -10, NF-κB2, Ifi-202, Ifi-204, Ifi-D3, and ADAM19. Two

other Notch target genes, NRARP and Deltex1, are shown to be negative regulators of Notch signaling itself and Notch1 and Notch3 have been reported as Notch itself target genes (Borggrefe and Oswald, 2009).

1.6 NOTCH SIGNALING IN CANCER

Given the range of processes that require normal Notch signaling, it is not surprising that a number of human diseases and cancer are caused by mutation or deregulation of different components of this pathway. In table 1.1 there are some examples of malignances in which Notch or its ligands are involved.

1.6.1 Notch as an oncogene

Notch deregulation is involved both in solid tumors as breast cancer, skin cancer, neuroblastomas, prostate cancer and cervical cancer (Allenspach et al., 2002), and in non-solid malignancies, such as leukemia (Weng and Aster, 2004) and multiple myeloma (Jundt et al., 2004).

From 90's to nowadays Notch signaling aberrations have been shown to be linked with several hematological malignancies such as T-cell acute lymphoblastic leukemia (T-ALL), acute myeloid leukemia (AML), lymphoma and multiple myeloma (MM).

The main oncogenic role of Notch can be found in T-ALL, an aggressive neoplasm of immature T-cells. Indeed, approximately 60% of T-ALL cases display activating Notch mutations (Weng and Aster, 2004).

The role of Notch signaling in AML is less clear than in T-ALL. Activating mutations of Notch have been reported but they seems to be a rare event (Palomero et al., 2006). Chiaramonte and colleagues demonstrate that AML primary sample show high levels of Jag-1 expression, despite low

Notch-1 pathway activation (Chiaramonte et al., 2005), thus suggesting a Notch-independent pathway driven directly by the Jag-1 ligand (Ascano et al., 2003).

Regarding B-cell malignancies, Notch deregulation has been detected in Hodgkin's lymphoma, large B-cell lymphoma, Burkitt's lymphoma, B-cell chronic lymphocytic leukemia, diffuse large B-cell lymphoma, primary effusion lymphomas associated with Kaposi's sarcoma herpes virus infection and in multiple myeloma (Mirandola et al., 2013).

The role of Notch in MM will be discussed in the following chapter.

The second most compelling evidence for a Notch oncogenic function comes from studies of breast and cervical cancer as well as melanoma. Molecular analysis reveal that Notch4 overexpression activates TGF-β and HGF signaling and promotes tumor invasion in the majority of breast ductal carcinoma *in situ* lesions (Meurette et al., 2009).

A role for aberrantly active Notch signaling has been proposed in cervical cancer, largely due to observation of intensive Notch 1 and 2 protein accumulation as well as consistent expression of Jag1 in which two oncogenic effector mechanisms are triggered by Notch: activation of PI3K/AKT pathway and up-regulation of Myc (Maliekal et al., 2008).

The Notch signaling is also up-regulated in primary human melanomas: the pro-oncogenic role of this receptor is linked with the activation of WNT signaling and to the promotion of N-cadherin expression(Koch and Radtke, 2007).

1.6.2 Notch as a tumor suppressor

The most emblematic example of Notch as a tumor suppressor comes from studies on the skin. The tumor suppressive effect of Notch 1 in the

epidermis is mediated by the induction of p21 (that blocks the cell cycle) and by the suppression of Wnt/β-catenin, leading to terminal differentiation by withdrawal of proliferating cell from the cell cycle (Rangarajan et al., 2001).

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 1.1 *Involvement of aberrant NOTCH signaling in a wide variety of cancers. NOTCH signaling may act as a tumor suppressor or a promoter depending on the cell type and cell context (Yin et al., 2010).*

CHAPTER 2

MULTIPLE MYELOMA

2.1 INTRODUCTION

Multiple myeloma (MM) is a malignant plasma cell disorder accounting for approximately 10% of all hematologic cancers.

This pathology is characterized by malignant plasma cells accumulations in the bone marrow (BM) associated to the aberrant production of Ig, usually monoclonal IgG or IgA. MM is also characterized by skeletal destruction, renal failure, anemia, and hypercalcemia. Although the recent advances in the therapeutic treatment, including the use of thalidomide and new drugs such as Bortezomib and CC-5013, MM is still incurable, with a median survival of 3-4 years after diagnosis (Kyle and Rajkumar, 2004).

The first pathogenetic step in the development of multiple myeloma is the emergence of a limited number of clonal plasma cells, clinically known as MGUS. Patients with MGUS do not have symptoms or evidence of end-organ damage, but they have an annual risk of progression to multiple myeloma or related disorder of 1% (Bergsagel and Kuehl, 2005).

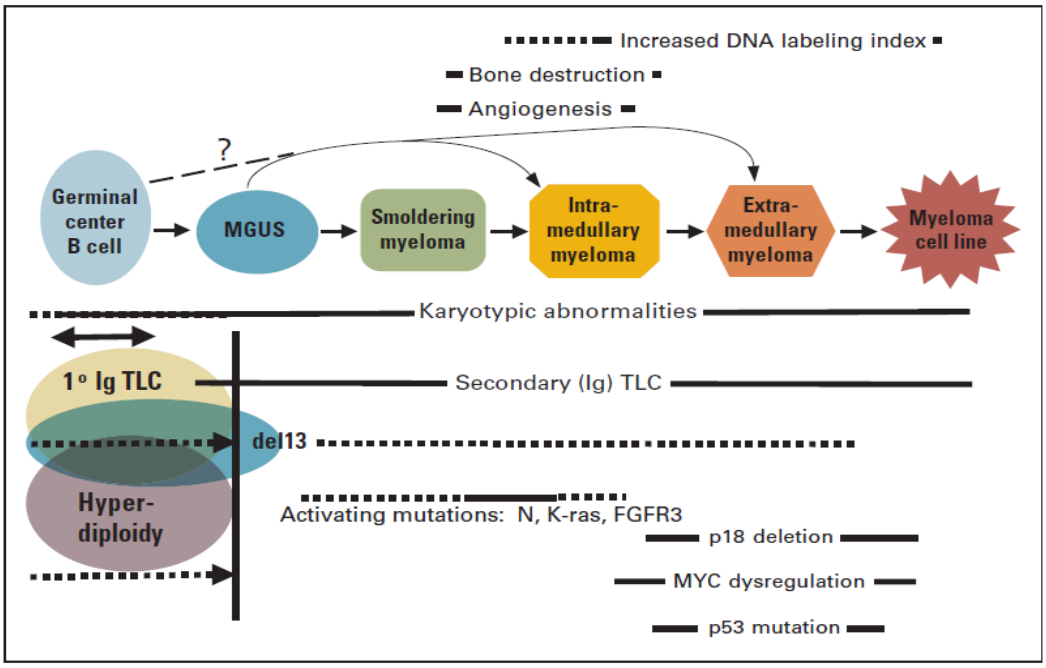

Figure 2.1 Stages of the disease and chronology of the oncogenics events *(Bergsagel and Kuehl, 2005).*

The progression of MGUS to myeloma is characterized by complex genetic events occurring in the neoplastic plasma cell (Figure 2.1). Changes also occur in the bone marrow microenvironment, including the suppression of cell-mediated immunity and the development of paracrine signaling loops involving cytokines such as interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) that promotes both angiogenesis and osteoclastogenesis (Bergsagel and Kuehl, 2005).

The development of bone lesions in MM is thought to be related to an increase of RANKL (receptor activator of nuclear factor-κB ligand) expression by osteoblasts and to a reduction in the level of its decoy receptor, osteoprotegerin. This increase in the ratio of RANKL to

osteoprotegerin results in activation of osteoclasts and bone resorption. Overexpression of RANKL is probably mediated in part by the release of macrophage inflammatory protein (MIP) 1α by neoplastic plasma cells, and in part by the IL6-VEGF loop (Roodman, 2010a).

The interactions between myeloma cells, bone marrow stromal cells, and microvessels contribute to the persistence of the tumor and to its drug resistance. These interactions involves the bone marrow stromal cells (BMSC) and others normal constituent of the bone marrow fibroblasts, osteoclasts, osteoblasts, endothelial cells and adipocytes (Mitsiades et al., 2006).

2.2 PATHOGENESIS

MM and non-IgM MGUS are exclusively post-germinal center (GC) tumors that have phenotypic features of plasmablasts (PB)/long-lived plasmacells (PC) and usually are distributed at multiple sites in the BM. The events that initiate myeloma transformation are unknown.

The GC or post-GC B cells have modified immunoglobulin (Ig) genes due to sequential rounds of somatic hypermutation and antigen selection, and sometimes to IgH switch recombination.

These two B cell–specific DNA modification processes, which occur mainly in GC B cells, sometimes can cause mutations or double-strand DNA breaks in or near non-Ig genes, including oncogenes like c-Myc, Ras, p53 and some genes of the Rb pathway, and genes involved in the regulation of the cell cycle, such as, for example, cyclins.

A failure to repair the double strand brakes can lead to mutation and neoplastic transformation.

Post-GC B cells can generate PBs that have successfully completed somatic hypermutation, antigen selection, and IgH switching before migrating to the BM, where stromal cells enable terminal differentiation into long-lived PCs.

A critical feature shared by MGUS and MM is an extremely low rate of proliferation, usually with no more than a small percentage of cycling cells until late stages of MM.

This implicates the existence of a malignant, self-renewing precursor cell as a result of oncogenic transformation and selection, but this population has not yet been identified.

Two kinds of cell populations are known in non-IgM MGUS or MM tumors:

- a small fraction of proliferative tumor cells have a phenotype that is similar to a PB or a pre-PB that might express some B-cell markers (CD19, CD20, CD45) but not some PC markers (CD138)
- non-proliferative cells that are not differentiated but have a phenotype similar to healthy, terminally differentiated, long-lived BM PCs. It is unclear if this second cell population retains the ability to revert to a proliferative phenotype.

Only a small number of tumor cells are detectable in peripheral blood. These cells represent an upstream population of late-stage B cells that may be a drug resistant reservoir of myeloma precursors, capable of expanding and differentiating into a malignant PC tumor (Bergsagel and Kuehl, 2005).

Clearly, the different nature of tumor cells found in MM supports the hypothesis of a complex multi-step transformation process, in which the malignant clones arise from a differentiation process closely tied to the normal B-cell differentiation pathway.

The first step of this multistep transformation process seems to be immortalization. The malignant PCs observed in MM are localized to the BM in the earlier stages of the disease and most closely resemble long-lived PCs. These cells have undergone antigen selection outside the BM, as evidenced by their isotype-switched and somatically hypermutated Ig genes.

Despite their similarities to long-lived PCs, myeloma cells have significantly lower rates of Ig secretion compared with normal PCs. Therefore, it appears that the critical neoplastic transformation events take place after or do not interfere with most of the normal B-cell differentiation process, leading to long-lived PCs (Davies et al., 2003).

During the development of the pathology the tumor cells depend on the bone marrow microenvironment, which provides the signals essential for their growth and survival (Vande Broek et al., 2008) (Figure 2.2).

Once immortalized, myeloma cells are necessarily resident in the BM, as early tumor growth is entirely dependent on the BM niche, especially from the paracrine support of IL-6 provided by BMSCs.

Bone marrow angiogenesis significantly increases in the advanced stages of monoclonal gammopathies end it has been hypothesized that also the dysregulation of various cytokines and growth factor might be involved in the transition from MGUS to MM.

In the BM, direct cell–cell contact through integrins trigger MM cell growth, survival and drug resistance by inducing different pathways: Ras/MEK/MAPK, PI-3K/Akt/mTOR, JAK/STAT3 and IKK-a/NF-kB (Blade and Rosinol, 2008; Mitsiades et al., 2006).

The interaction of MM cells with BMSCs is considered a critical component of the overall network of biological relationships established between the malignant cells and their BM.

BMSCs are described as a heterogeneous compartment of mesenchymal cells with morphological features reminiscent of fibroblasts that are able to support normal hematopoiesis.

So the support that BMSCs provide to MM cells in terms of their proliferation, survival and drug resistance may represent an abnormal and pathophysiologically unfavorable reprise of their intrinsic ability for providing support of normal hematopoiesis. For instance, adhesion of MM cells to BMSCs via adhesion molecules such as VLA-4 and ICAM-1 enhances MM cell proliferation and viability through several complementary mechanisms, which include cell adhesion-mediated stimulation of intracellular signaling pathways in MM cells and increased paracrine (BMSC-derived) and/or autocrine (MM cell- derived) release of cytokines/growth factors in the BM that increase the resistance to apoptosis (Mitsiades et al., 2007).

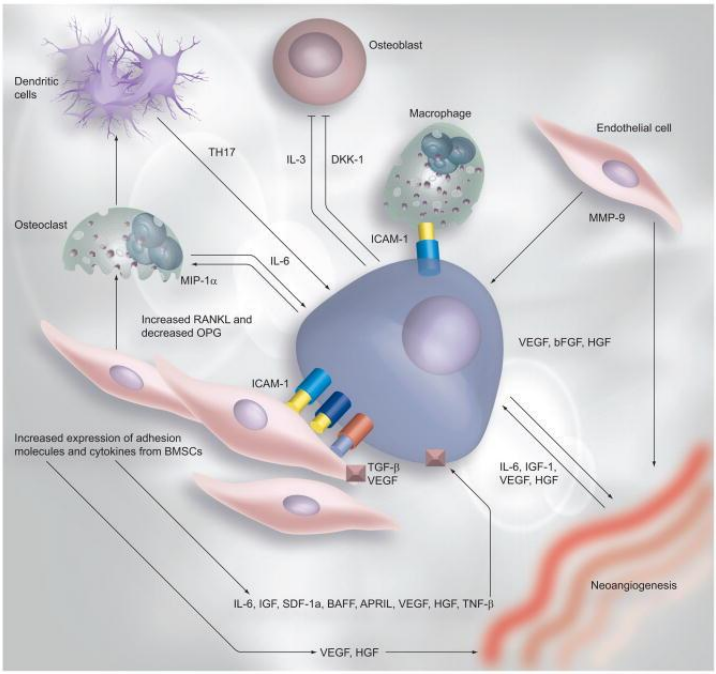

Figure 2.2 Bone marrow microenvironment and multiple myeloma cells (Balakumaran et al., 2010).

Another important feature of MM is the development of bone disease. MM cells must actively stimulate the recruitment of OCL precursors from the peripheral blood to the BM to induce osteolysis. Whereas a number of potential candidates have been suggested, recent studies suggest that the axis CXCR4/SDF1α is the best candidate for this role (Blade and Rosinol, 2008).

It has also been proposed that MM cells can stimulate RANKL expression in BMSCs and suppress OPG activity and stimulate production of multiple pro-osteoclastogenic cytokines in the BM.

This large constellation of osteoclastogenic stimuli includes IL-6, IL-1a, IL-1b, and IL-11;, chemokines such as MIP1a; TNF TNF-a, TNF-b and other soluble mediators, including M-CSF, PTHrP, or VEGF activity of increased levels of cytokines such as IL-6 (Colombo et al., 2013).

2.2.1 GENETICS OF MULTIPLE MYELOMA (Fonseca et al., 2004)

Multiple and complex chromosomal abnormalities are present in the clonal plasma cells (PCs) of multiple myeloma.

The rekindled interest in the role of specific genetic aberrations in the outcome of MM was sparked by reports showing clinical implication for karyotype abnormalities. MM is characterized by the frequent occurrence of aneuploidy, especially monosomies. The most common abnormalities are chromosome 13, 14, 16, and 22 monosomies and chromosome 3, 5, 7, 9, 11, 15, 19 and 21 trisomies.

Globally, aneuploidy analysis segregates patients into two categories: hyperdiploid and non-hyperdiploid (including hypodiploid, pseudodiploid, and near tetraploid). The nonhyperdiploid MM is characterized by a very high prevalence of IgH translocations (85%), while chromosome 13

monosomy and structural chromosome abnormalities are more common in patients with nonhyperdiploid karyotype. Deletions/monosomy of chromosome 13 are associated with a shorter survival. Translocations that involve both the immunoglobulin heavy-chain (IgH) and light-chain (IgL) genes are implicated in the pathogenesis of MM/MGUS. Unlike other B-cell tumors, for MM there is a marked diversity of chromosomal loci involved in immunoglobulin translocations. These include c-myc, FGFR3, c-maf and cyclin D3 that taken together are involved in immunoglobulin translocations in 40% of MM tumors while approximately 20 –30% have translocations that involve other chromosome partners that occur at a prevalence of 1% or less. IgH translocations may be primary genetic events but some variants will likely be progression events as secondary translocations. It has been proposed that most primary immunoglobulin translocations result from errors in B-cell-specific DNA modification processes, mostly IgH switch recombination or less often somatic hypermutation, and rarely VDJ recombination. These translocations are then predicted mostly to have translocation breakpoints within, or very near, IgH switch or J regions. By contrast, secondary translocations would not involve B-cell-specific DNA modification processes. The influence of IgH enhancers appears to extend over a long range, so after a traslocation oncogenes located hundreds of kilobases away from the enhancers can be under the influence of cis transcriptional up-regulation. It is not clear whether IgH translocations or aneuploidy occurs first in the PC neoplasms. Both genetic aberrations are seen in the very early stages of the PC disorders and no clear pattern is evident. The high prevalence of 13 among patients with the t(4;14)(p16.3;q32) and t(14; 16)(q32;q23) suggests primacy for 13, but it is also possible that these IgH translocations allow 13 to be “tolerated” by the cell. Other mutations detected in MM involved Ras

(35-50% of MM, but rare in MGUS, suggesting that this is a molecular marker if not causative in the progression from MGUS to MM for some tumors), the inactivation of p53 by either deletion or mutation, inactivation of the tumor suppressors gene p16/INK4a in the Rb pathway, inactivation of PTEN and complex abnormalities of c-myc that correlate with advanced stages of the disease.

2.3 MM THERAPY AND DRUG RESISTANCE

Initial treatment of multiple myeloma depends on the patient's age and co-morbidities. In recent years, high-dose chemotherapy with autologous hematopoietic stem-cell transplantation has become the preferred treatment for patients under the age of 65. Prior to stem-cell transplantation, these patients receive an initial course of "induction chemotherapy", this treatment induces hematopoietic stem-cell migration from the BM to the peripheral blood vessels; then blood-forming stem cells are removed from the patient's blood by a process called leukapheresis and are preserved.

After the high-dose chemotherapy stem cells are given back to the patient to reconstitute its BM. This treatment is not curative, but prolongs overall survival. Also allogeneic stem cell transplantation has the potential for a cure, but is only available to a small percentage of patients.

MM may be treated with a variety of drugs, including chemotherapics (for example, Melpahalan), corticosteroids (Dexamethasone), immunomodulating agents (Lenalidomide and Talidomide), proteasome inhibitor (Bortezomib and Carfilzomib), or a combination of them. Novel biologically based treatments target not only the MM cell, but also MM cell-host interactions and the BM microenvironment.

MM cells manifest intrinsic genetic mechanisms of drug resistance, (for example p53 mutations), or may acquire resistance following the exposure to conventional chemotherapeutic treatment. Furthermore, binding of multiple myeloma cells to extracellular matrix proteins induces cell-adhesion-mediated drug resistance to conventional chemotherapy and cell–cell contact with accessory cells [bone marrow stromal cells (BMSCs), osteoclasts, osteoblasts and endothelial cells] and the secretion of growth factors (TGF-β) further induce transcription and secretion of cytokines, which in turn confers drug resistance (Hideshima et al., 2007). New therapeutic agents, such as Carfilzomib, can overcome intrinsic drug resistance, as well as CAMDR and the protective effects of cytokines, and induce multiple myeloma cell cytotoxicity in the bone marrow milieu. These agents can also overcome clinical drug resistance to conventional and high-dose chemotherapies(Mateos et al., 2013).

2.4 NOTCH IN MULTIPLE MYELOMA

Notch receptors (Notch 1,2,3) are expressed on primary MM cells, BMSC and osteoclast (OCL) and Notch ligands (Jagged1 and Jagged2) are expressed on MM and BMSC and are able to activate Notch signaling through homotypic as well as heterotypic interactions. Probably are these interactions that allow tumor growth and survival.

Jagged1 (Jag1) and Jagged2 (Jag2) are overexpressed in myeloma cells because of promoter hypomethylation; this increased expression is related to the progression of the disease.

Jag1 promotes proliferation and inhibits apoptosis of MM cells, while Jag2 activates Notch signaling in BMSCs, leading to the secretion of interleukin-6, VEGF and insulin-like growth factor-1 that activated the MAPK signaling

pathway, increasing angiogenesis and promoting tumor growth and survival (Houde et al., 2004).

There is no evidence of a constitutive activation of Notch, suggesting that the increase of the Notch activity is caused by an overexpression of the receptor and his ligands.

Activation of Notch, which takes place during the MM cell interaction with BMSC, may result in the accumulation of the Notch target gene Hes1, which functions as a transcriptional repressor for the pro-apoptotic genes.

In MM cells Hes-1 is dose dependently downregulated upon Notch inhibition with γ-secretase inhibitors and concomitantly the proliferation rate of the MM cells was markedly reduced.

Different studies confirm the ability of the GSI to specifically inhibit Notch signaling in MM cells (there is a decrease of the Hes1 expression, but not of unrelated genes, showing that the effect is not due to a general inhibition of transcription) and the safety of the treatment (the activity of $CD34^+$ cells is unmodified). GSI rapidly and dramatically up-regulates the proapoptotic protein Noxa in MM cell lines and primary MM cells and the decrease of Hes1 expression causes an increase in the transcription of proapoptotic genes. Furthermore there are evidences that the inhibition of Notch signaling in MM cells may not only induce apoptosis of MM cells but may also substantially enhance the effect of chemotherapy *(Nefedova et al., 2008).*

A major hallmark of MM is the occurrence of severe bone lesions caused by the disturbed balance of OCL and osteoblast (OBL) activity. It is known that MM cells, growing within the BM in close contact to BMSCs, induce this imbalance. Moreover, the interaction with OCL further stimulates the growth of MM cells, thus generating a vicious circle of mutual activation between MM cells and OCL.

Some studies showed that Hes1 upregulation in OCL is accompanied by the increased tartrate resistant acid phosphatase-5 expression, indicative of higher OCL activity, suggesting an important role of Notch in the OCL/OBL imbalance.

The treatment with γ-secretase inhibitors is able to reverse the process, restoring OBL activity . Particularly, GSI XII induces apoptosis of myeloma cells and dramatically improves the sensitivity of myeloma cells to chemotherapeutic drugs such as Doxorubicin and Melphalan, representing a promising strategy for therapeutic intervention in multiple myeloma .

Taken together all these results suggest that the NOTCH pathway is a rational target for the therapy of multiple myeloma.

However, the role of Notch in B-cell tumors remains controversial. Several studies suggested that constitutively active Notch signaling leads to growth inhibition and apoptosis in malignant B-cells (Morimura et al., 2000; Nefedova et al., 2004; Romer et al., 2003; Zweidler-McKay et al., 2005). Zweidler-McKay demonstrated growth arrest and/or apoptosis as functional consequences of NOTCH activation in 13 cell lines representing multiple subclasses of B-cell neoplasia (murine and human preB-ALL, human Hodgkin, biphenotypic mixed-lineage leukemia and MM cells lines). This effect was observed by both expression of a ICN as well as ligand-induced Notch signaling activation. Furthermore, all four Notch members were able to induce growth inhibition and apoptosis(Kannan et al., 2011; Zweidler-McKay et al., 2005).

The presence of conflicting data makes necessary further studies to characterize the role of Notch in the development and maintenance of this pathology.

CHAPTER 3

THE BONE MARROW MICROENVIRONMENT IN MULTIPLE MYELOMA

3.1 INTRODUCTION

The close interaction between malignant cells and the local microenvironment where they reside is a feature that MM shares with a broad spectrum of solid tumors and hematological malignancies. The concept of tumor-microenvironment interplay can probably back up to “seed and soil” hypothesis by Stephen Paget in 1889.

The bone marrow microenvironment consists of cellular and non-cellular elements. Cell components include hematopoietic stem cells (HSCs), progenitor cells, immune cells, erythrocytes, BM fibroblast-like stromal cells (BMSCs), vascular endothelial cells, osteoclasts and osteoblasts. The non-cellular elements are represented by extracellular matrix (ECM) proteins, such as fibronectin, collagen, laminin and osteopontin.

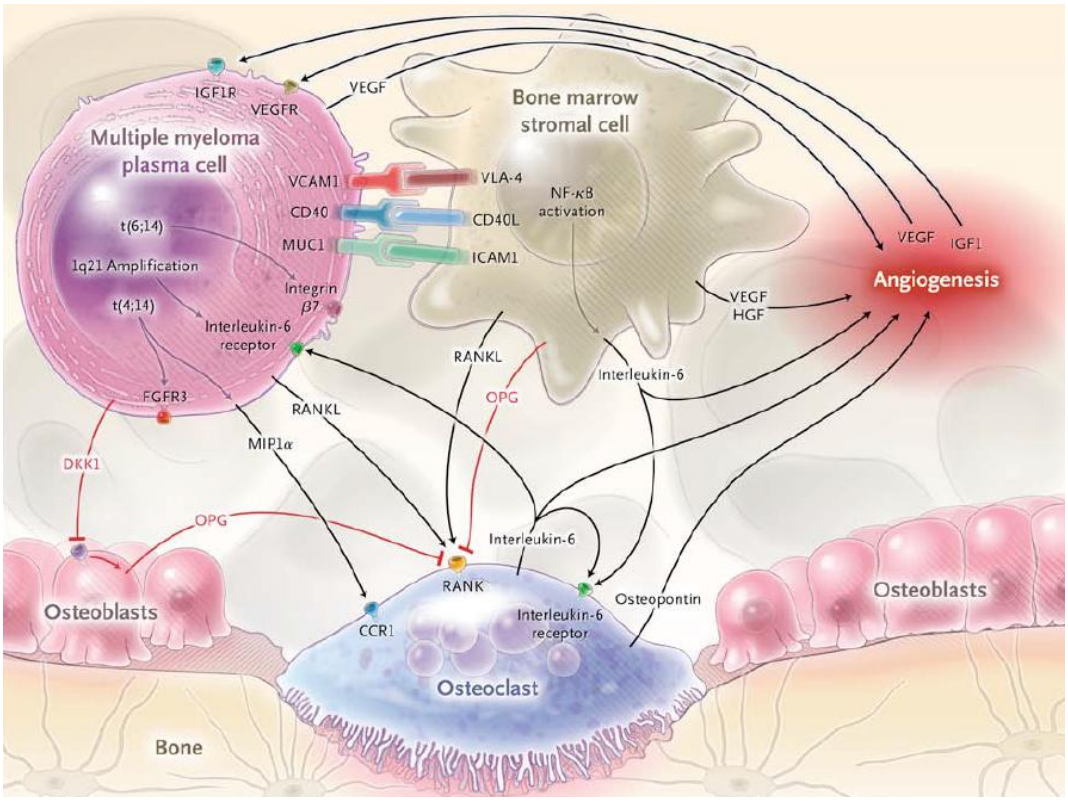

Figure 1.1. interaction between malignant plasma cells and bone marrow in MM (Palumbo and Magarotto, 2011).

The direct interaction of MM cells with BM microenvironment cells activate signaling pathway mediating growth, survival, drug resistance and the migration of MM cells, as well as osteoclastogenesis, angiogenesis and secretion of several soluble factors, such as interleukin 6 (IL-6), vascular endothelial growth factor (VEGF) ,stromal cell-derived factor 1 (SDF-1*)* and insulin-like growth factor (IGF1) (Colombo et al., 2013). Both homotypic and heterotypic adhesion of MM cells to either BMSCs or ECM are mediated through several adhesion molecules, i.e. CD44, very late antigen 4 (VLA-4),

VLA-5, intracellular adhesion molecule (ICAM-1), NCAM, syndecan 1 and MPC-1.

In the following paragraphs we will focus on three key elements of the interaction between myeloma and BM niche: the adhesion molecules, the soluble factors and their receptors, angiogenesis and skeletal destruction.

3.2 ADHESION MOLECULES

Adhesion molecules on MM cells were identified about two decades ago, and specific role in their adhesive interaction with the ECM were attributed to integrins *(Uchiyama, 1997).*

MM cells exhibit preferred adhesion to several ECM constituents, including laminin, collagens and fibronectin (FN), *via* β1 integrin-mediated adhesion.

Integrins are heterodimeric cell surface receptors that mediate adhesion to the ECM and immunoglobulin superfamily molecules. They are essentially expressed by all cell types, including cancer cells *(Neri and Bahlis, 2012).* A wide range of integrins is expressed by MM cell lines and primary MM cells, but about their specific functional roles still little is known.

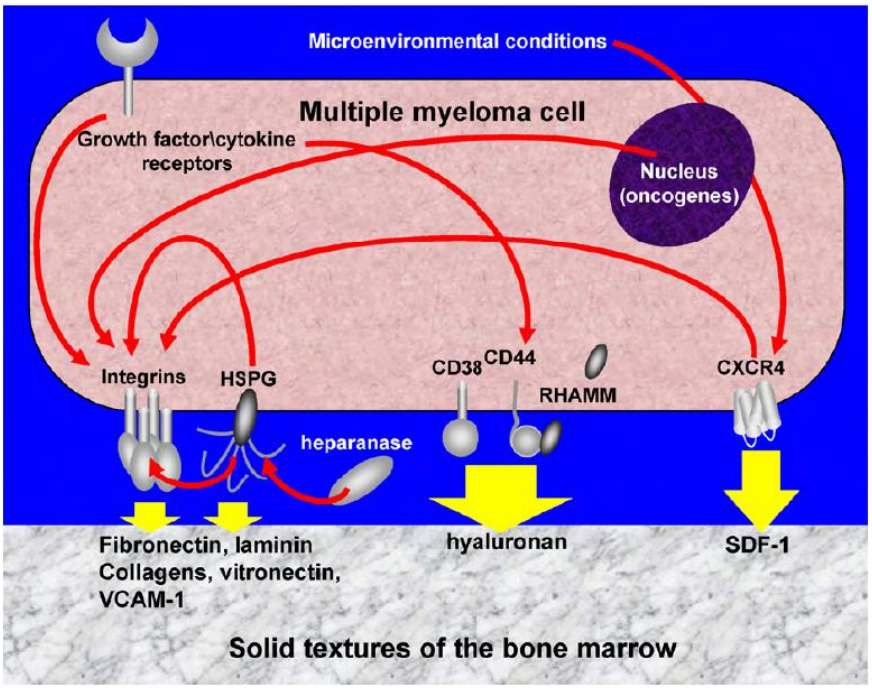

Figure 3.2. Adhesion molecules expressed by myeloma cells (Katz, 2010).

Others cell-surface molecules are involved in MM cells interactions with the bone marrow niche: one of the specific surface markers of MM cells is CD138, also identified as syndecan-1 *(Khotskaya et al., 2009).*

Adhesion molecules are responsible for the development of MM cells resistance to front-line chemotherapeutic drugs, such as Melphalan (alkylating agent) and doxorubicin (anthracycline), thus leading to treatment failure. This phenomenon is referred as cell adhesion mediated drug resistance (CAM-DR), and it suppresses drug-induced apoptosis *(Hazlehurst and Dalton, 2001).* The proteasome inhibitor Bortezomib was shown to overcome CAM-DR by selectively downregulating VLA-4 expression in MM cells *(Noborio-Hatano et al., 2009).*

3.3 SOLUBLE MEDIATORS

Since MM mainly progresses in the bone marrow, signals from this microenvironment play a critical role in the maintaining plasma cell growth, survival, migration, drug resistance and angiogenesis Reciprocal interactions between PCs and BM cells, namely HSCs, stromal cells, osteoblasts, osteoclasts, vascular endothelial cells and immune cells are mediated by an array of cytokines and receptors. PCs in the BM secrete tumor necrosis factor-α (TNFα), transforming growth factor-β (TGF-β), VEGF, Angiopoietin-1, FGF-2 and matrix metalloproteases (MMPs). Moreover, the cell-cell interactions mediated by adhesion molecules between PCs and BM cell trigger transcription and secretion by the latter of cytokines, such as IL-6, VEGF, SDF-1 (CXCL12), Hepatocyte growth factor-scatter factor (HGF-SF) and IGF-1 (Colombo et al., 2013).

The first cytokine described that placed the focus on BMSCs-MM interplay was probably IL-6: in 90's it was known that IL-6 induces *in vitro* growth of freshly isolated MM cells and that MM cells express the IL-6 receptor (IL-6R). Moreover, several MM cell lines have been described to be responsive and produce IL-6, thus leading to hypothesize an autocrine pattern.

In the same years, many studies showed that BMSCs are the major source of IL-6 and that, although all human MM-derived cell lines express IL-6R mRNA, only a subset express IL-6 mRNA. In 1996, Chauhan and colleagues finally showed that adhesion of MM cell lines to BMSCs and BMSC lines resulted in significant increase in IL-6 secretion by BMSCs, thus supporting tumor growth (Chauhan et al., 1996). Noteworthy, through gene reporter assays, they also indicate involvement of NF-κB in regulation of IL-6 transcription triggered in BMSCs. Various soluble factors have been

shown to mediate IL-6 secretion by BMSCs or MM cells, e.g. IL-1α, IL-1β, TNFα and VEGF (Chauhan et al., 1996).

In MM, VEGF is expressed and secreted by tumor cells as well as BMSCs. It induces proliferation through Raf-1-MEK-extracellular-signal-regulated protein kinase (ERK) pathway, it triggers migration of human MM cells through a protein kinase C (PKC)-dependent cascade *(Podar et al., 2001)* and it stimulates the expression of IL-6 by microvascular endothelial cells and BMSCs *(Dankbar et al., 2000).*

TNFα is expressed by BMSCs and PCs in myeloma patients and it is known to be a strong mediator of inflammation and bone resorption. TNFα induces proliferation, the expression of ICAM-1, VCAM-1 and VLA-4 and MAPK/ERK activation in MM cells, while IL-6 secretion, NF-κB activation and expression of ICAM-1 and VCAM-1 in BMSCs *(Hideshima et al., 2001)*

HGF-SF is a pleiotropic cytokine that induces complex biological responses in target cells, including motility and growth. Its biological effects are transduced *via* the transmembrane tyrosine kinase Met, while syndecan-1 (CD138) strongly promotes HGF-induced signaling through Met, thereby acting as a co-receptor *(Derksen et al., 2002).* MM cell lines and BM plasma cells express both HGF-SF and its receptor Met *(Borset et al., 1996).*

MM is a tumor with a high ability to degrade the bone matrix thanks to matrix metalloproteases (MMPs) expression *(Barille et al., 1997).* MMPs are a family of zinc-dependent endopeptidases with proteolytic activity for a large range of components of the extracellular matrix (ECM). MMPs are involved in physiologic ECM turn over, bone remodeling and angiogenesis, as well as in several pathologic processes, such as rheumatoid arthritis and tumor invasion. Human myeloma cells secrete constitutively MMP-9, while

BMSCs secrete MMP-1 and MMP-2, thus supporting the spreading of MM cells inside and outside the BM.

Chemokines are a complex superfamily of small secreted proteins (6-14kDa) that have many overlapping functions and are produced by a variety of cell types during the inflammatory response. These molecules play also different roles in physiological and pathological processes, for example, they have a key role in the angiogenesis regulation.

Chemokines are classified into two groups: the CXC family (or α) and the family CC (or β). This distinction is based on the presence or absence of an amino acid (X) between the first two cysteine residues at the N-terminal end. They bind to specific G-protein coupled seven-span transmembrane receptors (GPCRs). Most chemokines bind to multiple receptors, and the same receptor may bind to more than one chemokine. The chemokines functional classification includes three families: the constitutive or homeostatic chemokines (involved in physiological leukocyte migration), inflammatory or inducible chemokines (released following a damaging stimulus that generates an inflammatory response) and chemokines with homeostatic and inflammatory functions.

Several chemokine systems had a role in MM development and progression. Among these the SDF1α/CXCR4 axis was reported as key-regulator of MM cell homing, adhesion, growth and motility (Mirandola et al., 2013; Mitsiades et al., 2007). The axis CXCR4/SDF1α is also a strong candidate for regulating the mobilization and intravasation of primary cancer cells and their extravasation and formation of metastasis in bone because it is able to attract lymphocytes and monocytes and to retain these cells in the bone marrow environment (Colombo et al., 2013).

Another chemokine axis involved in MM progression are the CCR1/CCR5 systems. Indeed, CCR1, CCR5 and their ligand MIP1α can exert both a

direct effect on the MM cells and an indirect effect on the stromal cells. Over 70% of patients with multiple myeloma are characterized by high production of MIP1α by tumor cells and it has been shown in vivo and in vitro that blocking activity of MIP1α in multiple myeloma cells reduces the ability to migrate in the bone marrow, the growth of tumor masses and bone destruction *(Choi et al., 2003).*

3.4 ANGIOGENESIS

MM cells depends from BM microvasculature for the appropriate supply of oxygen and nutrient. Moreover, BM microvasculature provides to MM cells a route for homing and spreading. BM endothelial cells (BM-ECs) are also able to stimulate MM progression through direct cell contact or through the production of soluble factors as or VEGF, basic fibroblastic growth factor (bFGF), MMP-2, MMP-9, monocyte chemoattractant protein-1 (MCP-1) (De Raeve et al., 2004). BM-ECs stimulation with VEGF results in the secretion of other factors relevant for MM cells, such as stem-cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-6 (Dankbar et al., 2000). Therefore, it is not surprising that BM angiogenesis is a key step in MM progression from MGUS, or non-active MM to active MM, and predicts poor survival in patients at diagnosis (Kumar et al., 2004).

3.5 SKELETAL DESTRUCTION

The cellular interplay between MM cells and BM microenvironment mediates the formation of bone lesions. MM growth is associated with increased numbers of osteoclasts and suppression of osteoblastogenesis in areas adjacent to tumor foci. These effects are frequently described to establish a “vicious cycle” between tumor cells and surrounding

environment: myeloma induces osteoclastogenesis and osteoclasts induce myeloma growth *(Sezer, 2009).* The molecular mechanisms by which myeloma cells stimulates osteoclasts activity are multifactorial and involve osteoclasts differentiation and survival factors that are produced by microenvironmental cells and myeloma cells. Several osteoclastogenic factors have been described to be involved in MM-induced osteoclasts activity: receptor activator of NF-κB ligand (RANKL), inflammatory protein-1 alpha (MIP-1α) , SDF-1α, IL-3, IL-6 and TNFα.

RANKL is a member of the tumor necrosis factor superfamily and is produced mainly by osteoblastic lineage cells and stromal cells. Its receptor, RANK, is expressed on the surface of osteoclasts precursors and mature osteoclasts. RANKL indices differentiation, formation, fusion and survival of preosteoclasts. Osteoprotegerin (OPG) is a decoy receptor antagonist for RANKL, mainly secreted by osteoblastic lineage and stromal cells. MM cells induce stromal cells to upregulate RANKL and to downregulate OPG *(Giuliani et al., 2001).* A balanced RANKL/OPG ratio is essential for normal bone turn over: Qiang and colleagues demonstrated that myeloma cell production of Wnt antagonist Dickkopf 1 (DKK1) abrogates the canonical Wnt signaling to commit immature cells to osteoblastogenesis, ultimately increasing RANKL/OPG ratios *(Qiang et al., 2008).*

MIP-1α belongs to the RANTES family of chemokines and is chemotactic for osteoclasts precursors and promotes osteoclastogenesis by increasing production of RANKL and IL-6 *(Choi et al., 2001).* In addition to osteoclastogenic factor produced by MM cells, it has been reported that myeloma cells form themselves multinucleated cells capable of bone resorption *(Silvestris et al., 2009).* SDF-1α is directly responsible for chemotactic recruitment, development and survival of human osteoclasts

(Wright et al., 2005). Moreover, elevated serum levels of SDF-1α are associated with osteolytic bone lesions and increased osteoclasts activity in MM patients *(Zannettino et al., 2005).*

As mentioned above, osteoclastogenesis and osteoblastogenesis in the normal bone are finely balanced, but this equilibrium is disrupted in MM: mesenchymal cells (MSCs) isolated from MM patients are genetically and phenotypically abnormal, and have impaired osteogenic potential *(Corre et al., 2007).*

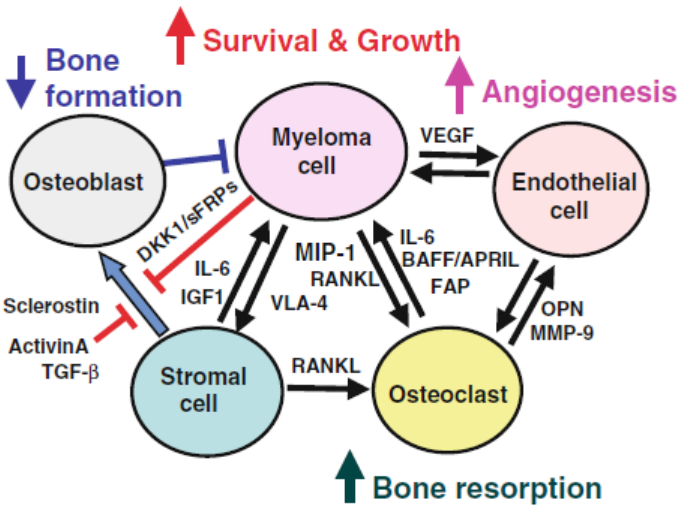

Figure 3.3. interplay between myeloma cells and BM microenvironment (Abe, 2011).

Osteolysis is a key element in MM cell progression, as well. OCLs play an active role in supporting MM cell long-term survival, proliferation and drug resistance (Yaccoby, 2010), and promote TGF-β release from the bone matrix, which plays a role in antagonizing patient's anti-tumor immune responses (Juarez and Guise, 2011). Moreover OCLs and vascular

endothelial cells interact and promote a vicious circle that leads to the progression of MM and to bone lesions formation (Tanaka et al., 2007).
Currently, MM bone disease is treated mainly by controlling the tumor burden and inhibiting osteoclast activity with bisphosphonates. Unfortunately, they reduce skeletal complications but the beneficial effects on myeloma progression are inconclusive

3.6 NOTCH AND BONE REMODELLING

The Notch pathway plays a key role in skeletal development and remodeling.
Notch activity is finely regulated during osteoclastogenesis and has distinct effects according to the different ligands and the receptor isoforms that are involved in the signaling. Notch1 and Notch3 have been reported to suppress osteoclastogenesis (Bai et al., 2008) while Notch2 is up-regulated during RANKL-induced osteoclastogenesis and plays a critical role in the late stage of OC differentiation (Fukushima et al., 2008). The role of the Notch ligands depends to the Notch isoform they engage. Dll1 inhibits osteoclast development through Notch1 (Yamada et al., 2003), while it enhances osteoclastogenesis by activating Notch2 (Sekine et al., 2012). These data suggest that MM cells may boost to the OCL differentiation process by the engagement of a particular Notch receptor (possibly Notch2) expressed by OCL precursors during osteoclastogenesis. Despite the complexity of Notch signaling modulation in osteoclastogenesis, it seems clear that Notch signaling activated by MM cells is necessary for OCL osteolytic activity. Schwarzer et al. demonstrate that the gamma secretase inhibitor GSI15 is able to impair OCL activation and to induce apoptosis in MM cells co-cultured with OCL, suggesting that the Notch

pathway can be a rationale target for the therapy of MM associated bone disease (Schwarzer et al., 2008).

Notch signaling is also involved in the maintenance of the early osteoblastic pool by inhibiting the Wnt/beta-catenin pathway in OB precursors, *(Zanotti et al., 2008)*. Therefore we can hypothesize that MM cell can activate the Notch pathway in the mesenchymal precursors in order to impair OB maturation.

These reports emphasize the complexity of Notch role in bone resorption triggered during MM progression, highlighting the need of further studies to elucidate the molecular mechanisms involved in this process.

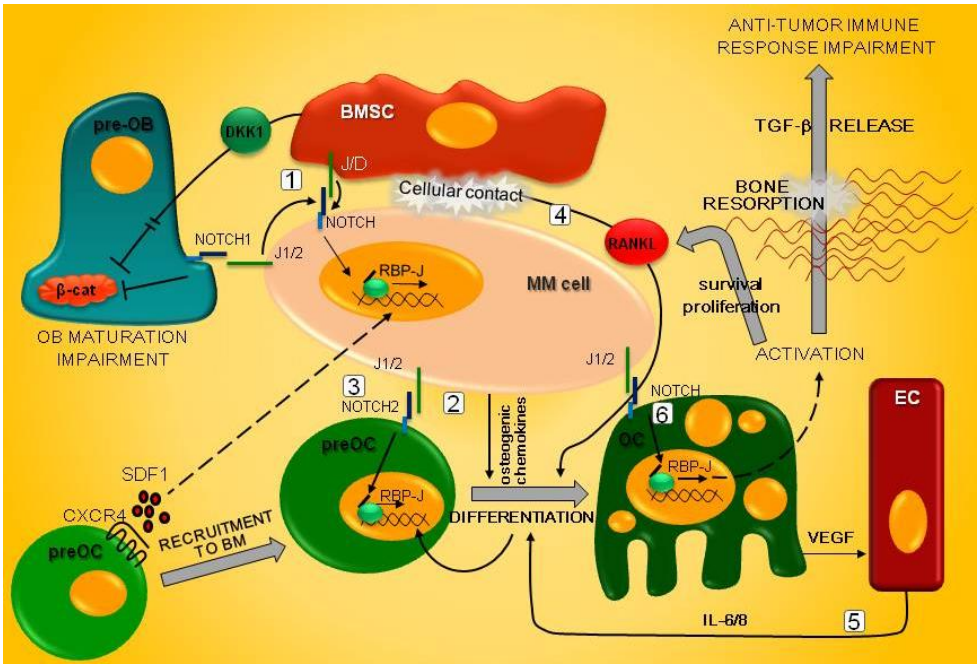

Figure 3.4. Model of Notch signaling involvement in MM cells directed bone resorption (Colombo et al., 2013).

AIMS

Osteoclastogenesis and the consequent osteolysis are major outcomes of MM cells localization to the BM. They produce fractures and bone disease significantly worsening patient's quality of life.

Moreover, skeletal destruction contributes to tumor progression and to the development of drug resistance.

Notch receptors are expressed by MM cells, BM stromal cells (BMSCs), and OCLs. Of note MM cells can activate the Notch pathway because they over-express the Jagged1 and Jagged2 ligands (Ghoshal et al., 2009; Skrtic et al., 2010). Evidences from our and other groups indicate that the active Notch signaling is involved in MM pathogenesis (Colombo et al., 2013) and that its inhibition induces apoptosis and inhibits MM cell drug resistance, and migration to the BM (Mirandola et al., 2013; Nefedova et al., 2008).

Recently the Notch pathway was also reported to play a key role both in bone tissue remodeling and skeletal development in collaboration with the NF-κB pathway (Bai et al., 2008; Fukushima et al., 2008; Sekine et al., 2012).

The aim of this study was to investigate the role of the Notch pathway in MM-driven regulation of OCL development and bone destruction.

To this I evaluated:

- the contribute of the Notch pathway on the ability of monocytes precursors to differentiate in mature OCLs;
- the role of the different Notch isoforms in OCL development;

- the mechanisms that drive MM-induced osteoclastogenesis and the contribute of the Notch pathway, specifically of its two ligands Jagged1 and Jagged2, to MM-mediated osteolysis.

If the hypothesis of a contribution of the Notch pathway in MM associated bone disease will be confirmed, the development of a Jagged1/2-specific approach will allow to eliminate the well-known toxicities caused by pan-Notch blocking agents, such as GSIs (Mirandola et al., 2011a; Searfoss et al., 2003; Wong et al., 2004). Moreover, targeting the Jagged ligands in MM cells could also prevent heterotypical activation of the Notch pathway in the BM microenvironment, resulting in interruption of the vicious cycle between MM cells and the BM niche.

RESULTS

1. Notch regulates OCLs differentiation and activity

In order to assess Notch contribution to osteoclastogenesis, Raw264.7 cells were induced to differentiate for 5 days using 50 ng/mL mouse RANKL (mRANKL) in the presence or absence 50 μM DAPT (a γ-secretase inhibitor), control cells were treated with an equal amounts of drug vehicle (DMSO). DAPT significantly reduced OCLs formation as shown by 65% reduction in TRAP+/ multinucleated cell number (Fig 1a).

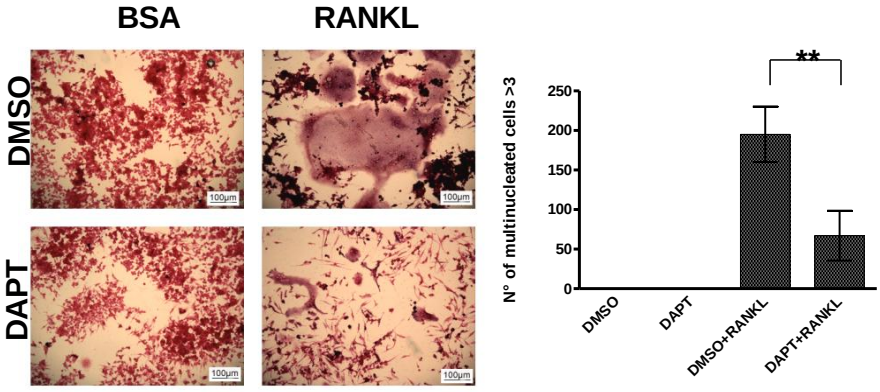

Fig 1a. Role of Notch in mRANKL-induced osteoclastogenesis*. TRAP staining (20x; left) and count of multinucleated cells (right) of Raw264.7 cells after 5 days of differentiation with RANKL with or without DAPT. Pictures are representative of at least three experiments with similar results. Error bars represent standard deviations calculated out of 3 independent experiments. Statistical analysis was performed by ANOVA and Tukey post-test; **= P <0,01.*

To assess the effects of Notch inhibition on OCL resorption activity, Raw264.7cells were cultured in calcium phosphate coated wells and treated with 50 ng/mL mRANKL and 50 µM DAPT or equal amounts of vehicle (DMSO). Notch inhibition impaired the ability of Raw264.7 cells to degrade the artificial bone matrix, resulting in 50% reduction of pit formation (Fig 1b).

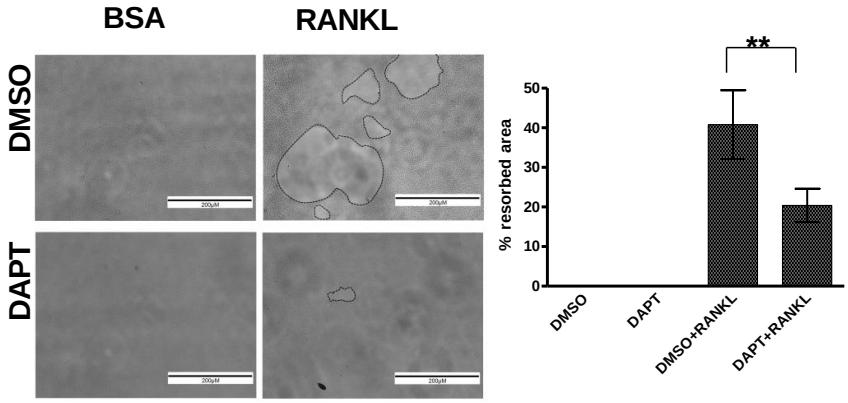

Fig 1b. Notch inhibition impairs OCLs activity. Pit formation assay. *The percentage of resorbed area was measured by using the Wimasis image analysis software (Wimasis GmbH) to process 20x pictures covering the whole well surface. Error bars represent standard deviations calculated out of 3 independent experiments. . Statistical analysis was performed by ANOVA and Tukey post-test; **= P <0,01.*

To investigate whether the effect of Notch inhibition previously observed on osteoclastogenesis and OCL activity was attributable to pre-OCL differentiation or depletion, we evaluated if DAPT influenced their proliferation and apoptosis rate. My results shown in figure 1c indicate that none of these biological features were affected by DAPT, indicating that Notch inhibition mainly affects OCL precursors differentiation without affecting their viability.

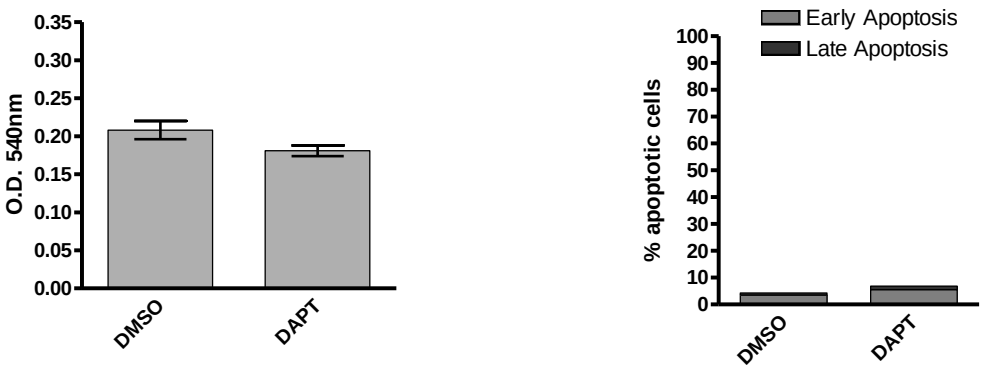

Fig 1c. DAPT has no significant effects on OCL precursors growth and survival. *MTT (left) and apoptosis assay (right) showed that DAPT do not affects Raw264.7 growth and survival. Error bars represent standard deviations calculated out of 3 independent experiments. Statistical analysis was performed by two tailed t-test, not significance; **= P <0,01.Apoptosis assay represent one of at least three experiments with similar results.*

To evaluate the effects of DAPT on Raw264.7 osteoclastogenic potential, cells were treated for 72h with 50 μM DAPT, or equal amounts of DMSO. Quantitative PCR (Fig 1d) showed that DAPT causes a downregulation in the expression levels of two key genes involved in OCL differentiation and activity: *Tartrate-resistant acid phosphatase* (TRAP) e *Receptor Activator of Nuclear Factor κ B* (RANK). The expression of a Notch target, HES5, were evaluated to confirm the treatment efficacy.

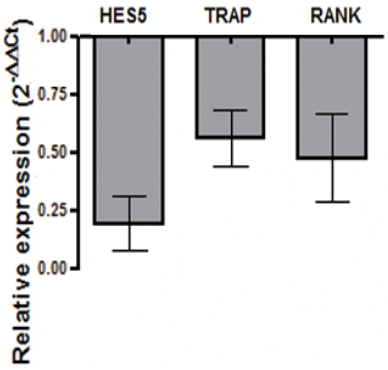

Fig 1d. Effects of DAPT on OCL precursors. *qRT-PCR on Raw264.7 following 3 days of DAPT treatment. Data are presented as the relative expression (control =1), calculated by the 2−ΔΔCt formula. Error bars represent standard deviations calculated out of 3 independent experiments. Two-tailed t-test confirmed statistically significant differences in the expression levels of the target genes.*

To further investigate the role of Notch in the regulation of TRAP and RANK during osteoclastogenesis, Raw264.7 cells were treated with DAPT for 3 and 5 days in differentiation condition with 50 ng/ml mRANKL. As expected, I observed along with differentiation a significantly increase in TRAP and RANK transcription levels (Fig 1e). Interestingly, this increase of the differentiation markers was hampered if Raw264.7 cells were treated with DAPT, confirming that Notch is necessary for osteoclastogenesis. (Fig 1e).

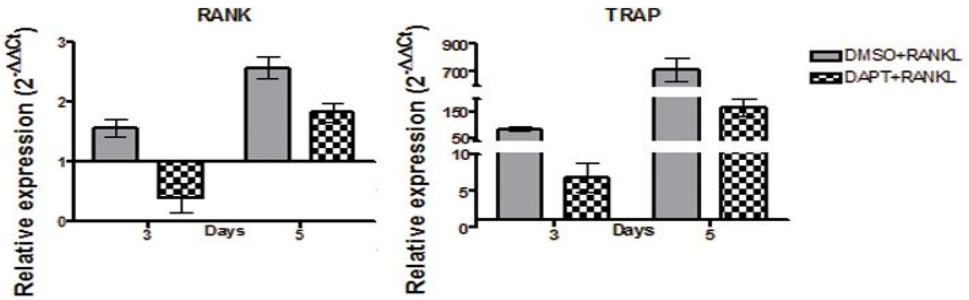

Fig 1e. The impaired OCL differentiation following DAPT treatment was confirmed at molecular level. *qRT-PCR on Raw264.7 following 3 and 5 days of differentiation and DAPT treatment. Data are presented as the relative expression (control =1), calculated by the 2−ΔΔCt formula. Error bars represent standard deviations calculated out of 3 independent experiments. Two-tailed t-test confirmed statistically significant differences in the expression levels of the target genes.*

2. Notch2 drives osteoclastogenesis, modulating RANKL expression

The evidence that Notch activity is required for OCLs differentiation, together with the presence of controversial data on the role of the different Notch isoforms in this process prompted me to evaluate the contribution of Notch 1 and Notch2 on OCL development. Raw264.7 were treated for 5 days with mRANKL 50ng/ml. qRT-PCR (Fig. 2a) showed that mRANKL-induced differentiation causes a switch between the two Notch isoforms expressed in Raw264.7. Specifically, data showed an upregulation of Notch2 that was associated to a decrease in Notch1 expression levels.

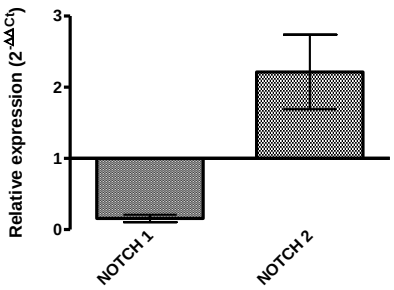

Fig 2a. Effects of mRANKL on the expression of Notch isoforms. *qRT-PCR on Raw264.7 after 5 day-treatment with DAPT. Data are presented as the relative expression (control =1), calculated by the 2−ΔΔCt formula. Error bars represent standard deviations calculated out of 3 independent experiments. Two-tailed t-test confirmed statistically significant differences in the expression levels of the target genes.*

To evaluate how Notch isoforms differently affected the osteoclastogenesis, Raw264.7 were transfected cells with plasmids carrying the constitutively active forms of Notch1 or Notch2 (ICN1 and ICN2, respectively, see Materials and Methods).

A western blot was performed in order to confirm the activation of Notch 1 and 2 in transfected cells (Fig 2b).

Fig 2b. Notch1 and Notch2 activation in transfected Raw264.7. *Western blot on Raw264.7 after 48h from the electroporation with two vector expressing the active form of Notch1 and Notch2. Pictures are representative of at least three experiments with similar results.*

At 48h post-transfection, the forced expression of Notch2 induced Raw264.7 differentiation, as confirmed by the increase in $TRAP^+$ multinucleated cells. On the contrary, Notch1 did not induced any change (Fig2c).

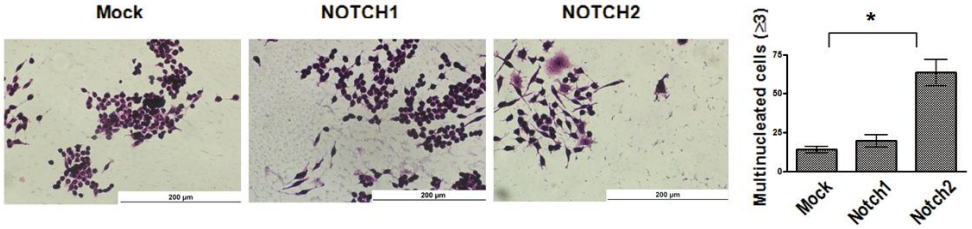

Fig2c. Role of Notch1/2 in osteoclasts differentiation. *TRAP staining (40x) and count of multinucleated cells. Pictures are representative of at least three experiments with similar results. Error bars represent standard deviations calculated out of 3 independent experiments. Statistical analysis was performed by ANOVA and Tukey post-test; *= P <0,05.*

A further study on the role of the activation Notch2 in the differentiation process, allowed me to discern that Notch2-transfected Raw264.7 cells were able not only to differentiate (as confirmed by the upregulation of TRAP expression levels), but also to autonomously secrete an RANKL, as shown by real time RT-PCR in Fig.2d on the left and by the ELISA on the conditioned medium, on the right of the same figure. On the contrary, ELISA assay indicate that there was no increase in RANKL secretion by Notch1-transfected cells (Fig2d, right panel).

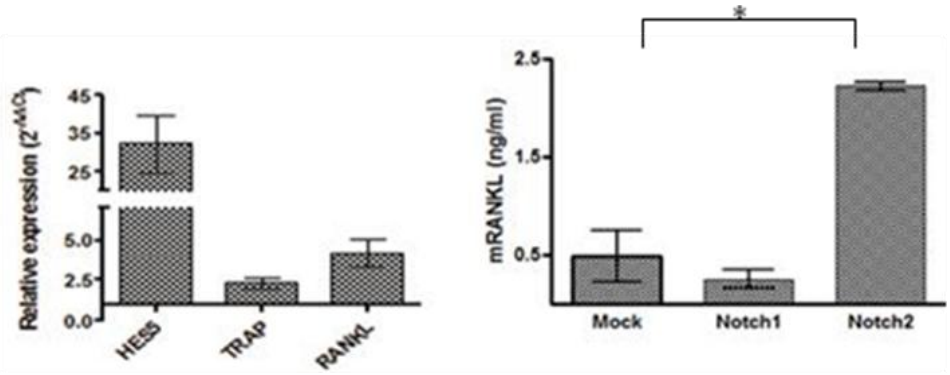

Fig2d. Notch2 promotes RANKL expression in OCL precursors. *qRT-PCR (left) shows that Notch2 is able to promotes differentiation (as confirmed by the increase in TRAP levels) and upregulates RANKL. The Notch-target gene HES5 was used in order to confirm the upregulation of this pathway. Data are presented as the relative expression (control =1), calculated by the 2−ΔΔCt formula. The increase in RANKL production were further confirmed by ELISA assay performed 48h after transfection (left panel). Error bars represent standard deviations calculated out of 3 independent experiments. Statistical analysis was performed by ANOVA and Tukey post-test; *= P <0,05.*

To test if Notch2-induced RANKL production was sufficient to induce osteoclastogenesis, Raw264.7 cells were cultured for 7 days with the conditioned medium (CM) from Raw264.7 cells transfected with empty vector (mock) or the vectors expressing Notch1 or Notch2. Notch1 did not induce osteoclastogenic differentiation (Fig2e). On the contrary, differentiation occurred when the conditioned medium was from Raw264.7

cells transfected with the vector expressing Notch2 as demonstrated by the increase in TRAP+/multinucleated cells (Fig2e)

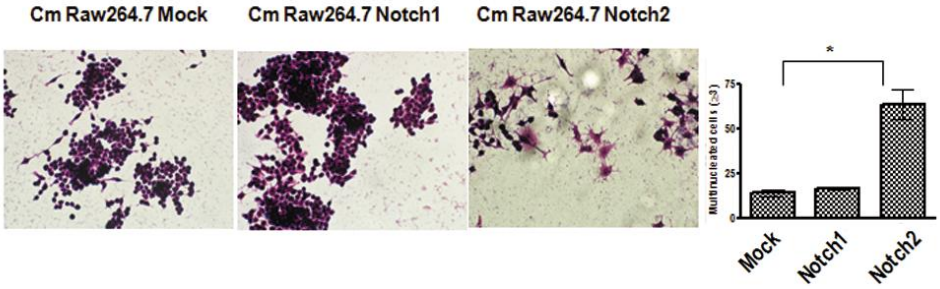

Fig2e. Role of Notch 2 in osteoclast differentiation. *TRAP staining (20x) and count of multinucleated cells. Standard deviations calculated out of 3 independent experiments are indicated by error bars. One-way ANOVA and Tukey post-test confirmed statistically significant variation in OCLs differentiation (*p<0.05).*

3. Notch signaling is required for Myeloma-mediated osteoclastogenesis

In order to evaluate the ability of MM cells to induce osteoclastogenesis and the contribution of Notch pathway in this process, the human osteoclastogenic MM cell line, U266, was co-cultured with Raw264.7 in the presence or absence of 50 μM DAPT for 7 days.

The presence of U266 cells promoted Raw264.7 cells differentiation (measured as $TRAP^+$ multinucleated cells), which was inhibited by DAPT addition (~70%) (Fig 3a). These results indicated that the pro-osteoclastogenic ability of MM cells depended upon Notch signaling.

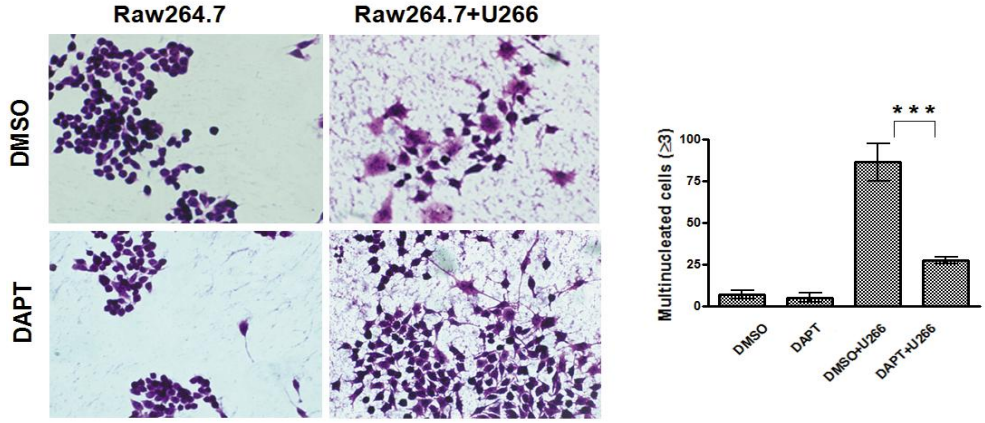

Fig 3a. MM cells are able to induce osteoclastogenesis. *TRAP staining (40x) and count of multinucleated cells. Standard deviations calculated out of 3 independent experiments are indicated by error bars. One-way ANOVA and Tukey post-test confirmed statistically significant variation in OCLs differentiation (***p<0.001). Pictures are representative of three experiments with similar results.*

To evaluate the role of Notch in OCLs activation, a bone resorption assay was performed with Raw264.7/U266 co-cultures induced to differentiate in the presence or the absence of 50 μM DAPT for 10 days. As expected, Notch inhibition impaired the pit formation ability of MM-induced OCLs as shown by the graph summarizing the obtained results in Fig 3b.

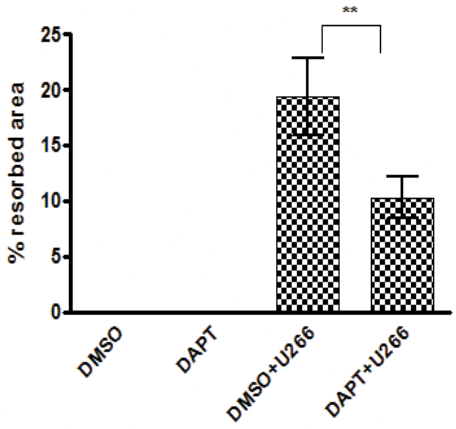

Fig 3b. Notch inhibition blocks MM-mediated OCL activity. *Pit formation assay. The percentage of resorbed area was measured by using the Wimasis image analysis software (Wimasis GmbH) to process 20x pictures covering the whole well surface. Results from 3 independent experiments are summarized in the graph with error bars representing standard deviations . Statistical analysis was performed by ANOVA and Tukey post-test; **= P <0,01.*

The qRT-PCR analysis of osteoclastogenic marker genes in Raw264.7 cells co-cultured for 7 days with U266 cells in the presence or the absence of 50 μM DAPT gave results similar to those obtained in mRANKL-differentiated cells: U266 cells promoted TRAP and RANK expression in

Raw264.7 cells (Fig3c) and DAPT significantly reduced TRAP and RANK levels, indicating that the MM-induced OCL differentiation required an active Notch signaling.

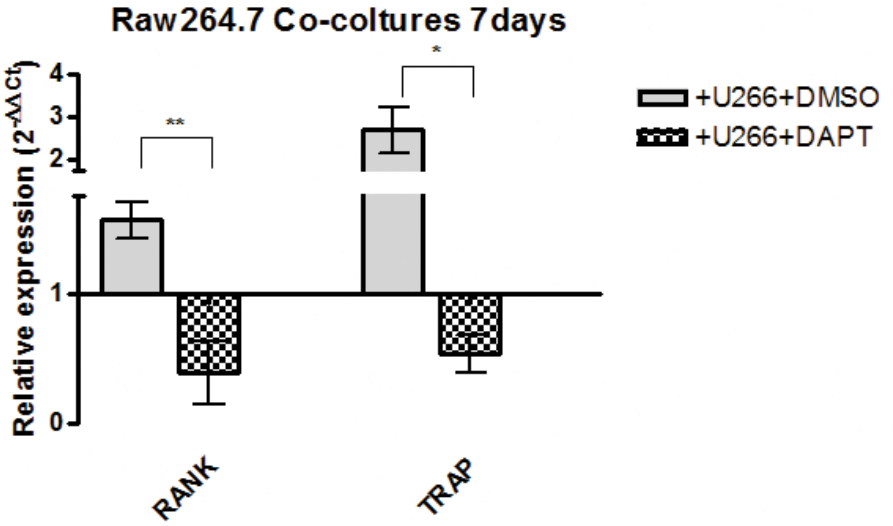

Fig 3c. Quantitative RT-PCR on RAW264.7 cells co-cultured with U-266 cells confirms their ability to induce express osteoclastogenic markers in a Notch-dependent manner. *U266 induced the upregulation of RANK and TRAP in co-culture Raw264.7 cells, Notch signaling inhibition significantly impaired this effect. Gene expression variations were evaluated comparing treated cells to untreated controls. Histograms represent mean ± SD and were calculated out of three independent experiments run in triplicate. Statistical analysis was performed by Two-wat t-test; *=p<0,05; **= p <0,01.*

4. MM cells induce OCLs formation in a contact-independent manner, through RANKL secretion

The evidence that MM cells are able to induce the Notch-dependent OCL differentiation prompted me to evaluate if differentiation required a direct contact between MM cells and pre-OCLs to trigger Notch activation in the last, or if MM osteoclastogenic ability depended upon the secretion of soluble factors triggered by Notch signaling.

Initially, to understand if the interaction with MM cells could activate Notch on Raw264.7 cells, I performed a dual luciferase assay (Fig 4a, left). Raw264.7 cells were transiently transfected with a plasmid carrying the firefly luciferase gene controlled by the DNA binding sequence of CSL, the transcription factor activated by the active Notch (13xCSL-RE). 24h-transfected Raw264.7 cells were cultured alone or with U266 cells for further 24 to 48 hours (Fig. 4a, left). As a positive control of Notch activation, Notch signaling pathway was forced in Raw264.7 cells by co-transfecting ICN1 and ICN2.

This first experiment showed that 24-48h of MM cells co-culturing with Raw264.7 cells were unable to simulate the Notch transcriptional activity in the last, since 13XCSL-RE activity did not changed when Raw264.7 were cultured alone or in the presence of U266 cells. Since Duan et al. previously reported that Notch signaling upregulation during osteoclastogenesis induced by RANKL (Duan et al., 2008), I wondered if U266 cells could indirectly activate the Notch pathway, at longer experimental times, through the expression of soluble mediators.

To address this issue, I measured by qRT-PCR the expression of HES5, a Notch target gene in Raw264.7 cells following osteoclastogenic stimulation induced through RANKL, direct contact with U266 cells or U266 CM. Results in figure 4a (right panel) showed that all the osteoclastogenic

stimuli failed to upregulate HES5 within 72h, on the contrary 5-days treatments showed Notch pathway activation. Moreover, the evidence that both U266 cells direct contact and CM induced a comparable level of HES5 transcription, confirmed that a direct contact between MM cells and osteoclasts is not essential for Notch activation.

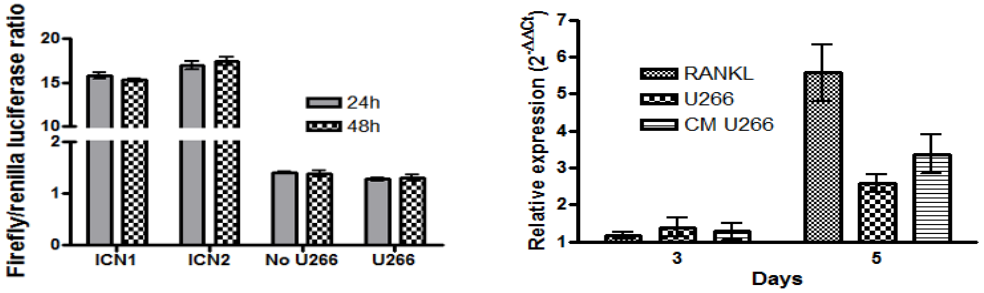

Fig 4a. *Left: Dual luciferase assay was performed in Raw264.7 cells cultured alone or co-cultured with U266 cell line. Raw264.7 cells co-transfected with vectors carrying the constitutively active Notch1 (ICN1) or Notch2 (ICN2) isoforms were used as positive controls of Notch activity. Histograms indicate the normalized luciferase activity (firefly luciferase activity/Renilla luciferase activity). Right: HES5 qRT-PCR was performed on Raw264.7 cells after 3 and 5 day of culture with the different osteoclastogenic stimuli reported. Data are presented as relative expression (control =1), calculated by the 2−ΔΔCt formula.*

To evaluate if OCL development was mediated by the Notch-dependent secretion of soluble factors from U266 cells, I set up differentiation experiments using the CM from U266 cells. At this purpose I prepared a 7-day culture of Raw264.7 cells in the presence of 20% V/V CM of U266 cells pre-treated with or without 50 μM DAPT. Results in Figure 4b (CM U266) show that U266 CM induced productive RAW264.7 cells

differentiation. As expected, the addition of DAPT to the co-culture dramatically reduced this effect (Figure 4b, CM U266 + DAPT). Finally, CM from DAPT-pre-treated U266 cells (Figure 4b, CM U266 treated with DAPT) was not able to induce OCL differentiation. These results suggested that MM cells also required Notch activity to produce the pro-osteoclastogenic soluble factors.

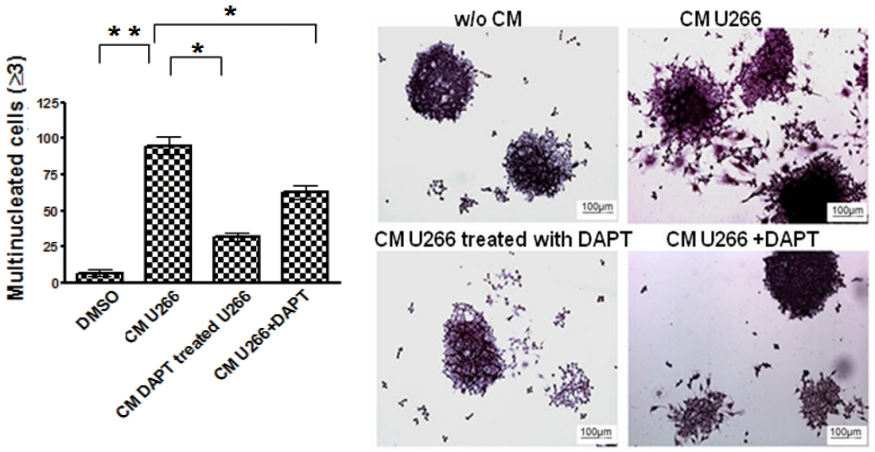

Fig4b. MM cells trigger OCL development by Notch-dependent soluble factors. *TRAP staining (20x) and counts of multinucleated cells in Raw264.7 cells exposed to CM from U266, CM obtained from U266 after DAPT treatment or CM from U266 together with DAPT treatment. Pictures are representative of at least three experiments with similar results. The graph summarizes all the obtained results. Bars represent standard deviations. Statistical analysis was performed using the ANOVA and Tukey test: *= P <0,01, **= P <0,001.*

The evidence that Raw264.7 cells differentiation was exclusively dependent upon RANKL stimulation, and that MM cells ability to produce pro-osteoclastogenic soluble factors was Notch-dependent, made me hypothesize that U266 cells produced RANKL in a Notch-controlled

manner. To test this hypothesis, I treated U266 with 50 μM DAPT for 48h and verified if changes occurred in RANKL gene expression and protein release in the medium. Fig 4c (left panel) shows that DAPT caused a significant decrease in RANKL gene expression levels. Variation in the expression of a Notch target gene, Hes6, was used to confirm the successful inhibition of Notch activity. The dependence of RANKL production on Notch was further confirmed by an ELISA on CM form U266 cells treated with DAPT for 48 and 96h (Fig. 4c, right).

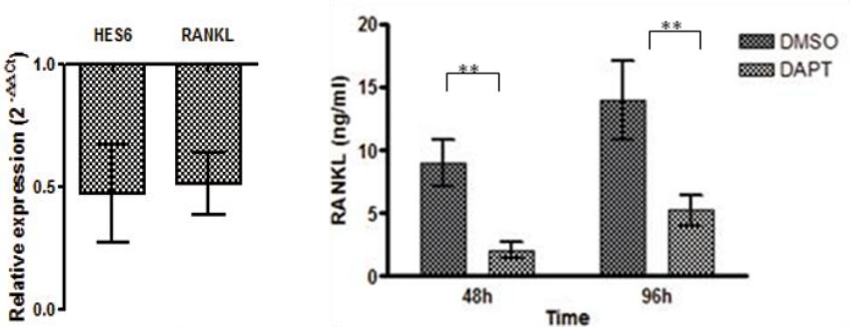

Fig 4c. RANKL production from MM cells is Notch dependent. *qRT-PCR of RANKL and the Notch gene target HES6 (left) and ELISA for RANKL (right) on CM from DAPT-treated U266. Gene expression variations were evaluated comparing DAPT treated cells to DMSO treated controls (ctrl=1). Statistical analysis was performed by Two-tailed t-test: **= P <0,01.*

Overall, this set of experiments allowed me to conclude that MM cells ability to induce osteoclastogenesis was cell contact independent and mediated by the secretion of RANKL. Moreover, MM cells osteoclastogenic potential, as well as OCL precursors ability to respond to the osteoclastogenic stimulus, are strongly dependent on the Notch pathway. Specifically, Notch is required from MM cells to secrete the osteoclastogenic factor RANKL.

5. RANKL expression in primary MM cells is correlated with Notch pathway activation

The dependence of RANKL secretion by Notch signaling activity observed in U266 cells, prompted me to verify if RANKL gene expression could be associated to Notch pathway activation in MM patients. To this purpose, a qRT-PCR analysis of RANKL and HES6 was performed on mRNA from $CD138^{+}$ cells from bone marrow aspirates of 17 MM patients. This analysis confirmed that the expression levels of HES6 and RANKL were strongly correlated in primary MM cells (Fig5), consistently with the reported *in vitro* evidence that in MM cells RANKL expression depends upon Notch activity.

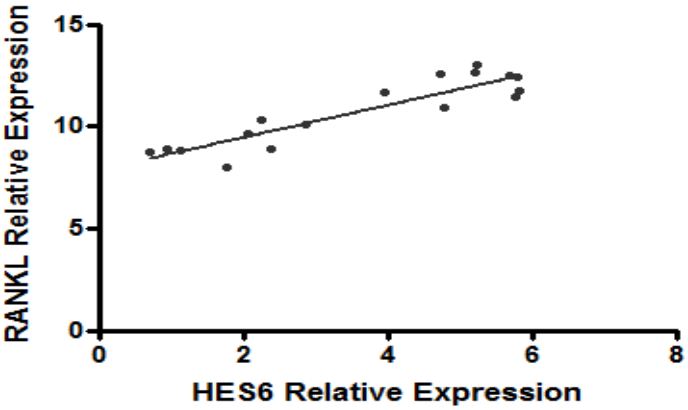

Fig5. Notch pathway activation is correlated with RANKL expression levels in MM patients. *qRT-PCR on primary MM cells from 17 patients for HES6 and RANKL genes (GAPDH expression levels =100). The graph shows the correlation between the two genes. Statistical analysis was performed with Pearson's product-moment correlation (p=0.0081).*

6. Jag1/2 silencing impairs MM-mediated osteoclastogenesis

Currently used drugs addressed to Notch pathway inhibition, such as γ-secretase inhibitors, induce high toxicity at gut level (Mirandola et al., 2011a; Searfoss et al., 2003; Wong et al., 2004), so my next step was to investigate the efficacy of a new and more selective approach that allows to inhibit the Notch signaling in MM cells, avoiding the side effects due to the contemporary inhibition of all the Notch isoforms.

In MM, the deregulation of Notch pathway is due to alterations of the expression of two Notch ligands, Jag1 and Jag2. This prompted me to assess if this two ligands represent possible targets for a Notch inhibitory approach directed to counteract the osteoclastogenesis and osteolysis associated to MM.

To this, Jag1 and 2 were simultaneously silenced in U266 cells by transfection of specific stealth shRNAs; cells transfected with scrambled shRNA represented a negative control. Every 48h cells were transfected again with Jag1/2 shRNAs (see Material and Methods). Cell count (Fig 6a, left) and apoptosis assay (Fig 6a, right) of silenced U266 cells indicated that Notch signaling inhibition had no significant effect on cell viability.

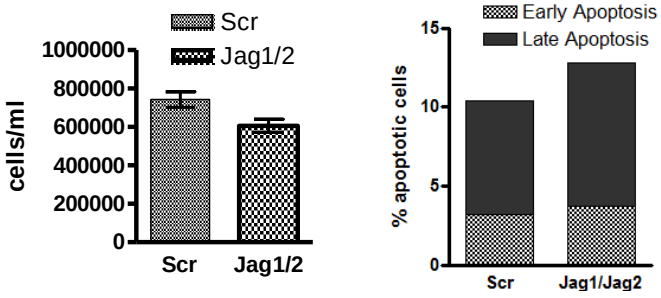

Fig 6a. Effect of Jag1/2 silencing on U266 cells. *DAPT does not affects Raw264.7 growth and survival of U266 cells after 96h Jag1/2 silencing. Left: cell count. Error bars represent standard deviations calculated out of 3*

independent experiments. Statistical analysis performed by two tailed t-test indicate not significant differences (P>0,05). Right: apoptosis assay represents one of at least three experiments with similar results.

The next step was to evaluate the molecular effects of Jag1 and 2 silencing on U266 cells. Results of qRT-PCR in Fig.6b show that 48h hours after transfection Jag1 and Jag2 gene expression was effectively reduced in U266 cells and, accordingly, the inhibition of Notch receptors activity is testified by the decrease in Hes6 expression. Two housekeeping genes (18s and HPRT1) were used to assess siRNAs specificity. Importantly, qRT-PCR revealed that the expression level of RANKL gene was significantly reduced in Jag1/2-silenced U266 cells (Fig. 6b, left). The impaired expression of RANKL in Jag1/2 silenced U266 cells was substantiated by ELISA assay on the CM analyzed after 48h or 96h from transfection (Fig. 6b, right). These results confirmed that Jag1/2-activated Notch was required from U266 cells to express RANKL and that Jag1 and 2 silencing was sufficient to repress U266 cells production of RANKL.

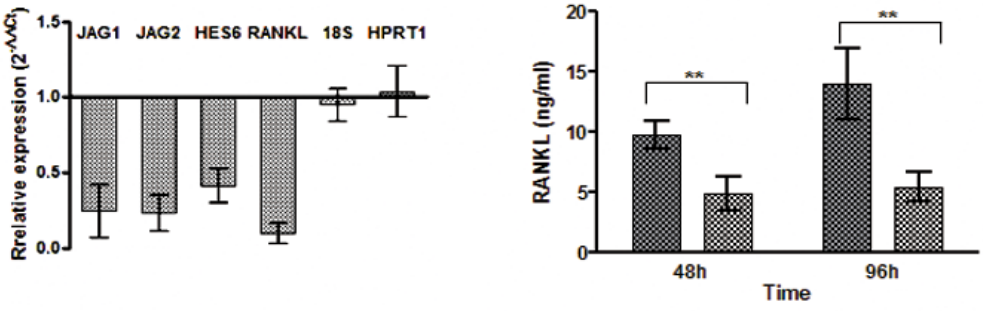

Fig 6b. Jag1/2 silenced U266 cells reduce RANKL expression. *48h post-silencing qRT-PCR of RANKL gene in Jag1/2-silenced U266 cells (left): Jag1, Jag2 and HES6 were analyzed to assess specificity and*

*efficacy, 18s and HPRT1 for selectivity ; 48 and 96h post-silencing ELISA for RANKL secreted protein (right), on Jag1/Jag2-silenced U266 cells CM. Gene expression variations were evaluated comparing silenced cells to cells transfected with scrambled shRNAs (Scr=1). Statistical analysis was performed using two-tailed t-test: ** = P <0,01. Standard deviations were calculated from 3 independent experiments.*

To test the contribution of Jag1 and Jag2 in MM-induced osteoclastogenesis, Raw264.7 cells were cultured for 7 days in the presence of CM from U266 transfected with Jag1/2 siRNAs or the negative control (scrambled siRNAs, Scr). TRAP staining and count of multinucleated cells (Fig. 6c) showed that the CM from Jag1/Jag2 silenced U266 cells displayed a reduced osteoclastogenic potential.

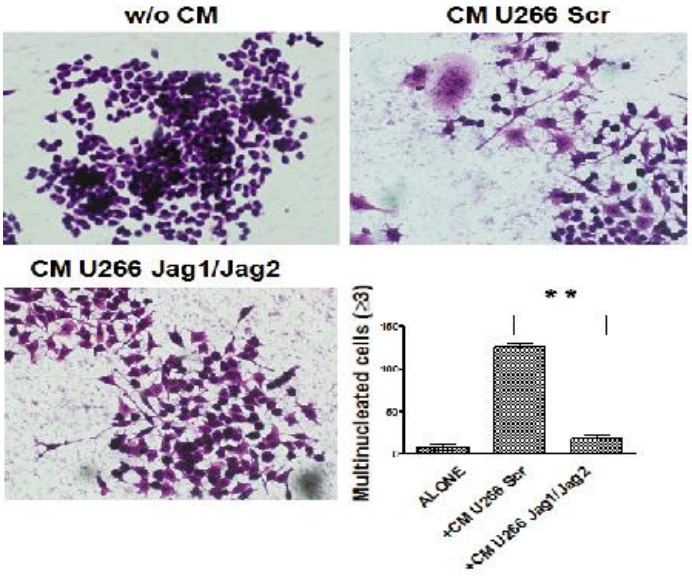

Fig 6c. Silencing of Jag1/2 impairs MM cell osteoclastogenic potential. *Raw264.7 cells were cultured for 7 days in the presence of CM from U266*

*cell line transfected with J1/J2- or Scr- shRNAs. On the left, TRAP staining (40x) and count of multinucleated Raw264.7 cells showed that U266 osteoclastogenic potential depends upon Jag1 and 2 expression. Pictures are representative of at least three experiments with similar results. The graph on the right summarizes the obtained results +/- standard deviations. Statistical analysis was performed using the ANOVA and Tukey post-test: ** = p <0,001.*

To evaluate the effect of Jag1/2 silencing on MM-induced osteolytic activity of Raw264.7 cells, a pit formation assay was performed. Cells were induced to differentiate with the CM from Jag1/2- or Scr-silenced U266 for 10 days. Jag1/2 downregulation in U266 cells impairs the ability of Raw264.7 to differentiate and resorb the calcium phosphate matrix, as indicated by the decrease in the degraded area represented by the pit (Fig 6d, left).

Moreover, Jag1/2 silencing impaired the modulations of the osteoclastogenic marker genes observed during OCL differentiation. Indeed, an analysis by qRT-PCR showed an upregulation of TRAP and RANK expression in Raw264.7 cells cultured with Scr CM, but not in Raw264.7 cultured with Jag1/2 CM (Fig. 6d, right).

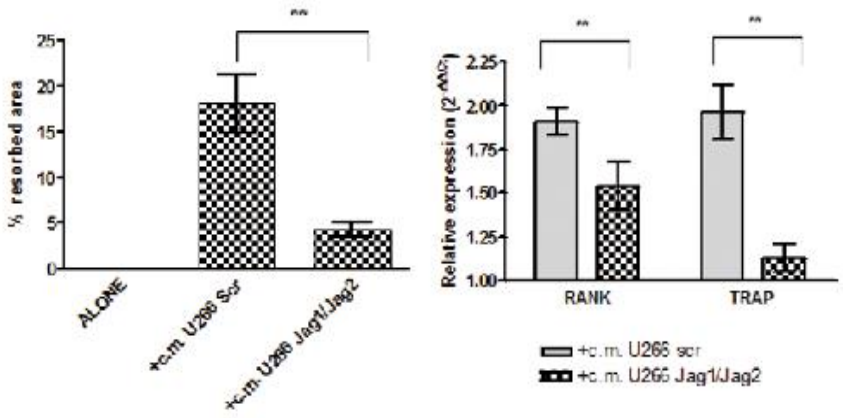

Fig 6d. Jag1 and 2 are required in MM cells to induce osteoclastogenesis. *Left: pit formation assay, the percentage of resorbed area was measured by using the Wimasis image analysis software (Wimasis GmbH) to process 20x pictures covering the whole well surface. Right: analysis of pro-osteoclastogenic genes, RANK and TRAP, by qRT-PCR showed that U266 pro-osteoclastogenic potential is Jag-dependent. Gene expression data are presented as relative expression (control =1) calculated through the* $2^{-\Delta\Delta Ct}$ *formula.*

*Statistical analyses were performed using the ANOVA and Tukey post-test (pit formation assay, **= P <0,01) and Two-tailed t-test (qRT-PCR, ** = P <0,01)*

7. Stromal cells stimulate RANKL secretion by MM cells in a Jag1/2-dependent manner

Several MM primary cells and cell lines, such as OPM2 cells have no osteoclastogenic potential since they express very low levels of RANKL.

The interaction of MM cells with BMSCs promotes the production of soluble factors that contribute to MM progression and to osteoclast development (Roodman, 2010a). Therefore, I wondered if BMSCs may interact with MM cells to increase their osteoclastogenic potential and if the Notch pathway could have a role in this process. To evaluate this hypothesis, OPM-2 cells were transfected with Jag1/2 or Scr shRNAs. After 48h cells were re-silenced and plated on a monolayer of NIH-3T3 cells (see Material and Methods). Co-cultures were maintained for 48h. CM (20% V/V) from co-culture or single culture were tested in differentiation assay of Raw264.7 cells.

Results showed that both the CMs from OPM2 and NIH3T3 cells had a slight intrinsic osteoclastogenic potential. On the contrary, the CM from OPM2 cells co-cultivated with NIH3T3 fibroblasts were able to induce a significant increase in OCLs differentiation. The increased osteoclastogenic potential was completely impaired by silencing Jag1 and 2 in OPM2 cells (Fig 7a).

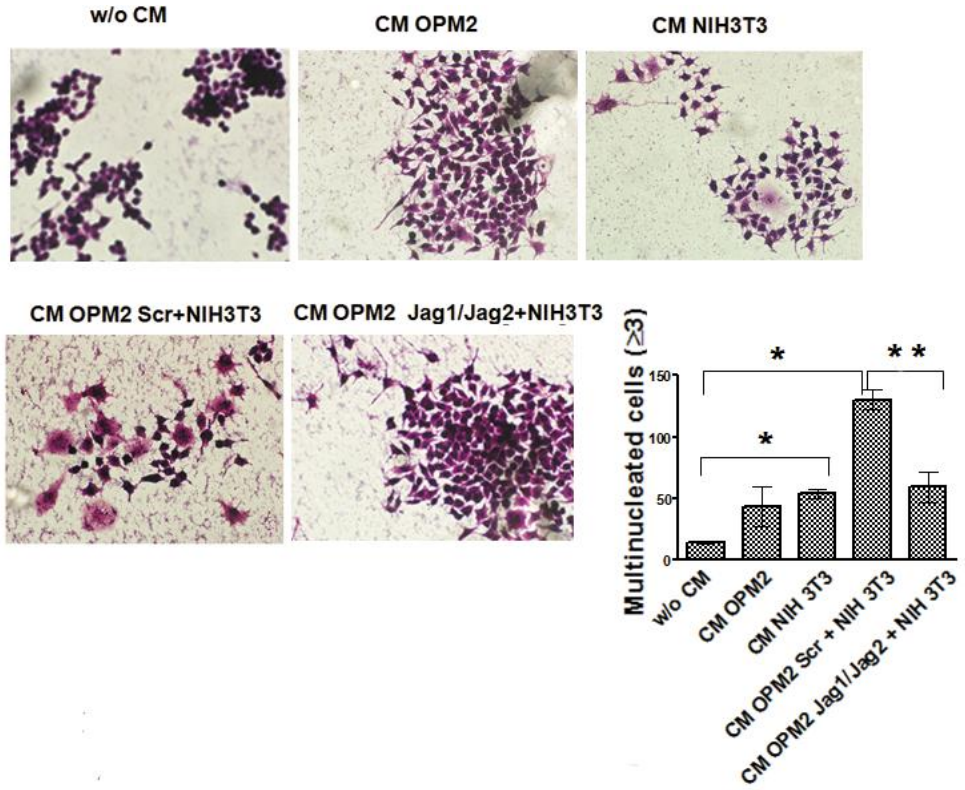

Fig 7a. NIH3T3 cells stimulates RANKL expression in MM cells in a Notch-dependent manner. *Raw264.7 cells were induced to differentiate with CM from Jag1/2 silenced-OPM2 cell line co-cultured with NIH3T3 cells. On the left, pictures representative of 3 independent experiments with similar results show TRAP staining of Raw264.7 cells (40x) after culturing in the different conditions. On the right, a graph with the mean values and standard deviations of counted multinucleated/TRAP+ cells Statistical analysis was performed using the ANOVA and Tukey post-test:* = P<0.05; ** = P <0.01.*

This evidence prompted me to assess by qRT-PCR and ELISA if variations occurred in the amount of RANKL secreted by OPM2 cells in the different culture conditions. I found out that when co-cultured with NIH3T3 cells, OPM2 cells increased Notch signaling activity (changes in HES6 expression in qRT-PCR, Fig 7b left panel) and this was associated to an increase in the secreted RANKL (qRT-PCR, Fig 7b left panel and ELISA, Fig 7b right panel). As expected, Notch pathway inhibition mediated by Jag1/Jag2 silencing in OPM2 cells dramatically impaired their RANKL production even when co-cultured with NIH3T3 cell line (Fig 7b). It is worthy to note that the low intrinsic osteoclastogenic potential of CM from NIH3T3 cells (previously reported) is due to the ability of these cells to secrete RANKL (2ng/ml in 48h CM, data not shown).

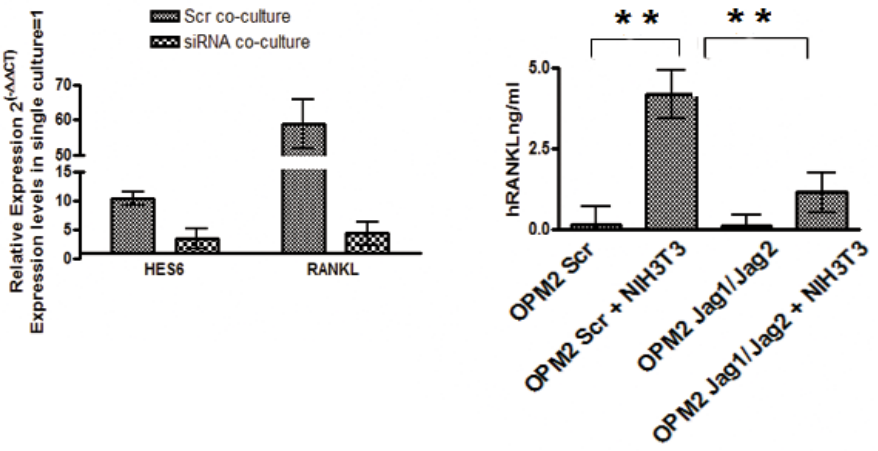

Fig 7b. Jag1 and 2 control MM cells ability to secrete RANKL in response to NIH3T3 cells stimulation. *The expression of RANKL produced by OPM2 cells co-cultured with NIH3T3 cells was measured. On the left qRT-PCR of RANKL and HES6 genes (to assess Notch activation variation in OPM2 cells). Bars represent the mean relative expression of the indicated genes measured by RT-qPCR (expression level in control cells was made equal to 1). Two-tailed t-test confirmed statistically*

*significant differences in the expression levels of the target genes (Scr vs. Jag1/Jag2 p<0.05). Standard deviations were calculated from 3 independent experiments. On the right ELISA of RANKL secreted in CM of OPM2 cell line alone or co-cultured with NIH3T3 cells, transfected with Jag1/2 or Scr shRNAs. Statistical analysis was performed using ANOVA and Tukey post-test: ** = P <0,01.*

Overall these results indicated for the first time that BMSCs might contribute to MM-induced OCL development by increasing the osteoclastogenic potential of non-osteoclastogenic MM cells via Notch signaling activation. BMSC-mediated Notch signaling activation seems to enable MM cells lacking osteoclastogenic potential to acquire it by secreting RANKL. Switching off Notch signaling in MM cells through Jag1 and 2 silencing could prevent both the intrinsic osteoclastogenic activity of MM cells and that induced by the surrounding BM microenvironment.

DISCUSSION and CONCLUSIONS

Osteoporosis and formation of bone lesions are common features of MM and affect almost the 80% of patients (Roodman, 2010b) .

Skeletal destruction not only negatively influences patients' quality of life, but also promotes tumor burden (Yaccoby, 2010), angiogenesis (Tanaka et al., 2007), drug resistance (Abe, 2011; Moreaux et al., 2011) and reduces the patient's anti-tumor immune response (Feyler et al., 2013; Juarez and Guise, 2011), finally supporting MM progression and contributing to the fatal outcome of this disease. Indeed, OCL activity occurs mainly adjacent to MM cells, supporting the hypothesis that neoplastic cells may be responsible for the increased bone resorptive activity and OCLs formation in MM. Soluble factors released by the increased bone resorptive process also support the growth of MM cells creating a "vicious cycle" in which bone resorption causes the release of growth factors that increase MM tumor burden that in turn results in increased bone destruction (Edwards et al., 2008). In addition, OCLs are able to promote microvessels formation through the production of pro-angiogenic factors (Tanaka et al., 2007) and to antagonize patient's anti-tumor immune responses promoting the release of TGF-β from the bone matrix (Juarez and Guise, 2011).

BM localization is extremely relevant in MM progression since MM cell is strongly dependent from the BM microenvironment and is able to unbalance the OCL/OBL ratio by increasing osteoclastogenesis and reducing OBL differentiation, finally resulting in MM-induced bone disease.

In the last years, the Notch pathway has been proposed as a promising therapeutic target in MM (Mirandola et al., 2013; Nefedova et al., 2008; Schwarzer et al., 2008). Notch receptors and ligands are reported to be dysregulated in MM and positively correlate with clinical stage (Ghoshal et al., 2009; Houde et al., 2004; Jundt et al., 2004; Schwarzer et al., 2008; Skrtic et al., 2010; Takeuchi et al., 2005).

MM cells simultaneously express Notch receptors and ligands, resulting in homotypic activation of Notch signaling within MM cells, as well as heterotypic Notch activation in the surrounding cells of the BM microenvironment. In accordance, Notch pathway affects the biology of MM cell and its pathological interaction with BM stroma. Indeed, evidences from our and other laboratories showed that Notch inhibition in MM cells resulted in reduced proliferation, increased apoptosis (Mirandola et al., 2013; Mirandola et al., 2011a; Nefedova et al., 2004; Ramakrishnan et al., 2011; Schwarzer et al., 2008; Vallet et al., 2007; Xu et al., 2012) and drug sensitivity (Chen et al., 2011; Nefedova et al., 2008).
Moreover, we recently described that Notch signaling controls malignant plasma cells localization in the BM by the regulation of the CXCR4/SDF1 chemokine axis (Mirandola et al., 2013).

The aim of this work was to study the role of Notch pathway in the key relationship between MM cells and the osteoclast precursors, to understand if Notch signaling deregulation in MM cells may contribute to MM-associated bone disease.
Notch is finely tuned during bone formation and modeling; however, signaling from the different Notch isoforms have different and still not completely understood outcomes (Bai et al., 2008; Fukushima et al., 2008).
The relevance of Notch activity in skeletal development and remodeling (Bai et al., 2008; Fukushima et al., 2008), prompted me to wondered if Notch signaling upregulation in MM cells may have a role in inducing OCL differentiation. Therefore, I initially investigated the contribute of Notch on OCLs precursors ability to differentiate using a murine cellular model of OCL precursors, the Raw264.7 monocyte cell line. I observed that Raw264.7 cells failed to differentiate in mature OCLs in the presence of the γ-secretase inhibitor DAPT, a commonly used Notch signaling inhibitor.

I deepened the possible causes of the impaired OCL differentiation by analyzing changes induced by DAPT administration on the expression of master genes involved in osteoclastogenesis, including *RANK* (Nakagawa et al., 1998) and *TRAP* (Abu-Amer, 2013; Tauchert et al., 2009). The usual upregulation of these genes occurring during osteoclastogenesis failed when murine OCL precursors were treated with Notch inhibitor. This results suggest that RANK and TRAP are two Notch downstream osteoclastogenic effector and their positive regulation by Notch signaling, at least in part, may be at the basis of osteoclast differentiation.

Indeed, the observed reduction in *RANK* expression associated to Notch withdrawal is noteworthy since this receptor not only represents a differentiation marker, but it also mediates pre-OCL response to RANKL stimulus which results in the activation of the NF-κB pathway, an essential step in the osteoclastogenic process (Abu-Amer, 2013).

The relevance of TRAP dependence on Notch signaling stems from the evidence that its enzymatic activity is directly involved in bone resorption (Scarnecchia et al., 1991) along with the associated degradation of type I collagen (Littlewood-Evans et al., 1997). Moreover, the reduction of its expression is in accordance with the decreased osteolytic activity of Raw264.7 cells upon Notch signaling withdrawal, confirming the results obtained by Schwarzer and colleagues who observed that in human OCLs TRAP expression was inhibited by treatment with GSI (Schwarzer et al., 2008).

The second issue was to understand the role of the different Notch isoforms expressed by Raw264.7 cells during osteoclasts differentiation.

As a matter of fact, the function of Notch1 is still controversial, since evidences from Choi and colleagues (Choi et al., 2013) indicate that RANKL-induced OCL differentiation is promoted by the Notch1 intracellular

domain, whereas data by Bai et al. not only report Notch1 inability to support osteoclastogenesis, but also describe its negative effect on this process (Bai et al., 2008).

My results indicated that Notch1 activity is neither necessary nor sufficient to induce osteoclastogenesis. As a matter of fact, Notch1 was downregulated during Raw264.7 cells differentiation and OCL precursors failed to differentiate in the presence of the constitutively activated Notch1. On the contrary, I observed that Notch2 had a relevant role in osteoclastogenesis. Specifically, its expression was increased during RANKL induced Raw264.7 cells differentiation and its forced expression alone induced OCL differentiation at least partially through the activation of an autonomous production of RANKL.

An interaction between the Notch2 and NF-kB pathways in directing the osteoclastogenic process had already been reported by Fukushima and colleagues who described a positive regulation of Notch2 expression due to a RANKL-triggered NF-κB signaling (Fukushima et al., 2008). My results further suggested that these two pathways synergistically reciprocally enhanced their activities, since Notch2-promoted RANKL secretion by Raw264.7 cells, which was sufficient to induce osteoclastogenesis.

The most interesting result presented here concerns the mechanisms by which the interactions between myeloma cells and the BM microenvironment promote bone resorption.

I demonstrate that MM cells were able to induce the differentiation of monocytes into OCLs and that this process required an active Notch signaling both in MM cells and in monocytes/OCLs.

Indeed, results showed that the U266 MM cell line was able to induce OCLs differentiation and that this process was inhibited by DAPT treatment.

DAPT hampered also the modulation of *RANK* and *TRAP* expression occurring during the differentiation process. These evidences suggested a role for Notch signaling in MM-induced osteoclastogenesis and supported the hypothesis that a treatment directed to Notch pathway inhibition could be effective in reducing MM-driven bone disease.

Since the used co-culture system did not allow to discriminate if the observed inhibitory effect of DAPT on osteoclastogenic differentiation was due to Notch inhibition on MM cells, OCL precursors or both, I needed to elucidate it .

This point was clarified when I demonstrated that Notch signaling needed to be active not only in OCL precursors but also in MM cells. Indeed, the osteoclastogenic ability of U266 cells was due to the secretion of RANKL, which in its turn was under Notch signaling control.

First of all, I demonstrated that U266 cells did not need a direct contact to trigger Notch signaling in Raw264.7 cells, but Notch activation occurring in OCLs during differentiation could be mediated by U266 cells conditioned medium. Accordingly, I found that U266 cells did not need to be in direct contact with pre-OCLs to induce differentiation, indeed U266 cells conditioned medium alone was able to induce osteoclastogenesis.

The evidence that RANKL is the only osteoclastogenic factor required for Raw264.7 cells differentiation, prompted me to verify if Notch activity promoted the osteoclastogenic ability of U266 cells enhancing RANKL secretion. Results indicated that Notch withdrawal resulted in the reduction of RANKL, both at mRNA and protein levels and, as expected, conditioned media from U266 cells treated with DAPT or with Jag1 and Jag2 siRNAs, were unable to induce Raw264.7 cells differentiation.

Interestingly, my study on cell lines was further confirmed in *ex-vivo* experiments by our collaborator, Dr. Carl S. Goodyear, at the University of

Glasgow, UK. Dr. Goodyear's group set up co-culture systems consisting of primary myeloma cells purified from patients BM and human monocytes and showed that DAPT significantly inhibited the ability of myeloma cells to induce osteoclastogenesis confirming the results obtained with Raw264.7/U266 co-cultures.

Overall, this is the first evidence that Notch signaling is necessary in MM-induced osteoclastogenesis and affects both the osteoclastogenic potential of MM cell as well as the pre-OCL ability to respond to this stimulus..

The new picture drawn from this work depicts the role of Notch in myeloma cell biology through some key points (detailed in figure 1): Notch signaling in MM cells promotes the release of RANKL, which in turn enhances the NF-KB signaling by engaging RANK on pre-OCL; RANKL-stimulated pre-OCL upregulates Notch2 expression which further reinforces the differentiation process by promoting the autonomous secretion of RANKL by OCL precursors. MM-triggered stimulation of RANK and Notch2 signaling pathways results in osteoclastogenesis and contributes to the osteolysis associated to this disease.

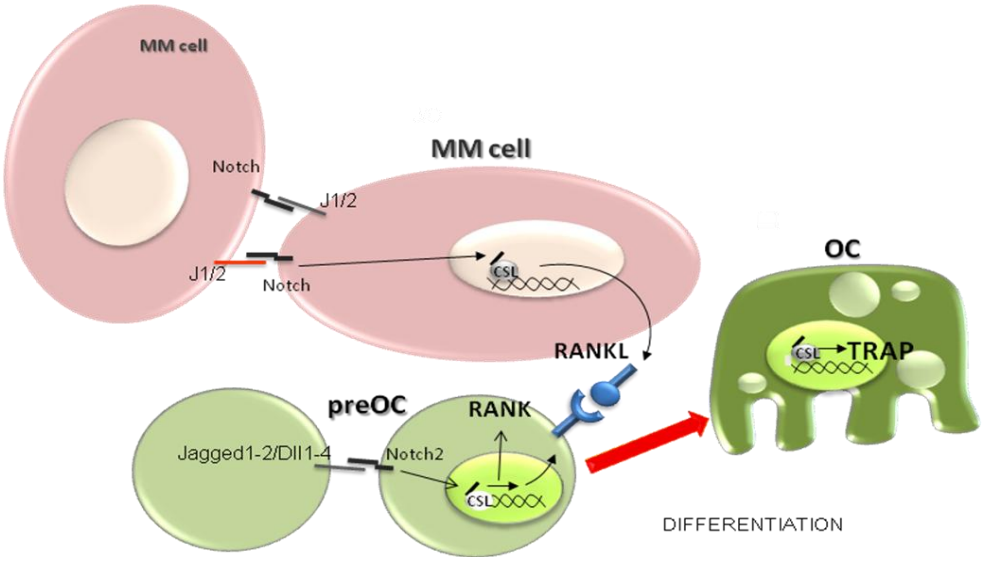

Fig 1. MM cells induces osteoclastogenesis in a Notch-dependent manner, through the production of RANKL.

The relevance of these findings arises from the key role of RANKL in MM-associated bone disease, indeed RANKL expression is not only increased in BMSCs from myeloma patients compared with those from healthy donors (Giuliani et al., 2001), but its expression levels in MM cells correlates with the level of skeletal involvement (Farrugia et al., 2003; Heider et al., 2003). In accordance, it has been reported that targeting RANKL can prevent the development of myeloma bone disease (Yaccoby et al., 2002).

Albeit the present results should be confirmed through in vivo experiments, I obtained a first confirmation of the clinical relevance of my findings about Notch signaling ability to promote osteoclastogenesis by identifying the existence of an association between the levels of Notch signaling activation and RANKL expression in MM patients. Indeed, I found a correlation between the expression of the Notch target gene, *HES6*, and the *RANKL* gene in purified $CD138^{+}$ cells from the BM of 17 MM patients.

An analogous result was obtained by our collaborator, Prof. Antonino Neri from the Department of Clinical Sciences and Community Health of the University of Milano. Indeed, by a Gene Expression Profile analysis performed on primary cells from 55 newly diagnosed MM patients, his group highlighted a significant correlation between *HES6*, and *RANKL* expression levels.

Notch signaling dysregulation in MM is often associated to alteration of Notch ligands expression or post-translational processing. Jag1 expression in malignant plasma cells arises upon progression of disease from MGUS to MM (Jundt et al., 2004). Jag2 dysregulation (Ghoshal et al., 2009; Houde et al., 2004; Takeuchi et al., 2005) seems even more important in MM pathogenesis since it is an early event preceding MGUS and positively correlated with stage (Houde et al., 2004). Jag2 dysregulation can be driven by promoter hypomethylation or constitutive core promoter acetylation (Ghoshal et al., 2009) or can be the result of the overexpression of Jag2 specific ubiquitin-ligase Skeletrophin (Takeuchi et al., 2005).
The evidence of Jag1 and 2 dysregulation in MM, prompted me to evaluate if they could be possible targets for a Notch pathway directed approach and if their selective inhibition could be sufficient to counteract the excessive osteoclastogenesis observed in MM patients.
To address this issue, an RNA interfering approach was used to silence Jag1 and Jag2 expressed by U266 cells. Jag1/2 silencing resulted in a reduced Notch activity and, notably, in the inability of MM cells to secrete RANKL induce osteoclastogenesis and the expression of RANK and TRAP in Raw264.7 cells.
Interestingly, I also found out that the mechanism of MM-induced osteoclastogenesis based on Notch-driven RANKL production is active also

in non osteoclastogenic MM cells . These are represented by OPM2 cells, which express a low RANKL level and are scarcely osteoclastogenic. Nonetheless, when these cells were co-cultured with NIH3T3 fibroblasts (used as mimics of BM stromal cells), they showed a strong increase in the amount of secreted RANKL, comparable to that released by the osteoclastogenic U266 cells. Accordingly, the amount of RANKL released in the medium by OPM2/NIH3T3 co-culture system was sufficient to induce Raw264.7 differentiation. Importantly, the ability of stromal cells to promote the osteoclastogenic properties of poor-osteoclastogenic MM cells, such as OPM2, required an active Notch signaling, since Jag1 and 2 silencing in OPM2 significantly reduced their osteoclastogenic potential induced by NIH3T3 fibroblasts.

The evidence that Notch signaling inhibition blocks MM-driven osteoclastogenesis makes the Notch pathway a promising therapeutic target to suppress the development of bone lesions in MM patients.

Moreover, the evidence that the inhibition of high levels of Jag ligands activity observed in MM cells strongly reduces osteoclastogenesis and osteolysis, suggest that the inhibition of the dysregulated ligands can be a selective Notch-directed therapeutic approach in MM patients to oppose to osteoclastogenesis, bone degradation and probably all the associated consequences, including increase in tumor burden (Yaccoby, 2010), angiogenesis (Tanaka et al., 2007), inhibition of patient's anti-tumor immune response (Juarez and Guise, 2011) and drug resistance (Abe, 2011; Moreaux et al., 2011).

The significance of this novel approach is evident if considering the high toxicity of the treatments with γ-Secretase inhibitors (Mirandola et al., 2011a; Searfoss et al., 2003; Wong et al., 2004), mainly due to the contemporary inhibition of the activation of all the 4 Notch isoforms. The

redundancy of Notch ligands and the effective possibility to reduce the excessive Notch signaling in MM cells by silencing only the dysregulated Jag1 and/or Jag2 ligands, may provide the rational for an effective and safer Notch-directed approach in MM therapy.

The future direction of this work should include a validation of the Jag1/2-directed therapeutic strategy on an *in vivo* animal model of MM. To address this issue I propose to xenograft a MM cell line conditionally expressing specific siRNAs for Jag1 and Jag2 by means of a lentiviral vector in busulfan-conditioned NOG mice (Choi et al., 2011; Mirandola et al., 2011b). To set up a situation analogous to that of MM patients, Jag1/2 knockdown should be induced in tumor cells by Doxycycline administration only upon BM infiltration and bone lesions establishment. Mice monitoring should include evaluation of tumor burden, survival and bone lesions development.

An *in vivo* confirmation of the role of Jag ligands upregulation in bone disease and the efficacy of their silencing, would ultimately provide the rationale for a new therapeutic strategy to reduce MM-associated skeletal destruction and improve the response to standard treatments, providing a valuable option for those patients who suffer from advanced disease and have no alternatives other than palliative cares.

MATERIALS AND METHODS

1. CELL CULTURES

1.1 Single cultures

The Multiple Myeloma (MM) cell lines used were:

U266: established from the peripheral blood of a 53-year-old man with IgE-secreting myeloma (refractory, terminal) in 1968; cells were described to produce IgE lambda; . Cells negative for CD3 , CD10, CD19 and CD20 and positive for CD138. They grow partially (loosely) adherent.

OPM2: cell line established from the peripheral blood of a 56-year-old woman with multiple myeloma (IgG lambda) in leukemic phase (relapse, terminal) in 1982. Cells negative for CD3 , CD10, CD80, CD19 and CD20 and positive for CD138. They grow in suspension.

All MM cell lines were maintained in 75 cm2 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 3x105cells/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. Cell line was 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% CO_2 at 37°C. Cells have a doubling time of 18-20h and were divided three times/ week.

Osteoclast precursors cell line is:

RAW264.7: Monocyte/macrophage cells established from a tumor induced by Abelson murine leukemia virus. These cells growth in adhesion and are able to differentiate in OCLs in presence of mRANKL or in dendritic cells (DCs) in presence of GM-CSF, IL4 and LPS.

1.2 Co-culture of MM/BMSC lines

NIH3T3 cells were plated in 24 multi-well plates at the concentration of 150000/ml. After 24h NIH3T3 medium was discarded and OPM2 MM cells were plated on top of NIH3T3 monolayer at the concentration of 350000/ml. All the co-cultures were maintained 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 for 48h.

1.3 Co-culture of MM/pre-OCL cell line

For co-culture experiments Raw264.7 and U266 cells were seeded on a 6-well plate at a density of 1×10^4 cells per well (about 8×10^3 Raw264.7 and 2×10^3 U266) and cultured for 7days in presence/absence of drugs.
For conditioned medium experiments, Raw264.7 cells were seeded on a 6-well plate at a density of 1×10^4 cells per well and allow to adhere for

3hours. Then, medium was replaced with 4/5 of DMEM and 1/5 of conditioned medium from U266 cells.

2. TREATMENTS AND DIFFERENTIATION ASSAYS

2.1 Notch inhibition DAPT-mediated

DAPT (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester, Chalbiochem), also called GSI IX, is an inhibitor of the enzyme γ-Secretase. It is dissolved in DMSO.

The cells were treated with the drug at a concentration of 50μM. The cells treated with the same amount of DMSO were used as control.

Solubility	Molecular Formula	Mol. Wt.
DMSO	$C_{23}H_{26}F_2N_2O_4$	432.5

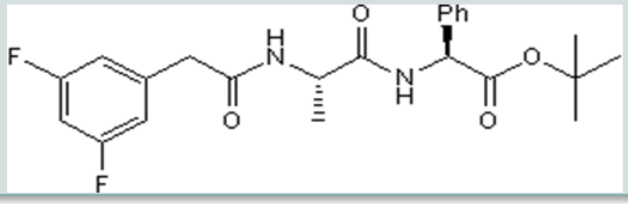

Figure 2.1. Molecular structure of DAPT.

2.2 Osteoclast differentiation from RAW264.7 cells

Raw264.7 were seeded on a 24-well plate at a density of 1×10^4 cells per well. Cells were treated for 5 days with 50ng/ml mRANKL in the presence/absence of DAPT.

3. TRAP Staining

On the day of harvest, cells were fixed on the culture plates with citrate-acetone solution and stained for tartrate-resistant acid phosphatase (TRAP) (Sigma-Aldrich) in order to identify mature OCLs.

Picture were taken by using a Leica microscope equipped with a DFC280 camera (Leica Microsystems), and analyzed with the LAS v2.8.1 software (Leica).

Osteoclasts were identified and enumerated under light microscopy by the presence of ≥3 nuclei.

4. BONE RESORPTION ASSAY

Raw264.7 were cultured on Osteo Assay Surface 24-wells plates (Corning) under differentiation conditions. After 7-10 days of culture, the plates were washed in 5% sodium hypochlorite solution to remove the cells. The resorbed areas on the plates were captured with *EVOS fl* microscope and the percentage of resorbed area was measured by using the Wimasis image analysis software (Wimasis GmbH) to process 20x pictures covering the whole well surface.

5. GENE EXPRESSION ANALYSIS

5.1 RNA isolation

The total RNA isolation was based on acid guanidinium thiocyanate-phenol-chloroform extraction *(Chomczynski P., 1987)*.The protocol is optimized for 10^6 cells:

- Cells were washed two times with cold 1X PBS.
- After centrifugation at room temperature, pellet was resuspended in 150 µl of D-solution.
- Sequentially it was added:
  - 15 µl Sodium Acetate 2M pH 4
  - 150 µl water-saturated Phenol
  - 30 µl Chloroform
- Sample was mixed by vortex and incubated at 4°C for 10'.
- After centrifugation for 10' at 14.000 rpm at 4°C, the aqueous phase was collected in a new tube.
- One volume of Phenol-Chloroform was added to the aqueous phase.
- Sample was mixed by vortex and centrifuged for 10' at 14.000 rpm at 4°C.
- The aqueous phase was collected in a new tube.
- One volume of cold isopropanol was added to the aqueous phase.
- Sample was incubated for 30' at –20°C.
- After centrifugation for 30' at 14.000 rpm at 4°C, the supernatant was discarded.
- Pellet was resuspended in 50 µl of D-Solution and precipitated with 1 volume of cold isopropanol.
- Sample was incubated for 30' at –20°C.
- After centrifugation for 30' at 14.000 rpm at 4°C, the supernatant was discarded.
- Pellet was washed with cold ethanol 70% .

- After centrifugation for 10’ at 14.000 rpm at 4°C, the ethanol was discarded.
- Pellet was dried with vacuum system and resuspended in 30 µl of H_2O DEPC.
- To obtain an homogenous solution, RNA was heated at 65°C for 5’.

1

PBS 1X pH 7,4

- 4,3 mM Na_2HPO_4
- 1,47 mM KH_2PO_4
- 137 mM NaCl
- 2,7mM KCl

D-Solution pH 7:

- 4 M guanidinium isothiocyanate
- 25 mM sodium citrate tribasic dehydrate
- 18.4 mM sodium lauroyl sarcosinate
- 100 mM β-mercaptoethanol
-

5.2 RNA quantification

RNA was quantified by spectrophotometric measure, using 2 µl of RNA in 700 µl of H_2O Milli-Q in quartz cuvettes at two different wavelengths: 260nm (A1) and 280nm (A2).

Since**:** 1 OD_{260nm} = 40 µg/ml

The concentration in µg/ml was calculated as:

$$A_{260} \text{ x } 40 \text{ ng/µl x dilution factor}$$

High quality RNA was used (A1/A2 ratio closed to 2).

5.3 Reverse transcription

The cDNA was obtained by reverse transcription with M-MLV RT KIT (Sigma-Aldrich Co.).

- A reaction mix of 20 µl was prepared with:
  - 2 µl of Random primers (250ng/µl)
  - 4 µl of 10 mM dNTPs (2.5mM each)
  - 2 µg RNA
  - H2O DEPC up to 10 µl
- The sample was heated at 65°C for 5'.
- After centrifugation it was added to the mix:
  - 2µl of 10x M-MLV RT Buffer
  - 1µl of M-MLV Reverse Transcriptase (200 U/µl)
  - 7µl of H_2O DEPC
- Sample was incubated 10' at room temperature, then at 37°C for 50 minutes and finally stored at –20°C.

5.4 PCR (Polymerase Chain Reaction)

Quantitative PCR reactions were carried out on a 7500 Fast Real-time PCR system (Applied Biosystems, Life Technologies Italia, Italy) using the GoTaq qPCR Master Mix (Promega, Italia s.r.l., Milan, Italy).

Each sample was analyzed in triplicate with no template controls. Calculations of the initial mRNA copy numbers in each sample were made according with to Ct (cycle-mix threshold) method and the copy numbers of the analyzed mRNA were normalized using GAPDH mRNA levels. Primer sequences used for cDNA amplification are displayed in Table 5.4.1.

RT-qPCR primers	Forward Primer 5'-3'	Reverse Primer 5'-3'
mGAPDH	TTGGCCGTATTGGGCGCCTG	CACCCTTCAAGTGGGCCCCG
mHES5	GGCTCACCCCAGCCCGTAGA	TCGTGCCCACATGCACCCAC
mCXCR4	AACCACCACGGCTGTAGAGCGA	TCCCGGAAGCAGGGTTCCTTGT
mTRAP	ACCGTGCCCTTCGCAACATCC	GACAGCTGAGTGCGGGCCAC
mRANK	TGCCCTGTGGCCCCGATGAG	TGGTAGCCAGCCGTGCAAGC
mRANKL	CCCAGCGAGGCAAGCCTGAG	TGCCGAAAGCAAATGTTGGCG
mNOTCH1	ACCGGAGTGGACGGGTCAGT	TGTGCGCCCATGCGGACATT
mNOTCH2	CTTGCTTGTGCCCCGTGGGT	GCCCGAGTGCTGGCACAAGT
hGAPDH	ACAGTCAGCCGCATCTTCTT	AATGGAGGGGTCATTGATGG
hHPRT1	GTAGCCCTCTGTGTGCTCAA	TTTATGTCCCCTGTTGACTGGT
h/m18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
hHES6	ATGAGGACGGCTGGGAGA	ACCGTCAGCTCCAGCACTT
hJag1	TTCGCCTGGCCGAGGTCCTAT	GCCCGTGTTCTGCTTCAGCGT
hJag2	CCGGCCCCGCAACGACTTTT	CCTCCCTTGCCAGCCGTAGC
hRANKL	AAGGAGCTGTGCAAAAGGAA	CGAAAGCAAATGTTGGCATA

Table 5.4.1 *Primer sequences.*

6. ELISA Assay

Flat-bottom 96-well polycarbonate plates were coated at 4°C overnight with 50 µL/well cell culture supernatants diluted 1:1 in carbonate coating buffer (0.1 M Na_2CO_3, 0.1 M $NaHCO_3$, pH=9.5). Standard curves were obtained with purified recombinant human RANKL (Merck-Millipore) serially diluted in coating buffer. After removing diluted supernatants or standards and blocking with PBS supplemented with 1% W/V BSA for 1 h at RT, plates were incubated with biotin-conjugated goat anti-human RANKL (Merck-Millipore) for 1 h at RT. Then, plates were washed twice with PBS containing 0.025% V/V Tween-20 (300 µL/well) and incubated at RT with Streptavidin-HRP-labeled secondary antibody (Invitrogen) for 30min. The plates were washed three times with PBS containing 0.025% V/V Tween-20 (300 µL/well), then the TMB substrate (Thermo Scientific, Inc) was added, and signal was measured using a microplate reader. All samples were run in triplicates.

7. FLOW CYTOMETRY ANALYSIS

A Beckman Coulter flow cytometer was used for apoptosis detection. $3x10^5$ cells/ml were washed with cold PBS1x, resuspended in “binding buffer 1X” (HEPES 0,01M, NaCl 0,14M, $CaCl_2$ 2,5mM) and incubated (or not for control) for 15’ at room temperature with Annexin-V FITC (Bender) + Propidium Iodide (2,5 ug/ml final, Sigma-Aldrich Co) in the dark. Finally, 400 µl of Binding Buffer 1x were added to the tube. 10.000 cells were acquired using FL1 and FL3 bandpass filter for Annexin-V FITC (λex=488 nm; λem=520 nm) and Propidium Iodide (λex=488 nm; λem=617 nm)

respectively. Cells were processed using “Cytomics FC500” BeckmanCoulter software program.

8. MTT ASSAY

Cells were incubated with 0,5mg/ml MTT suspension (Sigma-Aldrich Co) in the dark at 37°C 5%CO2 for 4h and finally resuspended in DMSO. An automated microplate reader was used to measure the optical density at a wavelength of 540nm (background wavelength= 620nm). Plates were processed using “Magellan” Tecan software program.

9.TRANSFECTION AND REPORTER ASSAY

Intracellular Notch1 (ICN1) and Notch2 (ICN2) constructs were previously described respectively by Prof. W.S. Pear and Prof. B. Bettler (Pear et al., 1996; Tchorz et al., 2009).

For the reporter assays, TK-pRL was from Promega Italia s.r.l. (Milano, Italy). The pGL3-based plasmid encoding the firelfly luciferase under the control of 13 repeats of the CSL-responsive element (13XCSL) was as described by Shawber C., et al. (32). Cells were harvested and resuspended (10^7/mL) in RPMI1640 without antibiotics. One hundred microliters of cell suspension were mixed with 5 µg DNA (1:25 firefly:renilla luciferase ratio), then transferred into a 2.0 mm-gap cuvette (BTX, MA, USA). Electroporation was performed using 250 V and 950 µF. Analyses were performed 72 hours after transfection. The dual luciferase assay was performed according to the manufacturer’s directions (Dual-Luciferase® Reporter Assay System, Promega).

10. WESTERN BLOT ANALYSIS

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 Na_3VO_4, 2µg/ml aprotinin, 2µg/ml leupeptin. After incubation on ice for 15 min, the lysate was clarified by centrifugation for 10 min at 4°C. Protein concentration was determined using the Bradford assay (Bio-Rad Laboratories). Protein samples (50–70 µg) were loaded and run on 8% denaturing SDS-PAGE gels, 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: cleaved-Notch1 (Val 1744) 1:1000, Notch 2 intracellular domain antibody-cleaved (Asp1733) 1:1000. Following washes, the filter was incubated with HRP-conjugated species-specific secondary antibodies (Santa-Cruz Biotechnology). Proteins were visualized with ECL reagents (Promega) according to the manufacturer's instructions.

11. RNA INTERFERENCE

To selectively inhibit Notch signaling in MM OPM2 and U266 cell lines a specific Jag1 and 2 knock-down was designed using a transient expression of specific siRNAs for Jag1-2. As negative control was used a "scrambled" siRNA, to discount any change in gene expression profile due to delivery method. Cells treated with fluorescent sdRNA "BLOCK-IT" (Life Technologies Italia, Milan, Italy) were used as positive control.

To address this issue, Stealth Select RNAi^{TM} siRNA system (Life Technologies Italia, Milan, Italy) was used according to the Manufacturer's guidelines.

Specific anti-Jag siRNAs were delivered following these steps:

- Cells were plated at 3x105/ml in medium without antibiotics;
- 24h later, cells were diluted to 3,6x105/ml in medium without antibiotics and plated in 0,5 ml of final volume;
- siRNAs (25 nM anti-Jag1 + 25 nM anti-Jag2 / or 50nM scrambled siRNA/ or 50nM fluorescent dsRNA) were diluted in 50µl of Opti-MEM medium (Invitrogen, Life Technologies Italia, Milan, Italy) without serum and antibiotics;
- 1µl of RNAi-MAX lipofectamine transfecting reagent (Invitrogen, Life Technologies Italia, Milan, Italy) was diluted in 50µl of Opti-MEM medium without serum and antibiotics;
- The two solutions (siRNA/lipofectamine) were mixed and incubated for 20' at room temperature;
- 100µl of lipofectamine/siRNA mix was added to the cells (final cells concentration 3x105/ml);
- Every 48h cells were diluted 1:1 with medium antibiotics-free and treated again with Jag1/Jag2 siRNA up to 8 days

Cells were maintained in RPMI 1640 medium (Sigma-Aldrich Co., St Louis, MO) supplemented with 10% (v/v) FBS (Gibco, Rockville, MD) and 2 mM L-glutamine (Invitrogen Corporation Carlsbad, CA, USA) without antibiotics and incubated in 5% CO2 at 37°C.

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 acquired with Beckman Coulter analyzer

using FL2 bandpass filter (λex=488nm; λem=575nm) for BLOCK-IT fluorophore conjugated sdRNA.

Jag1 and Jag2 effective silencing induced by specific siRNAs was assessed by quantitative PCR compared to scrambled siRNA-receiving cells.

12. STATISTICAL ANALYSIS

Data are represented as mean ± SD of at least 3 independent experiments. Statistical analysis on single culture and co-culture experiments on Raw264.7 and MM cell lines were performed using two-tailed Student's t-test to compare the means of normally distributed values and analysis of variance was performed by a one-way ANOVA with Tukey's post-test.

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Publications

- M Colombo, K Thümmler, L Mirandola, S Garavelli, K Todoerti, L Apicella, E Lazzari, M Lancellotti, M Chiriva-Internati, N Platonova, R Soutar, A Neri, CS Goodyear and R Chiaramonte- Notch signaling drives multiple myeloma induced osteoclastogenesis – Submitted to Leukemia in August 2013, currently in revision, resubmission due within 8 January 2014.

- Mirandola L, Apicella L, Colombo M, Yu Y, Berta DG, Platonova N, Lazzari E, Lancellotti M, Bulfamante G, Cobos E, Chiriva-Internati M, Chiaramonte R. 2013. Anti-Notch treatment prevents multiple myeloma cells localization to the bone marrow via the chemokine system CXCR4/SDF-1. Leukemia 27:1558-1566.

- M Colombo, L. Mirandola, N. Platonova, L. Apicella, E. Lazzari, E. Cobos, M. Chiriva-Internati, R. Chiaramonte. 2013 Notch-directed microenvironment reprogramming in myeloma: a single path to multiple outcomes- Review, Leukemia 27:1009-1018

Attendance to conferences:

- Chiriva-Internati M, Mirandola L, Lazzari E, **Colombo M**, Lancellotti M, Cobos E et al., "Promotion of human multiple myeloma cell growth in vitro and bone marrow invasion in vivo by Notch receptors and the CXCR4/SDF1 axis." Presented at ASCO annual meeting 2013, 31 May-4 June Chicago, USA

- Mirandola L, Chiriva-Internati M, Cobos E, Yu Y, Figueroa JA, Garavelli S, **Colombo M**, Lazzari E, et al. "Chemokine receptors as novel targets of the oncogene Notch1 in acute lymphoblastic leukaemia" Presented at ASCO annual meeting 2013, 31 May-4 June Chicago, USA

- **Colombo M**, Mirandola L, Apicella L, Platonova N, Lancellotti M, Lazzari E, Chiriva-Internati M and Chiaramonte R. "The role of Notch in the regulation of osteoclastogenesis in multiple myeloma". Presented at Cell Symposia "Hallmarks of Cancer 2012" 29-31 October 2012, San Francisco, USA.

- N. Platonova, **M. Colombo**, E. Vigolo, L. Apicella, L. Mirandola, GC Germisoni, M. Chiriva Internati, R. Chiaramonte - Cooperation between Notch and CXCR4/SDF1a axis in ovarian cancer. Presented at "Cell Symposium 2012-Hallmarks of cancer", San Francisco, CA, USA.

- Mirandola L, **Colombo M**, Apicella L, et al. - Anti-Notch treatment prevents multiple myeloma cells localization to the bone marrow via the chemokine system CXCR4/SDF-1. Presented at “17th World Congress on Advances in Oncology” October 11-13, 2012 Crete, Greece

- Platonova N, Vigolo E, **Colombo M**, Apicella L, Chiriva-Internati M, Comi P, Chiaramonte R “Notch promotes ovarian cancer cells migration and proliferation” Presented to “The Notch Meeting 2011”, Athens, Greece, 2-6 October 2011.

- L. Mirandola, L. Apicella, **M. Colombo**, S. Carluccio, N. Platonova, P. Comi,E. Cobos,W. Kast, M. Chiriva, and R. Chiaramonte “Notch1 produces a deregulation of CXCR4/SDF1 chemokine signaling in multiple myeloma cells”. - The Journal of Immunology, 2011, 186, 149.12

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